Genes and Cardiovascular Function
Preface

We are living in the era of molecular medicine and the influence of basic research on the clinical practice has never been more pronounced. Over the past 15 years, cardiovascular medicine has fully embraced the tools of modern molecular biology creating in effect a bridge between the traditional physiological and clinical discipline of cardiology and genetics, genomics and biotechnology. Moreover, there has been a widespread appreciation of the power of new advances in genetically engineered animal models and novel strategies for rapidly identifying mutations in candidate human genes for diverse cardiovascular diseases, both of which led to an exponential increase in our understanding of the molecular mechanisms that drive disease progression. Gene therapy is then one of the most fascinating consequences of the penetration of molecular biology and genetic engineering into cardiovascular medicine. It is, therefore, understandable that the interest of both experimental and clinical cardiologists in the role of genes in the heart is steadily increasing.

This book is based on the two Mendel symposia “Genes and the Heart”, organized in 2003 and 2008 in the Czech Republic as joint meetings of the Japanese and European Sections of the International Academy of Cardiovascular Sciences. The first one took place in Brno, well known for its industry, fairs, technological park, universities and rich cultural life. But this symposium was held in Brno for another reason: it is the birthplace of genetics. Here in the sixties of the nineteenth century an Augustinian friar and later abbot and, at the same time, a mathematician, physicist and biologist, Johann Gregor Mendel, discovered the principal laws of heredity. The abbey, the site of Mendel’s activities, has been recently restored and is ready to summon a limited number of scientists for their meetings. Thus, the participants of the first Mendel symposium had a unique opportunity, besides visiting an exhibition concerning Mendel’s discovery, to discuss the results of contemporary genetics in the genuine atmosphere of its true father founder.

The scientific and social success of this meeting exceeded the expectations of the organizers; we were repeatedly asked to continue and try to establish a new tradition. This request was strongly supported by our Japanese colleagues, both scientifically and financially. We have decided to invite the participants of the second Mendel symposium to another pleasant place of our country, the beautiful baroque castle Liblice near Prague. It was built between 1699
and 1702 as an aristocratic residence; now it is the property of the Academy of Sciences of the Czech Republic. The recent restoration has transformed the castle into a contemporary conference centre equipped with the latest technology. The castle is surrounded by beautiful French gardens with a ceremonial courtyard and offers exceptional stimulating environment for scientific events.

This book includes chapters which highlight the role of molecular biology and genetics in different areas of cardiovascular research; they are based on the selected contributions from the two Mendel symposia. The book is divided into six sections. The first, introductory, includes the short curriculum of Johann Gregor Mendel and the contemporary view on the possibilities and limitations of the gene therapy. The second section deals with the role of genes in cardiac development; the remaining four sections are devoted to the genetic approach of different cardiovascular disorders: mitochondrial diseases, ischemic heart disease, hypertension and arrhythmias, and cardiac hypertrophy and failure. It should stimulate the curiosity of cardiovascular scientists in gaining insight into the role of genes in the heart function in health and disease.

Twenty-four chapters in this book, written by established investigators, represent a wealth of material to emphasize the role of genetic factors in the genesis of different cardiovascular abnormalities. In addition, changes in gene expression, as a consequence of various pathological stimuli, have been identified to alter the protein content of different subcellular organelles in both cardiac and vascular myocytes and thus result in the development of cardiovascular dysfunction. It appears that a wide variety of gene expressions are excellent targets for gene therapy as well as pharmacological interventions to improve cardiovascular function in the disease state.

We are grateful to Prof. A. Kotyk and Mrs. M. Markova from Prague as well as Dr. Vijayan Elimban and Ms. Eva Little of Winnipeg for their help in the preparation of manuscripts for the purpose of editing. Cordial thanks are also due to Ms. Frances Louie and Mr. Ian Hayes, Springer USA, for their continuous advice and understanding during the editorial process. We hope this book will be of great value to cardiovascular students, fellows, scientists, clinicians and surgeons.

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Part I

Genes and the Heart
There is hardly any textbook of biology or encyclopedia in the world that would omit Johann Gregor Mendel. Unfortunately, the reality is often wrapped by romantic myths about an eccentric monk who happened to make a discovery due to his queer hobby and good fortune. In fact, there is nothing irrational in Mendel’s career and research (Fig. 1).

Johann Mendel was born in 1822 in a small farmer’s family in Hynčice, Silesia. The only way to offer a proper education to an obviously talented but poor boy were ecclesiastical schools. Once on such a track, Johann gradually became attracted by science, namely mathematics and physics. In 1843, Mendel suddenly appeared far from his home, in Brno, as friar Gregor of the Augustinian St. Thomas abbey. This location was quite natural. The Augustinian order was known to intentionally support science. Moreover, at that time, an enlightened abbot Napp headed St. Thomas, himself an amateur scientist, in then very popular field of agrology and breeding. Napp observed Mendel’s scientific aspirations with sympathy and allowed him to use the rich library of the abbey without restrictions and even to study his favorite disciplines, mathematics and physics, at the University of Vienna (1851–1853). It was a most fortunate decision since his later discovery was based on mathematical analysis, a completely novel approach in biology. Mendel was also kindly offered a piece of land and a greenhouse in the abbey’s garden for his experiments (Fig. 2).

The intellectual milieu of the abbey was quite exceptional. It became a meeting point of theologians, philosophers, scientists, and musicians.
It is well documented that the foremost Czech physiologist J. E. Purkyně, a name certainly familiar to cardiologists, used to be among the guests. For the sake of interest, during Mendel’s most intense research, a 10-year-old boy arrived as a choirboy in the affiliated basilica, who became a preeminent, world-famous composer half a century later, Leoš Janáček. Similarly, the academic environment of the rapidly developing city played a role in Mendel’s career. He presented a lecture on his results in 1864 at a meeting of a well-established society in Brno, “Gesellschaft für Beförderung des Ackerbaues, der Natur und Landeskunde,” and published them in the journal of the society under the title “Versuche über die Pflanzen-Hybride” 1 year later.

It is true that the attempts to verify the rules of heredity on other plant species were a bit disappointing. It may seem that Mendel’s original choice of pea was a mere lucky chance. But, more likely, owing to his exceptional observational talent he recognized the distinct features of the pea varieties ready to serve as a model. Obviously this was in the beginning of his idea to analyze these features in successive generations quantitatively, using statistics. This idea may be considered a prelude to currently so popular mathematical modeling in biology.

Johann Gregor Mendel was a remarkably many-sided scholar. Besides his ecclesiastical duties and research activities, Mendel taught physics at the German high school for a couple of years, systematically observed and recorded meteorological data, was a well-informed beekeeper (his beehive still exists), and even sat on the supervisory board of a prominent local bank. When abbot Napp died in 1867, Mendel, as the most revered candidate, became his successor. No wonder that the greenhouse was deserted and the experiments discontinued.
Mendel’s discovery was initially almost completely misunderstood and forgotten, like all premature discoveries used to be. He was aware of its significance and, at the same time, controversial character. He wrote to his friend – and a fierce opponent at the same time – Nägeli in 1867: “I have suspected that it is most difficult to reconcile my results with the current knowledge. With regard to the circumstances which followed the publication of such an unfamiliar experiment, they represent a dual peril: one for the experimenter and another one because of the consequences.” He was right. The Mendelian rules of heredity were doomed to unbelievable events to come. They were ignored for the next 35 years to be rediscovered independently by de Vries, Correns, and Tschermak in 1900. They became fully appreciated only gradually and with difficulty. In his homeland and eastward, they were doomed by the communist ideology for nearly two decades.

Johann Gregor Mendel died in 1884 at the age of 62 years. He did not live to see even a hint of recognition of his discovery. Today, he is appreciated as one of the most influential scientists of the nineteenth century, as the very founder of genetics.
Abstract
Gene therapy (GT) is one of the most fascinating consequences of the penetration of molecular biology and genetic engineering into medicine. Originally, it was assumed that monogenic genetic diseases will be the main field of its application. However, a great majority of the GT-based clinical trials in the last decade have dealt with acquired diseases. Still, its introduction into clinical practice is associated with serious problems. The main obstacle preventing a more rapid development in the field of GT is the imperfection of the vectors presently being used for gene transfer. At the present time, GT is predominantly being used in oncology where the barriers against its employment are weaker than in other medical disciplines. Among the acquired diseases, which are now in the focus of interest of GT, are also cardiovascular diseases. A number of different GT strategies have been developed. Their choice primarily depends on the disease to be treated. In addition to technical and strategic problems, ethical issues play a significant role in planning and performance of clinical studies.

Keywords
Cell therapy • DNA • Gene therapy • Oncolytic viruses • Plasmids • Transduction • Transfection • Transgene • Vectors

Introduction
Gene therapy (GT) is a modality whose therapeutic principle is the transfer of sequences of nucleic acids. It can be defined as a transfer of genetic material, which has a therapeutic effect, either because it supplements the cell with a new or missing function or because it suppresses its abnormal, pathological function. It can also be
employed for increasing the efficacy of other therapeutic modalities and for removing disturbing symptoms of various diseases, like pain. A strong support for GT development is provided by the progress of proteomics and, especially, ever-increasing understanding of the functioning of human genome. GT is definitely one of the most important and most hopeful, but also scientifically most demanding consequence of penetration of molecular biology and genetic engineering into medicine. At the same time GT, together with cell therapy (CT), which is being developed in parallel, represent two of the most controversial therapeutic modalities of contemporary medicine. Both call forth contradictory reactions in the lay and the medical communities alike.

The original aims of GT mainly comprised the treatment of monogenic hereditary diseases, but most of the clinical trials performed till now have dealt with acquired diseases. Among these dominate oncological diseases. The reasons for this are summarized in Table 1. As concerns over the number of clinical trials registered, cardiovascular diseases are on the second place.

The present problems, which GT is facing, are considerable. They can be divided into three categories: technical, methodological (strategic), and ethical. They should be dealt with, at least most of them, before GT can be raised to the level of routine therapy. The author feels that it might be useful to define some of the terms that will be used in the text below and that may not be familiar to some of the readers. A gene, which is being transferred, is a transgene. The carriers used for gene transfer are vectors, among which an important role is played by plasmids; these are small, circular genetic elements of bacterial origin. The term transfection describes the transfer of a foreign gene or its portion by means of the “naked” DNA or RNA. The process of transfer of a gene is called transduction, and the genetically modified cell is a transduced cell. The genetic material transfected frequently persists in the transduced cell in the form of episome, which is a circular, extrachromosomal element, replicating independently of the cell DNA in the transduced cells. Transposons are short segments of DNA capable of moving from one genetic location to another in a genome. They can replicate and can be integrated into random sites of the cell genome. MicroRNAs (miRNAs) are endogenous, highly conserved, short, non-protein-coding RNA molecules that mediate posttranscriptional gene expression by destabilizing target transcripts. They act by annealing to partly complementary sequences in the 3′-untranslated regions of target mRNA and thereby interfere with translation. It is assumed that miRNAs fine-tune at least 30% of protein-coding genes. Thus, miRNAs play a crucial role in the regulation of biological functions such as cell differentiation [1]. Ribozymes are RNA molecules, which possess sequence-specific cleavage activity. They occur naturally but can be synthesized to target-specific nucleic acid sequences.

### Technical Problems

When speaking about technical problems in GT, we usually have in mind problems with vectors. Vectors are of principal importance not only for the transduction efficiency and the properties and biological behavior of the transduced cell, but also for the risks associated with GT. The present imperfection of vectors is the main hindrance to rapid progress in GT.

The properties of an ideal vector are shown in Table 2. Vectors fulfilling all these requirements are not available so far. The presently used vectors are shown in Table 3. Extraordinary efforts are being devoted to the development of vectors that would approximate the ideal set down.

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**Table 1** Reasons for extensive use of gene therapy in oncology

1. It is easier to kill the cell than to repair it.
2. Transitory expression of the transgene may be sufficient for killing the cell.
3. A number of different efficient strategies are available.
4. Because of the nature of the disease, the barriers against its use are weaker than in other medical disciplines.
5. Oncological patients are generally willing to undergo experimental therapies.
Many investigators consider this endeavor to be the most important part of contemporary research in GT. The vectors are usually divided into non-viral and viral. The former group is nearly entirely represented by DNA in the form of non-linearized or linearized plasmids, which are introduced into the target cells by physical or chemical means or their combination. A number of different techniques have been developed for this purpose. Plasmids can be introduced into the cell directly, e.g., by microinjection, electroporation, ultrasound (s.c. sonoporation), bioballistics (by means of the gene gun), hydrodynamic method (based on an increase of intravenous pressure), or in the form of complexes. Especially popular is s.c. lipofection, usually based on the connection of DNA with cationic lipids. These lipoplexes fuse with the cell membrane and enter the cell. Another technique making use of the connection of anionic DNA with a variety of cationic polymers is sometimes called polyplexion. It is the aim of the latter manipulations to increase the stability of DNA and to facilitate the endocytosis mediated by cell receptors. The major advantage of the nonbiological methods is the relatively easy preparation of the genetic materials to be administered and their low toxicity and low immunogenicity. Among the disadvantages of the use of the plasmid-based gene transfer, the dominant are low stability in vivo, low efficiency of uptake by the target tissue, short term expression of the transgene, and a rare integration of the foreign DNA into the cell genome. A lot of effort is being exerted to overcome the disadvantages of the present nonviral vectors. The aim is especially to increase their stability outside the cell, their internalization, modification of intracellular trafficking from endosome to lysosome, facilitation of their dissociation from the carrier, and their entry into the nucleus. A number of sophisticated approaches are being employed for this purpose [2–4]. One of the most hopeful approaches is the construction of condensed particles of size less than 100 nm. Their preparation pertains to nanotechnologies and has been made possible by the recent major progress in the field of mechanics and physics. DNA is condensed and encapsulated, making use of the electrostatic interaction between anionic phosphate groups in DNA and the cationic carrier. In this form, DNA is protected against the action of endonucleases, and the cellular uptake is increased. Coupling cell – penetrating peptides and nuclear localization signals to the particle surface – can further facilitate it. Many researchers believe that particles prepared in this way, which are designated as synthetic virus-like particles by some of them, might, by their properties, approximate an ideal vector, as has been defined above. Hopes are also associated with the newly introduced transposon-based vectors [5]. Since the transposons present in mammalian genome have been inactivated millions of years ago, the gene carriers used are based on a reconstruction of active elements found in fish and amphibian genomes. Two of them carrying the fairy tale names of Sleeping Beauty [6] and Frog Prince [7] are being used for gene transfer.

In most of the GT clinical studies carried out so far, viral vectors (VV) have been used. The most frequently employed VVs and their basic properties are listed in Table 4. When compared with the nonviral vectors, VVs possess three major advantages. First, their surface structures predetermine their interaction with the cell receptors and penetration into the cell. It follows that the uptake of the transgene is much higher than in the case of nonviral vectors. Second, viral genome is equipped with regulation elements easily recognizable by mammalian cells. Third, some viruses

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<td>1.</td>
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<td>2.</td>
<td>It should penetrate into a large number of target cells.</td>
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<tr>
<td>3.</td>
<td>It should not be toxic either for the target cells or for other cells that might be hit unintentionally.</td>
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<tr>
<td>4.</td>
<td>The transgene must be transferred in a transcriptionally active state.</td>
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<td>5.</td>
<td>It should be capable of transferring large genes.</td>
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<td>6.</td>
<td>The expression of transgene should be sufficiently high and must persist long enough to achieve the effect desired.</td>
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<td>7.</td>
<td>Its immunogenicity should be low.</td>
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<tr>
<td>8.</td>
<td>It should not induce any serious systemic untoward reactions in the recipient.</td>
</tr>
<tr>
<td>9.</td>
<td>Its administration should not represent any risks for the recipient’s contacts.</td>
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V. Vonka

(virology, adeno-associated viruses (AAVs)) enable integration of the foreign genetic material into the cell genome, this ensuring a long-term expression of the transgene. On the other hand, VVs also have some disadvantages. First, the construction of VV is a challenging process practicable only in a well-advanced virus laboratory. Second, most of the viruses are strongly immunogenic. Preexisting antibodies may curtail or even block the expression of the gene delivered and the immunity developed after their administration may prevent repeated use of the same VV. Intensive investigations are under way for the preparation of VV with lowered immunogenicity. The “gutless” adenovirus can serve as an example of a significant success [8]. Third, the small size of viruses limits the size of the genetic material to be transferred. Fourth, the broad tissue tropism of some of the VVs is increasing the possibility of untoward off-target effects being induced. Therefore, considerable effort is being directed to such modification of VV as would increase their tissue specificity. There are quite a number of possible solutions [9]. One line of endeavor is to utilize specific receptors, which is attainable by adjusting the viral genome so that the requisite ligand is included in the surface structures of the virus particle. This has been termed transcriptional targeting. Another possibility is the inclusion of a promoter functional only in the target cell. Such targeting is called transcriptional. Fifth, there are biological risks associated with the use of VV. These risks are diminished, but not completely eliminated, by modifying the viral genetic material so as to disable the agent for replication.

This is usually achieved by deleting a portion of the virus genome [10]. Specifically genetically modified cell lines that complement the missing virus function are needed for the formation of virus particles from these defective virus particles. Such particles are capable of transferring the genetic material but are unable to replicate in the transduced cells. Still, all of the several tragic events that occurred recently (invariably cases of therapy of hereditary diseases, see below) were caused by virus particles incapable of reproducing in the cells transduced. It should be added that from the iron rule of non-infectivity of VV, two exceptions exist. The first relates to the oncolytic viruses, the effects of which are based on the replication of viruses in the tumor but not in non-tumor cells, this leading to their destruction. The other exception is represented by recombinant viruses, which are being used as experimental therapeutic vaccines.

In addition to the properties of VV shown in Table 4, they also differ by the time and duration of the expression of transgene, which influences the choice of the vector in any particular situation. In the case of adenovirus-based vectors the expression is relatively fast (within 1–4 days), which is of high advantage in situations where prompt expression is important. On the other hand, the expression of the transgene often ceases within a fortnight. If AAVs are used as vectors, the expression of transgene is not very efficient for weeks, but it may be sustained over months or even years. It should be kept in mind, however, that VVs are not the only factors responsible for the duration of transgene expression in vivo. It also depends on the tissue that is targeted and on the host response factors.

Table 3 Vectors used

<table>
<thead>
<tr>
<th>Vector</th>
<th>Technical demands</th>
<th>Efficacy of Transduction</th>
<th>Integration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DNA</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>2. RNA</td>
<td>Low</td>
<td>Low</td>
<td>-</td>
</tr>
<tr>
<td>3. Viruses</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>4. Synthetic VLP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>High</td>
<td>High</td>
<td>?</td>
</tr>
<tr>
<td>5. Bacteria</td>
<td>High</td>
<td>High</td>
<td>-</td>
</tr>
<tr>
<td>6. Transposons</td>
<td>High</td>
<td>?</td>
<td>High</td>
</tr>
</tbody>
</table>

<sup>a</sup>In the case of some viral vectors
<sup>b</sup>Virus-like particles

In addition to the properties of VV shown in Table 4, they also differ by the time and duration of the expression of transgene, which influences the choice of the vector in any particular situation. In the case of adenovirus-based vectors the expression is relatively fast (within 1–4 days), which is of high advantage in situations where prompt expression is important. On the other hand, the expression of the transgene often ceases within a fortnight. If AAVs are used as vectors, the expression of transgene is not very efficient for weeks, but it may be sustained over months or even years. It should be kept in mind, however, that VVs are not the only factors responsible for the duration of transgene expression in vivo. It also depends on the tissue that is targeted and on the host response factors.

There has been an ever-increasing interest in biological vectors other than viruses [11, 12]. These include bacteria genetically modified in such a way to make them nonpathogenic without losing their capability of penetrating into the target cells to be altered. These systems, properly genetically modified, could ensure a long-term expression of transgene without the risk of potent immune reactions against the vector developing. Bactofection might also permit the regulation of the production of the protein of interest, because
antibiotics could abolish it. There are two other great advantages of using gene-modified bacteria for GT. Their capacity for foreign genetic material is quite large and their preparation is inexpensive. Biological vectors include also the so-called biological liposomes, which are represented by spherical fragments of erythrocytes or exosomes.

An object of extraordinary interest is the development of vectors with whose aid it would be possible to direct the transgene to a particular position in the human genome. In spite of this aspiration not having been achieved so far, there is no lack of optimism among those who work on this difficult problem, and progress is evident. The mastering of this task would change the face of contemporary GT, in particular the treatment of monogenic hereditary diseases.

Methodical (Strategic) Problems

There exist some general principles for the application of GT. They can be outlined as follows: (1) understanding of the pathogenesis of the particular disease on the molecular level, (2) identification of the causative gene and knowledge of the nature of its aberration, (3) development of a therapeutic gene, (4) development of a vector that will ensure expression of the therapeutic gene over a desired time, (5) consideration of the off-target action of the gene and/or target effects that are different from the anticipated ones. However, these general principles acquire concrete and often very distinct forms, depending on the nature of the disease that is to be treated. Let us illustrate this through the examples of oncological and cardiovascular diseases (that together are responsible for some 80% of deaths in the developed countries), in whose treatment quite controversial intentions are sometimes involved. This is not surprising. In the case of malignant tumors the object is to destroy life-threatening tissue, while in cardiovascular diseases the aim, in great majority of cases, is to renew the functioning of an impaired organ, the regenerative capacity of which is low.

Still, there exist some general strategies aimed at blocking or tuning the expression of genes. One of the strategies being employed is to use antisense deoxyribonucleotides \[13\]. These sequences bind directly to the genes to be inactivated, blocking gene transcription, or to their mRNA, blocking gene translation. The latter event results in the formation of an RNA–DNA complex. Owing to the activity of the ubiquitous ribonuclease H, the RNA component of the duplex is destroyed. The antisense molecule remains untouched and can readily bind to another mRNA molecule. A major disadvantage of antisense nucleotides is their low stability. Replacing oxygen atoms with sulfur atoms can increase it. A significant enhancement of stability has been achieved by the introduction of the so-called PNA (protein nucleic acid). PNA is an analogue of the DNA molecule. Its backbone is made up of a peptide to which the individual bases are attached in a sequence, ensuring its binding to the target molecule. Another method of gene silencing, and also its fine-tuning, is based on synthetically prepared small interfering RNAs (siRNAs) mimicking the role of the endogenous double-stranded microRNA (miRNA).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Titre a (per mL)</th>
<th>Stability</th>
<th>Maximum capacity (kb)</th>
<th>Risks, disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retroviruses</td>
<td>$10^6$</td>
<td>Low</td>
<td>6–7</td>
<td>Oncogenicity, gene silencing</td>
</tr>
<tr>
<td>Adenoviruses</td>
<td>$10^{10}$</td>
<td>High</td>
<td>7.5</td>
<td>Toxicity, immunogenicity</td>
</tr>
<tr>
<td>AAV b</td>
<td>$10^{11}$</td>
<td>Very high</td>
<td>4.5</td>
<td>Low capacity</td>
</tr>
<tr>
<td>Herpesviruses</td>
<td>$10^8$</td>
<td>Low</td>
<td>&gt;30</td>
<td>Recombination c, activation of latent infection, immunogenicity</td>
</tr>
</tbody>
</table>

a Titres easily achievable  
b Adeno-associated viruses  
c Possible recombination with wild type virus
siRNAs, used as GT tools, are represented by synthetically prepared short, double-stranded, noncoding RNA molecules possessing a length of 19–22 ribonucleotides. Within the cell one of the strands is destroyed and the other one binds to complementary mRNA. This results in its degradation [14]. Much interest is devoted to ribozymes, which exhibit a strong antitranslational activity [15]. They can be introduced into cells by transfection or by means of VV.

There are a number of strategies, the value of which is markedly different in oncology and cardiology. Some of the differences will be outlined in the subsequent text.

**Oncology**

In the treatment of malignancies, GT has at its disposal a large number of different strategies. An attempt will be made to classify the approaches used, although we are aware that this is a task that cannot be fulfilled exactly, because a clear fundamentum divisionis is missing. Moreover, the individual strategies can be combined, they may overlap somewhat, and they have their say at different levels.

The strategies of the first group are based on direct modification of tumor cells. In addition to the approaches listed above, which primarily aim at inactivating the activated oncogenes, several others can be put to this group. These include, e.g., the introduction of fully functional tumor-suppressor genes [16]. Their expression may lead to the restoration of cell growth control or result in apoptosis of the tumor cells. Apoptosis can also be induced by the introduction of proapoptotic genes. All of these approaches are successful in the cell-culture systems and in some experimental models. A major problem comes in vivo. It is impossible to introduce the genetic material into all cells of the tumor being treated. The unmodified cells have a growth advantage over those whose malignant phenotype has been altered. They may soon become dominant in the tumor cell population. For successful treatment, combination with other treatment modalities is needed.

Therefore, two other direct modifications of tumor cells deserve more attention. The first one is based on the introduction of genes for immunostimulatory factors into tumor cells [17]. This ensures a high local concentration of such factors without any signs of toxicity that accompany their systemic administration. This raises the probability of a robust immune response that may have a clear therapeutic effect, without it being necessary to genetically modify all cells of the tumor. Similarly, it is not necessary to affect the whole tumor-cell population if the so-called suicide genes (SG) are used [18]. After prodrug treatment the toxic metabolites spread to neighboring cells (“bystander effect”), and the release of large amounts of tumor antigens may stimulate the development of a systemic antitumor immune reaction.

A second major group of GT strategies in the therapy of tumors is the one directed at gene modification of non-tumor cells. It includes, e.g., the introduction of the gene designated MDR-1 (multidrug-resistance-1), the product of which increases resistance of bone-marrow cells to toxic effects of chemotherapy [19] or the creation of conditions for the treatment of the life-threatening graft-versus-host disease by ex vivo introducing SG into donor T-lymphocytes [20].

Another group of GTs in oncology is procedures suppressing neoangiogenesis, a necessary precondition for tumor growth and metastasis formation. Precisely in this strategy, the difference between the GT of malignant tumors and the GT of the heart is the most marked. The introduction of, e.g., the gene for the angiogenesis-suppressing factor endostatin into tumor cells lowers their oncogenic potential and ability of metastasis formation [21]. However, an anti-angiogenetic effect can also be attained by a blockade of the functionality of important proangiogenetic factors, such as members of the families of VEGF (vascular endothelial growth factor) or FGF (fibroblast growth factor), e.g., by means of the corresponding antisense.

Oncolytic viruses, i.e., viruses replicating exclusively or predominantly in tumor cells, are also considered to be agents for tumor GT. Two groups of oncolytic viruses are distinguished: those
that are naturally oncolytic and mutants of other viruses. Taken *sensu stricto*, only the latter should be taken as GT agents, because their use was preceded by their genetic adjustment.

The last, viz. the fifth, group consists of genetic therapeutic anticancer vaccines. It is very likely that during the coming decade they will enter medical practice on a large scale. There are several distinct types of vaccines that are at the stage of development. Each of them has some advantages and some disadvantages. Considerable attention is being given to DNA vaccines [22], which are bacterial plasmids into which a gene for a specific tumor antigen has been incorporated. The gene must be in a form that ensures its expression in mammalian cells. Another type of genetic vaccines is recombinant vaccines. They are represented by recombinant proteins with the peptide carrying the immunodominant epitope of the tumor antigen inserted into another protein, known to produce a potent effect on the immune system [23]. A great endeavor is given to recombinant live viruses in which a certain gene that is not essential for replication is replaced by a gene for tumor antigen. Another type of genetic vaccines consists of cellular vaccines. They have a number of advantages. The first is that it is not necessary to know the immunodominant tumor antigens. These vaccines are prepared via modifying tumor cells by the introduction of genes for immunostimulatory factors. Both autologous and allogenic vaccines are under consideration. The development in the recent years rather favors the latter [24].

**Cardiovascular Diseases**

Interest in the use of GT in cardiology has been growing in the recent past, the reasons being several. The most important has been the gradual but rather fast recognition of the basics of physiological processes and the mechanisms that lead to the development of pathological states at the molecular level. Contributory to its development have been the quickly accruing successes in experimental systems. The growing interest in GT in cardiology has also reflected the slowdown in the development of efficient and safe new drugs.

Similarly as in other medical disciplines, a condition for the use of GT in cardiology is reliable and clinically relevant vectors, with safety aspects being more important than in oncology. The vectors most frequently used so far have been adenoviruses; however, it is probable that their place will gradually be taken over by adeno-associated viruses (AAVs) [25–27]. The recent discovery that some AAV serotypes are highly cardiotropic has been very helpful in this respect. However, a vector is still being sought that could be administered intravenously and that would have specific uptake by cardiomyocytes, with minimal off-target effects [28].

The use of a large spectrum of strategies is being considered and some are already in use. It is much more difficult to classify them in cardiology, because the efforts are less straightforward than in the field of oncology, in which the aim of the interventions is destruction of the unwanted tissue. Possibly the most marked differences are the absence of strategies influencing the immune system (with the exception of transplantations) and no use for SG. Another difference is a closer interconnection of GT and CT in cardiology. The choice of the strategy always depends on the purpose of the intervention. They are necessarily different when the therapy is meant just to serve as bridge to transplantation or bridge to recovery or whether a long-term expression of transgene is required [29]. To find optimal delivery system of the vectors is another important point. Among those which are under investigation is direct needle injection, pericardial delivery, catheter delivery into coronary arteries, and endocardial delivery.

From the literature available, it is apparent that special attention in heart GT is being paid to miRNA. This interest has been ever increasing with the gradual broadening of the recognition of the role played by the different miRNA species in the pathogenesis of cardiovascular diseases such as heart failure, cardiac hypertrophy, ischemia, arrhythmia, and atherosclerosis [30–32], and the recognition of potential approaches for miRNA-based interventions [33]. As has been summarized in a recent review, many cardiac patients can be treated by correcting their miRNA.
expression [34]. The fact that the involvement of certain miRNAs in several different heart diseases has been experimentally established brings the miRNA-based strategy closer to extensive clinical application.

There have been many applications of GT, with different strategies being used. In the next section some of them will be mentioned, the object not being to cover the entire field and its problems, but rather to document, on several examples, their diversity and the possibilities they offer.

GT is trying to break the classical dogma of heart regeneration, i.e., cardiomyocytes become postmitotic soon after birth. Recent findings of the research on myocardial regeneration suggest that it is possible to induce adult cardiomyocytes to reenter division by means of genes, the products of which are involved in the regulation of the cell cycle or act as pro-mitogenic growth factors, such as VEGF or FGF [35]. However, there seems to be a long way to go before these new observations are fully translated into clinical practice. Although in animal models of ischemic myocardium the administration of plasmids carrying VEGF or FGF have resulted in an increased collateral blood flow [36], similar studies in humans did not provide consistent results. The administration of a plasmid carrying VEGF gene into inoperable heart has been reported to result in increased perfusion and reduced angina symptoms [37], and favorable results have been reported also by another group [38]. However, they have not been confirmed by a more recent study [39].

One of the main topics of GT in cardiovascular diseases is the modification of ion channels. Their aberration is central to many cardiovascular diseases, including hypertension, heart failure, ventricular arrhythmias, or atrial fibrillation [40, 41]. There are also efforts aimed at developing biological pacemakers that might serve as an alternative to electronic devices [42]. A cell therapy approach using gene-modified human mesenchymal stem cells implanted into dog heart produced encouraging results [43].

The last-cited experimental study may serve as an example of interconnection of GT and CT. It is not the only instance. Quite a few other studies are under way that are based on the same principle, i.e., genetic manipulation of cells using cardiac stem cells, endothelial stem cells, bone-marrow stem cells, and adipose-tissue-derived stem cells, with this resulting in differentiation into cardiomyocytes. Sophisticated techniques for obtaining, for in vitro treatment, for implantation, and for in vivo activation of their growth, differentiation, and migration have been developed (for a review; see Madonna et al. [44]).

Another task for GT in cardiology is the prevention of rejection of heart transplant. In GT the aim is to inactivate genes that code for cytokines and adhesion molecules, the products of which are involved in rejection (for a review; see Suzuki et al. [45]).

To summarize, in spite of considerable efforts having been exerted and in spite of GT clinical trials representing the second largest group (after oncology) of clinical trials registered, the recent progress of GT in cardiology has been rather modest, more modest than anticipated 10 years ago. In their recent fine review Katz et al. [46] summarized the results of the recent experimental studies, described the advantages and disadvantages of the different approaches, and then defined the conditions that would lead to an optimization of the methods to be used. Notwithstanding the existing shortcomings, their conclusion is optimistic “the outlook remains promising.”

Ethical Problems

Ethical issues are of paramount importance for GT. Their importance is stressed by some serious events that took place in the past 10 years in the treatment of some genetic diseases. When a group of French scientists reported the successful treatment of children suffering from severe combined immunodeficiency (SCID) with a retrovirus carrying a therapeutic gene, a surge of enthusiasm followed. Unfortunately, 4 of the 11 children treated developed acute T-cell leukemia [47]. One of them died of leukemia. Another case of leukemia was reported in similarly treated British children [48]. The subsequent molecular analysis
Gene Therapy: Hopes and Problems

revealed that in at least four of these children, a similar pathogenic mechanism was involved. The retroviral vector was integrated in close neighborhood of the promoter of LMO2 gene, coding for a transcription factor whose overexpression was apparently involved in the pathogenesis of the disease. The phenomenon, called insertional mutagenesis, resulted in uncontrollable cell proliferation. Such risk is particularly associated with retrovirus integration. Theoretically, a similar risk may be coupled with AAV, the genomes of which are also readily integrated into cell genomes. However, their safety profile seems to be much higher than in the case of retroviruses because AAV DNA preferentially integrates into a certain locus of chromosome 19. Another death was reported from a trial aimed at GT treatment of ornithine-decarboxylase deficiency. It was caused by the use of a disproportionately high concentration of a recombinant adenovirus which produced a deadly toxic shock [49].

Another problem may be caused by the toxicity of siRNAs arising from competition with cellular miRNA processing [50] or from its off-target effects. Yet another possible source of untoward reactions may be chronic overexpression of the gene products, with uncertain consequences [51]. There is also a theoretical possibility that the VV used can recombine with a wild-type strain. The properties of such a recombinant cannot be anticipated.

The warning events call for carefulness in the use of GT and have stimulated a new ethical debate on GT. The result has been considerable toughening of the conditions for performing clinical studies.

Table 5 summarizes the principles that should be respected in all clinical GT studies.

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Guidelines for gene therapy clinical studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Every preparation must be produced in accordance with good manufacturing practice. Its quality and safety must be verified by all the methods specified by the law and must respect the newest achievements of biomedical sciences.</td>
</tr>
<tr>
<td>2.</td>
<td>The researchers must do their best to inform the patient about both the benefits and possible risks of the therapy. In these interviews they have to respect the education and the intelligence of the patient.</td>
</tr>
<tr>
<td>3.</td>
<td>Every clinical undertaking should unconditionally respect the rules of good clinical practice. The research group must have extensive experience in testing new pharmaceuticals.</td>
</tr>
<tr>
<td>4.</td>
<td>The protocol approved must be strictly adhered to. Patients who do not fulfill the criteria specified in the protocol must not be included.</td>
</tr>
<tr>
<td>5.</td>
<td>Any untoward or unexpected reaction must be reported without delay and thoroughly analyzed.</td>
</tr>
<tr>
<td>6.</td>
<td>The supervising authority should have enough resources for constant control of the undertaking.</td>
</tr>
<tr>
<td>7.</td>
<td>All undertakings should be double blind. There are two strong reasons for this. (1) The interest of the researchers in a positive outcome of the study, which may be subconsciously reflected in the process of evaluation. (2) The placebo effect, which is known to be strong in seriously ill patients.</td>
</tr>
<tr>
<td>8.</td>
<td>At this stage of knowledge, gene therapy should not be performed in patients suffering from diseases which can be successfully treated by other means. On the other hand, gene therapy should not be limited to patients in the terminal phase of their disease.</td>
</tr>
</tbody>
</table>

Conclusions

The ongoing development of GT and its gradual introduction into clinical practice embodies some serious problems, which I tried to characterize in the preceding parts of this brief review. However, their existence does not mean that GT research and applications should be calmed down. On the contrary, the breadth of the GT potential – its utility in combating not only genetic diseases but also acquired conditions that are beyond the possibilities of conventional cure – is a great promise for future medicine. Nevertheless, the up-to-now experience signifies that the road from the laboratory bench to the bedside should not be unidirectional. In the years ahead, researchers will be repeatedly returning from clinical studies to the laboratory to clarify the causes of unexpected events. Only in
this way will it be possible to fill up the vacancies in our knowledge, reduce the risks involved, and raise the effectiveness of the operations being performed. In the light of what we know at present, it might be expected that in the next decade the progress in the clinical utilization of GT will be faster in oncology than in cardiology.

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References

VEGFS, FGFS, and PDGF Coordinate Embryonic Coronary Vascularization

Robert J. Tomanek and Lance P. Christensen

Abstract
The formation of the coronary vasculature during embryonic and fetal life requires many signaling events that involve transcription factors, growth factors, and other molecules. Vascular precursor cells migrate to the heart from the proepicardium, and then differentiate and assemble to form the coronary vasculature. Several growth factors are required for coronary vasculogenesis, angiogenesis, and arteriogenesis, as documented in this chapter, based on both in vitro and in vivo studies in quail, rat, and mouse. Our data reveal that formation of the initial vascular, endothelial-lined channels is regulated by multiple VEGFs (especially VEGF-B), multiple FGFs, and angiopoietins. TGF-β inhibits at least two splice variants of VEGF, thus its expression attenuates endothelial cell proliferation during arteriogenesis. Our findings also document: (1) VEGF-B and VEGFR-1 as the key players in the formation of coronary ostia and stems, and (2) FGF-2 and PDGF as important regulators of coronary arterial formation. These conclusions are based on experiments in which these growth factors were inhibited by injecting neutralizing antibodies into the vitelline vein of quail embryos. Finally, we tested the hypothesis that embryonic mesenchymal stem cells (EMSCs) facilitate coronary tubulogenesis by adding these cells to mouse embryonic heart explants. These experiments revealed an increased tubulogenesis associated with a 22-fold enhancement of stromal-derived factor-1 (SDF-1), most of which was a product of the EMSCs. In conclusion, prenatal coronary vessel development requires temporally and spatially coordinated signaling processes, multiple growth factors, and the influence of progenitor cells.
Introduction

Prenatal myocardial vascularization requires the migration of precursor cells from outside the heart; their differentiation, proliferation, and coalescence; and then further growth by sprouting or partitioning (intussusceptive growth) and remodeling. These events are controlled by a variety of signaling events that are temporally expressed (reviewed in Tomanek 2005). [1] The first key event regarding myocardial vascularization is the formation of the epicardium by proepicardial cells that migrate from the proepicardium located in the dorsal body wall. The proepicardium, a transient, grape-like cluster of cells, develops from the splanchnic mesoderm and is under the control of many transcription factors, e.g., capusulin (epicardin), Is11, NKX2-5. Proepicardial cells migrate via multiple vesicles and cover the myocardium, thus forming the epicardium and subepicardium. Epithelial-mesenchymal transformation (EMT) of the epicardial cells results in various cell lineages, as these cells delaminate and change phenotype. This process involves signaling by pathways that include Wnt, Hedgehog, TGF-β, Notch, and the slug/snail family. Myocardial signaling plays an important role in coronary cell specification by expressing Vang12, a cell polarity gene which is required for the migration of epithelial-derived cells. Moreover, the myocardium provides growth factors that regulate the formation of the capillary plexus, formation of the coronary ostia, and development of the arterial hierarchy. This chapter reviews the roles of the key growth factors involved in these events.

Tubulogenesis (Vasculogenesis and Angiogenesis)

Progenitor cells proliferate, migrate, and form vascular tubes (vasculogenesis). These endothelial-lined tubes form branches (angiogenesis) and also coalesce to form larger channels. The regulation of coronary tubulogenesis has been explored in my laboratory by both in vitro and in vivo approaches. That VEGF and FGF-2 induce myocardial vascularization in the embryonic heart was documented by injecting these proteins in ovo into the vitelline vein of chicks [2]. In order to study which growth factors are required for coronary tubulogenesis, we used neutralizing antibodies in embryonic quail heart explants [3–7]. In this system, the tubular network is formed outside the explant in the collagen gel and is identified by antibodies that are specific for endothelial cells. These cells are derived from the epicardium and thus reflect the events that occur in vivo. To test the hypothesis that hypoxia provides a primary stimulus for VEGF expression and tubule formation, we incubated the heart explants in a hypoxic environment [3, 4]. Under this condition, VEGF mRNA was up-regulated; whereas in a hyperoxic environment, it was down-regulated. Although hypoxia stimulated several VEGF-A splice variants, VEGF165 was shown to be the main inducer of tubulogenesis. These data support the idea that the myocardium experiences a relative hypoxia as the ventricles develop a compact myocardium [8].

Multiple Growth Factors Regulate Coronary Embryonic Vasculogenesis

Our embryonic quail explant model was employed to test the hypothesis that several tyrosine kinase receptors contribute to vasculogenesis and, therefore, tube formation [5]. This hypothesis was based on our data documenting a total absence of vascular tubes when genestein was added to the explanted hearts in order to negate tyrosine kinase signaling. Tubulogenesis was attenuated...
VEGFS, FGFS, and PDGF Coordinate Embryonic Coronary Vascularization

(32–57%) by antibodies to FGF-2 and antibodies to VEGF-A, as well as by soluble Tie-2, a receptor for angiopoietins. When any two growth factors were inhibited, the amount of tubulogenesis was markedly inhibited, i.e., tube formation was only 8–20% of the controls. Additional experiments also documented the interdependence of the growth factors. Stimulation of tubulogenesis by VEGF-A was totally abolished when anti-FGF-2 was also added to the explant cultures. Similarly, soluble Tie-2 also negated VEGF-A induction of tube formation. Finally, the sixfold increase in tubulogenesis in response to FGF-2 protein was markedly reduced by the presence of anti-VEGF-A. Thus, these experiments provided evidence that at least three growth factors regulate tube formation in this embryonic heart model.

A focus on VEGFs and FGFs was deemed appropriate because these proteins are highly expressed in the developing heart. As seen in Fig. 1a, VEGF transcripts are selectively dense adjacent to the epicardium, the region in which tubulogenesis is first seen [6]. VEGF expression

Fig. 1 Immuno-histochemical staining for VEGF. (a) The epicardium (arrows) and cardiomyocytes stain variably with the greatest intensity of staining near the epicardium in the E7 quail embryo. (b) The atrioventricular groove is characterized by a loose network of subepicardial cells. Many of these cells stain intensely for VEGF (arrows).
then becomes more pronounced toward the endocardium as tubes form in a gradient in that direction. FGF-2 has also been shown to be abundant in fetal hearts of chick [9] and rat [10, 11]. Its relationship to coronary vascularization is indicated by data that reveal its peak expression at two key time points: early vascularization (embryonic days 14 and 15) and marked microvascular growth (birth) in the rat [11].

**VEGF Family Members and Tubulogenesis**

VEGF-A has been repeatedly shown to be important for vasculogenesis and angiogenesis. It binds to VEGFR-1 and VEGFR-2. Of the other VEGF family members (B, C, D, and E), the developing heart also contains VEGF-B [12, 13], VEGF-C [12], and VEGF-D [14]. VEGF-B and the related PIGF (placental growth factor) bind only to VEGFR-1, whereas VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3. Epicardial and subepicardial cells that function as endothelial cell progenitors are VEGFR-2 positive [15]. VEGF-C and VEGF-A become synergized to enhance plasminogen activator activity in endothelial cell lines [16], a finding that underscores their role in endothelial cell differentiation.

To test the hypothesis that VEGF-B and VEGF-C induce tubulogenesis in the heart, we added neutralizing antibodies in our quail heart explant assay [6]. Inhibition of VEGF-B reduced aggregate tube length in the explanted hearts by nearly 70%. Inhibition of VEGF-C was also effective, in that it attenuated aggregate tube length by 60%. In contrast, VEGF-A inhibition attenuated tubulogenesis by only 30%. Additional experiments were conducted in which soluble VEGFR-1 and soluble VEGFR-2 was added to the culture media. Addition of soluble VEGFR-1 (Flt-1) reduced tube formation to only 23% of controls, whereas in the presence of soluble VEGFR-2 (Flk-1) tube formation was 57% of controls. Thus, when VEGF-B and PIGF are bound, tubulogenesis is markedly inhibited. Subsequent in vivo experiments were conducted on quail embryos in which antibodies to VEGF-A and VEGF-B were injected into the vitelline vein [17]. These experiments also revealed that anti-VEGF-B is more effective than anti-VEGF-A in reducing embryonic myocardial tubulogenesis. Thus, based on these data, a key role for VEGF-B in embryonic coronary vasculogenesis is documented for the first time. This role is consistent with the high expression of VEGF-B in the embryonic heart.

**TGF-β Inhibits VEGF and Tubulogenesis**

The three main mammalian TGF-β isoforms, i.e., β1, β2, and β3, have both dissimilar and overlapping effects. Although TGF-β is important for vessel development, its specific effects have been controversial. Its major role is in arteriogenesis, which follows development of the initial vascular plexus consisting only of endothelial cells. We tested the hypothesis that some of the TGF-β isoforms inhibit tubulogenesis, considering the fact that endothelial cell proliferation ceases when the media of the vessel is being developed via smooth muscle recruitment. TGF-β1 added to our embryonic heart explants virtually negated tube formation, whereas anti-TGF-β1 neutralizing antibodies caused a twofold increase in tubulogenesis [5]. Subsequently, we found that TGF-β2 and TGF-β3 were similarly effective in negating tube formation [7]. Moreover, the three isoforms also attenuated tubulogenesis induced by VEGF-A or FGF-2. To determine whether anti-TGF-β would enhance tubulogenesis, we added neutralizing antibodies to each of the TGF-β isoforms. Neither anti-TGF-β1 nor anti-TGF-β2 had such an effect, whereas anti-TGF-β3 increased tubulogenesis by 50%. We then examined VEGF-A splice variants in the explants exposed to the three TGF-βs or their neutralizing antibodies. These experiments showed that: (1) VEGF190 and VEGF166 mRNA were enhanced by anti-TGF-β3 but not by the other anti-TGF-βs and (2) TGF-β1, β2, and β3 decreased the VEGFs. The data render the conclusion that TGF-βs inhibit embryonic heart tubulogenesis by limiting VEGF190 and VEGF166 mRNAs.
VEGFs, FGFS, and PDGF Coordinate Embryonic Coronary Vascularization

**FGF Family Members and Tubulogenesis**

Multiple FGFs, VEGF, and Hedgehog signaling. FGFs and Hedgehog signaling have been documented to drive coronary morphogenesis in an elegant study on engineered mice and organ cultures [18]. The data indicate that FGF signaling to cardiomyocytes regulates Hedgehog signaling, which then influences VEGF and angiopoietin expression. The work also revealed that FGF-9 is essential for coronary vessel formation since FGF-9−/− mice lack a complete vascular plexus. Blood vessel formation requires FGFR-1 signaling [19] and this receptor is over-expressed during epicardial-mesenchymal transformation and epicardial cell delamination [20].

Most recently, we tested several hypotheses that multiple FGF ligands are able to stimulate tubulogenesis and that embryonic mesenchymal stem cells (EMSC) will stimulate tubulogenesis via FGF signaling [21]. Our data, based on both quail and mouse explants, indicate that FGFs 1, 2, 4, 8, 9, and 18 are able to induce tubulogenesis and that FGF-2 is most effective at a lower dose. The effects of each of these FGFs were found to be VEGF dependent. In order to determine the role of FGF signaling in tubulogenesis, we added FGFR1-DN (an adenoviral construct encoding a cytoplasmic domain-deleted FGFR1 that inhibits signaling by all four FGF receptors) or soluble splice variant receptors of FGFR1 and FGFR3. Tubulogenesis and endothelial cell migration were inhibited by FGFR1-DN. Of the receptor splice variants, FGFR1-IIIc, which binds most of the FGF ligands, also decreased endothelial cell migration. Next, we tested the hypothesis that FGF signaling is required for VEGF-induction of tubulogenesis. When FGFR1-DN, FGFR1-IIIc, or FGFR3-IIIc were added to the heart explants, VEGF-induced tubulogenesis was negated. The C (EMSCs) isoforms, noted above, are usually specific for mesenchymal cells.

Embryonic mesenchymal stem cells. Finally, embryonic mesenchymal stem cells were co-cultured with the heart explants to determine their role in tubulogenesis. Endothelial cell density was increased 2.7-fold in the co-cultures compared to cultures containing only the heart explants. This response was completely blocked by the addition of FGFR1-DN, a finding that FGF signaling is required for this EMSC-induced response. ELISA analysis of the culture media revealed that SDF-1α (stromal-derived factor) was 22 times higher when EMSCs were added to the cultures. To determine whether the heart explant or the EMSCs were the source of SDF-1α, media from explants and EMSCs alone were compared. The data indicate that EMSCs provide more than 50 times the amount of SDF-1α than the heart explants. Not finding any evidence that EMSCs incorporate into the vascular tubes, we conclude that their effect is paracrine.

**Coronary Arteriogenesis**

Coronary ostia are formed when a capillary plexus penetrates the aorta at the left and right coronary cusps (Fig. 2). This phenomenon was first described in quail hearts by Bogers et al. [22]. Subsequent studies confirmed and expanded on this observation using chick-quail chimeras [23] and serial sections of chick [24] and rat [11] hearts. Some peritruncal tubes also enter the aorta at other sites, but fail to form a vascular channel (ostium) in the aorta [25]. The two coronary ostia that form via fusion of the peritruncal tubes require apoptosis of resident cells within the aorta [26]. As the ostia form, the attached vascular plexus concomitantly recruits smooth muscle cells that form the two main coronary stems [25].

The cells of the epicardium and those surrounding the root of the aorta or in the atrioventricular groove stain intensely for VEGF antibodies (Fig. 1). Moreover, VEGF receptors R-2 and R-3 are highly expressed at the base of the aorta, coinciding with the temporal and spatial formation of the coronary arteries [6]. Accordingly, we conducted in ovo experiments on quail embryos regarding the role of VEGFs in ostial and coronary artery stem formation [17]. Injection of VEGF-Trap, the VEGFR-1/VEGFR-2 chimera, prior to formation of the
main coronary arteries at E9 prevented formation of the coronary arteries in 11 of 13 embryos; the remaining two embryos had only one coronary artery. Injections of soluble VEGFR-1 or soluble VEGFR-2 usually limited coronary formation to one artery or prevented coronary artery formation. Antibodies to VEGF-A had little effect. In contrast, anti-VEGF-B most often prevented coronary artery stem formation or limited formation to one artery. These experiments established that VEGF-B and VEGFR-1 signaling are key regulators of coronary ostial and stem formation. This finding underscores the importance of VEGF-B and VEGFR-1 signaling in coronary arteriogenesis, as well as vasculogenesis and angiogenesis.

Based on the well-established roles of FGFs and PDGF in arteriogenesis, we tested the hypothesis that these growth factors regulate: (1) ostial, (2) stem, and (3) downstream development of coronary arteries by injecting quail embryos in ovo at embryonic (E) days E6, E7, or E8. Neutralizing antibodies to either FGF-2 or PDGF-limited coronary artery stem formation, especially when injected 2 days (at E6) before stem formation is under way. Anti-FGF-2 was more effective in this regard. If the neutralizing antibodies were administered when coronary artery stem formation may already be under way, the main effect was a delay in artery stem formation.

Muscularization of the endothelial-lined channels that form the coronary arteries proceeds in a base to apex direction. To study this process, we used serial sections to determine the extent of tunica media formation and the distance from the aorta that it progressed. The data revealed that when a coronary stem forms, despite being subjected to neutralizing antibodies, the process of muscularization is limited, compared to the controls. Thus, both FGF-2 and PDGF play a role in downstream development of the tunica media. Moreover, even in muscularized arteries, the tunica media is less developed compared to controls. We also showed that VEGF, in addition to its role in ostial and stem formation, is important in the development of the tunica media downstream from the coronary stems. These data are based on embryos injected with VEGF-Trap after the formation of the coronary stems. In conclusion, at least three growth factors (i.e., VEGF, FGF-2, and PDGF) regulate coronary artery formation and growth.

Fig. 2 A summary of growth factors signaling during embryonic/fetal development. Hedgehog signaling plays a key role in vasculogenesis and angiogenesis when activated by FGFs from the myocardium. This causes VEGFs and angiopoietins to be up-regulated and released from the myocardium and to regulate vascular tube formation and growth. The subsequent ostial and stem formation is VEGF-B dependent. Several growth factors contribute to medial formation of the coronary arteries.

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**GROWTH FACTOR SIGNALING CORONARY FORMATION**

**VASCULOGENSIS**

- Hedgehog
- FGFs
- VEGF-A, -B, -C
- Angiopoietins

**ANGIOGENESIS**

**ARTERIOGENESIS**

- Aorta
- Ostial + Stem Formation
- Main Coronary Arteries
- VEGF-B
- VEGFs
- FGFs
- PDGF
- TGF-β

FORMATION OF TUNICA MEDIA
Conclusions

All three of VEGF, FGF-2, and PDGF antibodies produced some common phenotypes. First, the sinusoidal area of the ventricles persisted, whereas the growth of the compact region was limited. Other anomalies included: (1) persistence of isolated multiple channels in the aorta, (2) occasional failure of the coronary ostia to enlarge sufficiently, and (3) massive accumulations of blood cells in the subepicardium or in the middle of the interventricular septum. In the study using VEGF-Trap [17], we found that the erythrocytes in the ventricular walls and blood islands of treated embryos were derived from the proepicardium, as documented by retroviral cell tagging. Thus, limitations in VEGF signaling induce proepicardial-derived hemangioblasts to differentiate into erythrocytes.

References


The E2F Pathway in Cardiac Development and Disease

Jennifer Rueger and Balwant S. Tuana

Abstract
Regulation of the cardiac cell cycle is an important and unique process in myocardial development. During embryonic growth cardiomyocytes rapidly proliferate, but shortly after birth they enter a final round of the cell cycle after which they permanently withdraw and cardiac growth depends on physiological hypertrophy. When the heart becomes stressed, it undergoes pathological hypertrophy to compensate for increased load on the heart. This process appears to involve the induction of genes involved in regulating the fetal gene program as well as the cell cycle which is largely controlled by the E2F family of transcription factors. In this review we summarize the current understanding of the E2F pathway and its contribution to normal and pathological cardiac development and growth.

Keywords
Cardiac growth • Cell cycle • E2Fs • Gene expression

Introduction

Proper cardiac development requires tight and coordinated expression of genes controlling both the cell cycle and differentiation. During embryonic development cardiomyocytes divide very rapidly, and thus approximately 70% of cells are synthesizing DNA. As cardiac precursor cells begin to differentiate they lose their proliferative capacity, resulting in only about 45% of cardiomyocytes synthesizing DNA [1]. Several days after birth cardiomyocytes undergo a final round of DNA synthesis without cell division, called acytokinetic mitosis, leaving many cardiomyocytes binucleated. Cardiomyocytes then permanently withdraw from the cell cycle and all postnatal cardiac growth depends on physiological hypertrophy. As the heart ages and is exposed to stressors and damage, this triggers abnormal or pathological cardiac hypertrophy often leading to heart failure. This abnormal growth is associated with a reactivation of the fetal gene program as well as the expression of genes involved in cell cycle reentry [2, 3]. Thus it appears that proper
regulation of the cell cycle is the key for both embryonic and postnatal cardiac development.

The Cell Cycle

The cell cycle is a complex and tightly regulated process which exists in five distinct phases and has numerous checkpoints to inhibit abnormal cell cycle entry and proliferation. The cell cycle begins in Gap 1 (G_1) in which the cell grows and prepares to enter the cell cycle. Cells which remain in G_1 for extended periods of time can exit the cell cycle and are said to be in a quiescent stage termed G_0. They may reenter the cell cycle or express genes necessary for terminal differentiation and become senescent. When the appropriate growth signals are received, cells can pass through a restriction point in G_1 after which they are committed to the cell cycle. The next stage is the synthesis (S) phase in which DNA is duplicated, followed by a second preparatory phase Gap 2 (G_2). Cells then enter mitosis (M) in which chromatin is condensed and assembled at the mitotic plate. DNA and cellular components are separated into two daughter cells which divide in a process called cytokinesis.

Cell cycle regulation is managed in large by cyclin-dependent kinases (CDKs) that, in association with cyclins, phosphorylate a wide range of proteins leading to the appropriate expression of genes for coordinated induction and passage through the cycle. Cyclin type and expression fluctuates throughout the cycle and different cyclins positively regulate CDKs which remain at stable levels throughout the cell cycle. Cyclin D associates with CDK4 and CDK6 during G_1 and is important for passage through the G_1/S restriction point. Cyclin E or A associates with CDK2 to create the S-phase-promoting factor (SPF). Cyclin B or A associates with CDK1 to promote mitosis by creating the M-phase-promoting factor (MPF). While the embryonic heart contains large amounts of cyclins and CDKs, they are down-regulated in quiescent adult cardiomyocytes [4].

In contrast to cyclins, CDK inhibitors (CDKIs) negatively regulate CDKs by competitive binding with cyclins to CDKs, thus blocking cell cycle progression. They exist in two groups: Cip/Kip (p21, p27, and p57) which broadly inhibit CDK4/6 and CDK1/2, and Ink4 (p15, p16, p18, and p19) which selectively inhibit CDK4/6 [5, 6]. In contrast to cyclins, Cip members are highly expressed in adult cardiomyocytes which have permanently withdrawn from the cell cycle [7].

Together cyclins and CDKIs respond to signaling pathways and mitogens to activate or inactivate the appropriate CDK complexes throughout the cell cycle which appears to be crucial for cardiac development. Deletion of CDK2 and CDK4 is embryonically lethal due to cardiac defects [8], and hyperplasia is observed in mice which overexpress cyclin D [9] and CDK2 [10]. These defects have been attributed to hypophosphorylation of the pocket protein pRb and consequently the activation of E2F-responsive genes. In fact, the pocket protein and E2F families are among the most widely studied targets of CDK complexes. This is because the E2F/pocket protein families play a pivotal role in the cell cycle by controlling the expression and repression of genes involved in cellular proliferation, differentiation, and death by apoptosis [11–13].

E2F Family Member Structure

The E2F family consists of nine transcription factors: E2F1, E2F2, E2F3a, E2F3b, E2F4, E2F5, E2F6, E2F7, E2F8 (Fig. 1). Each member shares a DNA-binding domain in common, consisting of a winged helix motif. In order to form functional DNA-binding complexes, E2F1–6 also share a dimerization domain containing a leucine zipper which allows them to heterodimerize with differentiation proteins DP1 and DP2 [14, 15]. DPs also share regions of homology to the E2F family in their DNA binding and dimerization domains. E2F7 and E2F8 lack a dimerization domain and instead have two DNA-binding domains and form homo and/or heterodimers with one another [16, 17].

E2F1–5 also share homology at their C-termini termed a transactivation domain, which is thought to recruit basal transcriptional machinery
E2F/Pocket Protein Pathway

Underscoring the importance of the E2F-RB pathway is its evolutionary conservation in *Drosophila melanogaster* [26], *Caenorhabditis elegans* [27], and *Arabidopsis thaliana* [28]. In more primitive animals, there exists only a single E2F activator and repressor and one pocket protein: pRb. The mammalian pocket proteins include pRb, p130, and p107, which interact with the “classical” E2Fs (1–5). E2F1–3 interact primarily with pRb [29], E2F5 with p130 and p107, and E2F4 interacts with all members [30]. Although the mammalian E2F/pocket protein family is much more diverse, E2F1–5 are regulated by pocket proteins in a fashion similar to that of invertebrates.

During G₀ and early G₁ stages of the cell cycle hypophosphorylated pocket proteins bind to E2Fs, masking the transactivation domain at their C-terminus leading to transcriptional repression [31]. Pocket proteins can recruit a number of histone-modifying enzymes including histone deacetylases (HDACs), histone methyl transferases (HMETs), and heterochromatin proteins (HP1) which recruit HMETs, in order to form heterochromatin and maintain gene repression [32, 33]. As cells receive growth signals CDK4 (in association with cyclin D) phosphorylates pocket proteins, causing them to become hyperphosphorylated and release the E2Fs (Fig. 2).
Subsequently, E2Fs can recruit chromatin-modifying enzymes, such as the histone acetyltransferases (HAT) p300 and Tip60, in order to relax chromatin architecture and activate gene expression [34, 35].

**E2F Family Member Function**

E2F family members are generally separated into two categories based on their capacity to drive quiescent cells into S phase and their presence at E2F-responsive promoters during the cell cycle. E2F1–3a are described as activators of transcription while E2F3b–8 are gene repressors. The “repressors” are usually subdivided into those that interact with pocket proteins (E2F3b–5) and those whose mechanism of repression is pocket protein independent (E2F6–8). These categories appear to be oversimplified as increasing evidence suggests that repressor E2Fs can activate and activator E2Fs can repress transcription (discussed in the next section). Further complicating the understanding of individual E2Fs is their large family size. When an individual member is deleted, other members have the capacity to compensate highlighting their redundancies [36]. In addition, over-expression and knockout of individual E2Fs will shift the balance of activating and repressing E2Fs as well as the availability of DP and pocket proteins; thus these types of studies should be interpreted with caution. Despite this, knockout and double knockout (DKO) mouse models have aided in determining some of the tissue- and temporal-specific functions of particular E2Fs and have contributed overall to our understanding and the importance of proper regulation of this pathway.

### Activating E2Fs

E2F1–3a represent the activator E2Fs which appear to have some overlapping and specific functions. When over-expressed, each member induced activation of E2F-responsive genes and had the capacity to cause cell cycle reentry in quiescent cells [29, 37]. Deletion of individual activators did not affect fibroblast proliferative capacity, but deletion of all three inhibited proliferation, suggesting a redundancy between members [38]. In spite of this their temporal- and tissue-specific expression points at nonredundant roles as well. E2F1 and E2F2 are up-regulated at the G1/S boundary of the cell cycle [39], while E2F3 is expressed very highly during mid G1 and remains stable through the S phase [40]. In addition, E2F1 is the only activator expressed in the...
brain after early embryonic development [15], while E2F1 and E2F3 are expressed in the heart as E2F2 is not [41].

E2F1 was the first E2F member to be identified and its function is the best described. E2F1⁻/⁻ mice are viable and their isolated fibroblasts have a normal proliferative capacity, but adult mice have a higher incidence of tumor development [42–44]. In support of its role in cancer, elevated levels of E2F1 have been found in breast [45], ovarian [46], and gastric [47] cancers. Furthermore, mutations of its inhibitor pRb have been implicated in dozens of cancers, and DKO of pRb and an activating E2F can reverse tumor incidence [48].

In addition to cancer, E2F1 has been demonstrated to participate in both p53-dependent and -independent apoptotic pathways. E2F1 can directly activate the expression of the CDKI p14ARF which in turn inhibits MDM2, an inhibitor of p53, resulting in the stabilization of p53 and activation of apoptotic pathways [49, 50]. It has also been suggested that E2F1 can directly interact with p53 through its cyclin-A-binding domain [51]. Thus, E2F1’s ability to activate p53 would depend on competitive binding between cyclin A and p53 [52].

Like E2F1 null mice, E2F2⁻/⁻ mice are also viable and fibroblasts proliferate at a normal rate [53]. Adult E2F2⁻/⁻ mice are more susceptible to infection and develop late-onset autoimmune disease. Although E2F2 is usually described as an activator and driver of cellular proliferation, its ablation also resulted in hyperproliferation in T-lymphocytes suggesting a repressive function in T-cell proliferation [53].

E2F3 has also been widely researched and its ablation is the only individual E2F knockout at a normal rate [54]. Adult E2F2⁻/⁻ mice are more susceptible to infection and develop late-onset autoimmune disease. Although E2F2 is usually described as an activator and driver of cellular proliferation, its ablation also resulted in hyperproliferation in T-lymphocytes suggesting a repressive function in T-cell proliferation [53].

E2F3 has also been widely researched and its ablation is the only individual E2F knockout which appears to affect embryonic viability. E2F3 KO leads to partial embryonic lethality which has been attributed to defects in proliferation [54]. The lethality of E2F3⁻/⁻ appears to be dependent on mouse strain. Pure strain 129/Sv mice have 100% embryonic lethality, while in mixed background mice (C57BL/6 × 129/Sv) 30% die in utero, 45% die within 24 h after birth, and 25% survive to adulthood, although 85% of these mice eventually die of congestive heart failure [44].

Interestingly, the authors noted that the major time points of death correlate with major proliferative events in embryonic development as well as perinatally during the final round of the cell cycle suggestive of a proliferation defect [55]. Upon histological analysis of E2F3 KO mice, several cardiac defects were discovered including hypoplastic septal walls and defects in septal development. A decrease in the proliferative index (measured by BRDU incorporation) in the embryonic heart of KO mice was observed. Thus it appears that E2F3 is necessary for proliferation and embryonic cardiac development. Additionally, autopsy of mice which lived into adulthood revealed dilated hearts and atrial thrombi in E2F3 knockouts [55]. The late-onset dilated cardiomyopathy is potentially due to the progressive accumulation of stress on the heart due to defects in sarcomere organization which were observed. Thus it appears that E2F3 is crucial for both embryonic and postnatal cardiac development and function.

Despite the array of studies depicting specific roles for individual activator E2Fs, in 2008 Tsai [56] provided evidence suggesting that, like Drosophila, mammals can survive with a single activating E2F. The authors created different combinations of compound knockout mice in order to more closely evaluate the individual and redundant roles of activator E2Fs. Their studies confirmed many previously published results with respect to adult phenotypes of individual E2F members, such as increased incidence of tumor development in E2F1⁻/⁻ mice. They also took a closer look at E2F3’s role by creating separate E2F3a and E2F3b KOs. Although mice with deletion of E2F1, E2F2, E2F3b were viable, deletion of E2F1, E2F2, E2F3a was lethal, suggesting that the crucial role of E2F3 in cardiac development can be attributed to E2F3a. Most interestingly, when E2F3b or E2F1 was expressed from the same genetic locus as the deleted E2F3a mice were rescued from the phenotype of E2F3a⁻/⁻ [56]. This suggests that the specificity of activating E2Fs has more to do with genetic context which controls spatial and temporal expression than a specific function of an individual family member.
Repressor E2Fs

E2F4 and E2F5

E2F4 and E2F5 represent the E2F class of repressors governed by pocket proteins. Both members are nuclear during early stages of the cell cycle and are thought to be necessary to repress cell cycle gene activity, but become exported from the nucleus as the cell cycle continues and cell cycle genes should be expressed [57, 58]. E2F4 is constitutively expressed throughout the cell cycle, while E2F5 is expressed more highly during mid-G1 [30]. Over-expression of E2F4 and E2F5 revealed that they did not have a similar capacity as activator E2Fs to drive cell cycle reentry in quiescent cells, and instead they tended to act as repressors of E2F-responsive genes [24]. This repressor effect may be tissue specific as other groups have found that E2F4 over-expression drove neonatal cardiomyocytes into S phase [59] and promoted cellular proliferation during fetal erythropoiesis [60].

Knockout mouse models of E2F4 and E2F5 are in agreement with early studies, depicting them as negative regulators of the cell cycle important in terminal differentiation. MEFs lacking E2F4 and E2F5 are unable to exit the cell cycle [61], and DKO mice die late in embryogenesis due to defects in differentiation [62]. E2F4−− mice display defects in differentiation of erythrocytes as well as gut epithelial cells and die very early due to increased susceptibility to infections [63, 64]. E2F5−− mice develop a non-lethal hydrocephalus due to excess cerebrospinal fluid production attributed to a defect in choroid plexus differentiation [57]. Like E2F1, E2F5 also appears to play a role in the development of cancer [65, 66].

E2F6

E2F6 was the first E2F family member identified which did not have a C-terminal activation domain and was a strong repressor of E2F activity [67]. When over-expressed, E2F6 has been demonstrated to inhibit cell cycle progression and cell cycle reentry in quiescent cells [67, 68]. The truncated C-terminus of E2F6 alluded to the fact that its mechanisms of regulation and its manner of repression are independent of the classical pocket protein pathway. Although not regulated by the cell cycle in the same manner as activating E2Fs, E2F6 expression does change throughout the cell cycle. Its expression is up-regulated at both the mRNA and protein level at the G1/S phase boundary. Our laboratory has shown that E2F6’s promoter contains two E2F-binding elements which when bound by other E2Fs, activates E2F6 expression [69]. This implies that when activating E2Fs are expressed early in the cell cycle, they up-regulate E2F6 expression to ensure there is no improper passage through the G1/S checkpoint. In support of this, ChIP studies demonstrated that E2F6 could be found bound to E2F-responsive gene promoters during G1 and S phase but not other phases of the cell cycle [22].

E2F6’s capacity to repress gene expression appears to involve the recruitment of polycomb group proteins (PcGs). PcGs include a wide variety of chromatin-modifying enzymes found in two distinct types of complexes which are epigenetic gene silencers during development [70]. The polycomb repressive complex 2 (PRC2) is important for the definition and trimethylation of genes to be repressed at histone 3 lysine 27 (H3K27). This marker recruits the PRC1 complex for long-term silencing marked by trimethylation at histone 3 lysine 9 (H3K9). E2F6 has been identified in complexes containing proteins from both types of complexes including Bmi1, Ring and YY1 binding protein (RYBP), EPC1, and EZH2 [24, 25].

Complimenting E2F6’s interaction with PcG proteins, deletion of E2F6 has a similar phenotype to the deletion of the PcG protein Bmi1, including posterior homeotic transformations of the axial skeleton [71]. Interestingly, MEFs from these mice did not display any defect in proliferative capacity. The authors have attributed this to functional compensation by E2F4. A similar effect was observed previously in ChIP experiments in which E2F4 replaced missing E2F6 at responsive genes in E2F6 KOs [36].
E2F6 has also been described in a unique complex in HeLa cells capable of binding to E2F sites during G0 [72]. This E2F6 complex includes DP1, HMET, HP1, various PcGs, as well as the transcription factors Max and Mga. This complex was capable of binding and repressing E2F, as well as Max and Mga-responsive genes (E-box and T-box sites), thereby implicating E2F6 in a complex which could be important for the repression of an expansive array of genes during cell-cycle arrest. Although this study differs with other studies suggesting only E2F4 is expressed and bound to promoters during G0 [22], the authors have attributed the observed differences to the use of different cell types and mechanisms of cell-cycle arrest. This appears plausible since ChIP studies done by Xu [73] and colleagues found that E2F1, E2F4, and E2F6 could be found bound to the same promoters in various cell types, yet E2F6 appeared to have some specific functions in Ntera2 cells in which it bound to its own set of genes. This suggests that E2Fs can share interchangeable roles, yet in specific cells and settings E2F6 (as well as other E2Fs) could play some unique roles in gene expression. These special roles probably involve interactions with other transcription factors and chromatin-modifying enzymes.

E2F7 and E2F8

E2F7 and E2F8 represent the last group of E2Fs that have been identified. Like E2F6 they are not regulated by pocket proteins and unlike all other E2Fs, they do not dimerize with DPs. They appear to be strong repressors of E2F activity which, unlike other E2Fs, are capable of inhibiting cellular proliferation when over-expressed [74, 75]. The two proteins appear to have overlapping roles in development since individual KO mice develop normally but DKO mice die very early during embryonic development due to mass apoptosis in various cell lineages [75]. Their redundancy is supported by their capacity to form heterodimers to repress gene expression.

Moon and Dyson [76] review a feedback loop, similar to what we described for E2F6 [69] in which E2F1 expression activates the transcription of E2F7 and E2F8 which, in turn, represses E2F-responsive genes including E2F1 itself. In fact, the lethal apoptosis in E2F7/E2F8 DKO mice has been attributed to elevated levels of both E2F1 and p53 [75]. Interestingly the mice also exhibit severe dilation of blood vessels which is not yet understood but may represent a specific function for these outlier E2Fs outside of repressing E2F1.

Counterintuitive to its role as a repressor of proliferation and E2F1-mediated apoptosis, E2F8 is up-regulated in human hepatocarcinoma (HCC) specimens [77]. Furthermore, its knockdown is sufficient to inhibit colony formation of HCC-derived cell lines and decrease tumorogenicity in vivo. The authors found that E2F8 over-expression caused an increase in DNA synthesis and cyclin D1 expression, which has been previously linked to HCC [78] while knockdown had the opposite effect. The authors have attributed this unexpected up-regulation of cyclin D1 (an E2F target) to excess E2F8, outcompeting E2F1 which would normally bind in association with pRb leading to gene repression [77].

This study is a perfect example of how E2Fs cannot be restricted to activators and repressors of gene expression and cellular proliferation, but the outcome of E2F activity will depend on the regulation of a delicate balance in E2F activity. Although not all E2F members are necessary for viability, the size of the family not only safeguards against mutation but also allows the tight regulation of development and function in complex mammalian tissues and organisms. E2F activity will depend on a variety of different factors including genetic context of E2Fs as well as their responsive genes, subcellular localization, tissue expression, and cell-cycle stage/signals.

E2Fs in Cardiac Growth and Pathology

Since proper cardiac development requires a very tight regulation of the cell cycle, it is likely that the E2F pathway will play a pivotal role in cardiac development and pathology, as demonstrated in
the E2F3 KO studies described earlier (summarized in Table 1). E2F1 was the first of the E2F family to display a role in cardiomyocyte cell-cycle control. When neonatal cardiomyocytes were transfected with E2F1, they displayed a marked increase in DNA synthesis accompanied by a high rate of apoptosis, thereby demonstrating for the first time the capacity of E2Fs to control cardiomyocyte cell cycle and death [79, 80]. Since neonatal cardiomyocytes still retain some proliferative capacity and differ greatly from post-mitotic adult cardiomyocytes, adult rat ventricular myocytes were transfected with E2F1 which induced DNA synthesis, but to a lesser extent (19% vs. 47%) which again was accompanied by apoptosis [80]. The effects of ectopic expression of E2F1 were also explored in vivo by injecting adenoviral-E2F1 into the myocardium of adult mice. Similar to the cardiomyocytes, an increase in DNA synthesis was observed and cardiomyocytes accumulated in G2/M but none were capable of overcoming the G2/M checkpoint to proliferate. Since E2F1 interacts with the p53 pathway, Agah [80] and colleagues tried transfecting E2F1 into the myocardium of p53 null mice. Surprisingly this did not alleviate the rate of apoptosis, indicating that E2F1 can induce cell death in a p53-independent pathway.

In addition to the p53 pathway of apoptosis, the mitochondria play an important role in programmed cell death. During hypoxic injury the mitochondrial permeability pore opens, causing a loss of membrane potential and cytotoxic protein release which activates apoptotic pathways leading to ventricular myocyte death [81]. It has previously been shown that the mitochondrial death protein Bnip3 plays a role as a sensor of oxidative stress during MI [82] and its induced expression leads to ventricular myocyte death [83]. Yurkova [84] showed that ectopic E2F1 directly activates the transcription of this death factor Bnip3. Protein levels of Bnip3 were not confirmed, but two inhibitors of Bnip rescued cells from the apoptosis incurred by E2F1 in cardiomyocytes [84]. Thus it appears that E2F not only plays a role in apoptosis by interacting with p53 but in cardiomyocytes also by directly controlling the levels of hypoxia-inducible pro-apoptotic factors.

In addition to regulating apoptosis in cardiomyocytes, it appears that the E2F family also plays a central role in regulating cell growth, a very important aspect of cardiomyopathic heart failure. Upon hypertrophic stimulation with phenylephrine (PE) E2F1–4 and E2F6 become up-regulated in neonatal cardiomyocytes [85, 86]. Vara and colleagues further demonstrated the importance of the E2F pathway in cardiac hypertrophy by inhibiting the pathway with specific inhibitors for E2F/DP heterodimerization, which resulted in a decrease in the intensity of hypertrophy as well as blocked the expression of hypertrophic markers ANP and BNP [85].

A few years later the capacity of E2Fs to induce cell cycle reentry in the heart was evaluated and

<table>
<thead>
<tr>
<th>Table 1</th>
<th>E2F pathway function in the heart. Knockout mouse models of E2F and partner proteins</th>
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<tbody>
<tr>
<td>Gene</td>
<td>Phenotype</td>
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<tr>
<td>E2F1</td>
<td>Cardiac apoptosis.</td>
</tr>
<tr>
<td>E2F3</td>
<td>Partial penetrant embryonic lethality due to cardiac developmental defects. Young adults develop congestive heart failure.</td>
</tr>
<tr>
<td>E2F1, E2F2, E2F3a</td>
<td>Partial embryonic lethal, perinatal lethal. Reduction in white adipose tissue deposits.</td>
</tr>
<tr>
<td>E2F1, E2F2, E2F3b</td>
<td>Viable, but have reduced body weight.</td>
</tr>
<tr>
<td>E2F7, E2F8</td>
<td>Embryonic lethal. Excess apoptosis (due to increased E2F1 and p53) and blood vessel dilation.</td>
</tr>
<tr>
<td>Rbα, p130</td>
<td>Cardiac hyperplasia.</td>
</tr>
<tr>
<td>Rbα, p107</td>
<td>Embryonic lethal. Increased proliferation in central nervous system, blood vessel endothelial cells, and heart defects (double-outlet right ventricle).</td>
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*Cardiac restricted deletion
indeed over-expression of E2F1–4 induced S phase in neonatal cardiomyocytes by inducing transcription of cyclins A and E [59]. In this study only E2F2 and E2F4 induced S phase without also causing apoptosis and only E2F2 over-expression resulted in mitosis. In order to determine if this was relevant in the adult heart, Ebelt and colleagues stably over-expressed E2F2 and E2F4 in adult mouse hearts by adenoviral infection [87]. Similar to what was observed in the neonatal cardiomyocytes, over-expression of both E2F2 and E2F4 resulted in the reentry of cardiomyocytes into S phase and cardiomyocyte hypertrophy. More importantly, expression of E2F2 also resulted in a modest increase in the number of mitotic adult cardiomyocytes. This is especially interesting since E2F2 is not normally expressed in the heart [41, 88]. Perhaps a lack of E2F2 hints at a protective mechanism against excess proliferation in the heart in order to maintain postnatal cardiac function. It also points to a therapy to stimulate cardiac regeneration post-myocardial infarction.

Recently van Amerongen [89] and colleagues demonstrated a specific function for E2F4 during cardiomyocyte mitosis. In this study E2F4 over-expression did not induce cell-cycle entry in cardiomyocytes. Although this differs from earlier work in which Ebelt [59] found that E2F4 over-expression induces DNA synthesis, different time points of cardiomyocyte isolation were used in each experimental study. In Ebelt’s [59] study cardiomyocytes were isolated from newborn rats, while in van Amerongen’s [89] study cardiomyocytes were isolated from 3-day-old rat hearts. At day 3 cardiomyocytes may have lost some proliferative capacity when taking into consideration that they permanently exit the cell cycle within a few days after birth. In this study both the expression and nuclear localization of E2F4 were correlated to cardiac development and the proliferative potential of cardiomyocytes, supporting its role in normal cardiac growth and development [89]. Unexpectedly, the authors found that E2F4 co-localized with kinetochores in cardiomyocytes and when knocked down by siRNA-restricted mitosis, suggesting a potential novel role for E2F4 in cell cycle regulation outside of transcriptional control. The relevance of these results in the adult myocardium is unknown as this was tested in postnatal cardiomyocytes which were artificially stimulated to proliferate (using FGF1 and a p38 inhibitor), but may be a useful tool in cardiac regeneration studies.

### Pocket-Protein-Mediated E2F Regulation in the Heart

In addition to a balance in levels of individual E2F family members, appropriate regulation of the E2F pathway by pocket proteins is crucial for normal cardiac development. Although cardiac-specific deletion of individual pocket proteins does not lead to specific cardiac defects, compound knockouts tell a different story. Since pRb−/− mice are not viable cardiac-restricted knockouts of pRb in conjunction with p130 or p107 knockouts have been utilized. Ablation of p130 and cardiac pRb led to a threefold increase in heart weight: body weight ratio due to abnormal hypertrophy [90]. In this model an increase in Myc, E2F1, and G1 CDK activity was observed, indicating that indeed the E2F pathway and cell cycle had been activated.

The importance of p107 in the heart was also highlighted in a study in which an embryonic lethal double knockout of pRb and p107 developed cardiac defects [91]. Mice lacking the two proteins developed a double outlet right ventricle (pulmonary artery and aorta exit from the right ventricle) and many embryos also displayed thinner myocardium, dilated atria, and septal defects [91]. Thus it appears that all three pocket proteins play an important role in cardiac development although, much like the E2Fs, they have the capacity to compensate for each other’s loss.

### Clinical Relevance and Conclusions

Accumulating evidence suggests that appropriate regulation of the E2F/pocket protein pathway is crucial to normal cardiac development. Highlighting this is the developmental regulation
of individual E2F and pocket protein members within the heart [4, 86, 90]. Recently, a direct link between E2Fs and congestive heart failure was demonstrated in humans. In this study patients with CHF displayed up-regulated levels of E2F1, pRb, p107, and p130 in comparison with control patients [92]. A positive correlation between pRb and p130 with cardiomyocyte diameter was also found, suggestive of their role in cardiomyocyte hypertrophy. Following unloading by left ventricular assistance device, a significant decrease in expression of E2F1 and pocket proteins was observed, indicating that ventricular unloading can reverse the process. Thus it appears that similar to studies in cultured cardiomyocytes and mouse models, the E2F/pocket protein pathway is a pivotal player in pathological cardiac hypertrophy. This fits well with recent studies which correlate an up-regulation of genes involved in cell cycle reentry (which are controlled by E2Fs) with cardiac hypertrophy and heart failure [2, 3].

In addition to playing an important role in regulating cardiac hypertrophy, the capacity of E2Fs to regulate cell cycle entry and exit may prove to be very important tools in cardiac regeneration. The capacity of E2F2 to induce proliferation in vivo is sufficient to warrant further investigation. Furthermore, the expression and role of the pocket-protein-independent E2Fs in the heart has yet to be addressed. The mechanisms of E2F6–8 are still quite poorly understood and will probably also prove important in cardiac development and disease.

Acknowledgments Funded by CIHR.

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Abstract

The sodium–calcium exchanger (NCX) is thought to be a critical protein in excitation-contraction (E-C) coupling in the heart through its regulation of intracellular [Ca^{2+}]. The exchanger removes Ca^{2+} from the cell in exchange for extracellular Na⁺ in the “forward mode” to induce cardiac relaxation. Although still controversial, NCX may also participate in cardiomyocyte contractile activity in a “reverse mode” by bringing Ca^{2+} into the cell in exchange for intracellular Na⁺. In addition to its important physiological role, the NCX has been associated with the pathology of ischemia-reperfusion injury, hypertension, cardiac hypertrophy, and heart failure. Therefore, it has the potential of being a valuable therapeutic target in the treatment of heart disease.

A limitation in the study of the exchanger has been the dearth of pharmacological blockers that specifically inhibit the NCX. Initially, therefore, the role of NCX in ischemic injury was elucidated with the use of blockers of the Na⁺-H⁺ exchanger, an upstream component of the NCX in the ischemia-reperfusion pathway. These drugs effectively inhibited the Na⁺-H⁺ exchange-NCX cascade during ischemia and early reperfusion to provide cardioprotection in isolated hearts and cardiomyocytes. Alternatively, the development of new genetic tools to increase or down-regulate the expression of the NCX has effectively characterized the role of the NCX in contractile activity and during ischemic injury. Compared to alternative molecular approaches to alter gene expression, the adenovirally delivered shRNA has been the most efficient method to alter gene expression in vitro. Cardiomyocytes with significantly depleted NCX through adenovirally delivered shRNA can still contract but are cardioprotected from ischemic injury.

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insult. Furthermore, the cardiac isoform NCX1.1 causes more severe Ca\textsuperscript{2+} overload during ischemia-reperfusion injury and glycoside toxicity than the renal NCX1.3 isoform of the exchanger when they are expressed in neonatal cardiomyocytes and HEK-293 cells. In summary, the data support an important but not a critical role for NCX in excitation-contraction coupling in the heart but an important, possibly critical role, for the NCX in ischemic reperfusion injury and drug-induced challenges. Overall, these results identify NCX as an important molecule to target to develop new strategies to influence heart function and dysfunction.

**Keywords**
Ca\textsuperscript{2+}-transport • Ischemia/reperfusion injury • Myocardium • Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger • Na\textsuperscript{+}-H\textsuperscript{+} exchanger

**Introduction**

Calcium is used within the cells as an intracellular messenger. Local and temporal changes in Ca\textsuperscript{2+} concentration activate a wide variety of cellular functions. Intracellular Ca\textsuperscript{2+} concentration, therefore, needs to be tightly regulated. A number of channels and transporters maintain calcium homeostasis. The sodium–calcium exchanger (NCX) is one of the transporters that is particularly important in the heart because of its involvement in the mechanism of excitation-contraction coupling. The NCX is a plasmalemmal protein. It is found in almost all cell types and is abundant in excitable tissues, like the brain and the heart. It transports Ca\textsuperscript{2+} in exchange for Na\textsuperscript{+} and is able to remove Ca\textsuperscript{2+} from the cell or transport it into the cell. The direction of this movement depends on the concentration gradient across the membrane for Ca\textsuperscript{2+} and Na\textsuperscript{+}, and also on the membrane potential. Generally, in the heart, the NCX plays an important role in Ca\textsuperscript{2+} removal from the cell during relaxation and may also contribute to Ca\textsuperscript{2+} influx during the peak of an action potential.

In addition to its important physiological role, the NCX has been associated with the pathology of ischemia-reperfusion injury, hypertension, cardiac hypertrophy, and heart failure. Therefore, it has the potential of being a valuable therapeutic target in the treatment of heart disease. Intensive study of the NCX would be of great value not only to advance our understanding of the function of the heart during normal healthy conditions but also to devise strategies to improve cardiac performance under pathological challenge as well.

**NCX Structure**

The 938 amino acids that form the mature cardiac NCX protein are arranged in 9 transmembrane segments (Fig. 1) [1]. Five transmembrane segments are present in the amino part of the protein and are separated from the other four by a large intracellular loop. A leader peptide, corresponding to the first amino acids of the protein, is removed during processing of the protein [2]. Once the leader peptide is removed, the resulting amino end is extracellular and glycosylated [3]. Two regions with homologous sequences are found within the protein: \(\alpha\)-1 is located between transmembrane segments 2 and 3, and \(\alpha\)-2, between transmembrane segments 7 and 8. The \(\alpha\)-1 and \(\alpha\)-2 sequences form reentrant loops that are involved in ion translocation [4–7]. These regions of homology are believed to have originated by gene duplication and can be found in other proteins of the NCX superfamily [8]. The large intracellular loop is involved in regulation of NCX activity. It contains the XIP (eXchanger
Cardiac Sodium–Calcium Exchanger Expression

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**Inhibitory Peptide** region, the regulatory Ca\(^{2+}\) binding site, and the region of alternative splicing. The region of alternative splicing will be discussed in the “NCX isoforms” section. The C-terminus is found on the cytoplasmic side of the protein.

The topology of the NCX that is currently accepted was determined by studying the ability of cysteine residues at different locations to form disulfide bonds [1, 9]. These studies indicated that the \(\alpha\) repeats are located on opposite sides of the membrane and close to each other in the folded protein. A previous model, based on hydropathy analysis of the cloned NCX, had established that the exchanger was composed of 12 transmembrane segments [10].

The XIP region corresponds to amino acids 218–238, and is located close to the N-terminus of the intracellular loop. The sequence of this region was found to be similar to a calmodulin-binding site [11]. It is rich in hydrophobic and basic amino acids. To test its regulatory properties, a peptide containing the sequence of this region was synthesized (XIP) and was found to inhibit the exchanger (see NCX inhibitors).

Mutational analysis of the endogenous XIP region indicated that it is involved in Na\(^{+}\) regulation of the exchanger [12].

Two calcium-binding domains exist within the intracellular loop: one spans amino acids 371–509 and binds calcium with high affinity [13] and another is located between amino acids 501–560 with lower calcium affinity. Calcium binding to these regions causes conformational changes that activate the protein, and mutations within these sequences affect the calcium regulatory properties of the exchanger [14]. The different calcium sensitivity would allow the exchanger to function over a wide range of intracellular calcium concentrations.

The variable region of the gene NCXI corresponds in the cardiac exchanger protein to amino acids 561–681, located toward the C-terminus of the intracellular loop. This region of the gene undergoes alternative splicing.

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**NCX Localization**

In cardiomyocytes, NCX is found on the cell surface, t-tubules, and intercalated discs [15]. Thomas et al., using confocal laser scanning microscopy, showed NCX staining in adult cardiomyocytes in the vertical t-tubules, but not in the longitudinal t-tubules. NCX was co-localized with the ryanodine receptor [16]. A previous study using the same technique had failed to show any co-localization of NCX with either the Na\(^{+}\) channel or ryanodine receptors but did show co-localization of ryanodine receptors with L-type Ca\(^{2+}\) channels [17]. Thomas et al. [16], looking for further resolution, used electron microscopy differential gold labeling and measured the distance that exists between the different transporters. They found that the...
distance separating the NCX from ryanodine receptors or L-type Ca\textsuperscript{2+} channels was the same as the distance between these latter two transporters (which were previously found to co-localize). It would be important to resolve this discrepancy in the future because the activity of the NCX in the Ca\textsuperscript{2+} entry mode would require physical proximity between these transporters to become physiologically important in excitation-contraction coupling.

### NCX Isoforms

Variability in NCX isoforms and their degree of expression and tissue distribution is, in mammals, under three levels of regulation: the existence of different genes, the use of alternative promoters, and, finally, alternative splicing of the RNA transcript.

### NCX Genes

Three genes code for the NCX in mammals: NCX1 [10], NCX2 [18], and NCX3 [19]. The three genes were probably generated by three duplication events and one deletion: only one gene has been found in invertebrates and four (NCX1–NCX4) are found in fish, amphibians, and reptiles. In mammals, whereas NCX1 is expressed in several tissues, NCX2 and NCX3 are both expressed mainly in brain and skeletal muscle [20]

### Alternative Promoters

The NCX1 gene contains three alternative promoters [21–23]. Depending on the promoter, three alternative exons 1 could be incorporated, creating transcripts that are: cardiac specific, kidney specific, and one that is ubiquitous. This third transcript has highest expression in brain. The alternative exon 1 is spliced to a common exon 2. Exon 2 starts at position –31 from the translational start codon (upstream). Because exon 1 is present in the 5’UTR (untranslated terminal repeat), its sequence will not affect protein sequence. The functional importance of the different promoters is, therefore, related to the level of expression of the protein (either constitutive or regulated by tissue-specific factors [24]). NCX gene expression has been reported to be regulated at the chromatin remodeling level by histone deacetylases.

### Alternative Splicing

Variations in the structure of the protein are generated by alternative splicing of the mRNA. NCX1 and NCX3 undergo alternative splicing of the primary transcript. The region that undergoes splicing corresponds to the carboxyl terminus of the intracellular loop of the protein. In NCX1, the part of the gene that codes for this region is composed of six exons (A, B, C, D, E, and F, corresponding to exons number 3–8 of the 12 exons that compose NCX1) [25]. NCX3 codes for exons A, B, and D. Exons A and B are mutually exclusive (only one of them has to be present). Inclusion of both exons A and B would produce a shift in the reading frame of the message. Exons A and B are followed by a combination of the other four cassette exons. Combinations of the different exons could lead to 32 isoforms. Up to now, 12 different isoforms of NCX1 have been identified. The isoforms are expressed in a tissue-specific manner [20, 21]. In general, excitable tissues contain isoforms with exon A, and all other tissues contain isoforms with exon B. The isoforms that relate to this study are NCX1.1 (ACDEF) and NCX1.3 (BD) (Fig. 2). The former is the only isoform found in the heart and is also present in skeletal muscle [26]. NCX1.3 is the most abundant isoform in the kidney and is expressed together with NCX1.7 in arterial smooth muscle cells [27]. Inclusion of exon B, for example, in NCX1.3 and 1.7, confers the exchanger forward mode (calcium efflux) inactivation and sensitivity to the NCX blocker KBR-7943. The origin of the different genes and splice variants was discussed by Quednau et al. [20]. NCX1 and NCX3 contain mutually exclusive
Changes in NCX During Development

The SR is not well developed at the time of birth. SERCA expression starts in the rat embryo at day 9 of development [31] and increases after birth [32]. Ryanodine receptor density is also relatively low at the end of gestation. Some authors found that ryanodine (a compound that blocks SR Ca$^{2+}$ release) had no effect in neonatal cardiomyocytes [33, 34], whereas others found a minor effect [35, 36] and still others have reported a more significant effect [37, 38]. Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) is less important early in development. Mature CICR is reached 3–4 weeks after birth in the rat [39, 40]. Before that time, the heart is more dependent on transsarcolemmal Ca$^{2+}$ fluxes to induce contraction.

Ca$^{2+}$ influx in the neonate is mediated primarily by L-type Ca$^{2+}$ channels ([41, 42], T-type Ca$^{2+}$ channels [43], and NCX [44, 45]. The changes in NCX expression during development oppose those of SERCA [46]. NCX mRNA and protein levels peak at birth and then decline [44, 47, 48]. Relaxation in newborn myocytes may occur predominantly through Ca$^{2+}$ extrusion through NCX as opposed to Ca$^{2+}$ uptake by the SR.

Another difference in E-C coupling between adults and neonates is that T-tubules are absent at birth. They develop around 10 days after birth in the rat. Previous to that time the smaller volume/surface relation is enough to provide Ca$^{2+}$ to the myofilaments for contraction.

Models of NCX Over-expression and Down-regulation

Over-expression

To clarify the role played by the NCX in the regulation of Ca$^{2+}$ homeostasis in the heart and to observe the changes in its expression in disease states, alternative approaches have been used to modify its expression level. NCX expression has been up-regulated by the use of...
transgenic mice and adenoviral vectors and has been down-regulated in knockout mice and through the use of antisense technology.

Philipson’s group developed a transgenic mouse for cardiac-specific expression of the exchanger. The canine NCX1.1 gene was expressed under the α-myosin heavy chain promoter in this model [8]. In heterozygous mice, 1.5–3-fold higher NCX activity was measured using isotope uptake and electrophysiological methods, respectively. No changes in intracellular Na+ concentration, resting Ca2+, Ca2+ transient amplitude, or adaptations in other Ca2+ regulatory proteins were observed [49, 50]. Increased NCX activity, however, accelerated relaxation and the decay of the caffeine-release Ca2+ transients (in intact cells and under voltage clamp). These observations support the role of the exchanger in Ca2+ efflux. One study showed higher SR Ca2+ content in NCX over-expressing cells [49]. To sustain an increase in Ca2+ efflux via the exchanger without a depletion of Ca2+ (since no changes in Ca2+ current were observed), an increase in Ca2+ entry through the same exchanger has been proposed. The importance of the reverse mode exchanger was shown in experiments where depolarization of the membrane to positive potentials caused SR Ca2+ release in some cells. It was also shown that Ca2+ entering the cell through NCX can be buffered by the SR. In Yao’s study, myocytes from heterozygous animals could maintain Ca2+ transients at a low frequency of stimulation after blockade of the L-type Ca2+ channel, showing that increased levels of NCX (working in reverse mode) can contribute to Ca2+-induced Ca2+ release from the SR [50]. In this last situation, SR function was required indicating that NCX can induce Ca2+ release from the SR but not in enough quantities to support contraction.

Heterozygous NCX transgenic mice did not show a cardiac disease phenotype. On the contrary, homozygous NCX over-expressors were found to exhibit mild hypertrophy by 3.5 months of age (22% increase in heart weight to tibia length ratio) [51]. Homozygous postpartum females and mice from both sexes showed more severe hypertrophy under stress conditions that resulted in heart failure [52–55]. The characterization of excitation-contraction coupling in ventricular myocytes from homozygous mice showed a decrease in gain. In other words, an increased L-type Ca2+ channel current activated a smaller Ca2+ transient without changes in SR Ca2+ content. The mechanism for increased and slower inactivation of the L-type Ca2+ channel current could not be completely explained by the authors, because no changes were observed in channel density and, in addition, NCX was shown to have no direct effect on channel activity (when NCX activity was transiently blocked, an enhanced L-type current was maintained).

NCX transgenic mice were shown to be more susceptible to ischemia/reperfusion injury [56]. The effect was observed only in males, probably due to the protective effect of estrogen in females. Estrogen prevented the rise of [Na+]i, that drives NCX in males [57]. The mechanism responsible for the gender difference in Na+ is not known. In this last study, after 30 min of ischemia, [Ca2+]i in myocytes from transgenic mice was 1.5-fold higher than in cells from wild-type mice. However, another study using the same transgenic mice showed that increased NCX expression had a protective effect. Transgenic hearts showed preserved Ca2+ transients during ischemia and hypoxia [58]. This surprising result probably is due to the short periods of ischemia or hypoxia used in the study.

The other approach to induce increased expression of the exchanger has been to use NCX adenoviral transfection vectors. The effects observed have varied with the species of myocyte studied. Adenoviral transduction of the exchanger in rabbit ventricular myocytes showed that Ca2+ efflux through the exchanger was dominant in this species. The changes observed included a depletion of SR Ca2+ content and a reduction of the amplitude of the Ca2+ transient [59]. In this case, relaxation and decay of the Ca2+ transient were prolonged. Conversely, in adult rat ventricular myocytes, the effect of increasing the NCX expression depended upon the extracellular Ca2+ concentration. SR Ca2+ content and Ca2+ transients decreased at low [Ca2+]o and increased at high [Ca2+]i [60].
Down-regulation

The first approach that was used to decrease the expression of the NCX was to employ antisense oligodeoxynucleotides (ODN). ODNs are synthetic DNA molecules with a complementary nucleotide sequence to a specific mRNA. RNA–DNA duplexes, formed by base-pairing between the mRNA and the ODN, activate cleavage of the corresponding mRNA by RNase [61]. ODNs may also interfere with the ability of the mRNA to access the ribosomes for translation. A limitation for this methodology is the low efficiency of transfection that can be achieved in cardiomyocytes. ODNs when modified, for example, as phosphorothioated ODN, are relatively stable molecules.

For the study of the effects of ODNs on NCX expression, 19 nucleotide long phosphorothioate ODNs targeting the 3' untranslated region of the RNA were initially used at a concentration of 3 μM in neonatal cardiomyocytes [62]. In this first study, ODNs nearly abolished NCX activity within 48 h. Another group used 0.5 μM of a pair of ODN sequences targeting the region around the start codon for the NCX [63]. These ODNs exhibited a significant effect after 4 days of treatment. NCX half-life was measured as 33 h [64]. This would indicate that when effects were observed just 24–48 h after treatment and using high concentrations of ODNs, these effects were probably due to a nonspecific action of the ODNs [62, 65]. The most recent study used 2 μM ODN to target one specific sequence near the NCX start site. This technique was used to demonstrate that decreased expression of NCX prevents Ca2+ overload in adult cardiomyocytes upon reoxygenation after anoxia [66].

To complement the data examining the effects of down-regulation of NCX expression using the ODN approach, transgenic NCX1 knockout mice have also been generated. Ablation of the NCX1 gene was performed independently by four laboratories [52–55]. Even though some differences were observed between the four knockout mice models, all of the mouse lines resulted in embryonic lethality.

The most significant characteristics of the knockouts were as follows: Homozygous NCX−/− mice died at 9.5–10 dpc [52, 55, 67] or 11.5 dpc [53]. Prior to death, the embryos were found to be smaller, with signs of necrosis in tissues other than the heart. The heart itself was also smaller in size, with an increased number of apoptotic cells [52, 55]. In one case, however, the incidence of apoptosis was normal and the heart was normal [53]. Spontaneous contractions of the heart and Ca2+ transients could be observed only in 30% of the embryos and at significantly lower frequency [55], or in one study they were not observed at all [53]. The heart tubes, however, responded to electrical stimulation, and surprisingly, Ca2+ transients and contractions were very similar to control responses [67]. The cardiomyocytes, therefore, appeared to be able to remove Ca2+ in the absence of NCX. However diastolic Ca2+ was significantly elevated when the stimulation frequency was increased [67]. The cardiomyocytes also showed myofibrillar disorganization. Reuter et al. observed almost complete depletion of SERCA protein without changes in PMCA in the homozygous null mouse [67]. The ability of the cells to extrude Ca2+ in the absence of NCX and without upregulation of PMCA expression (the other Ca2+ extrusion mechanism) would suggest that the activity or efficiency of the PMCA might be able to increase. Despite the decrease in SERCA protein, SR Ca2+ content was not altered [67].

The NCX1 −/+ heterozygous showed no cardiac defects. NCX protein expression in the heart and other tissues was 50% of wild type [52, 67], or was the same as in control [53]. NCX1 expression was restricted in only the heart before and at the time of lethality [68]. Consequently, lethality must have been due to the lack of NCX1 in the heart. The lack of heart contractile function would limit the perfusion of nutrients in the developing embryo to maintain growth. It is not clear what causes the lack of spontaneous contractions. It could be either a consequence of the myofibrillar disorganization, or an effect of the absence of NCX on the pacemaker activity of the heart. Reintroduction of cardiac expression of the NCX1 did not rescue the NCX knockout mouse [69].

Philipson and coworkers developed a cardiac-specific knockout of the NCX1 using the Cre-lox
system [70]. Cre recombinase activity is under the MLCv2 promoter. Therefore, ablation of the gene occurs in ventricular cardiomyocytes during development. The MLCv2 promoter activates at 8 dpc in mouse [71]. As opposed to the global knockout, the cardiac-specific NCX1 knockout survived to adulthood. Cardiac function was depressed 20–30% despite the NCX expression being inhibited by ~90%. However, the animals could not withstand stress (breeding). Animals also died at a younger age, probably due to heart failure. Approximately 90% of the cardiomyocytes in the knockout animal showed no NCX1 expression, whereas the remaining cells expressed normal levels of NCX1. No compensatory changes were observed in the other Ca2+ regulatory proteins. SR Ca2+ content was not affected and interestingly, no differences in the shape and magnitude of the Ca2+ transients were observed. The L-type Ca2+ current, however, was significantly decreased. Therefore, the authors hypothesized that the hearts were able to maintain Ca2+ fluxes by a combination of up-regulating the activity of the PMCA and decreasing the amount of Ca2+ that enters the cell through the Ca2+ channels. However, PMCA expression was not altered and PMCA activity was not measured.

**The Use of RNA Interference (RNAi) to Alter NCX Expression**

RNAi is the process of sequence-specific posttranscriptional gene silencing initiated by double-stranded RNA that is homologous in sequence to the silenced gene. This phenomenon was first observed in *Caenorhabditis elegans* by Drs. Fire, Mello, and coworkers in 1998 [72]. The impact of their discovery in biology and medicine was recognized a few years later with the 2006 Nobel Prize in Medicine. In plants and lower animals [73–78], RNAi serves as a natural mechanism of defense against viral infection and transposon elements. In vertebrates, it is a mechanism of gene regulation [79, 80]. In addition, RNAi is now being used as a powerful tool to study gene function.

Double-stranded RNA is processed within the cell by the ribonuclease III Dicer into 21–22 nucleotides long RNA duplexes (siRNA or small interfering RNAs) [79, 80]. siRNAs contain a phosphate group on the 5’ end, and 2 nucleotides overhang on the 3’ end. These characteristics are necessary for siRNA to be recognized by the next component of the RNAi pathway: the multinuclease complex RISC (RNAi-induced silencing complex). RISC unwinds the short duplex RNA and uses it to find mRNAs of homologous sequence (through base-pairing of the antisense sequence of the duplex with the mRNA). RISC then proceeds to cleave the mRNA (at the midpoint of the homologous sequence), preventing its translation (Fig. 3) [79, 80].

Double-stranded RNAs introduced into invertebrate cells (and also in mammalian embryonic cells) are processed by DICER to induce the RNAi pathway. In all other mammalian cells, however, long dsRNAs activate a nonspecific mechanism that leads to apoptosis. Double-stranded RNAs of more than 30 nucleotides long RNA duplexes (siRNA or small interfering RNAs) [79, 80]. siRNAs contain a phosphate group on the 5’ end, and 2 nucleotides overhang on the 3’ end. These characteristics are necessary for siRNA to be recognized by the next component of the RNAi pathway: the multinuclease complex RISC (RNAi-induced silencing complex). RISC unwinds the short duplex RNA and uses it to find mRNAs of homologous sequence (through base-pairing of the antisense sequence of the duplex with the mRNA). RISC then proceeds to cleave the mRNA (at the midpoint of the homologous sequence), preventing its translation (Fig. 3) [79, 80].
long trigger the synthesis of interferon. Interferon activates protein kinase R (PKR) and 2′–5′ oligoadenylate synthase that halts all protein synthesis and induces the degradation of all mRNAs in the cell, respectively.

Elbashir et al. [81] showed for the first time that RNAi can indeed be achieved in mammalian cell lines. They were able to bypass the interferon response by transfecting the cells with synthetic siRNAs. Today, siRNAs are widely used to down-regulate the expression of target genes. Multiple siRNAs have been pre-designed, some even pre-validated, and are commercially available from different sources.

An alternative approach to transfection of naked siRNA exists with the transfection of mammalian cells with DNA expression plasmids that code for RNA duplexes of 30 or less nucleotides long [82]. The RNA duplexes synthesized within the cell are also processed by DICER to form siRNA that induces gene silencing. The method could be achieved by placing the sense and antisense sequences under separate promoters (either on the same or on two separate plasmids), or by using a sequence that codes for a self-complementary short RNA hairpin (shRNA), where the sense and antisense sequences are separated by a loop of nucleotides (Fig. 4). In our laboratory, we utilized the shRNA approach, and to achieve high efficiency of transfection in cardiomyocytes, the shRNA construct was introduced into a replication-deficient recombinant adenovirus vector.

Adenovirus vectors are the vectors of choice for high efficiency of transfection in vitro for non-dividing cells. The advantage of adenoviral transfection vectors is that they remain episomal. They do not cause insertion mutations that could disrupt or alter the expression of important genes.

We employed adenoviral-shRNA in both neonatal and adult cardiomyocytes to effectively deplete NCX [83–86]. Other groups have utilized siRNA to target NCX expression in neurons and osteoclasts. In neonatal cardiomyocytes, ~95% of the NCX can be effectively depleted [84] whereas only ~60% can be depleted from adult cardiomyocytes through the use of shRNA delivered by adenoviruses. This is due to the limited time available to expose isolated adult cells to any intervention in a cell culture environment. Adult cardiomyocytes tend to change their morphology and functional characteristics after >48 h in culture. Neonatal cardiomyocytes do not and can then be exposed to interventions in culture for longer periods. Thus, the shorter exposure time to the Ad-shRNA and the relatively slow turnover time of the NCX (a half-time for turnover of 33 h) limits the depletion of the NCX that can be achieved in the adult cells before their morphological changes compromise the experimental results.

We have found that RNAi is one of the most effective methods available to inhibit NCX expression and activity [84, 86]. Despite ~95% depletion of NCX protein in the neonatal cardiomyocytes, only a few alterations in their contractile activity were observed [84]. Similar conclusions had been reached earlier from data obtained from knockout mice depleted of cardiac NCX by ~80% [87]. A large increase in PMCA expression appeared to compensate for the depletion in NCX [84]. This has brought into question the critical role of NCX in excitation-contraction coupling [84]. However, the role of the NCX in ischemic/reperfusion injury does not appear to be in question. Over-expression of NCX
in cardiomyocytes has resulted in augmented damage when cells were subjected to an ischemic insult [56, 85] and, conversely, cells survived better when the NCX expression was depressed [85, 87–89].

Unfortunately, some of these experiments [84, 85] have been conducted in neonatal cardiomyocytes, and adult and neonatal cardiomyocytes differ significantly in their excitation-contraction coupling process, activity, and expression levels of NCX [48, 89–91] and in their response to ischemic reperfusion challenge [92]. Although genetically modified mice provide excellent models to examine the effects of changes in NCX expression, the use of single cells can avoid potential influences from extra-cardiac factors (hemodynamic, hormonal, etc.). NCX expression was inhibited ~60% in comparison to the scrambled control infection in the adult cardiomyocytes. This degree of depletion of NCX protein over the 48-h incubation time was sufficient to induce a significant depression in NCX function. The adult cardiomyocytes that had a depressed NCX expression and activity maintained normal Ca2+ homeostasis upon electrical stimulation of contraction. This is consistent with studies in neonatal cardiomyocytes and transgenic mouse models where no dramatic loss of contractile activity and Ca2+ homeostasis was observed despite severe NCX depletion [84, 87]. There was, however, a slowing of cardiac contractile activity and Ca2+ homeostasis [84, 87].

Adult cardiomyocytes that have a depressed NCX expression and activity are less sensitive to ischemic/reperfusion challenge. NCX-depleted cells were significantly protected from the rise in cytoplasmic Ca2+, and this resulted in less damage than was observed in control cells during ischemia and reperfusion. This is consistent with previous work that has shown that increased expression results in greater damage [56] and, conversely, blocking NCX activity with drugs or reducing NCX expression protects the heart from ischemic/reperfusion injury [93]. The advantage of blocking the NCX instead of these two alternative Na+ transport pathways is twofold. First, it likely allows the transsarcolemmal H+ gradient to dissipate more quickly because the Na+/H+ exchanger should still be very active. This would allow the Na+ pump to activate faster as well and reduce the intracellular Na+ concentrations. Second, NCX inhibition is directly blocking the primary cause of ischemic damage – intracellular Ca2+ overload. It should be mentioned that NCX not only plays a critical role in ischemia/reperfusion injury but different forms of its isoforms are regulated by various pathophysiological conditions such as diabetes, hypertension, neurodegeneration, and bone resorption [94–104].

Conclusions

We can now conclude on the basis of increasingly persuasive evidence that NCX is extremely important in the Ca2+ overload and damage that accompanies ischemic/reperfusion insult in the heart. This protection is afforded to both neonatal cells and adult preparations as well. The strength of these conclusions about the role of NCX in ischemic injury is not transferred to its role in excitation-contraction coupling. Significant depletion of the NCX does not induce a loss of contractile activity in heart preparations. It would appear it is critical for modulating characteristics of the contraction/relaxation event but not for its maintenance.

Acknowledgments

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References


**Abstract**

Scleraxis is a transcription factor that appears to play a key role in both the development of extracellular matrix-rich tissues such as tendons, and in the synthesis of matrix itself by regulating matrix gene expression. Our understanding of how scleraxis works, how its activity and expression are regulated, and the specific role it plays in disease is largely incomplete. However, enough data have accumulated to date to identify scleraxis as a critical factor in tendon formation, and ongoing studies in our laboratory have implicated scleraxis as a previously unappreciated driver of cardiac fibrosis due to its role in regulating type I collagen formation. Scleraxis may in fact behave as a master regulator of fibrillar collagen formation in multiple tissues, and the development of therapies aimed at reducing scleraxis function may provide a novel means to control tissue fibrosis in multiple pathologies.

**Keywords**

Development • Extracellular matrix • Fibrosis • Gene expression • Heart • Heart valves • Tendons • Transcription factor

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**Introduction**

By regulating the expression of a multitude of target genes, transcriptional regulators have the potential to play dramatic roles in cell, tissue, and organ physiology and pathophysiology. Understanding how these regulators function, and identifying their target genes, is thus critical for better understanding disease and developing new treatments. Scleraxis is a member of the basic-helix-loop-helix (bHLH) superfamily of transcription factors, and was originally considered to be a highly specific marker for progenitor cells destined to form tendons, ligaments, and bronchial cartilage [1–3]. Recent studies, however, suggest that scleraxis is expressed in a much wider variety of tissues, such as cardiac fibroblasts, and thus may have a broader range of activity than originally believed [4].
To date, the target genes regulated by scleraxis are largely unknown. Studies carried out over the past several years by our laboratory and others suggest that scleraxis regulates expression of a variety of connective tissue genes, including those encoding fibrillar collagens [4]. Our data indicate that scleraxis is involved in cardiac fibrosis, and the expression of scleraxis in other tissues suggests the intriguing possibility that it may play a regulatory role in other forms of organ fibrosis. Additional work is needed to further our understanding of the mechanisms by which scleraxis works, to identify its target genes, and to better understand how expression and activity of scleraxis itself is controlled. Indeed, despite the fact that it was cloned well over a decade ago, at the time of this writing there are fewer than 120 papers referencing scleraxis in the literature [3]. The need for additional research on this intriguing protein is thus significant.

**Structure and Function**

The various members of the bHLH transcription factor superfamily play critical roles in such varied processes as cell proliferation, differentiation, and regulation of oncogenesis [5–7]. Structurally, these proteins contain a bHLH moiety consisting of a short stretch of hydrophilic residues followed by a set of mainly hydrophobic residues located in two short helices separated by a non-conserved sequence of variable length which constitutes the loop [8, 9]. The basic domain of bHLH proteins constitutes the actual interface for DNA interaction. One helix is slightly smaller than the other to provide additional flexibility to facilitate dimerization by folding and packing against the other helix. Typically the larger helix contributes to the DNA interaction interface [10]. The bHLH proteins bind to signature motifs consisting of a core hexanucleotide sequence, CANNTG (N represents any nucleotide), referred to as an E-box [3, 11, 12]. A transactivation domain, typically located in the C-terminal region, is responsible for the activity of these factors.

Small (~22 kDa), scleraxis is structurally very similar to other bHLH factors, including possession of an N-terminal bHLH motif and a C-terminal transactivation domain [3]. Scleraxis was first cloned in a yeast two-hybrid screen to identify interacting partners of E12 [3]. E12 is a ubiquitous Class A bHLH protein, also known as an E-protein [13]. Conversely, scleraxis is a Class B bHLH protein exhibiting a tissue-restricted expression pattern. Like other bHLH proteins, it binds specifically to E-boxes, and its ability to bind to oligonucleotides containing E-boxes from the muscle creatine kinase gene promoter is augmented by E12, suggesting that scleraxis heterodimerizes with E-proteins for full activity [3]. Using artificial promoter constructs, it has been shown that another E-protein, E47, also augments the ability of scleraxis to bind to E-boxes [14]. At present it is unclear how scleraxis “recognizes” specific E-boxes in target gene promoters. The identities of the two central nucleotides of the E-box hexamer are likely to be important, and it is possible that other nearby nucleotides also play a role in selectivity. Regardless of mechanism, it is clear that Class B E-box-binding bHLH proteins like scleraxis are not typically promiscuous. For example, while we noted that scleraxis strongly transactivated the human collagen Iα2 gene proximal promoter, which contains three E-boxes, the bHLH transcription factor MyoD had no effect on this promoter [4].

The Noda laboratory identified aggrecan as the first gene to be directly regulated by scleraxis, and noted that scleraxis was able to transactivate an aggrecan promoter reporter construct even without the addition of exogenous E-proteins E12 or E47 [15]. Deletion studies from our laboratory have shown that removal of the HLH domain, which mediates protein–protein interaction, resulted in an approximately 50% reduction in scleraxis activity [4]. This indicates that heterodimerization augments transactivation by scleraxis, but is not specifically required. This augmentation is likely to be context-specific, governed by the individual cell-type involved, the specific E-box sequence and the availability and identity of potential binding partners [15]. In contrast to the HLH domain, deletion of the basic DNA-binding domain completely attenuated transactivation by scleraxis, demonstrating an absolute requirement for DNA contact [4].
It is unclear whether scleraxis can interact with other proteins besides E12 and E47. However, specific experiments indicate that scleraxis does not appear to interact with the HLH inhibitory protein Id2 [14]. In our experiments, Id2 was able to inhibit scleraxis-mediated transactivation of the collagen Iα2 promoter, thus we hypothesized that Id2 may act by sequestering required scleraxis-binding partners [4]. Similarly, we have generated a mutant form of scleraxis lacking the basic DNA-binding domain and found that this mutant appears to act in a dominant negative fashion – also possibly by sequestration of binding partners. Nonetheless, it remains unclear whether these partners are E-proteins such as E12, or whether other critical partners exist. It is also unclear whether scleraxis is capable of forming homodimers to regulate gene expression. One novel binding partner for scleraxis is cAMP response element binding protein CREB2/ATF4, which represses scleraxis function in Sertoli cells [16].

Although the primary structure of scleraxis reveals a number of potential sites for posttranslational modification (e.g. phosphorylation), information is currently lacking as to whether such modifications may impact the transactivation activity, stability, or dimerization capabilities of scleraxis. It is thus difficult to theorize at this time exactly which intracellular signaling pathways may be important in mediating scleraxis function, although several such pathways have been demonstrated to be important. We have shown that scleraxis expression is up-regulated in response to TGF-β signaling, but it is not yet known whether this effect is mediated by the canonical TGF-β-Smad pathway or some other mechanism [4]. Furthermore, it is unclear whether scleraxis-mediated transactivation of gene promoters is modulated by this pathway independently of its expression level, although a study in ROS17/2.8 osteoblastic osteosarcoma cells suggested that TGF-β stimulated the DNA-binding activity of scleraxis [17]. Scleraxis expression is also up-regulated by cAMP or follicle stimulating hormone in Sertoli cells, although the mechanism is unclear [18]. Smith et al. have reported that scleraxis expression is regulated by ERK1/2 signaling [19]. FGFs also appear to be important regulators of scleraxis expression. The transcription factors Pea3 and Erm, which function downstream of FGF signaling, regulate scleraxis somite expression, and FGF4 was sufficient to increase scleraxis expression in developing avian heart valves downstream of ERK and in chick limb tendons [20–22].

**Gene Expression**

The human scleraxis gene is currently identified in the NCBI sequence database as two variants SCXA and SCXB; however, these sequences represent a single gene located on chromosome 8 in the region of 8q24.3. The scleraxis protein itself is highly conserved, with nearly 100% identity between rats and mice, and approximately 90% identity between humans and lower mammals (see Fig. 1). This high degree of identity suggests that the biological function of scleraxis may be critical for proper development. Supporting this idea, knockout of scleraxis is associated with a significant decrease in the number of viable pups at birth, with greatly reduced survival at 2 months [23]. Scleraxis gene homologs have been identified in a variety of other organisms, including chicken, frog, cow, horse, and zebra fish.

During murine embryonic development, scleraxis is widely expressed at the time of gastrulation around embryonic day (E) 6.0, but its expression pattern becomes restricted soon thereafter [24]. Its expression can be detected as early as E9.5 in the sclerotome compartment of somites from which the ribs and vertebrae arise [3, 24]. Scleraxis is highly expressed in a number of pre-skeletal mesenchymal cells prior to chondrogenesis, but a decrease in its expression has been noted during ossification. There is abundant expression of scleraxis in progenitor cells destined to form ligaments, tendons, and bronchial cartilage [3]. High levels of this gene have also been noted throughout the pericardium [3]. It has been shown to be expressed during valvulogenesis in the developing chordae tendinae proximal to the papillary muscles of the embryonic chick heart as well as in semilunar valve precursor cells [25].
**Physiological Role**

Since scleraxis is expressed in a variety of tissues, its specific physiological role may vary according to tissue type. However, insight into its role may be obtained by identifying genes that are regulated by scleraxis, of which several have been identified to date. Collectively, these data suggest that scleraxis may be a general regulator of extracellular matrix (ECM) formation, although the specific gene targets appear to vary by tissue type. Indeed, in some cases scleraxis appears to have opposing effects on gene expression depending on cell type.

In a number of studies, scleraxis expression has been associated with putative target gene expression in cardiac fibroblasts and myofibroblasts as well as in cardiomyocytes, i.e. it is expressed throughout the myocardium [4]. It remains unclear whether the various embryonic tissues that express scleraxis continue to do so in the adult, and whether new regions of scleraxis arise during maturation.
expression, although a direct causal regulatory mechanism has not yet been demonstrated. For example, several lines of evidence suggest that scleraxis may regulate type II collagen production in a variety of cell types. Scleraxis over-expression in ROS17/2.8 cells resulted in increased expression of collagen II and the cartilage marker osteopontin, while at the same time leading to a decrease in expression of osteoblast markers collagen I and alkaline phosphatase [15, 28]. The coordinated up-regulation of scleraxis, collagen IIb, and aggrecan marks the differentiation of embryonic stem cells to a chondrocyte phenotype [29]. Scleraxis expression also correlates with expression of collagen II and tenascin during development of heart valves, and addition of FGF4 to developing avian heart valves resulted in increased expression of both scleraxis and tenascin [21, 25]. It was recently shown that scleraxis and E47 appear to work synergistically with Sox9 to regulate collagen 2α1 gene expression, which is interesting since the expression patterns of scleraxis and tenascin overlap in early development [27, 30]. The expression of the tendon differentiation marker, tenomodulin, increases in cultured tendon-generating tenocytes in response to retroviral delivery of scleraxis, but whether scleraxis directly transactivates the tenomodulin gene or affects expression indirectly by acting on differentiation pathways is unknown. Both scleraxis and tenomodulin were concomitantly down-regulated in myostatin-null mice, suggesting they are co-regulated [31].

Scleraxis has also been demonstrated to directly regulate a number of target genes. Aggrecan 1, a major proteoglycan component of cartilage, is directly up-regulated by scleraxis in ROS17/2.8 cells, due to interaction of scleraxis with the aggrecan 1 promoter [15, 32]. In Sertoli cells, scleraxis has been shown to up-regulate the expression of transferrin and androgen-binding protein, which may contribute to regulation of Sertoli cell function [18].

Recently, evidence from our laboratory and others has demonstrated that scleraxis directly regulates expression of type I collagen. Both scleraxis and collagen I genes were concomitantly expressed in pluripotent tendon-derived cell lines [33]. Type I collagen comprises two subunits, each expressed from its own gene – collagen Iα1 and Iα2. Scleraxis appears to directly regulate expression of both of these genes. Rossert’s group recently demonstrated that the collagen Iα1 gene is directly regulated by scleraxis via a short promoter element that was required for expression in rat tendon fibroblasts, in conjunction with NFATc [34]. Our laboratory has reported that scleraxis is expressed by fibroblasts and myofibroblasts, the primary collagen synthesizing cells of the heart, and that scleraxis expression increases more than fourfold during the phenoconversion of fibroblasts to myofibroblasts [4]. We found that scleraxis directly transactivates the human collagen Iα2 gene promoter in both NIH 3T3 fibroblasts and primary rat cardiac fibroblasts. We also noted that cardiac fibroblast expression of scleraxis itself was strongly up-regulated by TGF-β1, a potent pro-fibrotic factor implicated in fibrosis of multiple tissues including the heart, which was previously shown to up-regulate scleraxis expression in ROS17/2.8 cells [17]. It thus appears that a major role for scleraxis is as a master regulator of collagen synthesis. Experiments to examine how scleraxis interacts with the canonical collagen synthetic pathways, including Smad transcription factors, are under way in our laboratory. While there is this clear evidence that scleraxis regulates type I collagen gene expression, the finding that scleraxis over-expression in ROS17/2.8 cells leads to a down-regulation of type I collagen expression indicates that cell context is critical [15].

Additional insight into the role of scleraxis has come from mouse knockout studies. The initial study reporting the generation of scleraxis knockout animals suggested that it was essential for early embryonic development, since embryos homozygous for a targeted scleraxis knockout allele suffered mortality in the early stages of embryogenesis [24]. Recently however, Schweitzer’s group produced a novel line of scleraxis-null animals, and found that the initial attempt had generated a hypomorph of an overlapping gene due to a neomycin-resistance selection cassette inserted into the scleraxis locus during generation of the mice. The scleraxis gene is located in the third
intron of Bop1, a housekeeping gene essential for biogenesis of ribosomes; thus the presence of a Neo minigene may alter Bop1 splicing [35]. Schweitzer’s laboratory performed elegant conditional recombination experiments in which they selectively excised the scleraxis coding region using a Cre/loxP approach, and excised the Neo cassette with FLP/frt [36]. Leaving the Neo cassette intact resulted in embryonic lethality similar to the previous knockout line but excision of the selection marker permitted the production of full-term pups.

Scleraxis knockout mice, though viable, had significant defects in load-bearing tendon formation [36]. These animals exhibited a dramatic disruption of tendon differentiation that was manifested in dorsal flexure of the forelimb paw, limited use of all paws, reduced functionality of the back muscles, and complete loss of ability to move the tail. Tendon defects were first noticed close to E13.5 in all tendons. Based on the phenotype observed in the scleraxis-null mice, it appears that scleraxis function is related to the incorporation of tendon progenitors into discrete tendons [36]. This is an important finding since there is little information on the molecular mechanisms that enable tenocytes to coordinate the secretion and organization of matrix structures during tendon genesis [37]. Scleraxis-null mice also showed alterations in their ability to produce tendon matrix, manifested in a dramatic decrease in the number of collagen fibres and their organization within the tendon matrix [36]. Significantly, there was a reduction or loss of collagen I expression in affected tendons, suggesting that scleraxis is required for normal collagen gene expression in agreement with our data in primary cardiac fibroblasts. However, since not all tendons were affected, and since type I collagen expression was relatively normal in some tissues, it is clear that scleraxis is not absolutely required for all such synthesis, and again suggests that cell context is likely to be critical. This study by Schweitzer’s group presented the first demonstration of a tendon differentiation phenotype and provided significant insight into the role of scleraxis. Mice in which the myostatin gene has been knocked out appear with a milder phenotype of scleraxis-null mice: tendons are smaller and have reduced fibroblast density [31]. Furthermore, myostatin-null mice have reductions in tendon expression of type I collagen, scleraxis, and tenomodulin.

Recent work has also highlighted a potential role for scleraxis in periodontal ligament formation. Scleraxis is expressed in human periodontal ligament cells (hPDLC) and gingival fibroblasts (hGF), with highest expression in hPDLC and lowest in hGF [38, 39]. Indeed, scleraxis is frequently used as a marker for periodontal ligament cells or cell lines committed to a PDL fate [40–42]. Notably, there was a decrease in hPDLC scleraxis expression with increasing passage number in culture [38]. Another study investigated the role of scleraxis in modulating the effect of high glucose concentration on differentiation of hPDLC [43]. Scleraxis expression was up-regulated in hPDLC cultured in high glucose medium in vitro, concomitant with inhibition of osteogenic differentiation in these cells [43]. These studies suggest that scleraxis expression persists in PDL cells, but is lost if these cells undergo osteogenic differentiation. However, in contrast to these results it was recently reported that scleraxis expression in a PDL-derived cell line did not decrease with passage number, and appeared to only diminish minimally with osteogenic induction [44]. The expression of tenomodulin decreased with passing or osteogenesis induction, even though scleraxis did not appear to change. This is surprising, since an earlier study had identified scleraxis as a regulator of tenomodulin expression [28]. It should be noted, however, that most of these studies were performed in immortalized cultured cell lines derived from PDLs; thus the specific conditions employed may alter cell phenotype and make broad conclusions difficult. Further research into the potential role of scleraxis in inhibiting PDL differentiation is warranted.

Pathological Role

With such limited information to date on the normal roles and mechanism of function of scleraxis, it is perhaps not surprising that little is known about its role in disease and pathological processes. A number of diseases of connective
tissue have been described, such as various classifications of Ehlers–Danlos syndrome in which various connective tissue components (particularly fibrillar collagens such as type III, V, and I) are mutated or otherwise incorrectly synthesized [45]. However, the possibility that scleraxis functionally affects initiation or progression of these diseases is completely unexplored to date. It was also previously reported that scleraxis expression is down-regulated in the brains of Down syndrome and Alzheimer disease patients, but this study reported only correlative data [46]. Nonetheless, potential roles for scleraxis in several pathologies are starting to be identified.

A recent study found concomitant increases in scleraxis and collagen Iα1 expression over several weeks in a mouse model of pathological patellar tendon injury [47]. Since scleraxis is expressed at much higher levels in cell cultures derived from patellar compared to Achilles tendons, it is possible that scleraxis plays a role specifically in the healing process of patellar tendons [48]. The possibility that scleraxis plays distinct roles in only a subset of tendons is further supported by the finding that scleraxis knockout largely affected only force-transmitting or intermuscular tendons [36].

The primary collagen constituent of the heart is type I fibrillar collagen, and this collagen is significantly up-regulated in cardiac fibrosis and in the formation of scar tissue following myocardial infarction [49]. We found a significant increase in scleraxis expression in the region of the infarct scar 4 weeks after surgically induced infarction in parallel with up-regulation of collagen Iα2 gene expression [4]. In contrast, these increases were not observed in distal non-infarcted myocardium or in sham-operated animals. We previously generated an acute heart failure model in transgenic mice by specifically expressing a constitutively active HDAC5 mutant in the heart [50]. Microarray analysis of these animals revealed a significant up-regulation of scleraxis expression several days prior to up-regulation of fibrillar collagens. Our data suggest that scleraxis may play a causal role in the induction of cardiac fibrosis. At this time, however, it is unclear whether scleraxis regulates type I collagen expression under basal conditions, in pathological conditions (e.g. post-infarct or during hypertension), or both.

Keloids are dermal thickenings or scars characterized by rampant over-expression of fibrillar type I or III collagen. Intriguingly, scleraxis expression is strongly induced in fibroblasts isolated from samples of dermal keloids [51]. In contrast, scleraxis is not expressed in normal dermal fibroblasts. Since our data indicate that scleraxis is sufficient to drive collagen Iα2 expression, it is intriguing to speculate that aberrant expression of scleraxis in dermal fibroblasts may contribute to the pathology of keloids. Since keloids represent a form of fibrosis, this finding in conjunction with our cardiac data suggests that scleraxis may be involved in the general induction of fibrosis, regardless of tissue type. If scleraxis is in fact a central player in multiple forms of fibrosis, then it may represent a convenient target for the development of novel anti-fibrotic therapies. The need for such therapies is significant, since pharmaceuticals specifically directed at reducing fibrosis are currently lacking [49].

Conclusions

The regulation of ECM formation plays a key role in mediating the function of many tissues. The association of scleraxis expression with that of a number of ECM genes suggests that it plays a central role in ECM synthesis, and thereby impacts ECM function through direct or indirect regulation of target genes. A clear role for scleraxis regulation of tendon development, structure, and function has been shown with the creation of scleraxis-null mice [36]. Data from our laboratory and others showing that scleraxis regulates type I collagen gene expression, that it is expressed in cardiac fibroblasts, and that its expression is associated with post-infarct cardiac remodeling indicate that scleraxis has other critical functions that are only beginning to be explored [4, 34]. Scleraxis may thus represent a candidate target for treatment of fibrosis in the heart and other tissues, but more work remains to be done before the promise of anti-fibrotic treatments based on interference with scleraxis function can be exploited.
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Introduction

Cardiovascular diseases are the leading cause of death in men and women in industrialized countries, with aging, hypertension, and metabolic disorders being the major risk factors. The profound impact of biological sex on cardiovascular physiology or pathology has long been known, but the biological mechanisms responsible for sex-related differences have emerged more recently. Thus, this chapter is aimed at bringing a comprehensive review of the sex-based differences in cardiac structure and function in adults, during aging, and on the cardiac adaptability to pressure overload. The analysis of the major molecular mechanisms involved highlighted the impact of sex-based differences in pathophysiology of the heart. It emerged from the review that the sex-based difference is a variable that should be dealt with in both basic science and clinical research.

Abstract

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Keywords
Cardiac function • Cardiac hypertrophy • Coronary artery • Estrogens • Gender • Heart failure • Myocytes
Sex-Based Differences in Cardiac Structure and Function in Adults and During Aging

Before puberty there is no difference between men and women in terms of heart size and number and size of cardiomyocytes. However, after puberty the male myocardium is 15–30% larger than that of female, the size of cardiomyocytes is higher in males than in females, whereas the myocyte number is similar (review in [1]). This indicates a greater hypertrophic growth of the cardiomyocytes in men than in women during the adult life. With aging, sex-based differences are also reported. There is a preservation of cardiomyocyte number and sizes in elderly females whereas a significant loss of cardiomyocytes is described in age-matched men (review in [1]). In men, the number of cardiomyocytes significantly decreases (64 million are lost per year) through different processes including apoptosis and necrosis (review in [2]) (Table 1), whereas in the remaining cells, cardiomyocyte volume increases through a hypertrophic process [1, 3]. Women demonstrate a marked increase in the incidence of left ventricular (LV) hypertrophy after menopause, when the prevalence of arterial hypertension increases [4]. This cardiac hypertrophy can be significantly prevented by hormonal replacement therapy [1]. The occurrence of interstitial fibrosis, another classical feature of aging of the heart, is differentially regulated according to gender. Indeed, cardiac interstitial fibrosis is dramatically developed in males when compared to females (27% vs. 18% in the males and females septa, respectively) [5]. These histopathological findings are in-line with the sex-based differences observed through echocardiographic analysis. The diameter of the left ventricle (LV) increases with age only in men. Young women have a better diastolic function than men. With age, ventricular filling is impaired in both sexes, but the systolic function deteriorates only in men [6, 7]. Exercise reveals clearly sex-related differences in both healthy subjects and patients with asymptomatic aortic stenosis (AS) despite similar hemodynamic properties of the heart at rest [8–10] (Table 2). During exercise, the ejection fraction tends to increase more in men than in women whereas the cardiac output increases similarly in both. These data reflect the sex-based mechanisms involved in the adaptive responses of the heart to exercise. Women tend to increase their cardiac output primarily by increasing end-diastolic volume index without significantly increasing the ejection fraction whereas men primarily decrease the end-systolic volume index and raise the ejection fraction (reviews in [1, 10]) (Table 2).

Table 1 Biological parameters of female cardiomyocytes compared to male

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<th>Biological parameters</th>
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<td>Ca²⁺ transients</td>
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<td>Gain of E-C coupling</td>
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<td>β-Adrenergic system</td>
<td></td>
</tr>
<tr>
<td>Density of β-adrenergic receptors</td>
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</tr>
<tr>
<td>Inotropnic response to β-adrenergic stimulation</td>
<td>Decreased</td>
</tr>
</tbody>
</table>

Molecular Mechanisms of Sex-Specific Differences in Adult Heart and During Aging

The candidates to mediate sex-specific effects in the cardiovascular system are receptors for estrogens, progesterone, and androgens (ERs, PRs, and ARs, respectively). The two known ERs – ERα and ERβ – have been described in the human and rodent heart, (review in [11]). ERs, ARs, and PRs act by a number of genomic and non-genomic pathways. They are transcription factors able to initiate the transcription of hormone-sensitive
genes or to modulate the activity of other transcription factors. Upon binding of hormones, ER, AR, and PR might activate or interfere with multiple signaling pathways including phosphatidylinositol 3-kinase (PI3K).

In addition, many cardioprotective genes, such as HSP72 or HSP70, are up-regulated either directly or indirectly by estrogens (review in [1]). One of the major cellular targets for the sex-based differences in the cardiovascular system are the endothelial cells, mainly through the modulation of the endogenous vasodilator nitric oxide (NO).

At baseline, the endothelial NO synthase NOS3 is present in both coronary vascular endothelium and cardiac endothelium. NOS3 is mainly targeted, through binding to caveolin-1 to plasmalemmal caveolae, microdomains that serve as sites for the sequestration of signaling molecules, review in [12]. Regarding the control of NOS3 activity in endothelial cells, the estrogens play a key role. Chambliss et al. [13] have shown in endothelial cells the non-genomic control of NOS3 via the estrogen receptor-α within the caveolae. Estrogen absence significantly affects the NOS3 activity [14, 15]. As reviewed by Fleming and Busse [15] chronic changes in estrogen status can differentially affect NOS3 and caveolin-1 protein levels in endothelial cells. In estrogen-depleted rat heart, a significant reduction in NOS3 activity without change in the NOS3 expression but with enhanced NOS3–caveolin-1 interactions has been described [16]. Thus, the estrogen-dependent NOS3–caveolin interactions play an important role in the control of NOS3 activity and in turn in the endothelium-dependent vasodilation. In addition to the action on NO, the modulatory effects of estrogens on artery myogenic tone appear to involve regulation of calcium-activated potassium (BKCa) channels [17, 18]. In-line with these results, sex-based differences in coronary artery function have been observed in response to moderate increase in cardiac aldosterone production without alteration of cardiac function [19]. Indeed, a coronary dysfunction in aldosterone synthase-transgenic mice is observed only in males [20] and is demonstrated to be related to altered (1) calcium-activated potassium (BKCa) channels’ expression in vascular smooth muscle and (2) coronary BKCa-dependent relaxation [21]. Recent results from our laboratory indicate that estrogens might counteract the effect of hyperaldosteronism on the BKCa-mediated coronary relaxation (Delcayre et al. unpublished data).

At the level of cardiomyocytes, there are sex differences in excitation-contraction (E-C) coupling in cardiac cells from adult rats; Ca\(^{2+}\) transients are smaller and the gain of E-C coupling is lower in female cardiomyocytes than in male

### Table 2 Sex-based differences in cardiac function in humans

<table>
<thead>
<tr>
<th></th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
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<tr>
<td>Healthy subjects [10]</td>
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<tr>
<td>LV stiffness constant β</td>
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<td></td>
</tr>
<tr>
<td>Heart failure with normal ejection fraction [10]</td>
<td>0.021</td>
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<tr>
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<td>63/64</td>
</tr>
<tr>
<td>Weight-adjusted peak O(_2) consumption (mL/kg/min)</td>
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<td>22.6</td>
</tr>
<tr>
<td>Ejection fraction at rest/during exercise (%)</td>
<td>+10%</td>
<td>+30%*</td>
</tr>
<tr>
<td>End-diastolic volume during exercise</td>
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<td>+33%*</td>
</tr>
<tr>
<td>Increase in cardiac flow during exercise</td>
<td>+2.33</td>
<td>+2.34</td>
</tr>
<tr>
<td>Ratio of exercise to resting O(_2) arteriovenous difference</td>
<td>8.0</td>
<td>5.4**</td>
</tr>
</tbody>
</table>

*\(p<0.05\); **\(p \leq 0.01\)
cells and, in addition, aging-induced alterations of cardiac E-C coupling are more prominent in cardiomyocytes from males than in cells from females [3]. The difference in gain of E-C coupling between male and female cardiomyocytes reflects differences in the mean amplitude of sparks and the time to peak, which are smaller in female cells than in the male ones [22]. Other sex-based differences in intracellular calcium handling have been reported, such as phosphorylation state of phospholamban and L-type Ca\(^{2+}\) channel density. Revisiting gender-related differences in Kv\(^+\)) currents in mouse ventricle, it has been recently proposed that down-regulation of Kv4.3 and Kv1.5 transcripts by estrogens is one of the mechanisms defining gender-related differences in ventricular repolarization [23].

In addition, sex-based differences are found also in the uptake of Ca\(^{2+}\) by cardiac mitochondria – mitochondria from female rat heart having lower Ca\(^{2+}\) uptake rates; for a review see [24]. In addition, female rats lower cardiac mitochondria content; they are more efficient and generate less H\(_2\)O\(_2\) than the males. Finally female myocytes have a lower density of β-adrenergic receptors and thus also a decreased inotropic response to β-adrenergic stimulation [24]. Of note, across the life span various biochemical characteristics (including telomerase activity and several components of the insulin-like growth factor system) vary differently in male and female cardiomyocytes [25].

In addition to the sex-based differences listed above, significant differences in the way male and female hearts respond to various challenges bring important insight into the mechanisms by which the female gender may influence favorably the remodeling and the adaptive response to myocardial insult.

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**Cardiac Adaptability to Pressure Overload and Impact of Sex-Based Differences**

Hypertrophy per se is an independent risk factor for heart failure and sudden death (review in [2]). Mechanical pressure overload being secondary to either hypertension or to aortic stenosis, we will refer to both etiologies in this review. We have known for a long time the sex-specific ability of the myocardium to adapt to mechanical overload [26, 27]; sex differences in cardiovascular disease have been receiving increasing attention in both experimental and clinical research in the recent past [1, 7, 11, 16, 24, 28–31].

It is well assessed that premenopausal women have a better prognosis than do men in response to hypertension and aortic stenosis. Indeed, a clinical study of 29 women and 53 men with asymptomatic aortic stenosis has clearly demonstrated that men show an earlier and more severe systolic dysfunction than women [9]. Based on a clinical trial, the lines of evidence indicated that heart failure with normal ejection fraction (HFNEF) is much more common in women than in men [10]. This predominance has been demonstrated in a large American study, including 79% of women after adjusting the data for all available information. The study focuses on 19,710 patients (over 65 years) with heart failure, 35% of them having heart failure, 35% of them having a normal ejection fraction. Of note, the diastolic function was not investigated in the cohort [32]. Similar results were found by Klapholz et al., [33] who conducted a prospective multicenter registry in a NY area to define the clinical characteristics, hospital course, treatment, and factors precipitating decompensation in patients hospitalized for HFNEF. A European study including 24 countries has led to similar conclusions, but again in the absence of left ventricular filling measures [34]. However, some authors have investigated the diastolic function by using echocardiography; review in [10]. Among them, “the Strong Heart Study” by Devereux et al. [35] deserves attention. This population-based study enrolled 3,184 patients aged between 47 and 81 years and was characterized by a high proportion of women (84%). It emerged that patients with HFNEF were old and overweight, in majority women, had renal dysfunction, impaired early diastolic LV relaxation, and concentric LV geometry. Conversely, patients with congestive heart failure and severe LV dysfunction were more often men, exhibiting a restrictive pattern of LV filling and eccentric LV hypertrophy. Other studies provide more nuanced
results, suggesting equal or greater prevalence of diastolic dysfunction in men (review in [10]). Thus, the greater rate of HFNEF in women might be related to sex-based differences in ventricular diastolic distensibility, in vascular stiffness and ventricular/vascular coupling, in skeletal muscle adaptation to HF, and in the perception of symptoms [10].

When focusing on patients with aortic stenosis (AS), [7, 36], women, and particularly elderly ones, develop a more concentric form of hypertrophy than men, with smaller ventricular diameters and less ventricular dilatation. In some, but not all studies, women had higher transvalvular gradients, greater relative wall thickness, and better systolic function. Furthermore, women with congestive heart failure have also been shown to survive better than men in some studies [7].

Interestingly enough, when analyzing hypertrophy regression after aortic valve replacement, LV hypertrophy reversed more frequently in women than in men [28].

Experimental data confirm and extend these sex-based differences in the development of cardiac hypertrophy to pressure overload. Different studies indicated that at the very early phase of a mechanical pressure overload, female rats develop more cardiac hypertrophy than male rats [27, 30] and that only males exhibit decreased signs of acute heart failure [30] (Fig. 1). However, in mice, the sex-based differences were observed only at later stages of cardiac hypertrophy (2 weeks after surgery) [37]. Thus, the differences in the adaptation of female and male hearts to pressure overload draw attention to the underlying mechanistic pathways.

**Fig. 1** Sex-related differences in responses to hemodynamic challenge. For a same level of aortic stenosis, LVEDP increases more and LV hypertrophy increases less in male rats than in females. In consequence, cardiac function is less depressed in females than in males (Redrawn from [30]).

**Sex-Specific Differences in Gene Expression and Cardiac Adaptation to Pressure Overload**

The sex-based differences in remodeling of the whole heart are mirrored by differences in intracellular signaling pathways and/or patterns of gene expression. One of the pioneer works on sex-based differences in cardiac response to severe pressure overload, such as thoracic aortic stenosis (TAC), demonstrated that β-MyHC expression is greater in male than in female hypertrophic...
hearts, whereas the sarcoplasmic reticulum Ca\textsuperscript{2+}−ATPase mRNA levels are depressed in males only, and the Na\textsuperscript{+}−Ca\textsuperscript{2+} exchanger mRNA levels are increased independently of the gender, these transcriptional changes being associated with a preserved contractile reserve in female hypertrophic hearts [38]. In contrast, mRNA microarray analysis using the transverse aortic constriction (TAC) model in mice does not show differences in the selection of hypertrophy markers such as α-actin, ANP, and BNP [37, 39]. However, several genes controlling mitochondrial functions including PGC-1 had a lower expression in males [37]. A whole female/male gene network analysis reveals that female-specific genes are mainly related to mitochondria and metabolism and male-specific ones to extracellular matrix and biosynthesis [37]. Of note, the number of differentially regulated genes in response to acute pressure overload is greater (greater than twofold) in males than in females, whereas the response to chronic pressure overload is similar in males and females [39].

Marked sex-based differences in the development of fibrosis associated with cardiovascular disease, and particularly with pressure overload, have been observed both in human [40] and in experimental models [37, 41]. In this microarray analysis [37, 41], genes associated with extracellular matrix remodeling exhibited a lower expression in female hearts (collagen 3, MMP 2, TIMP2, and TGF-β2) after TAC in mice. Although the molecular mechanisms underlying gender dimorphism are complex and are still not well understood, it emerged that steroid sex hormones (estrogens, progesterone, and testosterone) and their respective receptors play a key role (review in [31]). It is established that estrogens reduce the turnover of the extracellular matrix, especially the collagen network. Estrogens inhibit fibroblast proliferation and collagen synthesis of type 1, but activate the expression of genes encoding metalloproteinase [42, 43]. It is worthy of note the recent insights into the sex-specific regulation of fibrosis-related genes using genetic models and in vitro approaches. Indeed, β-estradiol significantly increased collagen-I and -III gene expressions in male fibroblasts and had opposite effects in female cells [28]. Using the genetic model ER-beta(−/−), it is demonstrated that sex-based difference in cardiac fibrosis after TAC was abolished in ER-beta (−/−) mice [41]. The sex-based differences observed in the regulation of genes encoding ECM proteins and metalloproteinase and, in turn, in the development of fibrosis in response to pressure overload might represent (1) one of the major mechanisms slowing the progression to heart failure in females and (2) a positive element allowing more rapid hypertrophy regression following surgery for aortic valve replacement.

Other lines of evidence of estrogen-induced cardioprotection were provided by studies devoted to NO bioavailability and/or endothelial dysfunction. It has been demonstrated that NO had direct systolic as well as diastolic myocardial effects (review in [12]). The reduction in the bioavailability of NO is a key feature of endothelial dysfunction, classically described during heart failure. This decreased dilatation in small arteries is mainly secondary to the chronic decrease in blood flow [44], which is probably involved in the decrease in the expression and/or the activity of NOS3 in the failing myocardium in human [45] and experimental [46–48] models. Besides, a role of the protein–protein interactions, or posttranslational modification controlling NOS3 activity, such as caveolin-1 binding, NOS3 phosphorylation has been proposed [49, 50] to modulate NOS3 activity. At least, the NO reduction could be due to the increased peroxynitrites or to NOS3 decoupling (review in [12]). During the development of cardiac hypertrophy, the NOS3 expression varies according to the hypertrophic stimuli. The development of physiological cardiac hypertrophy during gestation is associated with a transient increase of cardiac NOS3 expression (up to 14 days pc) that is paralleled by an increase of cGMP [51]. In response to a severe pressure overload secondary to a thoracic aorta coarctation (TAC), gender differences in changes in NOS3 activity are observed [30]. In female rats, the NOS3 activity in the hypertrophied heart remained constant, although the enzyme expression increased before appearance of HF signs [16]. After TAC, the absence of estrogen prevents the increase in
NOS3 expression and worsens the cardiac dysfunction without affecting the development of cardiac hypertrophy. These data highlight the role of NOS3 through estrogen in the cardiac adaptation to new load conditions [16]. One novel pathway to mediate the protective effects through NOS activity on the vasculature and heart may be the increase of protein S-nitrosylation in the vascular endothelium by estrogen and mainly β-estradiol [52].

Besides the putative role of NOS3-derived NO, the implication of NOS1-derived NO has been demonstrated during the development of cardiac hypertrophy and failure [53–55]. In addition, it was shown that male mice lacking both NOS isoforms NOS1/3(−/−) have a twofold higher mortality compared to females whereas gender does not affect survival when only one NOS gene was knocked out. Notably, the development of cardiomyocyte hypertrophy and interstitial fibrosis with age in NOS1/3(−/−) mice is independent of the gender [56].

Conversely to failing heart, in rats as well as in humans, in which NOS1 activity increased together with a redistribution of the enzyme at the sarcolemmal level through binding with caveolin-3 [53, 54], the subcellular relocalization of NOS1, particularly the translocation toward the sarcolemma, is not observed in hypertrophic hearts following TAC. Furthermore, the NOS1 activity is shown to depend upon enzyme expression level without influence of molecular partners such as caveolin-3 [16]. Sex-based differences have been observed: NOS1/caveolin-3 association is significantly higher in females versus males in response to cardiac injury in mice [57] or following pressure overload in rats [30]. In these models, β-estradiol per se modulates neither NOS1 expression nor activity, whatever the hypertrophy status [16]. Thus according to Murphy et al. [58], the increase in NOS1 near caveolin-3 in females under stress conditions (I/R) associated with increased calcium (which activates NOS) results in increased S-nitrosylation of the L-type calcium channel, lower calcium entry, and therefore less calcium loading, constituting a cardioprotective mechanism as previously discussed [52].

Lines of evidence indicated that mechanosensitive pathways trigger NOS1 expression and activity in muscle cells including cardiomyocytes (review in [12]). During the early phase after TAC, the higher parietal stress in males than in females [27] has been proposed to be one of the triggers for the early NOS1 induction in rat hearts after TAC [30]. In addition, it was demonstrated that NOS1 expression in cardiac muscle following TAC was independent of estrogen level [16]. Thus according to the pressure overload and estrogen status, there is a differential regulation of NOS expression and activity, the mechanotransduction pathway being mainly involved in the induction of NOS1, while the ER pathway regulated NOS3 activity and, in turn, cardiac function. This differential response in NOS expression and activity during development of cardiac hypertrophy and according to gender might affect the molecular mechanisms by which NO influences myocardial function, particularly cGMP-activated pathways [59]. In addition to NO, ANP is known to modulate cGMP levels in cardiomyocytes [60], and to alter their function. The association of natriuretic peptides (NPs), such as BNP and ANP, with gender was examined in several studies; despite disparity, some studies report a higher BNP concentration in females compared to males. Indeed in normal patients, NT-proBNP, like BNP, tends to be higher in female patients and older individuals, through mechanisms involving either the clearance receptor for BNP or increase in expression [61, 62]. On the other hand, a population-based study indicated that in women, LV mass and NP concentrations increase to a lesser extent and only with severe LV dysfunction when compared to men [63]. In the same way, experimental models indicate that ANP mRNA level is greater in male than in female TAC-induced hypertrophy rat hearts [38]. Regarding women, hormone replacement therapy has been associated with higher BNP levels, suggesting that BNP expression may be sensitive to estrogen regulation in humans [62]. The role of estrogens in the control of ANP expression during the development of cardiac hypertrophy has been evidenced in experimental models [64]. In-line with these findings, it has been shown
that in vitro estrogens exert antihypertrophic effects on cardiomyocytes, by transactivation of the ANP gene [65, 66]. Estrogen-induced ANP accumulation in the ventricular cardiomyocytes most likely results in ANP receptor activation in an autocrine/paracrine manner which, in turn, evokes cytoplasmic cGMP signaling downstream [65]. Furthermore, the increase in cGMP mediated by ANP, but not by NO, prevents cardiomyocyte hypertrophy [66]. Interestingly, during the development of pregnancy-induced cardiac hypertrophy, the down-regulation of cardiac NPs as BNP and receptors in LV during may be physiologically required to allow the development of physiological LV hypertrophy. Of note is the expression of NPs increasing postpartum, when the development of cardiac hypertrophy has to be stopped [51]. Taken together it emerged that the tight regulation of NP expression is of importance for the sex-based differences in the development of cardiac hypertrophy.

Conclusions

In summary, the differences in cardiac gene expression according to gender and/or female sex hormone described here would help to stress that gender is a variable that should be dealt with in both basic science and clinical research. It is clear that the response of humans and animals to various disease states can be profoundly affected by sex.

References


Part III

Mitochondrial Diseases
Mitochondrial DNA and Heart Disease

Chihiro Shikata, Masami Nemoto, Takanori Ebisawa, Akihiro Nishiyama, and Nobuakira Takeda

Abstract
The relationship between abnormalities of myocardial mitochondrial DNA and heart disease is reviewed. Myocardial mitochondrial DNA abnormalities can induce both hypertrophic and dilated cardiomyopathies. In mitochondrial encephalomyopathy, abnormalities of myocardial mitochondrial DNA can also induce cardiac involvement, for example, heart failure and arrhythmia. The influence of acquired mitochondrial DNA mutations is also discussed.

Keywords
Arrhythmia • Cardiomyopathy • Heart failure • Kearns–Sayre syndrome • MELAS • MERRF • Mitochondrial DNA • Mitochondrial encephalomyopathy

Introduction
Mitochondria have their own DNA besides nuclear DNA. Abnormalities of mitochondrial DNA decrease cellular energy production and induce impairment of organ function.

Mitochondria possess an energy-producing system composed of NADH dehydrogenase, cytochrome, and cytochrome oxidase, which are encoded and regulated by mitochondrial DNA as well as nuclear DNA. Human mitochondrial DNA is double stranded and circular, consisting of 16,569 base pairs and containing 11 structural genes for the subunits of respiratory enzyme complexes and two genes for two subunits of F0-ATPase, as well as for 22 tRNA molecules and two rRNA molecules [1]. Each cardiac myocyte contains 2,000–3,000 mitochondria, each of which possesses two or three DNAs.

Mitochondrial DNA mutations are inherited from the mother [2, 3]. This DNA is easily damaged because it is continually exposed to free radicals and contains neither histones nor introns, and the mitochondrial DNA repair system is relatively primitive. Oxygen-derived free radicals generated by the mitochondrial inner membrane convert deoxyguanosine (dG) in mitochondrial DNA to 8-hydroxy-dG, which is misread as another base during duplication. An increase of 8-hydroxy-dG of 16,569 base pairs and containing 11 structural genes for the subunits of respiratory enzyme complexes and two genes for two subunits of F0-ATPase, as well as for 22 tRNA molecules and two rRNA molecules [1]. Each cardiac myocyte contains 2,000–3,000 mitochondria, each of which possesses two or three DNAs.
is consequently synonymous with the accumulation of point mutations in mitochondrial DNA.

Ozawa and colleagues have succeeded in determining the entire mitochondrial DNA sequence using direct sequencing [4, 5]. Mitochondrial DNA mutation may lead to impairment of mitochondrial function, for example, deficiency of mitochondrial respiratory chain enzymes or ATPase related to the subunits encoded by mitochondrial DNA. The term “mitochondrial cardiomyopathy” has been coined to describe cardiomyopathy induced by mitochondrial DNA mutations [6].

**Cardiomyopathy**

Cardiomyopathy can have several forms, including hypertrophic, dilated, and restrictive cardiomyopathies, and arrhythmogenic right ventricular dysplasia. The etiologies of these conditions are still unknown, but recent developments in molecular biology have provided suggestive evidence, such as the detection of mutations of the genes encoding myocardial contractile proteins such as myosin, actin, tropomyosin, and troponin. Myocardial mitochondrial DNA mutations have also been detected by Ozawa et al. in cardiomyopathic patients [7–9], and a number of other reports have documented similar mutations and their potential role in cardiomyopathy [10–22].

A point mutation that alters adenine (A) to guanine (G) within the mitochondrial tRNALeu(UUR) gene is common in patients with the syndrome of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) [9]. PCR and Southern blot analysis have revealed multiple mitochondrial DNA deletions in a pedigree of inherited dilated cardiomyopathy [23], but the extent to which these mitochondrial DNA mutations are involved in the etiology of idiopathic cardiomyopathy remains to be elucidated.

All humans appear to have the potential to develop cardiomyopathy because the myocardium degenerates with age due to the accumulation of free radical-induced damage to the mitochondrial DNA. Abnormal acceleration of mitochondrial DNA mutations, especially those related to mitochondrial protein synthesis, can induce premature aging and severe mitochondrial cardiomyopathy [24]. An A-to-G point mutation at position 3,260 of the mitochondrial tRNALeu(UUR) gene has been found in a maternally inherited disorder that was manifested as a combination of adult-onset myopathy and cardiomyopathy [10]. An A-to-G substitution has also been found at position 4,269 in the tRNAIle gene of a patient with fatal cardiomyopathy [12], and an A-to-G mutation at position 15,923 of the mitochondrial tRNAThr gene is associated with neonatal cardiomyopathy [25].

Other patients with fatal infantile cardiomyopathy have also been reported [26, 27], as has the development of severe mitochondrial cardiomyopathy in young people with an A-to-G transition at position 827 of the mitochondrial 12S rRNA gene [28]. It has also been suggested that a G-to-A point mutation at 12,192 in the tRNAHis gene may be an evolutionary risk factor for cardiomyopathy [29]. Therefore, the detection of mitochondrial DNA deletions has been proposed as a new method for investigating sudden cardiac death in which ischemic damage is the primary cause [30].

No clear correlation has been found between the severity of clinical manifestations and the mutations detected by conventional analysis of limited regions of the entire mitochondrial DNA. In contrast, comprehensive analysis of mitochondrial DNA by the direct base sequencing technique has revealed a close correlation between the mitochondrial DNA genotype and clinical phenotype [5, 28]. Each cardiac myocyte contains 2,000–3,000 mitochondria, each of which possesses two or three circular mitochondrial DNAs. Thus, cells can contain normal and mutant mitochondrial DNA in varying proportions (heteroplasmy). This may result in marked differences of energy production among myocardial cells, which may induce arrhythmias.

If the mitochondrial DNA mutation is extensive, oxidative phosphorylation will be depressed, leading to a decrease of energy production and the development of heart failure. Mutations in
nuclear DNA encoding the mitochondrial respiratory enzyme complex subunits can also affect energy production. Furthermore, it has been suggested that mitochondrial DNA mutations might activate the mitochondrial apoptosis pathway, thus causing dilated cardiomyopathy [31]. The investigation of mitochondrial DNA mutations may therefore yield various clues to the etiology of arrhythmias and cardiac dysfunction.

Some characteristic phenomena have been observed in patients with mitochondrial cardiomyopathy: the level of lactic acid in serum or spinal fluid is over 1.5 times the normal upper limit; there is a defect of mitochondrial enzymes involved in the electron transport system, glycolysis, and lipid metabolism; and changes in mitochondrial morphology have been revealed in skeletal and cardiac muscles using Gomori trichrome staining, that is, the presence of ragged-red fibers. The diagnosis of mitochondrial cardiomyopathy can be confirmed by detection of mitochondrial DNA mutations in myocardial biopsy samples. Electron microscopy can show changes in the size or number of mitochondria. Mitochondrial DNA mutations are observed in hypertrophic and dilated cardiomyopathies. In fact, it has been reported that about 3% of dilated cardiomyopathies are induced by mitochondrial DNA mutations.

**Encephalomyopathy**

In patients with mitochondrial encephalomyopathy, abnormalities occur in the skeletal muscles and the central nervous system, and cardiac abnormalities are also sometimes present [32–38]

1. Kerns–Sayre syndrome (KSS)

Kerns–Sayre syndrome is characterized by chronic progressive external ophthalmoplegia, heart block, and pigmented retinopathy. The main cause is thought to be mitochondrial DNA deletions [39–44], although mitochondrial dysfunction induced by abnormal nuclear DNA may also be involved [45]. The cardiac manifestations of this disease are arrhythmias, such as atrioventricular block, premature ventricular contractions, supraventricular or ventricular tachycardia, sinus dysrhythmia, ST segment and T-wave changes, and cardiac dilatation and failure [46–52]. In many patients, implantation of a pacemaker is required to prevent sudden death, and some patients may acquire the need for a pacemaker at an advanced age.

2. Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episode (MELAS)

MELAS usually occurs at a young age and is characterized by headache, vomiting, and stroke-like episodes such as hemiplegia [53]. This disease is induced by point mutations of the mitochondrial tRNALeu(UUR) gene, from A to G at position 3,243 [54, 55] or from thymine (T) to cytosine (C) at position 3,271 [56]. Cardiac involvement results in the onset of cardiomyopathy [57, 58].

3. Myoclonic epilepsy with ragged-red fibers (MERRF)

Myoclonic epilepsy is the cardinal symptom of patients with MERRF, which is induced by an A-to-G point mutation at position 8,344 of the mitochondrial tRNALeu gene [59]. Cardiac involvement results in onset of cardiomyopathy. Pathogenic mechanisms of mtDNA mutations were described in literatures [60–62].

**Acquired Mutations**

Mitochondrial DNA mutations can occur after birth. An age-dependent increase of deletions in mitochondrial DNA has been observed in the myocardium of both humans and rats [63–68]; mitochondrial DNA deletions have been found in myocardial autopsy specimens from patients with diabetes or myocardial infarction [69, 70], and patients who have been treated with doxorubicin [70]. Autopsy and biopsy specimens of ischemic hearts are reported to show a higher degree of mitochondrial DNA damage than normal hearts [71]. In addition, myocardial mitochondrial DNA damage and deletions induced by doxorubicin have been demonstrated in experimental animals [72, 73]. Therefore, free radicals may play a role in inducing mitochondrial DNA mutations related to the pathological conditions described earlier.
Treatment

Some reports and review articles have indicated the possibility of L-carnitine and coenzyme Q10 therapy for patients with impairment of cardiac function due to mitochondrial DNA mutations, as well as the use of gene therapy [74–82].

References

A Novel Algorithm from Personal Genome to the Pathogenic Mutant Causing Mitochondrial Cardiomyopathy

Teruhiko Toyo-oka, Toshihiro Tanaka, Licht Toyo-oka, and Katsushi Tokunaga

Abstract
Amazing progresses in both human genome analysis and bioinformatics in silico have made it possible to reach whole genome profiling in a short period with a reasonable cost and time. In this review, we have introduced the next step after reading the full genome sequence of both nuclear and mitochondrial genomes to identify the pathogenic site(s) in several cardiomyopathies. Considering ~3 million sites of single nucleotide polymorphism (SNP) per person, it is difficult to reach not a personal variant but a pathogenic site. The current algorithm might be promising for the identification of responsible gene, even in the case of polygenic nature.

Keywords
Electron microscopy • Genome • Heteroplasmy • Magnetic resonance spectroscopy • Mitochondrial cardiomyopathy • Mitochondriosis • Open reading frame (ORF) • Oxidative phosphorylation • Pathogenic mutant • Revised Cambridge resequencing system (rCRS) • Risk factor • transgene

Introduction
Human whole genome was reported just 10 years ago and the aim of coming decades is addressed to the clinical translation of personal genetic background of each patient, searching for the precise mechanism of pathogenicity, gene counseling, and/or tailored medicine to provide most suitable option for treatment [1]. For the assessment of genetic origin of heart failure and/or dilated cardiomyopathy (DCM), the mitochondrial (mt) genome represents one of the most informative and cost-effective researches, because of (1) the abundant rate of exons over introns, not like a nuclear genome, (2) short genome size to determine the whole DNA sequence [2] to profile the progression of various diseases [3], (3) repeating the beating throughout life with consuming and producing huge amount of adenosine triphosphate (ATP) in
their own cells, and (4) continuous exposure to reactive oxygen species (ROS) produced in the oxidative phosphorylation with much fewer protective actions than nuclear genome.

The mt-genome includes abundant variants not related to the pathogenicity but reflecting the haplogroup or phylogeny to adopt extracellular environment [1]. Accordingly, mt-genome study is so meaningful and fascinating but it includes widespread problems, as follows: (1) ethical conflicts originated in a disclosure of patient’s privacy [2], (2) methodological arguments to sample considerable amount of living human cardiomyocytes to evaluate the mutant’s phenotype, (3) changes in the heteroplasmy rate during tissue culture, as is convenient for the analysis and the amplification [3], (4) environmental difference of cardiomyocytes in situ under mechanical and/or chemical stress(es) from cultured cells in vitro, and the resultant modification of the phenotype, (5) the case with no identical variant in rodent to prepare transgenic models [4], and (6) intrinsic problems to patents and licensing [5]. In this short review, we present a new scheme to overcome these dilemmas and to clarify the pathogenic mechanism of various mt-diseases, based on abundant sources of bioinformatics \textit{in silico}. The homology of mt-DNA sequence with that of nuclear-DNA is shown in Table 1.

### Table 1

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An Algorithm to Reach the Pathogenic Mutant in mtCM

Necessity for Full Sequencing of the mt-Genome

In the outpatient section of the Tokyo University Hospital, we have followed ~80 cases with hypertrophic cardiomyopathy (HCM) and DCM, of which diagnosis was based on clinical and laboratory data including morphological, physiological, biochemical, serological, and, most importantly, pathological characteristics of endomyocardial biopsy samples [6]. For the conventional measurement of gene survey, we have employed gene polymorphism using PCR (polymerase chain reaction)-amplified SSCP (single-stranded conformation polymorphism) or RFLP (restriction-fragment-length polymorphism, Fig. 1) for ~15 years. In the recent 5 years, we have shifted to more time-saving and accurate modality, sequence-specific primer cycle elongation-fluorescence correlation spectroscopy (SSPCE-FCS), as described previously [7]. As candidate genes, we have selected several variants popular in Japan [8] and detected three pedigrees with identical mutations [9].

The classical methods to utilize PCR-based gene amplification often cause misreading of not the responsible, but the pseudogene(s) located in the other site. Particularly, nuclear genes preserve an incredible amount of pseudogenes with the same sequence as the ancient mtDNA [NUMT, Ref. 10] in part, even when the original mtDNA has already altered adapting to a new environment (Fig. 2). Consequently, whole mt-genome sequencing is preferable over the classical methods and would be essential in future to avoid misdiagnosis.

New Modality to Read the Whole mt-DNA Sequence

The whole mt-DNA sequences of all three probands and 10 Japanese volunteer patients without CM or heart failure as an internal standard were determined with GeneChip® Human Mitochondrial Resequencing Array 2.0 [11, Toyo-oka et al., submitted]. The DNA sequences different from the

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**Fig. 1** Classical screening steps of wide variety of DCM/HCM in nuclear and mitochondrial genomes
Identification of Pathogenic Mutant in the ORF of the mt-Genome

For the evaluation of physiological significance of open reading frame (ORF), it would be reasonable to assume that the synonymous mutation has no or less meaning in the pathogenesis, except the modification of codon usage in nuclear or mitochondrial genome [12–14]. The mutation within ORF would directly cause the conformational change in the encoded protein (transgene) and accordingly result in the functional modification, if any. For the integration of all 13 polypeptides coded by the mt-gene into the inner membrane, most of mt-proteins abundantly include hydrophobic domains and are buried in the phospholipid bilayer. Among the mt-proteins, ATPase 6 is the most hydrophobic peptide and mutation of the current case from alanine to threonine...
occurred in the midst of the hydrophobic rigid structure (Toyo-oka et al., submitted). Thus, it would be conceivable to assume that the present mutation causes a serious alteration in oxidative phosphorylation at the final step to synthesize ATP. Another mutation to cause NARP (neuropathy, ataxia, and retinitis pigmentosa) confirmed the scheme described above in the same \( ATP6 \) gene [15].

The other mutations in ORF constitute the main source of the mitochondrial gene-related diseases and the predicted structure of the transgene, that is, LHON in \( ND1, ND4, \) or \( ND6 \) [16–19] or KSS with the large 5 kb deletion spanning from \( ATP8 \) to \( ND5 \) of [20, 21] a wait a more fine analysis like an ionic charge of the constituent amino acids, modulation of helical structure, and intragenic suppressor action in LHON \( ND1 \) gene [22]. For the functional prediction, the higher-ordered structure of the ND6 gene [23] or gene interference between nuclear and mt-genomes might be much more informative to estimate the mutant function (Toyo-oka et al., submitted).

### Pathogenic Mutation in tRNA of the mt-Genome

The tRNA is another large source of mt-gene mutations, because tRNA is situated at the critical step of protein synthesis and the defect will cause a serious problem in the production rate of each component protein in mitochondria. Several mutations have been reported on MELAS 3,243 in tRNA\(^{\text{Leu(UUR)}}\) [24, 25] and MERRF 8,344 in tRNA\(^{\text{Lys}}\) [26, 27] or dilated cardiomyopathy (DCM) in tRNA\(^{\text{Thr}}\) (Toyo-oka et al., submitted). McFarland et al. raised five criteria [28]:

1. ~Three-fourths of mutation sites in stem regions of the secondary structure
2. Pathogenic hot spots in both the acceptor and anticodon stems
3. Disruption of Watson–Crick base pairs
4. More common pathogenicity in C-G base pairing than A-T pairing secondary to the lower thermodynamic energy
5. Preferential pathogenicity in loop structure with unusual number of nucleotides that may affect the tertia structure. To these criteria, we add here the following three items more for pathogenicity:

6. Medical records describing the identical mutation in other mitochondrion-related diseases, especially in energy-consuming tissues, like neurodegenerative diseases in brain, inner ear, or retina; skeletal or cardiac muscles, like myopathy, HCM, or DCM; and endocrine organs, like diabetes mellitus with or without angiopathy

7. Conservation of the wild-type sequence in nonhuman primates, suggesting the biological significance

8. Pathological features of mitochondriosis in the electron microscopy of biopsy samples

When each criterion is precisely inspected, each item is not independent, but some overlap among these stratifications. Furthermore, each item may require scoring for the more exact prediction in future. Particularly, the morphological observation using fresh sample to avoid the postmortem degeneration is critical to proceed to an advanced step of an accurate diagnosis for the genetic diseases.

The endomyocardial biopsy samples provide several characteristic findings in mitochondria, involving accumulation of a huge number of bizarre-formed mitochondria, that is, mitochondriosis (Toyo-oka et al., submitted), concentric cristae [24], hypertrophic mitochondria within myocytes, and vessel walls with or without paracrystalline mitochondrial inclusions [25, 26].

### Other Comments on Gene Analysis

The DNA sequence in rRNA is meaningful for exact and efficient protein synthesis but the clinical significance of the mutant is still obscure, except the rare case of A1555g mutation in 12S-rRNA with sensory hearing loss or DEAF gene [29, 30].

As the initial step of assignment of the pathogenic mutations coding a mitochondrial gene, the nuclear gene should be separately or independently examined not to be mixed with each other. Then the combination of two analyses would yield unexpected results that show double or sometimes triple mutation, and pathogenesis
Less agreement of the microarray commercially available now, comparing NIH SNP/SNV data to our handmade database of MYH7 gene. Note that the overlap was very rare among these databases and that the detection rate between the two databases was 0.6% with Affymetrix, 900k-Microarray.

Fig. 3

Fig. 4 Human mtDNA migrations (cited from MITOMAP: A human mitochondrial genome database. http://www.mitomap.org, 2009)

of the complex familial disease is clarified or the classical “penetrance” is explained by the multiple gene defect with the different time course (Toyo-oka et al., submitted). The SNP microarray commercially available now is still at the primitive stage to cover pathogenic mutants or variants near the responsible locus, even when a 900 k gene chip is used for the analysis (Fig. 3).

In addition, magnetic resonance spectroscopy (MRS), together with 1H-magnetic resonance imaging (1H-MRI), will be promising for the
elucidation of an actual effect of mutant(s) on cardiac function, especially to detect the energy metabolism with $^{31}$P-MRS in the case with mtCM [31, 32], though both bore size and magnetic intensity of superconducting coils are insufficient for precise measurement of human hearts in vivo within a limited measuring time.

It is very meaningful to determine the belonging haplogroup as a risk factor. From the worldwide survey [32–37], we identified that the present mutation belonged to the B5b1a subhaplogroup (Toyo-oka et al., submitted). The same G15927A mutation in tRNA$^{Thr}$ has been reported to modify the pathogenesis of other neurological diseases [38]. In addition, the current sequence was restricted to the Japanese and no similarity to Chinese or Koreans has been reported [9], which may imply that the new haplogroup has branched from East Asians after their ancestors left the Eurasian continent (Fig. 4).

Conclusions

The sequence of both nuclear and mitochondrial genomes has been identified in different cardiomyopathies. The full resequencing together with comprehensive gene analysis would clarify the controversial results obtained from PCR analysis using a partial amplification in mitochondrial gene mutation.

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References


MELAS Syndrome: Mediated by Impaired Taurinomethyluridinuridine Synthesis

Stephen W. Schaffer and Chian Ju Jong

Abstract
Taurine (2-aminoethanesulfonate) is a ubiquitous \( \beta \)-amino acid found in a very high concentration in excitable tissue. One of its most important functions is its conjugation with uridine located in the wobble position of tRNA\(^{\text{Leu(UUR)}}\). Because the wobble modification stabilizes the UG base pairing, it facilitates the decoding of UUG codons. Consequently, taurine deficiency, which reduces the wobble modification, decreases the synthesis of proteins whose mRNA has a high UUG codon content. The synthesis of one such protein, ND6, plunges 60% after a 50% decline in taurine content. Because ND6 is a subunit of respiratory chain complex I, taurine depletion also leads to a decline in the activity of the electron transport chain. A similar sequence of events occurs in the mitochondrial disease, MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes). The initial event in most MELAS patients is the appearance of one mutation in tRNA\(^{\text{Leu(UUR)}}\), which in turn blocks the taurinomethyl modification of the wobble nucleotide. As a result, the synthesis of ND6 and other UUG-dependent proteins falls. As respiratory function declines, the generation of ATP is compromised and in some cases the mitochondria begin to produce oxidants. Because mutations in tRNA\(^{\text{Leu(UUR)}}\) trigger multiple events, the identification of which event causes mitochondrial dysfunction has been challenging. The taurine-deficient model has aided in the identification of at least one pathological pathway that contributes to the development of the MELAS disorder.
Introduction

Characteristics of Mitochondrial Diseases

Mitochondrial diseases are a heterogeneous group of disorders that arise from mutations in either mitochondrial or nuclear genomes and produce deficiencies in the mitochondrial respiratory chain [1]. Mutations in the nuclear genome can cause mitochondrial diseases by affecting the synthesis of mitochondria-encoded proteins, the encoding of respiratory chain subunits, the alteration of energy metabolism and the delivery of reducing equivalents to the respiratory chain, and the generation of proteins that regulate the integrity of the respiratory chain. On the other hand, mitochondrial mutations trigger mitochondrial diseases largely by affecting the synthesis of mitochondria-encoded proteins, which combine with other subunits to produce the respiratory chain complexes. While some mitochondrial mutations are localized to genes of individual mitochondria-encoded proteins and ribosomal RNA [1–3], most of the mitochondrial mutations occur in genes encoding mitochondrial tRNAs. Amazingly, 21 of the 22 mitochondrial DNA genes for tRNAs can harbor mutations [2, 3], with the gene for tRNA\textsuperscript{Leu(UUR)} experiencing the largest number of potential mutations (18 in 17 different positions). These mutations differ in origin and frequency. While point mutations of the mitochondrial genome are largely maternally inherited and relatively frequent, large-scale rearrangements (deletions and insertions) are fairly uncommon and often arise spontaneously.

Disorders attributed to mitochondrial DNA mutations are usually manifested as pathological lesions in the nervous system and skeletal muscle but can involve either a single organ (such as the eye in Leber hereditary optic neuropathy) or multiple organ systems. Because of the importance of electron transport chain flux and adenosine triphosphate (ATP) generation in the maintenance of myocardial contractile function, mitochondrial DNA mutations often lead to the development of myocardial remodeling and failure [4–6]. However, some mitochondrial mutations lead to catastrophic biochemical alterations that are incompatible with life and result in fatal infantile cardiomyopathies [6]. Because cells contain a mixture of normal and abnormal DNA, the severity of a given mitochondrial disorder depends upon the ratio of normal to mutant DNA.

Mutations in mitochondrial tRNA genes usually result in multisystem disorders, with the MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) syndrome being one of the most characterized multisystem disorders. The primary symptoms of the MELAS syndrome include lactic acidosis, episodic vomiting, seizures, hearing loss, migraine-like headaches, short stature, and recurrent cerebral insults that resemble a stroke. However, patients with the MELAS syndrome are also prone to the development of a cardiomyopathy [7, 8]. The primary lesions associated with the development of the MELAS syndrome are mutations A3243G and T3271C of the mitochondrial tRNA\textsuperscript{Leu(UUR)} gene. Nonetheless, other mutations in the tRNA\textsuperscript{Leu(UUR)} gene (G3244A, T3258C, T3291C) also trigger the development of the MELAS syndrome [9–12]. The syndrome can also arise from mitochondrial point mutations of respiratory chain components and from large-scale mitochondrial deletions [11]. Because cardiomyocytes of MELAS patients harbor both normal and mutant mitochondrial DNA, a threshold

Keywords
β-Alanine • Cardiomyopathy • MELAS syndrome • Mitochondria-encoded proteins • Mitochondrial disease • ND6 • Posttranscriptional modification • Respiratory chain activity • Taurine • Taurinomethyluridine • tRNA aminoacylation • tRNA\textsuperscript{Leu(UUR)} • Wobble nucleotide
percentage of mutant DNA must be present to initiate defects in respiration and the appearance of clinical symptoms.

Cybrid lines and tissue containing mutated tRNA$^{Leu(UUR)}$ genes generally exhibit decreased rates of mitochondrial protein synthesis, respiratory chain deficiency, reduced mitochondrial electrochemical potential, and diminished rates of respiration [13–16]. The onset of these events is initiated by malfunction of the mutated tRNAs. Three theories have been advanced to explain the effect of tRNA mutations on mitochondrial protein synthesis and respiratory chain activity. One theory attributes the decline in mitochondrial protein synthesis to impaired aminoacylation of the tRNA. The second theory attributes impaired mitochondrial function to reduced rates of transcription [17]. The other major theory implicates tRNA wobble modification deficiency in the development of mitochondrial dysfunction.

Role of Reduced Aminoacylation and Impaired Transcription in MELAS-Associated Mutations

The structure and function of cytosolic and mitochondrial tRNAs depend upon posttranscriptional modification of key nucleotides. Approximately 10% of the nucleotides in cytosolic tRNAs and 6–12% in mitochondrial tRNAs undergo posttranscriptional modification [18]. Figure 1 shows the clover-leaf structure and sequence of human mitochondrial tRNA$^{Leu(UUR)}$. Among the notable posttranscriptional modifications of tRNA$^{Leu(UUR)}$ are base methylation ($m^1$U, $m^2$G, etc.), dihydrouridine (D), pseudouridine ($\Psi$), taurinomethyluridine ($U^*$), ribothymidine (T). The tRNA assumes a clover-leaf configuration with several distinct regions. Shown are five MELAS mutations (A3243G, G3244A, T3258C, T3271C, and T3291C) that cause a wobble modification defect (failure to convert U to $U^*$).

![Fig. 1 Secondary structure of human tRNA$^{Leu(UUR)}$.](image-url) Human tRNA$^{Leu(UUR)}$ contains nine nucleotides that undergo posttranscriptional modification [methylation – $m^1$U, $m^2$G, etc.; dihydrouridine (D), pseudouridine ($\Psi$), taurinomethyluridine ($U^*$), ribothymidine (T)]. The tRNA assumes a clover-leaf configuration with several distinct regions. Shown are five MELAS mutations (A3243G, G3244A, T3258C, T3271C, and T3291C) that cause a wobble modification defect (failure to convert U to $U^*$).
S.W. Schaffer and C.J. Jong

Wobble Modification Defect in the MELAS Syndrome

One of the most important posttranscriptional modifications of tRNA^{Leu(UUR)} involves the taurinomethylation of a uridine base situated in the wobble position of the anticodon (Figs. 1 and 2). Using an E. coli cell-free translation system, Takai et al. [23] found that the modification of the wobble base of tRNA^{Ser} leads to enhanced wobbling, implying that the posttranscriptional modification of the wobble position affects the reading of the codon. This occurs because the posttranscriptional modification of uridine located in the wobble position affects the unusual base-pairing at the wobble position, allowing expansion of the decoding capability of the tRNA [24]. In the case of tRNA^{Leu(UUR)}, taurinomethylation of uridine in the wobble position permits equal decoding of UUA and UUG, while tRNA^{Leu(UUR)} lacking the taurinomethylation modification preferentially decodes UUA [25]. Interestingly, the posttranscriptional taurinomethyl modification of the wobble base is dramatically reduced in cybrid cells containing tRNA^{Leu(UUR)}, harboring one of five mutations associated with the MELAS syndrome (A3243G, G3244A, T3258C, T3271C, and T3291C) [12, 26]. This effect is important because mutated tRNA^{Leu(UUR)} lacking the wobble modification shows weak binding of the anticodon to the UUG codon but not to the UUA codon.

The formation of a stable and geometrically favorable codon–anticodon interaction allows translation to proceed. However, mismatches in the interaction alter the codon–anticodon mimetic structure, which slows the process of translation and can actually terminate it. Nonetheless, the ribosome accommodates most modifications in the wobble position. While the first two positions of the anticodon are restricted by Watson–Crick canonical base-pair interactions, the wobble position allows considerable flexibility. As a result, the synthesis of mitochondria-encoded proteins, whose mRNA is rich in UUG codons, proceeds uninterrupted in normal tissue. However, tRNA^{Leu(UUR)} mutations that restrict the wobble modification, such as those often seen in the MELAS syndrome, exhibit reduced rates in the synthesis of UUG-dependent mitochondria-encoded proteins. The respiratory chain complex I subunit, ND6, is unique among the mitochondria-encoded proteins because its mRNA is rich in UUG codons and its synthesis is highly dependent on the wobble modification. Thus, it is not surprising that Hayashi et al. [27] found that cybrid cells containing tRNA^{Leu(UUR)} harboring the MELAS 3271 mutation exhibit depressed respiratory chain complex I activity and impaired synthesis of ND6. It is noteworthy that overall protein synthesis is not appreciably altered by the T3271C tRNA^{Leu(UUR)} mutation, suggesting that the T3271C mutation only causes distinct changes in mitochondrial function triggered by defects in the wobble modification and the synthesis of proteins whose mRNA harbors a high UUG content [27]. These data support the view that tRNA^{Leu(UUR)}
MELAS Syndrome: Mediated by Impaired Taurinomethyluridine Synthesis

mutations initiate a sequence of events that begin with a reduction in taurinomethylation of the wobble base and proceed to the reduction in UUG-dependent protein synthesis, slowing in electron transport and the development of clinical symptoms of the MELAS syndrome [12].

Taurine Deficiency Triggers a MELAS-Like Condition

Taurine is a β-amino acid found in very high concentration in excitable tissues [28]. The reaction that links taurine to the MELAS syndrome is the taurinomethylation of uridine located in the wobble position of tRNA_{Leu}^{UUR}. The posttranscriptional modification of the wobble base stabilizes the UG base pairing of the codon–anticodon complex, thereby facilitating the decoding of UUG codons. Although the conjugation enzyme responsible for the taurinomethylation reaction has not been identified, it is reasonable to assume that the formation of taurinomethyluridine depends upon the ability of the conjugation enzyme to recognize taurine and tRNA_{Leu}^{UUR} as substrates and the availability of taurine to bind to the active site of the enzyme. Taurine depletion presumably reduces the levels of taurine below the Km of the conjugation enzyme, thereby diminishing the wobble modification. Like MELAS mutations, reductions in taurinomethyluridine content as a result of taurine depletion reduce UUG decoding and produce a MELAS-like syndrome. However, taurine depletion is unlikely to promote other complications of the MELAS syndrome, such as defects in aminoacylation and in other posttranscriptional modifications that alter the structure and stability of the tRNA.

Three models have been developed to examine the effects of cellular taurine depletion: a nutritional model of taurine depletion in certain species, a model produced by knocking out the taurine transporter, and a model dependent on the inhibition of the taurine transporter by taurine analogues. All three models lead to the development of a cardiomyopathy [29–31]. However, mitochondrial function has only been evaluated in the latter model. In that model, 50% of cellular taurine levels are lost upon treatment of isolated cardiomyocytes with medium containing the taurine analogue, β-alanine (5 mM) [32]. The decline in taurine content is associated with a decrease in the levels of specific mitochondria-encoded proteins, with ND6 suffering the greatest decline in cellular content (Fig. 3). However, there is a poor correlation between the number of UUG codons in a protein’s mRNA and the cellular level of that protein. While the number of UUG codons in rat ND6, ND5, and COI (cytochrome c oxidase I) is 6, 2, and 0, respectively, the decline in the levels of the three proteins in response to taurine depletion in rat cardiomyocytes is 40%, 30% and 0%, respectively [26]. Thus, like the MELAS syndrome, taurine depletion appears to reduce the synthesis of mitochondria-encoded proteins with a high UUG codon content.
The codon/UUR codon ratio, but the relationship between the protein synthetic rate and UUG codon content is not linear [2, 33]. One interesting feature of the taurine depletion models is that they serve as models of the MELAS syndrome because they mimic the defects arising from a wobble modification deficiency. However, taurine deficiency does not mimic the features of the MELAS syndrome caused by changes in the status of other posttranscriptional modifications or in aminoacylation defects. Although MELAS patients are prone to the development of a cardiomyopathy, the importance of the mitochondrial defects toward the development of the taurine-deficient cardiomyopathy remains to be established.

It is widely recognized that the rate of mitochondria-encoded protein synthesis is a determinant of respiratory chain activity [34]. Thus, it is not surprising that taurine depletion is associated with a 30% decrease in respiration, a 50% decline in complex I activity, and a 65% reduction in complex III activity (Figs. 4 and 5). It is significant to note that like taurine depletion, the A3243G tRNA<sup>Leu(UUR)</sup> MELAS mutation also leads to reductions in complex I activity and oxygen consumption [14, 33].

Although the mechanism underlying the development of the MELAS disorder and other mitochondrial diseases has not been definitively established, it is known that impaired electron transport chain function causes both a loss in ATP generation and enhanced production of reactive oxygen species [34]. Indeed, some mitochondrial mutations also lead to enhanced oxidative stress [35].

Conclusions

The MELAS disorder is associated with a complex web of interacting factors that contribute to the pathological and biochemical alterations of the mitochondrial disease. With so many events affecting respiratory function, it is not surprising that the identity of the distinct events that initiate the onset of the mitochondrial disorder remain to be established. Clearly, the use of a simplified model, such as the taurine deficiency model, helps in uncovering the identity of distinct pathophysiological pathways initiated by MELAS mutations.

Acknowledgments The present study was supported with a grant from the American Heart Association.
References


MELAS Syndrome: Mediated by Impaired Taurinomethyluridine Synthesis

transform


Abstract
Numerous teams are trying to find a genetic determination of individuals with high risk of myocardial infarction. Originally more attention was paid to candidate genes of atherosclerosis risk factors – hypercholesterolemia and hypertension. Out of several hundreds of potential genes involved, only effect of polymorphism of apolipoprotein E was reproduced in different settings and populations. Attention was focused to genes involved in monocyte behavior, arterial wall metabolism, and thromboembolic process more recently. Still the significant effect of few genes is low (OR 1.2–1.7), and in addition reproducibility is very low. Genome-wide scan has been used recently and most discriminative part was identified in chromosome 9 (but without any known protein function). Low reproducibility in different projects is probably related to low number of cases and controls. On the other hand, project with several thousands of individuals in compared groups suffered from inadequate characteristics of individuals, and gene environment interaction might also have substantial effect in reproducibility. Participation of high number of candidate genes with very small or negligible effect is supposed.

Keywords
Acute coronary syndrome • Candidate genes • Gene–environment interaction • Genetics • Myocardial infarction • Wide genome scan

Introduction
Myocardial infarction (MI) is the most common cause of death in almost all industrialized countries, and it is becoming the leading reason of death also in most developing countries. It is not only that this event is most prevalent but it is fatal usually for almost one-third of individuals when
it is first diagnosed. It is a complex clinical complication as a consequence of long-lasting development of atherosclerosis combined with an acute thrombosis. MI occurs when thrombosis is precipitated by ruptured atherosclerotic plaque leading to obstruction of an artery and consequently ischemia followed by necrosis of a part myocardium. Similar to other pathologies, these atherothrombotic events have a part in environmental effects but also a genetic background. Genetic predisposition has been known for almost a century as a positive family history of cardiovascular disease is one of the important risk factors of MI. It is well known that in addition to atherosclerosis, risk factors like dyslipoproteinemia, hypertension, insulin resistance, smoking and age, and family history of parental MI play very important role of MI risk even after adjustment for other risk factors. The importance of genetic compartment of MI was proved also in large twin study [1, 2].

Fast development of methods of molecular genetics resulted in enormous scientific activities searching for genes responsible for a genetic predisposition of MI. Several monogenic disorders have been identified with high relative risk of MI but they are very rare and represent a very small proportion of all MI. The most common form of MI are examples of multifactorial disease resulting as a combination of variants within many genes but each of them with a relatively small effect sometimes difficult to detect. Two main approaches have been used to identify genes involved in the genetic risk of MI – gene association studies and more recently genome-wide linkage.

### Genetic Component of MI Due to Genetic Determination of One of the Risk Factors

#### Dyslipoproteinemia and Hypertension

Three main risk factors for premature MI (dyslipoproteinemia, hypertension, and insulin resistance) have been known for almost half a century. Their genetic predisposition is of course in relation to all other effects. As increased concentration of atherogenic low-density lipoprotein (LDL) particles is one of the main and most frequently studied risks of MI, it is not surprising that its genetic determination attracts interest of geneticists. Familial hypercholesterolemia discovered in the sixties of the last century is the best example of substantial increased risk of MI. Individuals with familial cholesterolemia displaying high cholesterol concentration practically independently of diet and defects in LDL receptors are playing a dominant role in the appearance of high-risk phenotype. Homozygotes with this genetic defect displaying total cholesterol concentration of more than 20 mmol/L die at children age (without any other risk factors with the exception of high LDL) and they are the best example of genetic risk of MI. Fortunately the frequency of this metabolic defect is very small (1:1,000,000). But also heterozygotes of this disease (frequency 1:500) are at high risk of premature MI. Identification of families with a defect of the function of LDL receptor in the majority of industrially developed countries is successful, and its treatment starting in childhood and pharmacotherapy with very potent statins is one of the most important successes of application of genetics in practical medicine. Unfortunately, the majority of genetically predisposed high sensitivity to diet-increasing LDL cholesterol concentration is polygenic. Most frequently studied candidate genes for high intravasal LDL cholesterol concentration (apoB, apoE, HMgCoR, LDLr, PCSK9, ABCA1, LPL) were proved to be involved not only in the risk of hypercholesterolemia but also in the risk of MI [3]. Although numerous genes were identified to influence high-density lipoprotein (HDL) cholesterol concentrations, their effect on risk of MI has not been found with the exception of Copenhagen City Heart Study. In this large prospective study, a small but significant effect of polymorphisms in the ABCA receptor gene family (participating in HDL and reverse cholesterol transport) on MI risk was found [4].

Also genetic factors or other risk factors of atherosclerosis and MI have been studied for
almost three decades. Understandably, increased blood pressure is a complicated network of numerous pathophysiological defects involving numerous genes participating in this pathology. High number of candidate genes has been identified also for diabetes and insulin insufficiency [5], but similar to hypercholesterolemia and hypertension only few of the candidate genes were confirmed in different population studies.

Genetics of hypertension is the most frequent pathology in older age and represents a very important risk of MI. More detailed description of genetic background of dyslipoproteinemia and hypertension is presented in another chapter of this book.

**Obesity and Insulin Resistance**

The importance of visceral obesity and metabolic syndrome in acceleration of atherogenesis and risk of MI has increased during the last three decades. Cardiometabolic risk was defined as a combination of central obesity, high blood pressure, increased concentration of triacylglycerols and decreased HDL cholesterol concentration, and a decrease of insulin sensitivity [6]. It has been shown recently as a very strong individual risk for MI with hazard ratio of 2.40 for men and even 3.84 for women compared to individuals who do not have this phenotype [7]. We were able to document a shift from the dominating risk of LDL cholesterol concentration to substantial effect of low HDL and central obesity in the Czech population during last three decades. Numerous candidate genes have been found participating in the central obesity phenotype and high triacylglycerol and low HDL concentrations. For example, polymorphism in antipoietic-like protein is independent of lipid concentration, and a large prospective study demonstrated an increase of the risk ratio of MI to 1.48 [8]. FTO gene is one of the most documented genes participating in the increase of body mass index (BMI). Gene variants in this locus are also increasing risk in MI development but, in addition, it also increases total mortality independent of fatness [9].

**Genetics of Proinflammatory Status**

Presence of a proinflammatory status (and its genetic part) has been determined very frequently during the last decade after C-reactive protein (CRP) concentration was proved as an independent risk factor for MI. Although CRP (as a marker of proinflammation) concentration is related to numerous environmental and metabolic effects, namely obesity and visceral fat volume, it is also under genetic control and several genes might be involved. Central obesity is influencing a risk of MI not only due to insulin-resistance-related dyslipoproteinemia, but it can also influence a risk of MI more directly due to an increase of the proinflammatory status. The effect of proinflammatory status risk measured as high sensitivity determination of CRP (hsCRP) has been documented more than 10 years ago but its genetic component has not been discovered yet although its heritability is known.

We have documented that proinflammatory status is significantly increased in siblings (both sons and daughters) of young patients with coronary atherosclerosis (compared to age-matched controls) [10]. Analyzing polymorphisms in seven candidate genes related to cholesterol metabolism and proinflammatory status (including IL-6, TNF, IL-20, HMGCoR, and apoE) [11], Licastro identified individuals with low and high risk using a statistical model. Proinflammatory gene variants taken together determine an individual risk for MI, especially in young age. It is not due to a similar environmental effect in the family as patient’s wife did not differ in CRP concentration from age-matched controls. We were also able to prove participation of two genes determining proinflammatory status and influencing CRP concentration. Both genes were related to lipoprotein metabolism (genes for apoCI and apoE) [10]. Participation of three other genes related directly to the CRP gene was described in two large studies. With application of rather complicated statistical model, the other group of authors was able to prove the effect of participation of few other genes related to diabetes and proinflammation status (IL-6, IL-10, and TNF). Although no marker of proinflammatory
status was included in this study, the author concludes participation of these genes related to inflammation.

Because CRP concentration is influenced by BMI, it is not surprising that genes related to BMI play a role in MI risk [10]. Recently fatness-associated FTO gene variants were shown to increase mortality independently of fatness [9]. We have recently also documented that this gene polymorphism increases the risk of MI and this significant effect was also unrelated to BMI [13].

Looking for the Candidate Gene of MI

For more than 10 years, numerous research groups have searched for MI candidate gene using gene-association method [14]. On the other hand, low consistency and reproducibility of already published association of individual candidate gene alleles leads to a certain doubt if genetic epidemiology is able to bring additional information to family history in the identification of individuals with increased risk of premature MI.

An exponential increase in the number of MI candidate gene studies over this period would expect a substantial increase in the understanding of genetic predisposition of MI. The original idea of genetic epidemiology was to construct a substantial tool of clinical value of identifying individuals with higher risk of this disease. Now it is evident that there is only a relatively small effect of association of any single genotype with premature MI, ranging usually between 1.1 and 1.7 (OR, odds ratio) [14, 15]. It is understandable partly due to the multifactorial effect of coronary artery disease development combined with different methodological approach to its clinical complication. Genes related to different risk factors of atherosclerosis as well as genes related to thrombosis and arterial wall metabolism might be involved.

A list of most frequently studied candidate genes is in Table 1. This list of genes might document also a distribution of interest in different parts of atherothrombosis disease: its risk factors, reactivity of arterial wall, monocyte characteristic, and later thrombotic events. A dominant portion of candidate genes studied is related to lipoprotein metabolism (15%) and inflammation (16%), followed by genes determining arterial wall behavior after atherogenic stimuli (12%) and then to blood pressure regulation, monocyte activity, oxidation, and coagulation (6–8%). Whereas in the late nineties attention was focused on lipoprotein metabolism [1, 15] and blood pressure regulation, more attention has been paid to inflammation and atherothrombosis-regulating genes [11, 16].

Our experiences are similar to other groups applying epidemiology genetics to study a genetic background of MI. We compared 1,399 patients with transmural and non-transmural premature MI (under 65 years in men and 75 years in women) with quite complete clinical, biochemical, anthropometric, and socioeconomic data, and we compare them with sex and age-matched individuals selected from a large representative sample of the same (Czech) population. Data obtained from patients and the control group are identical and their collection is based on the WHO MONICA Study. (More than 20 populations over the world were followed for development of cardiovascular risk factors using the same methodology.) Out of numerous polymorphisms analyzed till now, significant influence of only two genes – apolipoprotein E and FTO (related to obesity) – were proved to participate in the individual risk in this case-control study [13, 17].

Fast development of molecular genetics finally drives attention to constructing genetic chips that allow to analyze hundreds or thousands of variants within genes in one study. Although this method is rather expensive, an engagement of private companies already helps to solve this disadvantage. Recent analysis of possible participation of more than 17,000 SNPs in MI was published by a group of Cardiovascular Research Institute, UCSF [18]. They used analysis of pooled DNA samples in three independent case-control studies and identified 5 SNPs in four genes associated with MI. One is related to thrombus formation which is critical to this clinical complication, and two others related to mitochondrial oxidation, and the last one with high Lp(a) concentration or probably to “more deleterious” form of Lp(a). Unfortunately, none of these genes was confirmed by the first whole gene scan [19].
Table 1  List of the most frequently analyzed candidate genes for premature myocardial infarction

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name : gene function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette A1 participating in HDL metabolism</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1 : vasodilator</td>
</tr>
<tr>
<td>ADD1</td>
<td>Adducin 1 (α) : binds calciomodulin</td>
</tr>
<tr>
<td>AGT</td>
<td>Angiotensinogen : cofactor</td>
</tr>
<tr>
<td>AGTR1</td>
<td>Angiotensin II receptor, type 1</td>
</tr>
<tr>
<td>ALOX5AP</td>
<td>Arachidonate 5-lipoxygenase-activating protein</td>
</tr>
<tr>
<td>ANGPT L 40 K</td>
<td>Angiogenic-related protein participating in fatty acid metabolism</td>
</tr>
<tr>
<td>APOA1</td>
<td>Apolipoprotein A-I : participates in reverse cholesterol transport</td>
</tr>
<tr>
<td>APOA5</td>
<td>Apolipoprotein A5 participating in triglyceride-rich lipoproteins</td>
</tr>
<tr>
<td>APOE</td>
<td>Apolipoprotein E : ligand for apoB/E receptor</td>
</tr>
<tr>
<td>CCL11</td>
<td>Chemokine (C-C motif) ligand 11 : inflammatory reaction</td>
</tr>
<tr>
<td>CCR2</td>
<td>Chemokine (C-C motif) receptor 2 : chemikinese receptor</td>
</tr>
<tr>
<td>CCR5</td>
<td>Chemokine (C-C motif) receptor 5 : immune response</td>
</tr>
<tr>
<td>CD14</td>
<td>CD14 molecule: LPS receptor on monocyte membrane</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesteryl ester transfer protein : LPS exchange in plasma compartment</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-methyltransferase : inactivation of catacholamine hormones</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>Chemokine (C-X3-C motif) receptor 1 : mediates adhesive and migratory function</td>
</tr>
<tr>
<td>CYP11B2</td>
<td>Cytochrome P450 : corticosterone metabolism</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Cytochrome P450 : monoxygenase</td>
</tr>
<tr>
<td>ENPP1</td>
<td>Ectonucleotide pyrophosphatase/phosphodiesterase 1 : soft tissue calcification</td>
</tr>
<tr>
<td>ESR1</td>
<td>Estrogen receptor 1 : cellular proliferation</td>
</tr>
<tr>
<td>F12</td>
<td>Coagulation factor XII : blood coagulation, fibrinolysis</td>
</tr>
<tr>
<td>F13A1</td>
<td>Coagulation factor XIII : stabilazing fibrin clot</td>
</tr>
<tr>
<td>F2</td>
<td>Coagulation factor II (thrombin) : blood homeostasis, inflammation</td>
</tr>
<tr>
<td>F5</td>
<td>Coagulation factor V : activation of prothrombin</td>
</tr>
<tr>
<td>FGB</td>
<td>Fibrinogen β-chain : cofactor of platelet aggregation</td>
</tr>
<tr>
<td>FTO</td>
<td>Fatness-associated gene</td>
</tr>
<tr>
<td>GJA4</td>
<td>Gap junction protein : alfa connexon</td>
</tr>
<tr>
<td>GP1BA</td>
<td>Glycoprotein Ib (platelet) : formation of platelet plaques</td>
</tr>
<tr>
<td>GRL (NR3C1)</td>
<td>Nuclear receptor subfamily 3 : inflammatory response</td>
</tr>
<tr>
<td>GSTT1</td>
<td>Glutathione transferase participating in lipoprotein oxidation</td>
</tr>
<tr>
<td>HFE</td>
<td>Hemochromatosis : binding to transferrin receptor</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone-sensitive lipase regulating fatty acid release from adipocytes</td>
</tr>
<tr>
<td>HNF1</td>
<td>Hepatic nuclear factor, inflammation, influence on CRP gene</td>
</tr>
<tr>
<td>HTR2A</td>
<td>5-Hydroxytryptamine (serotonin) receptor 2A : involved in contraction</td>
</tr>
<tr>
<td>ICAM1</td>
<td>Intercellular adhesion molecule 1 : adhesion molecule for monocytes</td>
</tr>
<tr>
<td>IL1B</td>
<td>Interleukin 1, β : inflammatory response</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin 6 (interferon, β 2) : monocyte differentiation and inflammation</td>
</tr>
<tr>
<td>IL18</td>
<td>Interleukin 18, immune response</td>
</tr>
<tr>
<td>ITGA2</td>
<td>Integrin, α 2 : adhesion of platelets to collagen</td>
</tr>
<tr>
<td>ITGB3</td>
<td>Integrin, β 3 : binding of metalproteinase</td>
</tr>
<tr>
<td>KIF6</td>
<td>Kinesin-like protein 6 involved in cytokinesis and microtubule transport</td>
</tr>
<tr>
<td>LDL R</td>
<td>LDL receptor, cell-surface receptor binding apoB/E</td>
</tr>
<tr>
<td>LIPC</td>
<td>Hepatic lipase : important for HDL metabolism</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase : involved in triglyceride-rich lipoproteins</td>
</tr>
<tr>
<td>LRP1</td>
<td>Low-density lipoprotein-related protein 1 : clearance of lipoprotein remnants</td>
</tr>
<tr>
<td>MGP</td>
<td>Matrix Gla protein : associates with organic matrix of bone</td>
</tr>
</tbody>
</table>

(continued)
as none of them was genotyped. It is a similar situation to participation of gene for apoE \([19, 20]\) as this most frequently proved candidate gene for MI was not included in the chip for the whole gene scan in any study published yet.

A new approach to analyze the genetic background of MI was presented recently. These authors stated that SNPs in some biomarkers provide new tools for investigating a causal relationship with the disease \([21]\). Analyzing almost a thousand of SNPs, they found in addition to the effect of already proved lipid relation genes (apoB, LDL-R, CETP, apoAIV, and PCSK9) an association with several gene polymorphisms related to a biomarker of inflammation and for CRP concentration.

### Gene Score

An alternative approach is to construct a set of selected candidate genes with a well documented effect and to calculate certain gene score to increase the informative value of molecular genetics for clinical practice. The best example was published by the group of University College London \([21]\). Humphries and his coworkers selected genes repeatedly proved to participate in the risk of MI. This set of candidate genes makes it possible to calculate a score which is as useful in analyzing individual risk as the complete set of traditional risk factors (LDL-C, HDL-C, BP, sex).

Steve Humphries and coworkers from London followed more than 2,000 men in the prospective Northwick Park Heart Study II for 10 years. In stepwise multivariate risk analysis, they included 12 genes previously associated with MI risk \([21]\). Four of them remain in the model at the end – uncoupling protein 2, apolipoproteins A4 and E, and lipoprotein lipase. The combined discriminative effect of all these four genes was almost identical compared to the effect of most important traditional risk factors (age, triacylglycerols, and cholesterol concentration and blood pressure). Combining the effect of candidate risk factors with genotype improved significantly dissociation of cases and controls \((p=0.001)\). This approach seems to be potentially possible for application in practical medicine.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name : gene function</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP3</td>
<td>Matrix metalloepitidase (stromelysin1) : degradation of collagen and proteoglycans</td>
</tr>
<tr>
<td>MTHFR</td>
<td>5,10-Methenyltetrahydrofolate reductase : homocysteine metabolism</td>
</tr>
<tr>
<td>MTP</td>
<td>Microsomal triglyceride transfer protein : regulation of apoB containing LP in liver</td>
</tr>
<tr>
<td>MTR</td>
<td>5-Methylenetetrahydrofolate-homocysteine methyltransferase : homocysteine metabolism</td>
</tr>
<tr>
<td>OLR1</td>
<td>Oxidized low-density lipoprotein receptor 1 : internalization of ox-LDL</td>
</tr>
<tr>
<td>p22-PHOX (CYBA)</td>
<td>Cytochrome b-245, α polypeptide : generates superoxide</td>
</tr>
<tr>
<td>PAI1 (SERPINE1)</td>
<td>Serpin peptidase inhibitor, plasminogen activator inhibitor : regulation of fibrinolysis</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet/endothelial cell adhesion molecule : transendothelial migration of monocytes</td>
</tr>
<tr>
<td>PON 1 + 2</td>
<td>Paraoxonase 1 and 2 : prevents LDL oxidation</td>
</tr>
<tr>
<td>PPARG</td>
<td>Peroxisome proliferator-activation glucose homeostasis, adipocyte differentiation</td>
</tr>
<tr>
<td>PTGS2</td>
<td>Prostaglandin-endoperoxide synthase 2 : mediator of inflammation</td>
</tr>
<tr>
<td>RECL (WRN)</td>
<td>Werner syndrome : cell junction</td>
</tr>
<tr>
<td>SELE</td>
<td>Selectin E : immunoadhesion</td>
</tr>
<tr>
<td>SELP</td>
<td>Selectin P : inflammation, monocyte reaction</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)</td>
</tr>
<tr>
<td>THBD</td>
<td>Thrombomodulin : endothelial cell receptor, cell-to-cell interaction</td>
</tr>
<tr>
<td>THPO</td>
<td>Thrombopoietin : regulator of circulating platelet reaction</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4 : immune response to lipopolysaccharides</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor : inflammation factor secreted by macrophages</td>
</tr>
<tr>
<td>TNFRSF1A</td>
<td>Tumor necrosis factor : signal function in inflammation reaction</td>
</tr>
<tr>
<td>UCP-2</td>
<td>Uncoupling protein 2 participating in lipoprotein oxidation</td>
</tr>
</tbody>
</table>
Genetics of Myocardial Infarction

The Whole Genome Scan

As attempts of identifying genetic variants that affect the risk of MI have been hampered by poor reproducibility of identified candidate genes in one population, an alternative approach was completed during the last years [19, 22, 23]. Technological advances have led to the development of relatively inexpensive genotyping arrays that contain thousands of variants covering the majority of the genome.

Genome-wide scan has been used to study several pathologies recently and this approach has reached a really exponential gradient. While there were only two GWS published in 2000, 1,252 papers were published till now. More than 50% of these papers studied genetic predisposition of risks factors related to cardiovascular disease, but only 10% were related to atherosclerosis and its clinical complications. Only a few of them tried to analyze the individual risk of MI as it is understandable that this problem of multifactorial disease is much more complicated to elucidate.

Well-powered genome-wide scan has now identified several novel putative loci that increase the risk of MI. In 2007 two independent genome-wide scan completed in two populations presented strong association of a common variation in the position of chromosome 9p21[21–23] with MI. Another scan of whole genome in two European studies identified 7 chromosome loci with statistically significant association with coronary artery disease displaying a certain association with classical risk factors of MI (e.g., locus on chromosome 1 is strongly associated with LDL cholesterol concentration). The most significant effect was identified on chromosome 9 and this result is in agreement with earlier published studies. This association in chromosome 9 has not demonstrated any relation to hypercholesterolemia, hypertension, and diabetes. Recently two large genome-wide scans analyzing individual risk of MI were published [19, 21]. They identified four positions highly significantly different from the control group. The first one is in position 9p21.3 with OR 1.20 (CI 1.16–1.25). The other one with similar OR displays a signal in positions 1p13.3, 1q41, and 10q11.21.

Reproducibility of Results

The most important problem of case-control association studies is their rather low reproducibility. Very often a significant difference in genotype frequencies of MI survivors group and controls published is not reproduced in other studies [14]. What might be the reason for this low reproducibility? One possible reason (applied mainly to earlier studies) is in the very small size of groups compared. A significant difference after comparison of a low number of cases with the same size of “healthy controls” might be only a product of small number of genotypes in each of the compared groups. Recently, the majority of case-control studies comprise around several hundreds of individuals, so the probability of random positive effect is much smaller [18]. On the other hand, large studies of several thousands of MI survivors and controls are negatively influenced by inadequate quality and non-homogeneity of data of individuals included that might be another source of false results. This type of studies is usually based on a collection of different projects analyzed in the same laboratory. Also, construction of a control group might influence the results of case-control gene-association studies. So called “healthy control” selected from different databases need not be the real control group. Probably a better way is to select age- and sex-matched control from a large representative population sample of the same population as cases-MI survivors. Large prospective studies in homogeneous populations represent probably the best methodological approach if the number of cases ranges in several hundreds and controls are matched from the same project.

Gene–Environment Interaction

Gene–environment interaction has been intensively studied during the last decade [24–26]. Numerous interactions at different gene locuses have been documented leading to a new era of nutrigenomics and pharmacogenomics.

It is understandable that different lifestyle conditions in different populations might also
influence a lower reproducibility of different candidate genes participating in early onset of MI. For example, low saturated fatty acid intake in Japanese population together with high intake of protective n3 fatty acids and high vegetable intake are potentially able to cover differences in candidate genes related to lipoprotein metabolism. On the other hand, a large prevalence of smoking in this population might influence the comparison to the results of population with lower smoking prevalence but high LDL concentration and less healthy diet.

In our analysis of genetic predisposition to acute coronary syndrome, we compared 1,399 consecutive patients from five coronary care units in Prague to controls selected from 1% population sample of the same Czech population polymorphism in four genes. Polymorphism in connexin-37, stromelysin-1, PAI-1, and lymphotixin-α were proved in predisposition to MI in Japanese population. Although our groups were larger and well defined, no association of any of these four genes was documented in our project [27].

We analyzed environmental interaction with the most frequently studied candidate gene for MI—apolipoprotein E [20, 28, 29]. Apolipoprotein E is a cell-surface protein of triacylglycerol-rich lipoproteins, and it is important ligand for apolipoprotein B/E receptor (LDL receptor) on the surface of human tissue cells [17]. Polymorphism of this gene (alleles 2, 3, and 4) influences lipoprotein metabolism, and individuals possessing E4 allele display higher LDL concentration. Genotypes with this disadvantage allele (E3/4 and E4/4) are at higher risk of premature MI and this fact was confirmed in several already published studies [20, 28, 29], whereas some of them failed to find this negative effect. We compared the prevalence of E4+ genotypes in 1,066 male individuals admitted for MI in five coronary care units in Prague under 65 years of age and compared them with the same number of age-matched controls selected from 1% population sample [17]. Methods and protocols of the study from the WHO MONICA Project were applied.

Frequency of the apoE4+ genotype was significantly higher (22.38%) compared to controls (16.76%). Then we gradually decreased the patient group size, eliminating smokers in the first step, diabetics in second one, and hypertones in the last step. Number of individuals included decreased to 350 (without smokers), then to 277 (after diabetics elimination), and to final group 129 (after additive elimination of hypertones). OR increased gradually from 1.36 for the whole group to final 1.71, and this trend was statistically significant. The final group where most genetic influence was expected displays significantly higher prevalence of family history of cardiovascular disease (measured as CVD mortality in the first-line relatives). It is an example of interaction of environmental and metabolic effects with apolipoprotein E as a candidate gene in the risk of premature MI, and it might explain different results in different populations.

Conclusions

After almost two decades looking for “the gene” for premature MI, it is evident that this gene has not been identified yet. There is also very low chance to use molecular genetic methods to identify individuals with a high risk of MI at this moment and to apply this tool to clinical practice. Probably a gene score might be partly informative for individual risk bringing additional information to analysis of risk factors including family history of cardiovascular disease. On the other hand, with all data obtained and published we are able to understand that MI is a complex pathology with participation of a large number of candidate genes with a relatively small effect. Molecular genetics data enlarge our understanding of this problem and we learned a lot. In addition, further progress of whole gene scan might be useful to understand an adaptation of human genes to substantial change of the environmental long-lasting effects [30]. Still we lack a useful tool of molecular genetics for medical practice in MI predisposition.

References

Genetic Background of Myocardial Infarction

Kouichi Ozaki and Toshihiro Tanaka

Abstract
Myocardial infarction (MI) is a common disease whose pathogenesis includes genetic factors, and it is among the leading causes of death. In 2000, we started a genome-wide association study (GWAS) for MI using nearly 90,000 gene-based single-nucleotide polymorphisms (SNPs), and identified lymphotoxin-a (LTA) conferring risk of MI in Japanese population. This was the first GWAS that identified a disease susceptibility gene in the world. Moreover, through examining the LTA cascade by combination of biological and genetic analyses, we have identified additional MI-susceptible genes, LGALS2, PSMA6, and BRAP, so far. We present here our recent work focused on identification and functional analyses of genes that confer risk of MI.

Keywords
Genetic risk factors • Myocardial infarction • Signaling molecule • Single-nucleotide polymorphism

Introduction
Coronary artery diseases (CADs), including myocardial infarction (MI), have been the major cause of mortality and morbidity among late-onset diseases in many industrialized countries with a Western lifestyle [1, 2]. MI often occurs without any preceding clinical signs and is followed by severe complications, especially ventricular fibrillation and cardiac rupture, which might result in sudden death. Although recent advances in treatment and diagnosis have greatly improved the quality of life for patients after MI, its morbidity is still high. MI is a disease of the vessel that feeds the cardiac muscle, called the coronary artery. Abrupt occlusion of the coronary artery results in irreversible damage to cardiac muscle. Plaque rupture with thrombosis is a well-established critical factor in the pathogenesis of MI [3, 4]. Although detailed mechanisms of plaque rupture are unknown, inflammation is thought to play an important role in its pathogenesis.
Inflammatory mediators like cytokines are involved in atheroma formation; rapid evolution of the atheromatous injury, leading to rupture of the plaque; and intraluminal thrombosis [5]. Epidemiological studies revealed that coronary risk factors include type 2 diabetes mellitus, hypercholesterolemia, hypertension, and obesity. Some studies reported a genetic factor; one reported that first-degree relatives of patients who have had an acute MI before age 55 have a two to seven times higher risk of MI [6]. A twin study indicated an eight-fold increase in risk of death from MI when a first twin dies of MI before age 55 [7]. Common genetic variants are believed to contribute to the genetic risk of disease [8–10]. In this context, we started genome-wide association studies (GWAS) of this disorder using nearly 90,000 gene-based SNPs (http://snp.ims.u-tokyo.ac.jp/) [11] by high-throughput multiplex PCR invader assay system [12], and identified several genes conferring risk of MI including LTA [13–15]. Although the roles of these susceptible genes in MI pathogenesis are under investigation, these findings showed the potent power of GWAS, which is hypothesis-free, to identify unexpected anchors to further understand the disease. Through examining the LTA cascade by combination of biological and genetic analyses, we have identified additional MI-susceptible genes [16–18]. In this chapter, we focus on our genetic association results and show that our initial hypothesis-free strategy unexpectedly revealed the importance of inflammation in the pathogenesis of MI.

**Genome-Wide Association Study (GWAS): Identification of LTA as a Susceptibility Gene for MI**

Through a large-scale case-control association study using 92,788 SNP markers, one SNP in the LTA, encoding an inflammatory cytokine lymphotoxin-a, (6p21.3) was identified as a candidate susceptibility locus for MI [13]. Following linkage disequilibrium, haplotype mapping and further functional analyses revealed that two functional SNPs (LTA intron 1 252A>G and exon 3 804 C>A) were in complete linkage disequilibrium in this locus and conferred risk of MI. Recently, we have further confirmed an association between MI and LTA exon 3 804 C>A SNP using larger sample sizes (approximately 2,833 case and 3,399 control subjects), and we obtained a similar association result ($P<0.001$; recessive association model) (Table 1). Furthermore, among white Europeans (in the Precocious Coronary Artery Disease [PROCARDIS] study), a transmission disequilibrium test analysis of 447 trio families with CAD demonstrated that the LTA 804 C allele (26 N-LTA) was excessively transmitted to affected offspring ($\chi^2 = 8.44, P=0.002$, recessive association model) [19].

### Table 1 Confirmation of association between MI and LTA exon3 SNP

<table>
<thead>
<tr>
<th>Genotype</th>
<th>MI(%)</th>
<th>CO(%)</th>
<th>$\chi^2$ (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon3 804 C/A, T26N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>1,028 (36.3)</td>
<td>1,333 (39.2)</td>
<td>AA vs. CC+CA</td>
</tr>
<tr>
<td>CA</td>
<td>1,318 (46.5)</td>
<td>1,630 (48.0)</td>
<td>23.31</td>
</tr>
<tr>
<td>AA</td>
<td>487 (17.2)</td>
<td>436 (12.0)</td>
<td>(0.0000014)</td>
</tr>
<tr>
<td>Total</td>
<td>2,833</td>
<td>3,399</td>
<td></td>
</tr>
</tbody>
</table>

**Association of the SNP in LGALS2: Encoding Galectin-2 that Interacts with LTA**

After identifying LTA as a novel genetic risk factor for MI, we searched for proteins that interact with LTA to better understand its role in the pathogenesis of this disease. Using both the *Escherichia coli* two-hybrid system and a phage-display method, we identified a protein, galectin-2, as a binding partner of LTA [16]. Because galectin-2 was shown to bind to LTA, we examined whether variations on *LGALS2* (encoding galectin-2) were also associated with susceptibility to MI. We found one SNP (3279 C>T) in intron 1 of *LGALS2*; this substitution represses the level of galectin-2 expression and shows a significant association with MI [16]. This genetic substitution seemed to affect the transcriptional level of
galectin-2, which led to altered secretion of LTA, thereby affecting the degree of inflammation. We also found that galectin-2 binds to tubulins, which are important components of microtubules, suggesting a role in intracellular trafficking. It is likely that LTA is another molecule that uses the microtubule cytoskeleton network for translocation, and galectin-2 mediates LTA trafficking through binding to microtubules [20, 21], although the precise role of galectin-2 in this trafficking machinery complex has yet to be elucidated (Fig. 1).

**A Functional SNP in PSMA6: Encoding Intercellular LTA Signaling Molecule Associated with MI**

Because LTA binding to its receptor strongly activates nuclear factor κB (NFκB) by proteosomal degradation of its inhibitory partner (IκB) protein [22], we hypothesized that the variation(s) in the genes encoding proteasomal proteins could confer risk of MI. The 20S proteasome, which is composed of 7α- and 10β-subunits, is the core particle for 26S proteasome system [23]. We selected tagSNPs with minor allele frequencies of more than 10% that covered most of haplotypes in the genomic region of genes encoding these subunits and found that one SNP (rs1048990) in the 5′-untranslated region of exon 1 (5′UTR -8 C>G) of PSMA6, encoding proteasome subunit alpha type 6 was significantly associated with MI (Table 2) [17]. The SNP, located within 5′UTR of exon 1 in this gene, enhanced the transcriptional level of PSMA6. Moreover, suppression of PSMA6 expression level using siRNA in cultured coronary vascular endothelial cells as well as T-lymphocyte cell line reduced activation of NFκB, a central mediator of inflammation, by stabilizing phosphorylated IκB. Thus, the levels of PSMA6 protein influence the degree of inflammation, indicating that PSMA6 SNP is a novel genetic risk factor for MI [17].

**SNPs in BRAP Associated with Risk of MI**

To further understand the molecular mechanism that confers risk of MI, we searched proteins that interact with galectin-2. By means of tandem affinity purification, followed by matrix-assisted
laser desorption/ionization-time of flight (MALDI/TOF) mass spectrometry analysis, we identified the BRAP, BRCA1-associated protein, as a possible binding partner of galectin-2 (Fig. 2a) [18]. We confirmed their interaction by coimmunoprecipitation experiments (Fig. 2b) and examined whether genetic variation in BRAP was associated with susceptibility to MI by resequencing of genomic DNA and followed by association study of selected tag SNPs. As an initial association study, we compared genotype frequencies of these tag SNPs in about 450 individuals with MI and 450 controls and found that one SNP rs3782886, in exon 5 (90A>G, R241R) of BRAP was significantly associated with MI ($P=0.0014$). Haplotypes based on these tag SNPs showed a less significant association with MI. rs11066001 was in very strong LD ($r^2=0.96$) with rs3782886, so we examined these two SNPs in 2,475 cases and 2,778 controls, and found strong associations with MI ($\chi^2=83.6$, $P=3.0 \times 10^{-18}$, OR = 1.48, by comparison of allele frequency of rs11066001; Table 3). To further confirm the association, we examined two additional panels, 862 cases and 1,113 controls from the Japanese population, and 349 cases and 994 controls from the Taiwanese population, and confirmed the association of rs11066001 and rs3782886 with MI in these two sets (Table 3).

According to HapMap data (http://www.hapmap.org) [24], minor allele frequencies of rs3782886 were 0.239 in Japanese in Tokyo and 0.148 in Han Chinese in Beijing, but this allele was observed in neither CEPH individuals (Utah residents with ancestry from northern and western Europe) nor Yoruba individuals from Ibadan, Nigeria. No information was available for rs11066001. We additionally examined a panel of 50 CEPH individuals and found that there was no variation at these two SNP loci. These results indicate that these SNPs are likely to be present only in Asian populations. However, the possibility cannot be excluded that other variations in this gene confer risk of MI in other populations. Because rs1041981 in LTA and rs7291467 in LGALS2 were associated with MI, as described above, we also performed logistic regression analysis for the combinatorial effect of rs11066001 (BRAP), rs1041981 (LTA exon 3 804 C>A SNP), and rs7291467 (LGALS2) on MI susceptibility. We did not find any evidence of gene–gene interactions as addition of a statistical interaction term showed no significance. The combinatorial effect was consistent with a multiplicative odds ratio model [18]. We also examined the possibility of confounding effects by age, gender, and classical risk factors within cases and found no obvious relationships between genotype and these factors. These results indicated that the significant SNP in BRAP is an independent risk factor of MI [18]. Using antibodies directly labeled with fluorescein (a-galectin-2) or rhodamine (a-BRAP), we examined subcellular localization of native galectin-2 and BRAP in human coronary artery smooth muscle cells (HCASMC) and found that these proteins colocalized in the cytoplasm and nucleus (Fig. 2c).

We also investigated whether the BRAP protein is

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Association between MI and exon1 SNP in PSMA6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>Numbers of MI</td>
</tr>
<tr>
<td>PSMA6 exon1–8 C&gt;G (rs1048990)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>1,134</td>
</tr>
<tr>
<td>CG</td>
<td>1,137</td>
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<td>GG</td>
<td>321</td>
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<td>$\chi^2$</td>
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<tr>
<td>$P$</td>
<td></td>
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<td>Odds ratio</td>
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<tr>
<td>95% CI</td>
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<tr>
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<tr>
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<td>Additive model</td>
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</table>
Fig. 2 Galectin-2 interacts with BRAP. (a) Isolation of TAP-tagged galectin-2 and interacting proteins. (b) Co-immunoprecipitation of Myc-tagged galectin-2 and S-tagged BRAP in COS7 cells. (c) Co-localization of endogenous galectin-2 with BRAP in HCASMC. (d) Expression and co-localization of galectin-2 and BRAP in the coronary atherectomy specimen. Single-labeled immunohistochemistry of serial sections of primary atherosclerotic lesions from human coronary arteries obtained by directional coronary atherectomy, stained with antihuman BRAP or antihuman SMC-actin. Magnification, ×65.5 (BRAP) and ×90 (SMC-actin). (e) Double-labeled immunohistochemical staining with anti-BRAP (brown) and galectin-2 (blue) antibodies. Magnification, ×89.5
in fact expressed in the myocardial infarction lesion, that is, the atherosclerotic lesion of the coronary artery. Immunoreactivities for BRAP were detected in the SMCs and macrophages in atherosclerotic plaques (Fig. 2d). Co-expression of BRAP and galectin-2 was also observed in the majority of polymorphic SMCs and activated macrophages by double-labeled immunohistochemistry (Fig. 2e).

The two SNPs in BRAP showing very strong associations did not cause amino acid substitutions. Therefore, we investigated whether these SNPs, rs11066001 (intron3 270A>G) and rs3782886 (exon5 90A>G; R241R), would affect BRAP expression by reporter gene analysis. A clone containing the intron3 270A allele showed approximately half of the transcriptional activity of the 270G allele or that of the BRAP promoter only. No allelic difference was observed in constructs containing the exon5 SNP [18]. To confirm these results, we cloned three tandem copies of the genomic segment including these SNPs, and obtained similar results. These results indicate that the substitution in intron3, but not the one in exon5, affected the transcription level of BRAP. We subsequently looked for nuclear factor(s) that might bind to oligonucleotides corresponding to genomic sequences of the 270A allele. No known protein was predicted to bind to this DNA segment by TFSEARCH program based on the TRANSFAC database. Using nuclear extracts from HCASMC, we observed one band in the lane corresponding to the A allele, indicating binding of a nuclear protein(s) to the A allele [18]. This result suggested that an unidentified nuclear factor(s) interacting with this genomic region might suppress transcription of BRAP and thereby play a role in the pathogenesis of MI. BRAP was originally identified as a protein that binds to the signal peptide of breast cancer suppressor protein BRCA1 [25], and is known to be an E3 ubiquitin ligase that associates with Ras and modulates mitogen-activated protein (MAP) kinase signaling through regulation of the scaffolding activity of KSR (kinase suppressor of Ras) [26]. The MAP kinase signaling pathway is well known as a regulator of cell survival, growth, differentiation, transformation, and production of proinflammatory factors [27, 28]. Galectin-2 was also implicated in the inflammatory pathway through interactions with lymphotoxin-α and tubulin proteins [16]. Thus, we examined whether the cellular level of BRAP protein could influence NFκB, a central transcription factor of inflammation [29]. An siRNA against BRAP significantly suppressed BRAP mRNA, and resulted in inhibition of NFκB activation in HCAEC [18]. This result implied that altered expression of BRAP might influence the expression levels of genes involved in the NFκB-dependent inflammatory pathway. Thus, it is conceivable that a higher expression level of BRAP with a minor allele of intron3 (G allele) might enhance the degree of inflammation through activation of NFκB protein, thereby playing an important role in the

<table>
<thead>
<tr>
<th>Study population</th>
<th>rs11066001</th>
<th>Comparison of allele frequency</th>
<th>rs3782886</th>
<th>Comparison of allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAFa</td>
<td>ORb (95% CI) P value c</td>
<td>MAFa</td>
<td>ORb (95% CI) P value c</td>
</tr>
<tr>
<td>Japanese</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First panel</td>
<td>0.34</td>
<td>1.48 (1.36–1.61) 3.0×10−18</td>
<td>0.35</td>
<td>1.42 (1.31–1.54) 2.8×10−15</td>
</tr>
<tr>
<td>Replication panel</td>
<td>0.34</td>
<td>1.46 (1.27–1.67) 4.4×10−4</td>
<td>0.36</td>
<td>1.50 (1.31–1.71) 1.8×10−7</td>
</tr>
<tr>
<td>Combined</td>
<td>0.34</td>
<td>1.47 (1.37–1.56) 1.3×10−24</td>
<td>0.35</td>
<td>1.44 (1.34–1.55) 7.0×10−23</td>
</tr>
<tr>
<td>Taiwanese</td>
<td>0.33</td>
<td>1.31 (1.09–1.58) 4.7×10−3</td>
<td>0.33</td>
<td>1.26 (1.05–1.52) 1.5×10−2</td>
</tr>
</tbody>
</table>

a Minor allele frequency
b Odds ratio
c Adjusted for Bonferroni’s correction in Japanese cohorts
pathogenesis of MI, although the relationship of BRAP protein with activation of coronary artery endothelial cells involved in plaque instability and/or rupture remains to be tested.

**Conclusion**

To date, we have identified two other loci (SNPs in MIAT, ITIH3) [14, 15] that confer increased risk of MI, and also have replicated the association for SNP locus on 9p21 previously identified by European GWAS [30, 31]. Each odds ratio of these genetic risk factors described here is relatively small (Fig. 3); however, by combination of these genetic risk factors, the odds ratio has risen by about 11 (Fig. 3). This result suggests that the combination of genetic risk factors strongly contributes to the pathogenesis of MI. Coronary artery disease attributable to atherosclerosis is a leading cause of MI in many countries. We believe that knowledge of genetic factors contributing to its pathogenesis provides a useful clue for development of diagnostic methods, treatments, and preventive measures through combinations of risk variants (for diagnostic methods) and clarification of the molecular mechanism in the pathogenesis of causative genes (for therapeutics) for this common but serious disorder.

**References**

Abstract

The renin–angiotensin system (RAS) plays an important role in regulating the main characteristics of cardiovascular functions. The aim of the study is to test possible associations of ACE I/D polymorphism with coronary artery disease (CAD) and diabetes evaluated together in 600 persons with coronaryography. Four groups of patients (the CAD + DM + patients with both CAD and diabetes, the CAD + DM – patients with CAD, the CAD – DM + with only diabetes, and the CAD – DM – without CAD as well as diabetes) were compared in ACE I/D polymorphism, intermediate phenotypes (hemodynamic and metabolic parameters), and pharmacological therapy. We proved a number of significant differences especially between the CAD + DM + and CAD – DM – groups. Although the patients had been treated according to their clinical state, we were able to prove significant differences between ACE I/D genotypes (in the model of heterozygote advantage) in these groups (hypertension, obesity, BMI, renal insufficiency, more cardiovascular risk factors, some inflammatory factors, glycemia, and lipid profile). The drugs were administrated more frequently to the DD + II carriers, which further supports the heterozygote advantage hypothesis tested in the study. We proved a heterozygote advantage model for ACE I/D polymorphism, CAD, and diabetes mellitus confirmed by associations with intermediate phenotypes and by therapy schedule.

Keywords

CAD • Diabetes mellitus • ACE I/D
Introduction

In many biological processes, evolutionary adaptations, and thus also complex diseases, there are certain gene pattern combinations together with environmental factors that correspond to what we can call a general predisposition. This makes the understanding of the underlying mechanisms very complicated. In contrast to monogenic disorders, the basic principles of genetics of complex disorders still remain to be elucidated. This is why the attitude of clinicians toward the results of genetic studies focused on genetics of complex diseases often suffers from unsubstantiated expectation over suspect “big factor genes” to severe scepticism to genetic background in diseases, such as essential hypertension or ischemic heart disease. It can be rather supposed that a certain allelic combination of frequent polymorphisms will be found in the population (possibly various loci in different patients with the same disease) that will be possible to be successfully associated with a disease.

Pathophysiological Features of the RAS System

The renin–angiotensin system (RAS) plays an important role in regulating the main characteristics of cardiovascular functions, arterial pressure, and blood volume [1]. In the classical (systemic) system, the enzyme renin is released into the circulation from kidney juxtaglomerular cells in response to sympathetic stimulation, renal artery hypotension, or decreased levels of sodium in the distal tubules [2]. Renin converts angiotensinogen (AGT) from the liver to the decapptide angiotensin (Ang) I, which in turn undergoes proteolytic cleavage to the biologically active octapeptide, Ang II. The latter step is carried out by the angiotensin-converting enzyme (ACE), which is highly expressed in vascular endothelium, particularly in the lungs. Alternatively, a recently identified carboxypeptidase, ACE2, cleaves one amino acid from either Ang I or Ang II [3–7], decreasing Ang II levels and increasing the metabolite Ang 1–7, which has vasodilator properties. Thus, the balance between ACE and ACE2 seems to be an important factor controlling Ang II levels. Ang II is further degraded by aminopeptidases to Ang III (Ang 2–8) and Ang IV (Ang 3–8) with their specific receptors Mas and IRAP [3–7]. Ang II has many important functions, including increasing arterial pressure by constricting resistance vessels, stimulating thirst center in the brain, stimulating aldosterone release from the adrenal cortex, thus increasing sodium and fluid retention by the kidney; stimulating release of antidiuretic hormone from the posterior pituitary to increase fluid retention; enhancing sympathetic adrenergic function by facilitating norepinephrine release from sympathetic nerves and inhibiting its reuptake; and causing cardiac and vascular remodeling [1, 8].

These actions mostly result from activation of AT1, a 7-transmembrane (7-TM) receptor that mainly couples to the Gq/G11 family of heterotrimeric G-proteins and activates multiple signaling pathways. AT1 can function independently of Ang II as a mechanical stress receptor [9]. Binding of Ang II to its receptors mediates intracellular free radical generation which promotes mitochondrial dysfunction [10]. The type 2 receptor, AT2, couples to hetero-trimeric Gi/Go proteins. Although originally thought to simply oppose the physiological actions of AT1, AT2 seems to act in some conditions like AT1 in having growth stimulatory and proinflammatory actions [11]. Insulin receptor transinactivation by AT2 was recently shown to require expression of a novel protein, AT2-interacting protein (ATIP1) that constitutively interacts with the C-terminal tail of AT2 [12].

In addition to the systemic RAS, “local” RAS that generates Ang II to act in an autocrine and paracrine manner, was described for a number of tissues in the 1990s, including heart, vessels, adipose tissue, adrenals, brain, and kidney [13, 14]. The local production of Ang II plays an important role in regulating a number of physiological processes, including blood pressure, vasopressin release, drinking behavior, sodium reabsorption, and sympathetic outflow. Activation of a local RAS has been implicated in cardiac remodeling.
Mechanical stress, for instance, enhances expression of cardiac renin and AGT genes [15]. The local hematopoietic bone marrow (BM) renin–angiotensin system (RAS) was shown to mediate alterations of hematopoiesis [16]. A close interrelationship between cardiac RAS and the hematopoietic bone marrow RAS was reported earlier [17]. In relation to atherogenesis, Strawn et al. proposed a lipid–angiotensin system connection within the bone marrow that participates in atherosclerosis initiation [18, 19].

The “bone marrow response to lipid” hypothesis supposes that pro-atherogenic properties of hematopoietic and non-hematopoietic progenitors are determined by the local actions of modified low-density lipoprotein (LDL) on the expression of local RAS genes [19]. According to our results, ACE inhibitors are able to modify the red blood cell count as well as mean corpuscular volume of erythrocytes in patients with essential hypertension, which also seems to reflect involvement of local RAS system in bone marrow [20, 21].

Angiotensin-Converting Enzyme (ACE)

ACE is a zinc metalloendopeptidase, which functions as a C-terminal peptidyl dipeptidase and converts the biologically inactive Ang I to the potent vasoconstrictor and cardiovascular trophic factor, Ang II. A single gene encodes two different ACE proteins through transcription from alternative promoters [22]. The ubiquitous somatic ACE is highly expressed in vascular endothelial cells and has two tandem, independent catalytic sites with distinct properties. Somatic ACE is produced by various other cells as well, including activated macrophages, tubular epithelium, and proximal gut epithelium. Developing male germ cells produce a different ACE isozyme, germinal or testicular ACE, which has a single active site [23].

Somatic ACE is a major contributor to cardiovascular homeostasis, and randomized studies have shown that ACE inhibitors improve symptoms and survival in all grades of heart failure when given with diuretics [24]. The beneficial effects of ACE inhibitors are in large part due to the prevention of events downstream of Ang II formation, such as elevated vascular tone, heart and vessel remodeling, and salt and water retention. However, ACE also hydrolyzes bradykinin, a potent vasodilator and cardioprotective agent. Since bradykinin is more readily hydrolyzed by ACE than Ang I [25], the net therapeutic effect of ACE inhibitors may reflect both diminished Ang II and increased bradykinin levels. In addition, the tetrapeptide N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP), which is normally present in mammalian plasma and many tissues, is hydrolyzed almost exclusively by ACE [26]. AcSDKP is generated from the N-terminus of thymosin β4 (the main G-actin sequestering peptide in eukaryotic cells) and has antifibrotic actions in the heart and kidney that may result in part from inhibition of cellular proliferation and TGF-β signaling [27, 28].

ACE Gene (17q23)

In the last decades, ACE gene has been cloned that allowed the identification (1) of two isoenzymes, one called somatic ACE resulting from gene duplication and primarily expressed in endothelial cells, and the other, called germinative or testicular ACE, resulting from the transcription in the male reproductive system of a more simple gene, (2) of an hydrophobic C-terminal peptide for membrane-anchoring and specifically cleaved by a metalloprotease to release soluble forms of both isoenzymes, and (3) of several allelic polymorphisms, one of them consisting of an insertion/deletion (I/D) polymorphism in a short intronic Alu sequence that could account for half the variance in plasma ACE level and resulting in a large interindividual variability; moreover this I/D polymorphism was proposed as a genetic marker for identifying individuals at high risk of ischemic heart disease and of anticipating in one individual the efficacy of the antihypertensive therapy [29]. In 1992, PCR method for simple detection of ACE I/D polymorphism was published [30].
Case-Control Studies: ACE I/D and Cardiovascular Diseases

No evidence was found to support an association between ACE genotype and heart failure [31]. ACE genotype distributions were similar between the patients with and without asymptomatic CHF in CAD [32]. There was no evidence that ACE gene I/D polymorphism plays a role in the development of CHF in CAD or any influence on exercise capacity in treated patients with ischemic CHF [33].

ACE DD genotype is associated with decreased exercise tolerance in CHF, possibly mediated by altered pulmonary function. Pharmacological strategies effecting more complete inhibition of serum and tissue ACE and/or potentiation of bradykinin may improve exercise capacity in patients with CHF and ACE DD genotype [34].

The higher incidence of hypertension in 684 healthy subjects, which had completed 6 years of follow-up, was observed in the older age groups (36–45 and 46–55 years) with DD and ID genotypes [35].

A total of 4,264 normotensive and 2,174 hypertensive participants of the Rotterdam Study (a population-based prospective cohort study) were available for follow-up from 1990 until 2000. Incidence rates (IR) of heart failure in normotensive subjects were the same over all genotype strata (10 per 1000 person-years) in ACE I/D polymorphism. In hypertensive subjects, the IR increased with the number of D-alleles present. Hypertensive subjects carrying the II-genotype did not have an increased risk of heart failure compared to normotensive II subjects. However, hypertensive subjects carrying one or two copies of the D-allele did have a significantly increased risk of heart. The findings suggest that the ACE I/D polymorphism may play a modifying role in the development of heart failure in hypertensive subjects [36].

The ACE gene polymorphism distribution was found to be similar in CHF patients and control subjects. However, ACE gene DD polymorphism was associated with a more severe condition, greater LVDD, and higher plasma Ang II level [37].

Case-Control Studies: ACE I/D and Diabetes Mellitus

Recently, a meta-analysis of results from 14 studies with 1,985 patients with T2D and 4,602 controls was performed [38]. The D allele carriers in ACE I/D polymorphisms were evaluated to have an increased risk for T2D compared to the I allele carriers, with the highest significance for recessive model for DD genotype [38].

Genotype-Phenotype Studies: ACE Activity in Plasma

Plasma ACE activity measured in 197 unrelated healthy Caucasian (Czech) subjects was correlated with sex, ABO blood groups, and ACE I/D polymorphism. Marked differences in plasma ACE activity levels were observed both among the blood groups and among ACE phenotypes; the highest activity was observed in DD genotype and blood group B. The I/D polymorphism and the ABO system turned out to be two independent (additive) factors influencing plasma ACE activity. Together, they are responsible for 9.56% of the phenotypic variability of ACE in Czech population [39].

A marked difference in the plasma Ang II levels was observed among the three genotypes (DD, ID, and II). The levels of plasma Ang II in the DD genotype class were the highest [37]. ACE gene DD polymorphism was associated with greater LVDDs, higher plasma Ang II levels, and the greatest decreased magnitude of plasma Ang II levels after treatment in patients with chronic heart failure where ACE gene DD polymorphism might be a marker of a higher level of activation of the renin–angiotensin system (RAS) [37].

Genotype-Phenotype Studies: Systolic and Diastolic Dysfunction

Higher heart rate variability was associated with ACE DD genotype [40]. Both systolic and diastolic cardiac dysfunctions coexist in various
degrees in the majority of patients with heart failure. Endogenous bradykinin seems to contribute to the cardioprotective effect of the ACE inhibitor, improving left ventricular diastolic dysfunction rather than systolic dysfunction, via modification of NO release and Ca²⁺ handling and suppression of collagen accumulation [41]. In 1,200 male military recruits, LV dimensions and mass were compared at the start and end of a 10-week physical training period. LV mass increased with training by 8.4 g overall, but with DD men showing roughly three-fold greater growth than II men. It clearly demonstrates the importance of the ACE–renin–angiotensin system in determining LV dimensions in situations of high cardiac demand, which may also be important in pathology such as hypertension and heart failure. The use of these “stress-the-genotype” approaches to explore gene–environment interactions are likely to be the key to understanding the causes determining both coronary artery disease and other multifactorial disorders [42].

DD significantly and early affected myocardial diastolic properties in the total group of 684 healthy subjects, which had completed 6 years of follow-up, also when stratified for age [35]. The ACE genotype was determined in 171 patients selected with idiopathic dilated cardiomyopathy (IDC) in New York Heart Association functional class II to III heart failure and with a LV ejection fraction of 40%. The frequency of ACE gene ID alleles was not different in the study versus non-age-matched and age-matched control groups. The ejection fraction was found to be worse in patients with the DD genotype. The LV end-systolic and end-diastolic diameters were increased in patients with the DD genotype. Multifactor regression analysis showed the ACE genotype to be an independent predictor of both ejection fraction and end-diastolic diameter. This indicates that the DD genotype of the ACE gene is independently associated with both a reduced LV systolic performance and an increased LV cavity size in patients with IDC [43].

The ACE gene polymorphism was evaluated in 90 patients after heart transplantation because of end-stage dilated cardiomyopathy compared to the population sample. A decrease of heterozygote ID carriers in the patients was proved [44].

ACE I/D, Coronary Artery Disease, and Diabetes: Continuing Story

Abnormality of the microvascular system causing impaired tissue perfusion is referred to be common among cardiovascular risk factors, including hypertension, diabetes, and obesity [45]. If it is so, reading descriptive characteristics of cardiovascular association studies, everybody must mention different numbers of comorbidities and/or risk factors which can unpredictably modify their results. Therefore, we decided to evaluate possible association of ACE I/D polymorphism with CAD and diabetes evaluated together. In this manner we have obtained four groups of patients (CAD + DM + patients with both CAD and diabetes, CAD + DM − patients with CAD, CAD − M + with only diabetes, and CAD − DM − without CAD as well as diabetes). Further, significant differences in intermediate phenotypes (hemodynamic and metabolic parameters, drug therapy) were analyzed in the groups.

Methods

Patient Population

The study comprised 600 consecutive patients with suspected or known CAD referred to the 1st Department of Internal Medicine/Cardioangiology for coronary angiography between October 2005 and February 2007, 417 men (median age 63, range 25–87 years) and 183 women (median age 67, range 27–91 years). During the short-term hospitalization, the patients underwent full cardiological investigation (history, physical examination, electrocardiography, laboratory examination, coronary angiography, echocardiography in patients with unclear diagnosis). Coronary angiography was performed using the standard technique. The coronary lesions were visually analyzed in multiple projections. Based on the morphology of coronary arteries, two patient groups were defined: (1) patients with significant coronary atherosclerosis (at least one coronary artery with luminal diameter narrowing by 50% or more, CAD group) and (2) patients with normal smooth coronary
arteries. Patients with insignificant coronary atherosclerosis (stenoses with luminal diameter narrowing below 50% or luminal irregularities) were excluded from the study (N = 59).

Other diseases, such as diabetes, hypertension, obesity, and hyperlipidemia, were evaluated at the same time according to clinical guidelines. The study was approved by the institutional ethics committee; informed consent of all patients is archived.

Genotyping

Genomic DNA was isolated from peripheral leukocytes by a standard technique using proteinase K. The ACE I/D genotypes of all subjects were detected using a PCR method according to Rigat (1992). The reaction mixture contained 12.9 μL of PCR water, 2.5 μL of KCl buffer, 2.5 μL of MgCl₂, 1.25 μL of each primer (P1: 5'-CTGGAGACCACCTCCCATCTTT, P2: 5'-GATGTGGGCACTCACCATTCGT), 0.5 μL of dNTPs, 0.1 μL of Taq polymerase 5U, and finally 4 μL of genomic DNA. The amplification products were separated on 2% agarose gel, containing ethidium bromide (EtBr, 1.2 μL per 10 mL) and visualized under UV light. The PCR yields amplification products of 490 bp/490 bp in case of I/D genotype; in DD genotype it is a 190 bp/190 bp pattern and heterozygous ID genotype gives products of 490 bp/190 bp.

Statistical Analyses

In all groups of subjects, distributions of genotype and allelic frequencies and their differences were calculated using χ² tests and Fisher’s exact test. Consistency of genotype frequencies with the Hardy–Weinberg equilibrium was tested using the χ² test on a contingency table of observed versus predicted genotype frequencies.

Kruskal–Wallis ANOVA test by Ranks was used to compare continuous parameters in groups. Odds ratio (OR) and 95% confidence interval were calculated to estimate the risks related to detected polymorphisms. To calculate the significance of OR, Fisher’s exact test was used. The program package Statistica v. 8.0 (Statsoft Inc., Tulsa, OK) was employed.

Results

We did not observe any difference in genotype distribution (Pg) and allelic frequency (Pa) in ACE I/D polymorphism between patients with and without CAD (Table 1). Similarly, no differences were observed after patients’ division according to diabetes (Table 1). No significant results were observed when the presence of both diseases has been evaluated (Table 2).

Another possibility, how to search expected interaction of CAD, DM, and ACE I/D polymorphism, was to test different models of inheritance. No significant difference was observed either in the DD-recessive model, or in the (DD + ID) codominant models (data not shown).

Finally, a “heterozygote advantage” model was calculated. No difference for CAD has been found (Table 3). However, when the groups of patients with and without diabetes were been compared, a significant difference between these two groups was observed (Table 3). Odds ratio (OR) for the ID

<table>
<thead>
<tr>
<th>Table 1</th>
<th>ACE I/D polymorphism, CAD, diabetes: case-control study</th>
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</thead>
<tbody>
<tr>
<td>CAD+</td>
<td>N</td>
</tr>
<tr>
<td>484</td>
<td>162</td>
</tr>
<tr>
<td>CAD-</td>
<td>116</td>
</tr>
<tr>
<td>All grps</td>
<td>601</td>
</tr>
<tr>
<td>DM+</td>
<td>171</td>
</tr>
<tr>
<td>DM-</td>
<td>430</td>
</tr>
<tr>
<td>All grps</td>
<td>600</td>
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</table>

Pg = probability of a difference in genotype distribution
Pa = probability of a difference in allelic frequency
Table 2  ACE I/D polymorphism, CAD and diabetes: case-control (CAD − DM −) study

<table>
<thead>
<tr>
<th>CAD DM</th>
<th>N</th>
<th>ACE – DD</th>
<th>ACE – ID</th>
<th>ACE – II</th>
<th>Pg</th>
<th>D allele (%)</th>
<th>Pa</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD + DM+</td>
<td>153</td>
<td>58</td>
<td>53</td>
<td>42</td>
<td>0.09</td>
<td>55.2</td>
<td>0.880</td>
</tr>
<tr>
<td>CAD − DM −</td>
<td>331</td>
<td>104</td>
<td>153</td>
<td>74</td>
<td>0.924</td>
<td>54.5</td>
<td>0.997</td>
</tr>
<tr>
<td>CAD − DM+</td>
<td>17</td>
<td>3</td>
<td>8</td>
<td>6</td>
<td>0.357</td>
<td>41.2</td>
<td>0.149</td>
</tr>
<tr>
<td>CAD − DM −</td>
<td>99</td>
<td>30</td>
<td>48</td>
<td>21</td>
<td></td>
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<td>195</td>
<td>263</td>
<td>143</td>
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</tr>
</tbody>
</table>

Pg = probability of a difference in genotype distribution
Pa = probability of difference in allelic frequency

Table 3  ID advantage model, CAD and diabetes: case-control study

<table>
<thead>
<tr>
<th>CAD</th>
<th>N</th>
<th>ACE – ID (%)</th>
<th>ACE – (II + DD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD+</td>
<td>484</td>
<td>206 (43)</td>
<td>278</td>
<td>0.156</td>
</tr>
<tr>
<td>CAD−</td>
<td>116</td>
<td>56 (48)</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>All grps</td>
<td>601</td>
<td>263</td>
<td>338</td>
<td></td>
</tr>
</tbody>
</table>

Diabetes

<table>
<thead>
<tr>
<th>DM+</th>
<th>N</th>
<th>ACE – ID (%)</th>
<th>ACE – (II + DD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM+</td>
<td>171</td>
<td>62 (36)</td>
<td>109</td>
<td>0.01</td>
</tr>
<tr>
<td>DM−</td>
<td>430</td>
<td>201 (47)</td>
<td>229</td>
<td></td>
</tr>
<tr>
<td>All grps</td>
<td>600</td>
<td>263</td>
<td>338</td>
<td></td>
</tr>
</tbody>
</table>

P = probability of a difference between ID and (II + DD) frequencies

Table 4  ID advantage model, CAD and diabetes: case-control (CAD − DM −) study

<table>
<thead>
<tr>
<th>CAD DM</th>
<th>N</th>
<th>ACE – ID</th>
<th>ACE – (II + DD)</th>
<th>ID genotype (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD + DM+</td>
<td>153</td>
<td>53</td>
<td>100</td>
<td>53.0</td>
<td>0.02</td>
</tr>
<tr>
<td>CAD − DM −</td>
<td>331</td>
<td>153</td>
<td>178</td>
<td>46.2</td>
<td>0.389</td>
</tr>
<tr>
<td>CAD − DM+</td>
<td>17</td>
<td>8</td>
<td>9</td>
<td>47.1</td>
<td>0.561</td>
</tr>
<tr>
<td>CAD − DM −</td>
<td>99</td>
<td>48</td>
<td>51</td>
<td>48.5</td>
<td></td>
</tr>
<tr>
<td>All grps</td>
<td>600</td>
<td>263</td>
<td>143</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P = probability of a difference between ID and (II + DD) frequencies

The heterozygote advantage model was used for comparison of eight groups (including ACE I/D genotype as ID or II + DD). We proved several highly significant differences in some hemodynamic (EF LV, plasma creatinine), proinflammatory (leukocyte number and fibrinogen), and especially in metabolic parameters (BMI, glycemia, total cholesterol, LDL, HDL, triglycerides). The significance of these differences is sufficiently high to “survive” multiple comparisons (Tables 5 and 6), although the parameters had to be modified by different therapy.

Table 7 summarizes comorbidities and risk factors in all groups. Generally, in most cases, we proved significant differences between the CAD + DM + and CAD − DM − groups. Moreover, we observed a higher frequency of ID genotype in CAD + DM + with hypertension compared to CAD − DM − group (OR = 6.42, 95% CI 2.17–19.04,
<table>
<thead>
<tr>
<th>Group × ACE</th>
<th>N</th>
<th>EF LV median (range, %)</th>
<th>Leu median (range, ×10⁹/L)</th>
<th>Fibrinogen median (range, g/L)</th>
<th>BMI median (range, kg/m²)</th>
<th>Creatinine median (range, μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD+DM+ (ID)</td>
<td>53</td>
<td>50 (18–70)</td>
<td>7.7 (4.3–13.9)</td>
<td>4.4 (2.8–5.6)</td>
<td>30 (22–40)</td>
<td>94.5 (5.6–180.0)</td>
</tr>
<tr>
<td>CAD+DM+ (II+DD)</td>
<td>100</td>
<td>50 (20–75)</td>
<td>7.5 (3.5–12.7)</td>
<td>4.1 (2.5–5.6)</td>
<td>29 (21–43)</td>
<td>104 (70–200)</td>
</tr>
<tr>
<td>CAD+DM− (ID)</td>
<td>153</td>
<td>55 (20–75)</td>
<td>7.6 (4.0–16.9)</td>
<td>4.0 (2.1–218.0)</td>
<td>28 (18–43)</td>
<td>98 (71–176)</td>
</tr>
<tr>
<td>CAD+DM− (II+DD)</td>
<td>178</td>
<td>55 (18–75)</td>
<td>7.9 (3.6–127.0)</td>
<td>4.0 (1.05–5.90)</td>
<td>28 (18–43)</td>
<td>99 (66–189)</td>
</tr>
<tr>
<td>CAD−DM+ (ID)</td>
<td>8</td>
<td>60 (55–70)</td>
<td>7.0 (4.9–9.8)</td>
<td>4.1 (2.6–5.4)</td>
<td>31 (20–38)</td>
<td>107 (69–117)</td>
</tr>
<tr>
<td>CAD−DM+ (II+DD)</td>
<td>9</td>
<td>50 (20–60)</td>
<td>7.5 (2.8–10.6)</td>
<td>3.7 (3.1–5.4)</td>
<td>30 (21–42)</td>
<td>92 (83–116)</td>
</tr>
<tr>
<td>CAD−DM− (ID)</td>
<td>48</td>
<td>60 (15–75)</td>
<td>6.35 (3.1–10.0)</td>
<td>3.7 (2.24–5.60)</td>
<td>29 (21–36)</td>
<td>94 (60–161)</td>
</tr>
<tr>
<td>CAD−DM− (II+DD)</td>
<td>51</td>
<td>60 (37–70)</td>
<td>6.75 (3.8–10.9)</td>
<td>3.6 (2.7–5.4)</td>
<td>27 (21–42)</td>
<td>91 (66–131)</td>
</tr>
<tr>
<td>All groups</td>
<td>600</td>
<td>55 (15–75)</td>
<td>7.5 (2.8–127)</td>
<td>4.0 (1.05–218)</td>
<td>29 (18–43)</td>
<td>98 (5.6–200)</td>
</tr>
</tbody>
</table>

Kruskal–Wallis: H=50.98161, P=0.0000
ANOVA by Ranks: H=33.97776, P=0.0000

H=37.81505, P=0.0000
H=25.39687, P=0.0006
H=35.49770, P=0.0000
### Table 6  Significant differences among groups and ACE genotype (heterozygote advantage model)

<table>
<thead>
<tr>
<th>Group × ACE</th>
<th>N</th>
<th>Glycemia (median, range, mmol/L)</th>
<th>Cholesterol (median, range, mmol/L)</th>
<th>LDL (median, range, mmol/L)</th>
<th>HDL (median, range, mmol/L)</th>
<th>Triglycerides (median, range, mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD+DM+(ID)</td>
<td>53</td>
<td>7.4 (3.3–13.9)</td>
<td>4.49 (2.12–9.80)</td>
<td>2.52 (0.86–101.00)</td>
<td>1.06 (0.63–1.83)</td>
<td>1.63 (0.53–6.33)</td>
</tr>
<tr>
<td>CAD+DM+(II+DD)</td>
<td>100</td>
<td>6.8 (3.4–17.4)</td>
<td>3.97 (2.61–9.43)</td>
<td>2.12 (1.19–4.96)</td>
<td>0.98 (0.59–1.89)</td>
<td>1.87 (0.68–10.50)</td>
</tr>
<tr>
<td>CAD+DM–(ID)</td>
<td>153</td>
<td>5.2 (3.5–10.7)</td>
<td>4.29 (2.51–8.18)</td>
<td>2.40 (0.91–102.00)</td>
<td>1.08 (0.58–2.38)</td>
<td>1.40 (0.54–6.64)</td>
</tr>
<tr>
<td>CAD+DM–(II+DD)</td>
<td>178</td>
<td>5.1 (3.6–8.1)</td>
<td>4.40 (2.25–8.39)</td>
<td>2.48 (0.65–101.00)</td>
<td>1.09 (0.60–2.18)</td>
<td>1.44 (0.46–6.36)</td>
</tr>
<tr>
<td>CAD–DM+(ID)</td>
<td>8</td>
<td>6.8 (5.1–12.1)</td>
<td>4.67 (2.95–7.47)</td>
<td>2.40 (1.19–4.85)</td>
<td>1.13 (0.86–1.76)</td>
<td>1.96 (0.80–3.00)</td>
</tr>
<tr>
<td>CAD–DM–(ID)</td>
<td>9</td>
<td>5.6 (4.2–8.0)</td>
<td>3.97 (2.97–6.11)</td>
<td>2.82 (1.12–4.10)</td>
<td>1.09 (0.73–1.52)</td>
<td>1.09 (0.93–2.72)</td>
</tr>
<tr>
<td>CAD–DM–(II+DD)</td>
<td>48</td>
<td>4.9 (3.9–6.8)</td>
<td>4.65 (2.25–6.40)</td>
<td>2.79 (0.83–4.15)</td>
<td>1.28 (0.50–2.54)</td>
<td>1.19 (0.43–3.02)</td>
</tr>
<tr>
<td>CAD–DM–(II+DD)</td>
<td>51</td>
<td>4.9 (3.8–8.3)</td>
<td>4.98 (3.49–7.90)</td>
<td>2.87 (1.67–5.54)</td>
<td>1.38 (0.81–2.59)</td>
<td>1.37 (0.63–4.23)</td>
</tr>
<tr>
<td>All groups</td>
<td>600</td>
<td>5.6 (3.3–17.4)</td>
<td>4.42 (2.12–9.8)</td>
<td>2.47 (0.65–102.00)</td>
<td>1.09 (0.50–2.59)</td>
<td>1.48 (0.43–10.5)</td>
</tr>
</tbody>
</table>

**Kruskal–Wallis**
- H = 150.2524
- P = 0.0000

**ANOVA by Ranks**
- H = 30.08967
- P = 0.0001
- H = 30.80595
- P = 0.0001
- H = 79.14303
- P = 0.0000
- H = 35.63787
- P = 0.0000
Table 7  Significant differences among groups and ACE genotype (heterozygote advantage model)

<table>
<thead>
<tr>
<th>Group × ACE</th>
<th>N</th>
<th>Hypertension (%)</th>
<th>Obesity (%)</th>
<th>Hyperlipidemia (%)</th>
<th>Renal insufficiency (%)</th>
<th>No risk factors - more than 1 (%)</th>
<th>Current smoking (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD+DM+(ID)</td>
<td>53</td>
<td>49 (92)</td>
<td>24 (45)</td>
<td>33 (62)</td>
<td>17 (32)</td>
<td>11 (21)</td>
<td>4 (8)</td>
</tr>
<tr>
<td>CAD+DM+(II+DD)</td>
<td>100</td>
<td>84 (84)</td>
<td>37 (37)</td>
<td>55 (55)</td>
<td>26 (27)</td>
<td>28 (28)</td>
<td>6 (6)</td>
</tr>
<tr>
<td>CAD+DM−(ID)</td>
<td>153</td>
<td>120 (78)</td>
<td>41 (27)</td>
<td>91 (59)</td>
<td>26 (17)</td>
<td>25 (16)</td>
<td>32 (25)</td>
</tr>
<tr>
<td>CAD+DM−(II+DD)</td>
<td>178</td>
<td>137 (77)</td>
<td>43 (24)</td>
<td>97 (54)</td>
<td>27 (15)</td>
<td>28 (16)</td>
<td>29 (16)</td>
</tr>
<tr>
<td>CAD−DM+(ID)</td>
<td>8</td>
<td>7 (88)</td>
<td>4 (50)</td>
<td>5 (63)</td>
<td>2 (25)</td>
<td>1 (13)</td>
<td>1 (13)</td>
</tr>
<tr>
<td>CAD−DM+(II+DD)</td>
<td>9</td>
<td>7 (78)</td>
<td>4 (44)</td>
<td>6 (67)</td>
<td>1 (11)</td>
<td>2 (22)</td>
<td>1 (11)</td>
</tr>
<tr>
<td>CAD−DM−(ID)</td>
<td>48</td>
<td>29 (60)</td>
<td>9 (19)</td>
<td>30 (63)</td>
<td>3 (6)</td>
<td>8 (17)</td>
<td>7 (15)</td>
</tr>
<tr>
<td>CAD−DM−(II+DD)</td>
<td>51</td>
<td>39 (76)</td>
<td>14 (27)</td>
<td>38 (75)</td>
<td>1 (1)</td>
<td>4 (8)</td>
<td>5 (10)</td>
</tr>
<tr>
<td>All groups</td>
<td>600</td>
<td>472 (79)</td>
<td>176 (29)</td>
<td>355 (59)</td>
<td>103 (17)</td>
<td>107 (18)</td>
<td>85 (14)</td>
</tr>
</tbody>
</table>
Table 8 Differences among groups and ACE genotype (heterozygote advantage model) — drugs

<table>
<thead>
<tr>
<th>Group × ACE</th>
<th>N</th>
<th>Aspirin (%)</th>
<th>Other antiaggregation t. (%)</th>
<th>ACEI (%)</th>
<th>Beta-blockers (%)</th>
<th>Statins (%)</th>
<th>Other antilipid t. (%)</th>
<th>AT1R antagonists (%)</th>
<th>Antivitamin K t.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD+ DM + (ID)</td>
<td>53</td>
<td>46 (87)</td>
<td>28 (53)</td>
<td>31 (58)</td>
<td>50 (94)</td>
<td>52 (98)</td>
<td>2 (4)</td>
<td>23 (43)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>CAD+ DM + (II + DD)</td>
<td>100</td>
<td>89 (89)</td>
<td>57 (57)</td>
<td>73 (73)</td>
<td>97 (97)</td>
<td>100 (100)</td>
<td>2 (2)</td>
<td>18 (18)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>CAD+ DM − (ID)</td>
<td>153</td>
<td>130 (85)</td>
<td>98 (64)</td>
<td>107 (70)</td>
<td>143 (93)</td>
<td>145 (95)</td>
<td>5 (3)</td>
<td>19 (12)</td>
<td>5 (3)</td>
</tr>
<tr>
<td>CAD+ DM − (II + DD)</td>
<td>178</td>
<td>160 (90)</td>
<td>109 (61)</td>
<td>124 (70)</td>
<td>166 (93)</td>
<td>168 (94)</td>
<td>1 (1)</td>
<td>21 (12)</td>
<td>6 (3)</td>
</tr>
<tr>
<td>CAD− DM + (ID)</td>
<td>8</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>5 (63)</td>
<td>2 (25)</td>
<td>2 (25)</td>
<td>0 (0)</td>
<td>2 (25)</td>
<td>1 (13)</td>
</tr>
<tr>
<td>CAD− DM + (II + DD)</td>
<td>9</td>
<td>2 (22)</td>
<td>0 (0)</td>
<td>6 (66)</td>
<td>6 (66)</td>
<td>3 (33)</td>
<td>0 (0)</td>
<td>2 (22)</td>
<td>2 (22)</td>
</tr>
<tr>
<td>CAD− DM − (ID)</td>
<td>48</td>
<td>13 (27)</td>
<td>0 (0)</td>
<td>17 (35)</td>
<td>27 (56)</td>
<td>12 (25)</td>
<td>0 (0)</td>
<td>5 (10)</td>
<td>3 (6)</td>
</tr>
<tr>
<td>CAD− DM − (II + DD)</td>
<td>51</td>
<td>6 (12)</td>
<td>0 (0)</td>
<td>22 (43)</td>
<td>25 (49)</td>
<td>15 (29)</td>
<td>0 (0)</td>
<td>11 (22)</td>
<td>3 (6)</td>
</tr>
<tr>
<td>All grps</td>
<td>600</td>
<td>446 (74)</td>
<td>292 (49)</td>
<td>385 (64)</td>
<td>516 (86)</td>
<td>497 (83)</td>
<td>10 (2)</td>
<td>101 (17)</td>
<td>23 (4)</td>
</tr>
</tbody>
</table>
Similarly, a significant increase of ID carriers in obese CAD+DM+ compared with obese CAD−DM− group was proved (OR = 3.59, 95% CI 1.45–8.86, P = 0.004) which was not observed between (II+DD) carriers. A higher risk of renal insufficiency between CAD+DM+ and CAD−DM− group was also observed. However, the highest OR was calculated for the (II+DD) genotype (OR = 17.69, 95% CI 2.32–134.73, P = 0.00007) in CAD+DM+ group.

The CAD+DM+ patients with genotype (II+DD) had more cardiovascular risk factors compared to (II+DD) CAD−DM− subjects (OR = 4.57, 95% CI 1.51–13.87, P = 0.003). No differences for hyperlipidemia and current smoking among the groups were observed; the state of lipids had to be substantially influenced by therapy.

Then we evaluated the drug administration schedule in the groups (Table 8). Aspirin was applied much more frequently in patients with CAD+DM+ compared to those with CAD−DM−, especially in the (II+DD) carriers (OR = 60.68, 95% CI 21.08–174.60, P = 0.0000001). No differences in other anti-aggregation therapy administration were observed. ACE inhibitors were prescribed more frequently to CAD+DM+ patients, especially with II+DD genotype (OR = 3.56, 95% CI 1.75–7.24, P = 0.0003). A similar situation was repeated in beta-blockers [OR for CAD+DM+ and (II+DD) ACE genotype was 33.63, 95% CI 9.41–120.15, P = 0.0000001], statins [OR for CAD+DM+ (II+DD) = 237.6, 95% CI 30.29–1864.03, P = 0.0000001], and warfarin prescription (OR = 14, 95% CI 1.71–114.86, P = 0.03). On the opposite, the AT1R antagonists were administrated more frequently to patients with the ID genotype and CAD+DM+ compared to patients with CAD−DM− with the same genotype (OR = 6.38, 95% CI 2.18–18.63, P = 0.0002). It is necessary to mention that drug prescription could not be influenced by genotype knowledge because genotyping was performed independently of clinical decisions.

When we evaluate the number of affected coronary arteries, diabetes, and ACE I/D polymorphism, a highly significant heterozygote advantage can be observed in patients with two affected coronary arteries and diabetes mellitus compared to those without diabetes. In these patients, OR for ID genotype, CAD with two affected coronary arteries and diabetes is 0.27, 95% CI 0.12–0.59, P = 0.0005.

Conclusions

The main task of the study was to summarize clinical and genetic results and to do it as simply as possible to prevent statistical artefacts (or doubts about statistical correctness).

We proved a lot of significant differences especially between CAD+DM+ and CAD−DM− groups. Although the patients had been treated according to their clinical state, we were able to prove significant differences between ID and (II+DD) ACE genotypes in hypertension, obesity, and renal insufficiency prevalence, in BMI values, in the number of cardiovascular risk factors, in some inflammatory factors, glycemia, and lipid profile. Generally, the drugs were administrated more frequently to the II+DD carriers that support the heterozygote advantage hypothesis tested in the study. We were able to find only one study with a similar design [46] which did not confirm association of ACE I/D with diabetes mellitus type 2. Unfortunately, a different evaluation of a significance of coronary artery stenosis for patients’ exclusion of the study was used. The results of both studies are not fully comparable; in Grammer’s study, the heterozygote advantage hypothesis has not been tested.

The most important conclusion of the study is the necessity to define generally accepted association study design for complex diseases with respect to precise description of the clinically valid parameters, comorbidities, and risk factors including their “standard” prevalence.

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References

Abstract

Peroxisome proliferator-activated receptors (PPARs), ligand-activated transcription factors, belong to the nuclear hormone receptor superfamily regulating expression of genes involved in different aspects of lipid metabolism and inflammation, and all three isoforms of PPAR (α, β/δ, and γ) detected so far modulate cardiac energy production. The activation of PPAR-α by its natural ligands, long-chain fatty acids (FAs) and eicosanoids, promotes mitochondrial FA oxidation as the primary ATP-generating pathway in the normal adult myocardium. Moreover, under physiological and pathological conditions associated with acute or chronic oxygen deprivation, PPAR-α modulates the expression of genes that determine myocardial substrate selection (FA vs. glucose) aimed at the maintenance of energy production to preserve basic cardiac function. However, whether PPAR activation plays a beneficial or detrimental role in myocardial response to ischemia/reperfusion (I/R) is still a matter of debate. Although PPAR-α and PPAR-γ agonists, hypolipidemic and antidiabetic drugs, have been reported to protect the heart against I/R, the role of PPARs in cardioprotection, in particular in pathological models, is not completely elucidated. This chapter reviews some findings demonstrating the impact of PPAR activation on cardiac resistance to ischemia in normal and pathologically altered heart. Specifically, it addresses the issue of decreased susceptibility to ischemia in the experimental model of streptozotocin-induced diabetes, with particular regard to the role of PPAR gene expression and its modulation by concomitant pathology, such as hypercholesterolemia. Finally, the involvement of
PPAR in the mechanisms of pleiotropic lipid-independent cardioprotective effects of some hypolipidemic drugs in both normal and diseased heart is also discussed.

**Keywords**
Cardioprotection • HMG-CoA reductase inhibitors • Hypolipidemic drugs • Myocardial ischemia • Pleiotropic effects • PPAR

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### Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily regulating expression of genes involved in different aspects of lipid metabolism [1, 2], energy production [3], and inflammation [4]. All three isoforms of PPAR detected so far, α (alpha), β/δ (beta/delta), and γ (gamma), encoded by separate genes, are expressed in many species including rodents [5] and humans [6]. They differ in their tissue distribution, ligand specificity, and cofactor interactions [7, 8]. Therefore, PPAR isoforms regulate different sets of genes, and there are different biological consequences of their stimulation. PPAR-α has been recognized as the central regulator of mitochondrial fatty acids (FAs) catabolism, whereas PPAR-γ is thought to regulate lipid anabolism or storage. Until recently, the function of PPAR-β/δ was relatively less explored. However, several lines of evidence suggest that all three isoforms modulate cardiac energy metabolism [1, 2]. Nevertheless, it is still a matter of debate whether PPAR activation plays a beneficial or detrimental role in the setting of ischemia/reperfusion (I/R), in particular in pathologically altered myocardium. Conflicting findings have documented both the negative impact of PPAR-α up-regulation on myocardial functional recovery upon I/R [9, 10], in particular during early reperfusion [11], and beneficial effects of PPAR-α and PPAR-γ agonists on I/R damage [12–15]. This contradiction is apparently related to the fact that PPAR activation may improve myocardial function via metabolic or other, metabolism-independent, activities.

---

### Tissue Distribution and Function of PPAR

Table 1 illustrates the main tissue distribution and physiological effects of PPAR isoforms. Two of the three PPAR isoforms, PPAR-α and PPAR-β/δ, are abundantly expressed in tissues with high level of FA oxidation (FAO) including heart, liver, kidney, skeletal muscle, and pancreas [7, 16]. PPAR-γ (and its splice variants) is mainly associated with adipose tissue and macrophages, with a low level of more ubiquitous expression in liver, heart, skeletal muscle, and bone marrow [8]. PPAR-β/δ is abundantly and ubiquitously expressed at much higher levels than PPAR-γ and PPAR-α [5]. It is important to note that tissue expression of all three PPAR isoforms may vary under different physiological and/or pathological conditions. Figure 1 demonstrates

---

<table>
<thead>
<tr>
<th>Tissue Distribution and Physiological Effects of PPAR Isoforms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tissue distribution</strong></td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>PPAR-alpha</td>
</tr>
<tr>
<td>Liver, skeletal muscle, heart, kidney, adipose tissue</td>
</tr>
<tr>
<td>PPAR-beta/delta</td>
</tr>
<tr>
<td>Ubiquitously</td>
</tr>
<tr>
<td>PPAR-gamma</td>
</tr>
<tr>
<td>• gamma1</td>
</tr>
<tr>
<td>All tissues including heart, muscle, kidney</td>
</tr>
<tr>
<td>• gamma2</td>
</tr>
<tr>
<td>Adipose tissue</td>
</tr>
<tr>
<td>• gamma3</td>
</tr>
<tr>
<td>Macrophages</td>
</tr>
</tbody>
</table>

**PPAR** peroxisome proliferator-activated receptors, FA fatty acids
that all three PPAR isoforms are present in the myocardial tissue of rats under normal conditions and that their gene expression is modified by ischemia/reperfusion (I/R) [17].

Heart tissue normally uses FA as the major energy source, and PPAR-α regulates genes encoding enzymes of FA transport/uptake and utilization via β(beta)-oxidation in mitochondria [18]. Activation of PPAR-α by its natural ligands (long-chain FA, eicosanoids) promotes mitochondrial FAO as the primary ATP-generating pathway in the normal adult myocardium [3, 18]. Moreover, under physiological and pathological conditions associated with acute or chronic oxygen deprivation, PPAR-α modulates expression of genes that determine myocardial substrate selection (FA vs. carbohydrates) in order to maintain adequate production of energy and preserve basic cardiac function [19]. In addition, involvement of PPAR in anti-inflammatory response in different tissues has also been recognized [20, 21].

**Mechanisms of Action of PPAR**

**Natural and Synthetic PPAR Ligands**

Upon binding to PPAR, different ligands can induce stimulatory or inhibitory responses depending on the nature of the specific target gene and its cellular location. Both natural and synthetic compounds have been recognized as PPAR ligands. Although many FA are capable of activating all three PPAR isoforms, some preference for specific FA by each PPAR has been demonstrated [22]. The long-chain polyunsaturated FA and their oxidized derivatives, especially eicosanoids such as 8-S-hydroxyeicosatetraenoic acid (8-S-HETE), leukotriene B4 (LTB4), and arachidonate monoxygenase metabolite epoxycosatrienoic acids, have been shown to potently activate PPAR-α with high affinity [23]. PPAR-γ can be activated by several prostanoids, such as 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) and 12- and 15-hydroxyeicosatetraenoic acid (12- and 15-HETE), which are derivatives of arachidonic acid synthesized through the lipoxigenase pathway [24]. Prostaglandin 15d-PGJ2 is not only the most potent natural ligand for PPAR-γ identified to date, but also by far the most commonly used naturally occurring PPAR-γ agonist [25]. In addition to PPAR-γ naturally occurring agonists produced by human body, flavonoids w-baptigenin and hesperidin found in plants were identified as strong PPAR-γ agonists [26].

With respect to the synthetic ligands, hypolipidemic drugs fibrates (e.g., fenofibrate, clofibrate) are well-known ligands for PPAR-α [24]. Fibrates activate PPAR-α leading to increased expression of lipid-metabolizing enzymes that
effectively lower serum lipid levels, in particular triacylglycerols, in humans.

The most widely used PPAR-\(\gamma\) agonists belong to the thiazolidinedione (TZD) or glitazone class of anti-diabetic drugs used in the treatment of type-2 diabetes. The two available TZDs, rosiglitazone and pioglitazone, are currently used alone or in combination with other oral antidiabetic agents [24]. These drugs are known as insulin sensitizers, stimulating tissue uptake of glucose in the diabetics [27]; however, their action extends far beyond their hypoglycemic activity and involves limitation of lethal ischemic injury in the nondiabetic heart [13, 28–30]. Table 2 summarizes the most important PPAR modulators.

![Table 2](image)

<table>
<thead>
<tr>
<th>Modulators</th>
<th>PPAR-(\alpha)</th>
<th>PPAR-(\gamma)</th>
</tr>
</thead>
</table>
| Naturally
 occurring agonists | fatty acids eicosanoids leukotriene B4 | fatty acids eicosanoids prostaglandin flavonoids \(\psi\)-baptigenin, hesperidin |
| Synthetic
 agonists     | fibrates        | thiazolidinediones (glitazones)  |
|                  | • fenofibrate   | • rosiglitazone                  |
|                  | • clofibrate    | • pioglitazone                   |
|                  | WY 14643        | • ciglitazone                    |
|                  | GW7647          |                                  |
| Antagonists       | MK-886          | GW9662                           |

**PPAR** peroxisome proliferator-activated receptors

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**Regulation of PPAR Activity**

Many proteins act as co-activators or corepressors that regulate the ability of PPAR to either stimulate or repress gene transcription. In the unbound state, PPAR/RXR heterodimers are associated with corepressors, which prevent gene transcription. However, once a ligand binds to the receptor, a conformational change occurs which not only facilitates corepressor dissociation, but also the recruitment of several positive co-activators that initiates a sequence of events ultimately leading to gene transcription [31].

Although co-activators and corepressors appear to be the major factors responsible for regulation of PPAR activity, these receptors can also be modulated by MAPK-induced phosphorylation. In fact, phosphorylation by extracellular signal-regulated kinases (ERKs) has been found to repress PPAR-\(\alpha\) activity [3, 32], while phosphorylation induced by p38-MAPK enhances PPAR-\(\alpha\)-mediated gene expression [33].

**Transcriptional Transactivation**

Upon activation by endogenous or synthetic ligands, PPARs form obligate heterodimers with the 9-cis-retinoic acid receptors (retinoid X receptor, RXR). The resulting complex undergoes a conformational change which allows binding of the heterodimer to a DNA sequence in the promoter region of target genes known as the peroxisome proliferator response element (PPRE), followed by the induction of gene transcription [25, 34] and synthesis of the respective gene products. When both PPAR and RXR are activated simultaneously, it results in significant synergistic enhancement of gene transcription [34]. The search for PPAR target genes with identified PPREs has led to the identification of numerous genes involved in lipid metabolism, oxidative stress, and the inflammatory response [1, 18, 20, 35].

**Transcriptional Transrepression**

In addition to PPAR transactivation, stimulation of PPAR can also negatively regulate gene expression in a ligand-dependent manner by inhibiting the activities of other transcription factors, such as activated protein-1 (AP-1), nuclear factor-kappaB (NF-\(\kappa\)B), nuclear factor of activated T-cells (NFAT) or signal transducer and activator of transcription (STAT) via a mechanism known as ligand-dependent transrepression [36]. In contrast to transcriptional activation, transrepression does not involve binding of PPAR to the response element of the target genes but direct interaction with other transcription factors and corepressors or modulation of kinase activity.

Research suggests that PPAR may exert beneficial effects by negatively regulating the expression of
pro-inflammatory genes in inflammation-related diseases including myocardial I/R [36]. Several mechanisms have been suggested to account for this activity including ligand-independent repression of the transcription of target genes via binding of PPAR to response elements in the absence of ligands and recruitment of the corepressor complexes [22].

**PPAR Function and Outcome of Myocardial Ischemia/Reperfusion Injury**

Delivery of oxygen and metabolic substrates via coronary circulation is essential for normal cardiac function, and its cessation leads within minutes to irreversible cellular injury. The duration of ischemia and the extent of metabolic and structural alterations in the myocardium are the main factors that determine the progress toward cell death (by mechanisms of necrosis or apoptosis) or cell survival. Restoration of blood flow in the previously occluded coronary arteries is undoubtedly the main prerequisite of the heart rescue. However, reperfusion may have injurious components and limit the recovery of the tissue through the induction of “reperfusion injury” [37]. I/R injury represents a clinically relevant problem associated with reperfusion therapy, such as thrombolysis, percutaneous coronary intervention, and coronary artery bypass graft surgery [38, 39]. I/R injury is a complex cascade of events, where oxidative stress and inflammatory response play the pivotal role [40] and, besides other factors, it involves activation of nuclear factor-κB (NF-κB) as one of the central processes [41], in particular in the ex vivo perfused heart [42].

The role of PPARs in the pathogenesis of a variety of heart disorders, including myocardial damage due to acute myocardial I/R, is a matter of controversy and still remains unclear. Gene expression of PPAR-α declines in chronically hypoxic heart resulting in a substrate switch from FA to glucose, and down-regulation of PPAR-α has been considered as an adaptive response [32, 43]. In-line, experimental overexpression of PPAR-α was found to be related to impaired cardiac recovery after ischemia [10]. It appears that in long-term processes, such as myocardial hypoxia and/or hypertrophy linked with limitations in oxygen supply, glucose as a fuel may be beneficial for the heart by decreasing oxygen consumption [32]. Moreover, chronic activation of PPAR-α (and increased rates of FAO at the expense of glucose oxidation) may be detrimental to the heart during postischemic reperfusion possibly due to FAO-induced oxidative stress [10].

On the other hand, other studies indicated that targeted deletion of PPAR-α resulted in increased serum levels of free FA and larger size of infarction in PPARα−/− mice subjected to ischemic challenge [14]. In acute settings, decrease of PPAR-α, in conjunction with the metabolic effects, was observed in a rat ex vivo model of 30-min ischemia/2-h reperfusion [44] and in the setting of acute I/R in the in vivo mice, in which reversal of down-regulation of PPAR-α and its target genes responsible for the metabolic fuel shifts (decreased FAO and increased glucose oxidation) improved posts ischemic myocardial contractile recovery and reduced the size of infarction [14]. Similarly, in our study of the isolated rat heart, we have also found that 30-min ischemia significantly decreased mRNA levels of all isoforms of PPARs and their further decline was observed following 2-h reperfusion (Fig. 1b) accompanied by the development of irreversible myocardial injury [45].

There is no clear consensus on whether attenuation of I/R-induced down-regulation of PPAR-α and FAO is beneficial or detrimental to the heart. The discrepancy in the results may arise from the different substrate availability in the different experimental models (ischemia/reperfusion, in vivo/in vitro protocols). Although FAO is an important source of energy production during the basic conditions, glucose uptake may be crucial during ischemia. It is believed that partial inhibition of FAO and a substrate switch from FA to glucose [3] improves functional recovery of the heart upon reperfusion [46] while overexpression of PPAR-α impairs posts ischemic cardiac recovery [10]. Thus, pharmacological interventions that increase glucose oxidation and suppress FAO appear to be beneficial for the recovery of the myocardium previously subjected to I/R [9, 11].
In the long term, however, this switch may become detrimental as less ATP is generated per mole of glucose oxidized, and lipid accumulation and lipotoxicity of the myocardium may develop [3]. The controversy regarding the role of PPAR-α in the heart suggests that the function of this transcription factor might not be the same in different cardiac pathologies or in their different stages and that effects other than lipid metabolism might also be involved.

**PPAR and Endogenous Cardioprotection**

The role of PPAR in the mechanisms of endogenous protection against I/R injury is less documented, although Takeda et al. [47] demonstrated that PPAR-γ agonists activated the ERK1/2 pathway of MAP-kinases in vascular smooth muscle cells through phosphatidylinositol 3-kinase (PI3K). Since ERK1/2 cascade and PI3K and its effector protein kinase B (Akt) are implicated in protective mechanisms of ischemic preconditioning and other forms of cardioprotection [48, 49], it has been hypothesized that pretreatment with the PPAR-γ agonist pioglitazone could confer preconditioning-like protection to the myocardium when given prior to myocardial I/R [50]. Indeed, pioglitazone induced significant anti-infarct protection comparable with the effect of classical ischemic preconditioning in the diabetic myocardium [55–58]. In addition, several other protective mechanisms, such as reduction in the levels of pro-inflammatory cytokines, increase in the cell survival factors (HIF1-α, VEGF) and angiogenesis, along with reduced fibrosis, have been found to be activated in the acute phase of streptozotocin (STZ)-induced diabetes [59].

Since the role of PPAR in cardioprotection, in particular in pathological models, such as the experimental model of STZ-induced diabetes mellitus, has not been sufficiently elucidated, we explored a potential link between cardiac response to I/R and gene expression of PPAR in the hearts of diabetic rats. One week after STZ administration (65 mg/kg, i.p.), despite high blood glucose levels (> 20 mmol/L) and reduced baseline functional parameters in the diabetic hearts [60], susceptibility to I/R in these hearts was decreased similarly to the effect of preconditioning in the normal nondiabetic hearts documented by reduced size of infarction, suppressed arrhythmogenesis, and lower myocardial ROS production during ischemia [60–62]. This was coupled with significantly enhanced baseline mRNA levels of all isoforms of PPAR that were preserved after
I/R in contrast to their marked down-regulation in nondiabetics (Fig. 2a, b), indicating that maintenance of enhanced PPAR gene expression during I/R may contribute to improved outcome of myocardial I/R injury in the diabetic heart [17]. The latter might possibly involve not only metabolic effects of PPARs but also their anti-inflammatory and anti-oxidative effects [20, 21] through negative regulation of NF-κB [36].

**Effect of Hyperlipidemia on Ischemic Tolerance in the Diabetic Myocardium**

Hyperlipidemia, especially hypercholesterolemia (HCH), is regarded as an independent risk factor for the development of ischemic heart disease. However, the controversy still remains on whether experimental HCH influences the severity of myocardial I/R injury per se and whether it interferes with the cellular mechanisms of endogenous cardioprotection. Most of the studies show that the major effect of preconditioning (infarct size limitation) may be lost in cholesterol-fed animals [63], although molecular mechanisms of this effect are not completely clear. Recently, Kocsis et al. [64] demonstrated that HCH diet leads to alterations in preconditioning-induced gene expression in the mouse heart resulting in an enhanced oxidative/nitrosative stress signaling which, in turn, attenuates the cardioprotective effect of preconditioning. We addressed the issue of ischemic tolerance in the hearts of STZ-induced diabetic and simultaneously HCH rats (high fat-cholesterol diet, 1 week; 62) with the aim to explore a potential relationship between cardiac response to I/R in this model and gene expression of PPARs. Similar to the effect of HCH in the nondiabetic heart that abrogated the cardioprotective effect of preconditioning [63, 64], this comorbidity appeared to be one of the reasons for the loss of anti-infarct protection and the impaired post-ischemic recovery of ventricular function in the acutely diabetic Langendorff-perfused rat hearts, as well as the exacerbated severe ventricular arrhythmias in the

![Fig. 2](image-url)
open-chest in vivo diabetic animals [62, 65], indicating that HCH might blunt endogenous cardioprotection in the diabetic heart. In addition, in this “double disease” model, we found that concurrent HCH suppressed gene expression of PPAR-γ below those detected in normal controls both at baseline and after I/R (Fig. 3a, b). Cardioprotective effects (infarct size limitation, reduced arrhythmogenesis, and improved contractile recovery) and up-regulation of PPAR-γ mRNA levels in the diabetic hearts blunted by concurrent HCH were restored by simultaneous treatment of rats with simvastatin, a very effective hypolipidemic drug, (Fig. 3b, c, d) without affecting the plasma cholesterol levels [65, 66]. These findings indicate that changes in PPAR gene expression might be involved in the adaptive protective mechanisms activated in the diabetic myocardium in the acute phase to counteract metabolic disorders, while loss of protection might be potentially related to concomitant HCH and down-regulation of PPAR promoting detrimental pro-inflammatory effects. Furthermore, cardioprotective effects of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, statins, independent of cholesterol lowering (pleiotropic effects), may be probably associated with the regulation of PPAR in this diabetic/hypercholesterolemic model since research has revealed that the positive

![Diagram](image.png)

**Fig. 3** Effect of 5-day treatment with simvastatin (S) on myocardial I/R injury and gene expression of PPAR-γ in the Langendorff-perfused hearts of diabetic and diabetic-hypercholesterolemic rats. (a) Expression of PPAR-γ mRNA in the heart of non-diabetic control (C), diabetic (D) and diabetic-hypercholesterolemic (DH) rats at baseline (BL). (b) Effect of simvastatin on the PPAR-γ gene expression at baseline and after 30-min global ischemia and 2-h reperfusion (I/R). The results are presented as means ± S.E.M for at least four different hearts. *P<0.05 relative to respective control groups; * P<0.05 relative to BL in the respective groups. (c) Effect of simvastatin on the size of myocardial infarction after 30-min regional ischemia and 2-h reperfusion in the hearts of D and DH rats. Infarct size (IS) is expressed in% of the area at risk (AR) size. Values are means ± S.E.M. from 12 hearts per group. * P<0.05 vs. untreated group. # P<0.05 relative to BL in the respective groups. (d) Recovery of left ventricular developed pressure (LVDP) after 30-min global ischemia and 2-h reperfusion expressed in% of preischemic values in simvastatin-treated and untreated hearts of D and DH rats. Values are means ± S.E.M. from 10 hearts per group. * P<0.05 vs. untreated group.
impact of statins on inflammatory processes may be mediated through the activation and an increase in PPAR-α and PPAR-γ levels [67, 68].

Cardioprotective Effects of PPAR Agonists

Activation of PPAR-α with synthetic ligands has been shown to be cardioprotective in a setting of I/R, as manifested by a reduced infarct size and improved post-ischemic recovery of contractile function in different in vivo and ex vivo models of I/R [13, 14, 44]. In this context, treatment with PPAR-α selective and potent agonist GW7647 that reversed I/R-induced down-regulation of PPAR-α and its target genes attenuated myocardial contractile dysfunction and reduced the size of infarction [14]. Similar cardioprotective effects, in conjunction with the metabolic effects, were observed in a rat ex vivo model of 30-min ischemia/2-h reperfusion after treatment with the PPAR-α agonist clofibrate [44]. These studies do not support the view of the beneficial role of FAO inhibition in the mechanisms of protection against acute I/R, at least in this experimental setting.

Fibrates as PPAR-α agonists have shown protection against myocardial I/R injury beyond their lipid-lowering properties [13]. Other potent hypolipidemic drugs, statins, are being also intensively studied in this respect. By inhibition of the enzyme HMG-CoA reductase, statins have been shown to prevent the synthesis of isoprenoid intermediates of the cholesterol biosynthesis pathway involved in posttranslational modification of small GTP-binding proteins, such as Ras, Rho, and Rac, which modulate a variety of cellular processes [69], such as oxidative stress and inflammation [70–72], thrombogenesis [73], atherosclerotic plaque formation [74], vascular endothelial dysfunction [69], and the outcome of myocardial response to I/R [65, 75]. It is hypothesized that preconditioning-like effects of statins are attributed to up-regulation of “survival” pathways, such as PI3K/Akt, ERK1/2, and eNOS [76–78].

To get further insight into the potential regulatory effects of statins on PPAR activity, we focused on PPAR-α gene and protein expression in the hearts of normocholesterolemic rats exposed to I/R after 5-day treatment with simvastatin [66]. A remarkable elevation in PPAR-α gene expression coupled with an enhanced protein expression (3.3-fold and 2-fold increase in mRNA and protein levels, respectively) was observed in the myocardium of these animals that was maintained after both ischemia and reperfusion and accompanied by a significant reduction of the infarct size, improved contractile recovery, and attenuation of severe ischemia- and reperfusion-induced ventricular arrhythmias [79]. In support of the view that PPAR-α activation may underlie the mechanisms of beneficial effects of statins against lethal myocardial injury in the hearts of normocholesterolemic animals, our studies confirmed that anti-infarct protection conferred by 5-day treatment with simvastatin in a rat ex vivo model of 30-min global ischemia and 2-h reperfusion was comparable with the effect of WY14643 (WY; Fig. 4), one of the most potent and selective PPAR-α agonists [25], a hypolipidemic compound that has been shown to protect rat myocardium against I/R injury [80].

Although statins are not specific PPAR ligands, they have been reported to up-regulate PPAR-α in some cell types, such as human HepG2 hepatoma cells [81] or mouse peritoneal macrophages [82], and to increase both PPAR-α expression and its protein levels in primary endothelial cells [67]. Our findings provide evidence of the
up-regulation of PPAR-α by statins in the myocardium, perhaps not via a direct agonistic mechanism. This is in agreement with the data documenting a beneficial effect of PPAR-α activation on cardiac I/R injury [13, 14, 44] and may indicate that preserved FAO is important for the maintenance of adequate energy production under the conditions of restored coronary flow, when oxygen supply is no longer rate-limiting.

**PPAR and Inflammation**

Beneficial effects of PPAR agonists may be attributed not only to modulation of cardiac metabolism but also to inhibition of inflammation with the salutary effects on the cardiac muscle [21, 83]. In fact, in the experiments that have demonstrated beneficial effects of PPAR agonists on the myocardial, cerebral, and hepatal I/R injury, protection was attributed to the attenuation of oxidative stress and inflammatory response via inhibition of the activation of NF-κB [14, 15, 20, 52, 84–86]. Research indicates that the acute anti-inflammatory effect of simvastatin occurs through a mechanism involving inhibition of PKCα-induced phosphorylation (and inactivation) of PPAR-α and activation of PPAR-α (and PPAR-γ) via a cyclooxygenase (COX)-2–dependent increase in the levels of natural PPAR ligand 15d-PGJ2, as well as decreased transactivation of NFκB [68, 82, 87, 88]. Figure 5 summarizes the potential mechanisms of PPAR activation as a part of pleiotropic effects of statins (including anti-inflammatory and antioxidant effects) and the agonists of PPAR-α—fibrates, which share a number of pharmacological properties with statins, suggests a mechanistic link between these two classes of drugs and similarity in their effects on PPAR-α [44, 89].

Thus, it is conceivable that an improved outcome of I/R injury in statin-treated normal and diabetic/hypercholesterolemic animals in our studies was linked to the anti-inflammatory effects of PPAR activation as well. In support of this, Planavila et al. [90] have reported that atorvastatin treatment prevented both the fall in the protein levels of PPAR-α and NF-κB activation in pressure-overload-induced cardiac hypertrophy.

**Conclusions**

The experimental data reviewed here suggest that changes in gene expression of PPARs are involved in the pathophysiological mechanisms of myocardial injury and may modulate it in a distinct way, dependent on the type and duration of cardiac pathology. Collectively, these data indicate that up-regulation of PPARs may underlie mechanisms of lipid-independent cardioprotective effects of hypolipidemic drugs in both normal and diseased heart. Thus, PPARs might represent an important therapeutic target in the management of ischemic heart disease in patients with or without metabolic disorders. However, a more detailed elucidation of the role of PPARs in myocardial ischemic injury and cardioprotection requires further investigation.

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Genes and Plasma Lipids in Czech Slavic Population

Jaroslav A. Hubacek and Rudolf Poledne

Abstract
Dyslipidemias are between the most important risk factors of the cardiovascular diseases. Studies with different designs show that the dyslipidemic pattern is clearly heritable and the heritability was estimated to be around 50%. We have analyzed the genetic background of elevated plasma lipids in three studies with different design, all originating from Czech Slavic population. We have examined two small groups of children (N~90) selected from opposite part of the distribution curve of total cholesterol of 2,000 children; large population of 2,500 individuals selected according the WHO MONICA study criteria and finally 8 years cohort of 250 individuals where substantial decrease of the plasma lipids, as a consequence of the socioeconomic changes, was observed. Altogether more than 100 variants within different candidate genes were analyzed. Our results showed that especially the variants within the genes for apolipoprotein E, apolipoprotein A5, hepatic lipase and HMGCo-A reductase play a significant role in the determination of the plasma lipid levels. Nutrigenetic analyses suggest an important role of gene environment interactions for variants within apolipoprotein A4, apolipoprotein A5, and cholesterol 7-alpha hydroxylase. However, we were not able to confirm some recent candidates, detected through the genome wide approach. For example the MLXIPL variants exhibit just very marginal effect on plasma triacylglycerols in our Slavic population. Our results point to some generally valid associations between genetic polymorphisms and plasma lipids and underline the importance of the analysis of the gene-gene and gene-environment interactions, as well as the intra-ethnic differences.

Keywords
Cholesterol • Dyslipidemia • Interaction • Nutrition • Polymorphisms • Slavic population • Triacylglycerols
**Introduction**

Cardiovascular diseases are the main cause of mortality in industrial countries, and the dyslipidemias (elevated plasma levels of total or LDL-cholesterol and triacylglycerols, lower levels of HDL-cholesterol) are among the major risk factors of their development. External/environmental causes of dyslipidemias (at first unhealthy diet and low physical activity) have been known for decades. However, family and twin studies clearly show that a substantial part of the dyslipidemias have a significant genetic background [1]. The heritability (the proportion of phenotypic variance attributable to genetic variance) of the dyslipidemias was estimated to be around 50%, however, despite the intensive research and methodological efforts achieved recently, the exact causes remain undetected. Despite the fact that there are mutations (rare deleterious single-hit changes) in some genes (for example LDL receptor, apolipoproteins A5 and B, PCSK9, LPL maturation protein) causing the hypercholesterolemia/dyslipidemia [2], the vast majority of this pathology is based on a polygenic background [3].

Soon after the first publications concerning the association between the individual genetic polymorphisms appeared [4], we have designed the first study. As the hypercholesterolemia is a result of both external and genetic factors, we supposed that pre-pubertal children are the best individuals to study the genetic predisposition – exposure to the negative external factors is shorter than in adults, they are non-smokers without alcohol consumption, and without lipid lowering medications; thus the genetic background could be more expressed.

**Prague Children Study**

In the late eighties, we have examined a total of 2,000 ten- to eleven-year-old children from complete biological families with at least one sibling, living in ten schools in the Prague 4 district [5].

Nonfasting total cholesterol was analyzed once from the blood obtained from the finger tip by dry chemistry method, with written agreement of the parents. The distribution curve of total cholesterol was constructed, to analyze the genetic predisposition of hypercholesterolemia; two groups, high-cholesterol (HCG, total cholesterol between 95 and 100 percentile) and low-cholesterol (LCG, total cholesterol between 5 and 10 percentile) were created. Individuals with the lowest total cholesterol, below 5-th percentile, were excluded because of the possibility of undetected and not examined metabolic disturbances, such as high level of thyroxine. These groups were repeatedly reexamined at intervals of 9–12 months and we excluded probands with at least two decreases (from the HCG group), or increases (from the LCG group) of total cholesterol over the desired values. Further, one proband with clinically confirmed diagnosis of familial hypercholesterolemia was also excluded from the HCG group. The final numbers of the probands within the groups were as follows: 82 probands in HCG with stable high total cholesterol levels and 86 probands in LCG with stable low total cholesterol levels.

Based on the candidate gene approach [6], almost 30 polymorphisms within the 13 genes with known functions in the metabolism and/or transport of plasma lipids were selected for the analysis during two decades. The overview of the variants is listed in Table 1.

As some of the polymorphisms were analyzed more than 20 years ago, Southern blotting and restriction fragment length polymorphism approach was used for a couple of variants. This makes the exact localization of the analyzed variant unclear and many of such variants are not characterized by the recent exact and unambiguous rs coding system. Later, we have analyzed the variants using the polymerase chain reaction and restriction analysis.

From the recent point of view, the protocol of the children study was underpowered – it was recognized that the relative by low number of individuals make such studies generally prone to false positive or false negative results. However, on the other hand, the several strengths of the study outweigh this problem. The unique and
careful selection of the examined individuals from the opposite parts of the distribution curve with repeatedly confirmed phenotype allows us to detect the real effects of some of the analyzed variants who were simultaneously or later confirmed in large population studies.

The first analyzed gene was the gene for apolipoprotein E (APOE) [7]. Firstly, we have detected almost a four times higher frequency of the carriers of the APOE4 allele in HCG compared to LCG and probands with APOE2 allele were less common in this group. Importantly, analysis of the large population sample (see in more detail below) reveals the frequency of the APOE4 allele carriers between the HCG and LCG group. This association between the APOE4 allele and elevated plasma LDL-cholesterol was 100 times confirmed in studies with different design and this confirms that our study is well designed and able to detect the real and significant effect of individual polymorphisms.

Table 1: Complete list of the genes and variants analyzed in our studies

<table>
<thead>
<tr>
<th>Gene</th>
<th>Variant</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOE</td>
<td>E2, E3, E4</td>
<td>Protein component of VLDL and HDL particles</td>
</tr>
<tr>
<td>APOB</td>
<td>XbaI, C-516&gt;T</td>
<td>Major constituent of LDL particles, major ligand for the interaction with LDL receptor</td>
</tr>
<tr>
<td>APOA1</td>
<td>G-75&gt;A and C83&gt;T</td>
<td>Major protein in HDL particles, cofactor for LCAT</td>
</tr>
<tr>
<td>APOA2</td>
<td>(CA)_n repeat</td>
<td>The second most represented protein in HDL particles</td>
</tr>
<tr>
<td>APOA4</td>
<td>Thr347&gt;Ser and Gln360&gt;His</td>
<td>Structural component of chylomicrones and HDL particles</td>
</tr>
<tr>
<td>APOA5</td>
<td>T-1131&gt;C, Ser19&gt;Trp, Val153&gt;Met</td>
<td>Modulates the activity of lipoprotein lipase and influenced the affinity of lipoprotein particles to cellular receptors;</td>
</tr>
<tr>
<td>APOC1</td>
<td>CGTT insertion/deletion</td>
<td>Constituent of triacylglycerol-rich particles, inhibitor of lipoprotein particles binding to cellular receptors</td>
</tr>
<tr>
<td>APOC3</td>
<td>C3238&gt;G</td>
<td>Component of VLDL and HDL particles</td>
</tr>
<tr>
<td>CETP</td>
<td>TaqI</td>
<td>Transfer insoluble cholesteryl esters among different lipoprotein particles</td>
</tr>
<tr>
<td>LPL</td>
<td>Asp291&gt;Ser</td>
<td>Key enzyme in lipoprotein metabolism, hydrolyses triacylglycerol in VLDL and chylomicrons</td>
</tr>
<tr>
<td>LDL receptor</td>
<td>PvuII</td>
<td>Major cell surface receptor that plays an key role in cholesterol homeostasis</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>A-204&gt;C *</td>
<td>The key enzyme in catabolism of cholesterol, catalyze the first step in bile acid synthesis</td>
</tr>
<tr>
<td>ABCG5</td>
<td>Gln604&gt;Glu</td>
<td>Responsible for the secretion of cholesterol into bile, potentially influence the responsiveness to dietary changes</td>
</tr>
<tr>
<td>ABCG8</td>
<td>Asp19&gt;His, Tyr54&gt;Cys, Thr400&gt;Lys, Ala632&gt;Val</td>
<td>Responsible for the secretion of cholesterol into bile, potentially influence the responsiveness to dietary changes</td>
</tr>
<tr>
<td>HMGCoA reductase</td>
<td>(TTA)_n repeat</td>
<td>The key enzyme in cholesterol synthesis</td>
</tr>
<tr>
<td>Hepatic lipase</td>
<td>C-480&gt;T</td>
<td>Hydrolyses triacylglycerol in TG-rich particles</td>
</tr>
<tr>
<td>MLXIPL</td>
<td>His241&gt;Gln</td>
<td>Cellular transcription factor</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>Leu72&gt;Met</td>
<td>Ligand for the growth hormone secretagogue receptor, regulates growth hormone release</td>
</tr>
<tr>
<td>INSIG2</td>
<td>G-102&gt;A</td>
<td>Regulates lipid synthesis by blocking the activation of SREBPs by SCAP</td>
</tr>
</tbody>
</table>

*aAnalyzed in the children study
*bAnalyzed in MONICA study
*cAnalyzed in the nutrigenetic study
reductase – these genotypes were almost twice more frequent in HCG than in LCG children [8]. However, this result was never confirmed or refitted in further studies.

Within the APOB gene, three variants have been investigated and strong, but not complete linkage disequilibrium (LD) was demonstrated between the variants within the regulatory part of the gene. Haplotype analysis suggests that deviations from LD are much higher in LCG children than in HCG children [9]. Namely, the presence of the I/I genotype together with at least one T-516 allele was more than five times higher in LCG than in HCG.

Interestingly, we have detected a significant difference between the HCG and LCG group also within the APOA5 gene. This gene, involved in the metabolism of triacylglycerol-rich particles was of outstanding interest, as we were participating on the description of the gene and have

<table>
<thead>
<tr>
<th>Gene</th>
<th>Variant</th>
<th>Different genotype frequency between the HCG and LCG</th>
<th>Association between polymorphisms and lipid traits within the HCG or LCG a</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOE</td>
<td>E2, E3, E4</td>
<td>E4 carriers more common in HCG</td>
<td>No</td>
</tr>
<tr>
<td>APOB</td>
<td>XbaI</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>C-516&gt;T Ins/del at signal peptide</td>
<td>Not individually, yes in linkage – Carriers of the II genotype and T allele are more common within LCG</td>
<td>No</td>
</tr>
<tr>
<td>APOA2</td>
<td>(CA)_n repeat</td>
<td>No</td>
<td>Carriers of the five and eight repeats have higher apoB and plasma cholesterol in HCG group</td>
</tr>
<tr>
<td>APOA5</td>
<td>T-1131&gt;C</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Ser19&gt;Trp</td>
<td>Yes, Trp carriers more common in HCG</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Val153&gt;Met</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>APOC1</td>
<td>CGTT insertion/deletion</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>APOC3</td>
<td>C3238&gt;G</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>CETP</td>
<td>TaqI</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>LPL</td>
<td>Asp291.Ser</td>
<td>Yes, carriers of the Ser291 were detected just within the LCG group</td>
<td>No</td>
</tr>
<tr>
<td>LDL receptor</td>
<td>PvuII</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>A-204&gt;C b</td>
<td>No</td>
<td>In HCG, CC individuals have higher total cholesterol than AA homozygotes</td>
</tr>
<tr>
<td>ABCG5</td>
<td>Gln604&gt;Glu</td>
<td>No</td>
<td>In LCG Gln604 carriers have lower LDL cholesterol than Glu homozygotes</td>
</tr>
<tr>
<td>ABCG8</td>
<td>Asp19&gt;His</td>
<td>No</td>
<td>In HCG, His19 carriers have lower total cholesterol than AspAsp homozygotes</td>
</tr>
<tr>
<td></td>
<td>Tyr54C&gt;ys</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Thr400&gt;Lys</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Ala632&gt;Val</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>HMGCoA reductase</td>
<td>(TTA)_n repeat</td>
<td>Yes, carriers of the genotypes with odd alleles</td>
<td>No</td>
</tr>
</tbody>
</table>

*aRecently specified as -203  
*bPossible gene-gene or gene-environment interactions necessary for the gene effect expression
focused our research on this gene in a wide range of different aspects.

In HCG, there was more than a twice higher number of APOA5 Trp19 carriers than in LCG, despite the fact that at the time of the first examination LCG and HCG did not differ in plasma triacylglycerol levels. It was a surprising result, as this gene was primarily detected as determinant of plasma triacylglycerol and not cholesterol. However, we have later confirmed this result [10] on a large population MONICA study (see below). In the population, the variant was associated with non-HDL cholesterol but not with total and/or LDL cholesterol – this fact can explain why this association was not detected before; despite the better risk prediction value, non/HDL cholesterol is usually not analyzed.

The gene for lipoprotein lipase is another gene primarily associated with triacylglycerol levels and exhibits also a different distribution of the variants between HCG and LCG groups. The carriers of the Ser291 allele seem to be protected from development of hypercholesterolemia.

The genotype of allelic frequencies of all other polymorphisms did not differ between the HCG and LCG significantly. For some of them, the exact role in the determination of plasma lipids remains unclear.

Interestingly, despite the fact that our groups represented a relatively narrow part of the population, for some analyzed variants we have observed also an association with plasma lipids within one group. From a recent point of view, it is likely that such observations represent a false positive result, but we cannot exclude (and we have not confirmed) that it could reflect the so-called variability gene effect. The variability gene effect [11] could be attributed to equilibrium with recently used epistasis (gene-gene) or gene-environment interactions [12]. We suppose that some other markers are necessary to be present for the expression of the allelic effect (for example, the consumption of a distinct kind of food in sufficient amount) on plasma lipids.

According to our results, especially APOA2 [13] and CYP7A1 genes seem to be involved in such interactions. Similar effects at the APOC1 locus almost surely reflect its allelic association (linkage disequilibrium) with different APOE alleles.

Based on the recent level of knowledge it is clear that the effects of individual polymorphisms on lipid traits need to be analyzed on large, well-selected population samples. The small studies are prone to both false positive and false negative results (type I and type II errors) and did not allow the adjustment to environmental and non-genetic factors (for example sex, age, smoking, BMI, alcohol intake, medication, dietary habits, physical activity…).

Large-Population-Based Study

To analyze the genetic determination of the plasma lipids in Czech Slavic population (primarily, later this sample was used also for the analysis of genetic predisposition to obesity, renal failure, osteoporosis,…), we have used the well defined and selected representative WHO MONICA (Multinational MONIstoring of trends and determinants in CArdiovascular diseases) [14] study. The examined individuals were selected and collected in nine Czech districts – largest cities were avoided – as a 1% population sample to examine the cardiovascular risk factors. This study is based on the population register and we have reached very high response rate at the first examinations – between 70% and 85%. The DNA bank based on MONICA study includes DNA samples from 2,559 individuals (1,191 males and 1,368 females, aged 25–65 years at the time of the first examination – 1997/1998, response rate 63%).

More than 100 biochemical and anthropometric characteristics were collected for all individuals. All individuals were examined twice within the 3 years; the first examination was performed in 1997/8, the second in 2000/1. The availability of two independent values further enhances the strength of the genetic association studies, if the obtained results are the same or similar in both years.

During the last 10 years of research, we have analyzed more than 100 variants within about 30 genes. The first focus was to analyze the genes selected on the candidate gene approach (with known functions of gene products). Recently also the confirmatory studies of the SNPs detected
through genome wide analyses (GWAs) approach were performed.

In the Czech MONICA study we have so far analyzed the following genes/variants where we have expected some associations with plasma lipid traits – apolipoprotein E (E2, E3 and E4 alleles), apolipoprotein CI (promoter insertion/deletion), apolipoprotein A5 (T-1131 > C, Ser19 > Trp), hepatic lipase (similarly to lipoprotein lipase, hydrolyses triacylglycerols in TG-rich particles; C-480 > T), and through GWAs discovered MLXIPL (cellular transcription factor; His241Gln). For more detail about some of the analyzed SNPs see table 1.

In this population based study, we have confirmed major associations between the APOE and APOA5 genes and plasma lipids. As expected, the plasma levels of total or LDL cholesterol were the lowest in carriers of the APOE2 allele and highest in carriers of the APOE4 allele but it needs to be mentioned that the lipid-lowering effect of the APOE2 allele was more pronounced than the enhancing effect of the APOE4 allele.

We were between the first, who described the effect of the common variants within the APOA5 gene on the plasma levels of triacylglycerols [15, 16] and later also on plasma non-HDL cholesterol levels. For the APOA5, we have proved that the plasma levels of triacylglycerols are lowest in individuals with just common alleles and that there is linear enhancing effect of the less common alleles (C-1131 and Trp19), which increase the plasma TG levels by about 0.25 mmol/L. The third examined APOA5 variant (Val153 > Met) was associated with HDL-cholesterol levels, in sex-dependent manner with ValVal homozygotes showing higher levels in females but not in males [17].

In addition, there is also a couple of variants, analyzed in the studies not primarily related to lipid metabolism but the association with a lipid parameter or another risk factor associated with CAD was detected in the “second phase data mining”. This includes the gene for ghrelin (ligand for the growth hormone secretagogue receptor which also regulates growth hormone release) where the Leu72 > Met variant is significantly associated with plasma levels of HDL-cholesterol both in males and females [18], promoter variant (G-102 > A) within the INSIG2 gene (regulates lipid synthesis by blocking the activation of SREBPs by SCAP) which exhibit a sex-specific effect on HDL-cholesterol levels – it is affected by the polymorphism just in females.

Surprisingly, we were not able to confirm the association between the His241 > Gln variant within the MLXIPL gene and triacylglycerol levels [19], despite the fact that this GWA-approach-detected SNP was suggested to be the strongest genetic determinant of plasma triacylglycerol levels [20], even stronger than APOA5 (for a brief summary of the effects of individual polymorphisms see Table 3).

**Table 3** Summary of the genes and variants analyzed in Czech MONICA sample with confirmed associations with plasma lipids

<table>
<thead>
<tr>
<th>Gene</th>
<th>Variant</th>
<th>Detected association</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOE</td>
<td>E2, E3, E4</td>
<td>E4 allele carriers have higher, and E2 allele carriers have lower, levels of total and LDL cholesterol</td>
</tr>
<tr>
<td>APOCI</td>
<td>CGTT Ins/ Del</td>
<td>Insertion allele is associated with elevated plasma TG levels</td>
</tr>
<tr>
<td>APOA5</td>
<td>T-1131 &gt; C</td>
<td>Carriers of the less common allele C have higher TG and non-HDL cholesterol levels</td>
</tr>
<tr>
<td></td>
<td>Ser19 &gt; Trp</td>
<td>Carriers of the less common allele Trp have higher TG and non-HDL cholesterol levels</td>
</tr>
<tr>
<td></td>
<td>Val153 &gt; Met</td>
<td>Higher HDL cholesterol was found in Val/Val homozygotes in females but not in males</td>
</tr>
<tr>
<td>Hepatic lipase</td>
<td>C-480 &gt; T</td>
<td>Carriers of the T allele have higher levels of HDL cholesterol</td>
</tr>
<tr>
<td>MLXIPL</td>
<td>His241 &gt; Gln</td>
<td>Borderline association of the Gln allele with low plasma TG levels</td>
</tr>
<tr>
<td>INSIG2</td>
<td>G-102 &gt; A</td>
<td>Females, carriers of the GG genotype have higher levels of HDL cholesterol</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>Leu72 &gt; Met</td>
<td>Met carriers have lower plasma HDL cholesterol</td>
</tr>
</tbody>
</table>
Nutrigenetics in Population Based Study

One of the promising fields in the recent population-genetic research is the nutrigenetic. Here, the question how the individual genetic variants interact with diet in the determination of the plasma lipid levels is analyzed. More or less by chance we have the possibility to study the decrease of plasma lipids in the population after significant nation-wide changes in dietary habits. The possibility to study the interaction between the dietary changes and genes and plasma lipid changes was based on an unusual coincidence.

The 135 unrelated adult males and 155 adult females included in the “interventional” study represented a part of an 8-year cohort selected in 1988 and then reinvestigated in 1996 in one district according to the protocol of the MONICA study. All individuals have the lipid levels in both 1988 and 1996. Both data of cholesterol concentrations from the whole MONICA study and data of food consumption obtained from the Institute of Agricultural Economy in the Czech population were used as evidence of the changes between the years 1988 and 1996. From 1988 to 1996, the food intake and dietary composition in the Czech population changed dramatically. The major reason was probably that during the post communist economy transition, the subsistence on meat and dairy products was canceled in 1991. The most pronounced changes were found in the intake of animal fat and lard which decreased by 45%. Whereas the consumption of egg and beef and pork meat dropped by about 20%, the consumption of chicken and fish increased by 10%. Also the consumption of vegetables, fruits and vegetable oils increased – in 1988, the consumption was ~133 kg per capita and year, in 1996 it was already ~150 kg per capita and year. Reflecting these dietary changes, the mean cholesterol concentrations decreased in the population by ~one-half mmol/L (about 10%). Importantly, there was no evidence for increase of physical activity and the body mass index remains unchanged between 1988 and 1996 [21].

As well as in the entire Czech population (results from the whole MONICA study), the cholesterol concentration in individuals form the district analyzed in detail decreased over the 8-year period by ~0.6 mmol/L. Interestingly, the decrease was not observed in all individuals. In fact, in 65% individuals total cholesterol decreased by more than 5%, in 25% remained unchanged and in 10% an increase by more than 5% was detected. Unfortunately, as we cannot in 1988 expect the political, socioeconomically and inferential life style changes, there are just the basic lipid/anthropometrical parameters available.

At this small cohort we have analyzed almost 30 variants within different genes (mostly the same variants as for the previous studies were selected) – apolipoprotein E, apolipoprotein C1, apolipoprotein A1 (major protein in HDL particles, cofactor for LCAT, which forms most of the cholesterol esters in plasma; G-75 > A and C83 > T), apolipoprotein A4 (structural component of chylomicrons and HDL particles; Thr347 > Ser and Gln360 > His) apolipoprotein A5, apolipoprotein B (just insertion/deletion within the signal peptide), hepatic lipase (C-480 > T), lipoprotein lipase (Ser447 > Ter), ABCG5 and ABCG8 transporters and finally cholesterol 7 alpha hydroxylase.

Of these analyzed variants, just five significantly influenced individual differences in the decrease of the plasma LDL-cholesterol. Interestingly, in all cases, the possible diet-induced changes were gender-specific [22–25]. According to our study greater profit from the dietary changes will be found in the male carriers of the following alleles (genotypes/haplotypes)
- Trp19 at APOA5 gene
- C-204C at CYP7A1 gene
- Gln360Gln+ at least one TrpThr347 allele at APOA4 gene
- Thr400Thr at ABCG8 gene
- Tyr54 allele within the ABCG8 transporter seem to have a better response to dietary changes.

In females, just the carriers of the Tyr54 allele within the ABCG8 transporter seem to have a better response to dietary changes.

Under the borderline of the statistical significance remains the apolipoprotein E variant where, in accordance with most of the published results, carriers of the APOE4 allele seem to profit more from dietary interventions.

Other polymorphisms were not associated with significant changes in lipid parameters.
Again these negative results could be the result of the relatively low number of analyzed individuals and cannot be excluded as a candidates for the future nutrigenetic research.

Conclusions

Genetic polymorphisms within almost 100 genes encoding different proteins have been in the center of our attention for at least 20 years. A substantial part of them was analyzed in an effort to detect the putative association with plasma lipid traits. Apolipoproteins E and A5 genes and their variants belong to the most studied genetic factors in search of the genetic basis of dyslipidemia, and are undoubtedly linked to plasma levels of triacylglycerols and cholesterol. The hepatic lipase promoter is repeatedly associated with HDL-cholesterol not just in our study but also in other studies. Further, our results suggest that also genes for \textit{HMGCoA} reductase, \textit{CYP7A1} and \textit{APOA4} could have some effects of plasma lipid levels of cholesterol and triacylglycerols. In the near future, we will focus on the analysis of the impact of gene – environmental interactions (most importantly, physical activity and dietary habits but also psychosocial factors and alcohol intake seem to be very important factors) on plasma lipids as well as on the detection of the significant gene-gene interactions.

References


Part V

Hypertension and Arrhythmias
Genetic Basis of Salt-Sensitive Hypertension in Humans

Frans H.H. Leenen, Md. Shahrier Amin, Alexandre F.R. Stewart, and Frederique Tesson

Abstract

The genetic network responsible for blood pressure (BP) variation in the general population and specifically the hypertensive population remains elusive. Several recent genome-wide association studies (GWAS) have identified and confirmed loci associated with BP and hypertension. However, only a small fraction of the trait is currently explained. This apparent deficit relative to the estimates for heritability can be due to several factors, a major one being the poor assessment of the phenotype, i.e. BP. All GWAS have used so far office BPs, which are notoriously variable. Imputation of BP in treated subjects is done by a fixed number. In addition, BPs are usually being “adjusted” for e.g. age, gender or body mass index under the assumption that these do not alter BP systematically by genotype, an assumption that appears no longer valid.

A better approach may include more specific assessment of the actual BP level in a given individual for both cases and controls, preferably by 24-h ambulatory BP monitoring, for cases off antihypertensive therapy, and to stratify for factors such as sex, age and body mass index. This approach will likely provide better insight into the distinct genetic architectures contributing to different hypertension phenotypes and explain a substantially larger part of the BP variance. Further improvements will likely emerge when other environmental/lifestyle factors are incorporated, such as extent of alcohol intake, stress, salt intake or physical activity. In the ongoing Ottawa GWAS for BP response to salt, we hope to identify loci and genes associated with salt-sensitive versus salt-resistant hypertension.

Keywords

Blood pressure phenotypes • Candidates genes • Dietary salt • Genetics • Genome-wide association studies • Heritability • Hypertension • Salt resistance • Salt sensitivity • 24-h ambulatory blood pressure monitoring
Introduction

Primary or essential hypertension affects 20–30% of the adult population in North America [1, 2], and represents a major risk factor for cardiovascular morbidity and mortality. There is a significant genetic contribution in the susceptibility to hypertension. Estimates for heritability – the proportion of variance in a trait that can be attributed to genetic variance – of systolic blood pressure (BP) range from 0.18 in the general population, 0.19–0.45 within families, 0.74 in young adult twins [3–5] to 0.80 in hypertensive adolescents with a family history of hypertension [6]. As will be reviewed in the next section, in spite of numerous studies the genetic network responsible for BP variation in the general population and specifically in the hypertensive population remains elusive.

Genome Scans for Essential Hypertension

A number of genetic linkage analyses have been performed in different populations around the world. Studies have used different sets of markers for genotyping and most were widely spaced (average inter-marker distance \( \sim 5–10 \) cm) which will miss variations in intermediate chromosomal segments. In the Family BP Program, the results from four individual studies incorporating >6,000 individuals of multiple ethnic origins were analyzed. In Whites with hypertension, high linkage was found on 2p11, 3p14, 14q32 and 16q12 for systolic BP and 3p14, 9q31, 14q32 and 16q12 for diastolic BP [7]. Interestingly, genetic linkage to BP has been weak in the larger studies for Caucasians, but strong when performed in small, possibly more homogeneous, populations (Framingham study – 17q; Dutch – 4p; Icelandic – 18q; Sardinian – 2p). Genome scan meta-analysis of nine studies in Caucasians demonstrated significant linkage to 2p12–q22.1 and 3p14.1–q172.3 loci [8]. The detection of BP alleles by genome-wide scans may also depend on their age. A hypertension allele that originated 100,000 years ago would by now be ubiquitous across the populations, making its detection unlikely. For example, the aldosterone synthase (8q21) alleles C-344 and T-344 exist at an equal frequency in Caucasians (T/C \( \sim 0.53/0.47 \)), TT homozygotes showing a 1.45 times higher risk of hypertension in Caucasians [9]. However, none of the linkage analyses showed significant linkage to this locus. On the other hand, regions with a significant impact might be found in a small population if the genes have emerged recently.

Linkage analysis, however, is best suited for monogenic disorders in which genes are more highly deterministic, with a major gene contribution and a minimum environmental contribution. Association studies are considered to be statistically more powerful than linkage analysis for complex traits.

The Wellcome Trust Case Control Consortium (WTCCC) study was the first attempt to identify SNPs associated with essential hypertension using a genome-wide association (GWA) approach [10]. The Affymetrix GeneChip 500 K Mapping Array Set was used. This study identified several loci for six common diseases but failed to spot one for hypertension [10]. The same year, the Framingham Heart Study was also negative for hypertension but the SNP density was low (~70,000) and probably insufficient to tag enough linkage disequilibrium blocks [11]. However, since the beginning of 2009, eight studies have yielded positive results in different populations including Caucasian [12–14], African-American [15] and Asian [16, 17] populations (Table 1). A few identified loci were reproducible; for example, eight loci associated with BP were found in both the Global BPgen and the CHARGE studies [12, 13]. Four were also associated with BP and hypertension in the Korean population [18] and two among Han Chinese [19]. Some genetic variations associated with BP and/or hypertension were located within genes making those genes sound as candidate genes [20, 21]. However, only a few of these polymorphisms have so far been associated with a function. A SNP associated with BP and located within an intronic conserved element of the STK39 gene has been shown to influence the expression of STK39 in vitro [22, 23].
Table 1  Most significant GWA findings with BP and hypertension phenotypes

<table>
<thead>
<tr>
<th>Study population</th>
<th>Phenotyping</th>
<th>Major findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Wellcome Trust Case Control Consortium</td>
<td>History of hypertension diagnosed before 60 year of age, BP the mean of 1–3 BP recordings (BP not imputed for treatment)</td>
<td>Negative study</td>
</tr>
<tr>
<td>Framingham Heart Study 100 K Project</td>
<td>For most subjects, BP the mean of 2 recordings (BP imputed for treatment)</td>
<td>Negative study</td>
</tr>
<tr>
<td>NFBC1966. 4,730 subjects with European ancestry</td>
<td>BP the mean of 2 recordings (BP not imputed for treatment)</td>
<td>Negative study</td>
</tr>
<tr>
<td>Global BPGen</td>
<td>BP the mean of 2–4 recordings (BP imputed for treatment)</td>
<td>rs17367504 (CR1) and SBP 11191548 (CR10) and SBP 12946454 (CR17) and SBP 16998073 (CR4) and DBP 1530440 (CR10) and DBP 653178 (CR12) and DBP 1378942 (CR15) and DBP 16948048 (CR17) and DBP</td>
</tr>
<tr>
<td>CHARGE</td>
<td>BP the mean of 2 recordings (BP imputed for treatment)</td>
<td>rs1004467 (CR10) and SBP 381815 (CR11) and SBP 2681492 (CR12) and SBP 2681472 (CR12) and DBP § HT 3184504 (CR12) and SBP and DBP 9815354 (CR3) and DBP 11014166 (CR10) and DBP 2384550 (CR12) and DBP 6495122 (CR15) and DBP</td>
</tr>
<tr>
<td>KORA/HYPEST</td>
<td>BP the mean of 2 recordings, subjects did not receive BP lowering medication</td>
<td>rs12153297 (CR5) and SBP 11646213 (CR16) and HT</td>
</tr>
<tr>
<td>HUFS</td>
<td>BP the mean of 2 recordings (BP not imputed for treatment)</td>
<td>rs5743185 (CR2) and SBP 16877320 (CR6) and SBP 17365948 (CR8) and SBP 12279202 (CR11) and SBP 11160059 (CR14) and SBP</td>
</tr>
</tbody>
</table>

HT Hypertension
*Replicated in another population

However, a replication study did not confirm the association of this SNP with BP [23]. A recent study looked at the association of 1,180 non-synonymous SNPs with systolic BP and hypertensive status [24]. Two SNPs, one in the arginine decarboxylase and another in the phospholipase D2 genes, were associated with both systolic BP and hypertension [24]. However, altogether, only a fraction of the trait (1–3%) is currently explained by haplotype-tagging SNPs [25].

Structural variations, such as CNVs, have been more recently characterized. CNVs are common in the human genome [26] and might alter gene dosage, disrupt coding sequences, or perturb long-range gene regulation [27]. SNPs and CNVs have been shown to capture 83.6% and 17.7% of the total detected genetic variation in gene expression, respectively [28]. The signals from the two types of variations have little overlap [28]. Therefore, both types of variants should
be tested. So far, very few studies have explored the potential association of CNVs with BP/hypertension phenotypes [29, 30]. One of them found an association between a CNV and endothelin-1 expression [29].

**Evaluation of Candidate Genes for Essential Hypertension**

Loci suggested from a genome scan need to be further verified both by increasing the number of markers and assessing their physiological roles. Evaluation of candidate genes is therefore essential to investigate the effects of variants in relation to presence or absence of hypertension or more preferably to BP as a continuous variable. To date >150 genes have been studied for possible association with BP. We will only provide a brief summary of the main studies focusing on genes of possible interest for salt-sensitive hypertension particularly in Caucasians.

**Single variants:** A number of studies evaluated candidate genes in the RAAS, the sympathetic nervous system and relevant ion channels. Some variants in each category show major ethnic variability. The difference in frequency between normotensive and hypertensive populations is usually modest. Among the RAAS components that have been most widely studied, a meta-analysis for the angiotensinogen (AGT) M235T polymorphism including 45,267 subjects showed a stepwise increase in angiotensinogen levels and increased risk of hypertension by 8% in MT and 19% in TT homozygote Caucasians [31]. Homozygosity for the T allele of C-344T in aldosterone synthase promoter conferred a ~1.45 fold higher risk of hypertension in Caucasians [9]. A Mineralocorticoid Receptor gene variant showed marked protection [32], but this has not yet been reproduced. For other RAAS components (ACE, AGTR1, AGTR2) association has been weak or not always reproducible. In the sympathetic nervous system, variants contributing to hypertension have been identified in almost all steps. Most of these variants impart mild (<2 mmHg difference between groups) to moderate (2–6 mmHg difference) effects. Many ion channel mutations are causes of rare Mendelian forms of hypertension or hypotension. Recent studies showed that many common and rare variants of genes involved in sodium transport are present in the general population and loss-of-function mutation carriers exhibit lower BP [33]. The large number and diverse types of channels in neurons still need to be evaluated. Interestingly, variants of cytoskeletal components such as adducin, that might modulate ion channel activity have shown fairly consistent association with BP in different populations. The alpha adducin G460W substitution was found to be an important risk factor for hypertension in Caucasians [34, 35]. Variants of ion channel regulatory or interacting proteins have also been identified, and linked to greater change in BP over time.

**Haplotypes:** For several genes non-significant effects of individual variants attained significance when studied with co-inherited variants. In White siblings that shared two microsatellite marker alleles in chromosome 16p12 including the epithelial sodium channel (ENaC) β and γ subunits genes, the difference in systolic BP was half (7 mmHg) of those that did not share any alleles (14 mmHg) [36]. The estimated frequency of the SCNN1G haplotype (rs13331086, rs11074553, and rs4299163) was substantially greater in subjects with high (13.3%) than low (0.6%) systolic BP [37]. Homozygosity for the intron 6CC and exon 8CC/TT of the serum/glucocorticoid regulated kinase 1 gene (SGK1) compared to other genotypes was found to be responsible for 4/2 mmHg higher BP as well as a greater increase in BP [2.1/0.8 vs 1.6/0.4 mmHg/year] over a mean follow-up period of ~11 years [38]. In the same cohort, individuals with the NEDD4L haplotype (rs4149601GG + rs2288774 CC/CT) had 0.8/0.7 mmHg higher BP as well as greater increase in BP [1.7/0.4 vs 1.5/0.3 mmHg/year] [39]. Furthermore, multiple rare and common WNK1 variants in haplotypes can contribute substantially to BP variation and hypertension [40, 41].

**Gene-gene interactions:** Few studies have assessed the effect of co-inherited mutations in different genes on BP. Genetic combinations
might increase the risk of hypertension, nullify each other’s effects or be protective and contribute to resistance to hypertension. For example carriers of the MM(AGT), AA(AGTR1), CC(CYP11B2), DD/ID(ACE) combination were found to have a substantially higher probability of being hypertensive [42]. In a cohort of 226 Chinese parents and 253 of their offsprings, synergism was noted for ADD1 Trp and ACE I/D genotypes: 9.3 mmHg higher systolic BP in (ADD1 TrpTrp+ACE DD) versus (ADD1 TrpTrp+ACE II) [43]. Clear synergism for the ADD1 variant and variants in other genes such as ADD3, NEDD4L, WNK1 and AS have also been reported [34].

Assessment of Hypertensive Phenotype

Association studies indicate the possible contribution of a number of loci and genes to higher BP, but so far only a small fraction of the trait can be explained. Several factors may contribute to this apparent deficit relative to the estimates for heritability. A major factor may be the rather poor assessment of the phenotype, i.e. BP, particularly in the GWAS. In all GWAS, the BP was only quantified by office measurements (see Table 1). Even if measured properly, these BP values are notoriously variable and considering BP as a quantitative trait can markedly differ from the actual BP for a given individual. When considering BP as a qualitative trait (i.e. presence vs. absence of hypertension), office BP can readily lead to misdiagnosis, i.e. over-diagnosis (“white-coat hypertension”), or under-diagnosis (“masked hypertension”), and lead to high percentage of false positives or false negatives in cases and controls [44, 45]. No GWAS has so far employed the current gold standard for assessment of BP: 24 h ambulatory BP monitoring (ABPM).

A second confounder relates to the different approaches used to deal with the impact of antihypertensive drug therapy on BP in GWAS (Table 1). Tobin et al. [46] and Cui et al. [47] reviewed why some approaches are fundamentally flawed. These include ignoring the problem altogether and analyzing observed BP in treated subjects as if it were underlying BP, or excluding treated subjects from the analysis. In studies evaluating BP as a quantitative trait, no appropriate correction for use of antihypertensive therapy can lead to substantial shrinkage in the estimated effect of the genetic determinants of BP as well as a marked reduction in statistical power. Correction relies upon imputation in treated subjects of the underlying BP from the observed BP. Newton-Cheh et al. [12] for the Global BP Gen consortium imputed BPs by adding 15 and 10 mmHg for systolic and diastolic BP. Levy et al. [13] for the CHARGE consortium added 10 and 5 mmHg to observed systolic and diastolic BPs. To account for the number of drugs, Cui et al. [47] proposed stepped increments of 8/4, 14/10 and 20/16 mmHg to the measured BPs of treated subjects taking 1, 2 and ≥3 drug classes. This appears to be a better approach rather than adding a fixed number irrespective of the intensity of antihypertensive therapy. However, none of these approaches take into account the marked inter-individual variability in BP responses to antihypertensive therapy. Figure 1 is an example of this large variability in BP response, in this particular study to hydrochlorothiazide. In addition, this study highlights that a major part of this variability can depend on the genetic background of the individual, in this study variants in three genes involved in sodium transport [34]. These variants may not only influence the antihypertensive response to the thiazide diuretic, but also may contribute to salt-sensitive increases in BP [34]. Imputing all treated BPs by adding a fixed number clearly would cause substantial shrinkage if not disappearance of the effect-size of these variants.

A third confounder relates to the presence of hypertension in controls for case control studies. Using an unphenotyped control sample from the general population may save resources [10], but clearly causes misclassification. According to the authors of the WTCC “the effect this has on power is modest unless the extent of misclassification bias is substantial: for example, if 5% of controls would meet the definition of cases as the
same age, the loss of power is approximately the same as that due to a reduction of the sample size by 10%” [10]. Using a non-phenotyped control population may indeed be a valid approach for many diseases that are relatively rare in the general population, but not for hypertension. Figure 2 shows the increasing prevalence of hypertension by age, reaching up to 30–40% in the 50–59 year age-group, and 50–60% above 60 years of age [2, 48]. Zhang et al. [49] recently re-estimated the power for the WTCC hypertension case control study and estimated that the statistical power was likely minimal. In late onset diseases such as hypertension, additional ambiguity will arise when individuals with putative variant alleles develop hypertension later in life. Are these young individuals “controls”?

Taking the above three issues all together, we propose as a more optimal strategy to minimize misclassification bias that (a) 24-h ABPM be performed
in both cases and controls; (b) antihypertensive therapy be discontinued for a short period if possible at all, and (c) controls not being age-matched, but rather selected from the older (>65–70 years), normotensive population, who less likely may still develop genetically determined hypertension. This approach will provide a more specific assessment of the actual BP level in a given individual, thereby providing a more accurate, and likely larger, effect-size of the genetic determinants of BP variation in the population. This approach will likely also provide higher explanatory power for the heritability of hypertension and, a closer step toward the understanding of the underlying genetic architecture.

**Genotype by Environment Interactions**

Whereas proper assessment of the BP phenotype appears an essential step toward understanding of the underlying genotype, there are a variety of factors which modulate this relationship. These factors are often ignored, as stated by Clark et al. [50]: “Despite a plethora of editorials and perspectives that expound on its importance, most studies make an early and prominent assumption that no genotype – environment interactions exist.” Alternatively, BPs are being “adjusted” for parameters such as age, gender or body mass index [12, 13] under the assumption that these do not alter BP systematically by genotype. This assumption appears no longer valid. Shi et al. [51] re-evaluated the genome-wide linkage analysis of hypertensive siblings conducted by the HyperGEN study for gene-age interaction using a newly developed variance components method that incorporates age variation in genetic effects. Substantially improved linkage evidence, in terms of both the number of linkage peaks and their significance levels, was observed. Gene-gender interactions have been demonstrated as well. Rana et al. [52] reported gender-specific effects of single nucleotide polymorphisms (SNPs), haplotypes, and gene-by-gene interactions that determine BP in white Americans. In females, polymorphisms in beta-1 and alpha-2A adrenergic receptor genes contributed to BP, whereas in men, polymorphisms in beta-2 adrenergic receptor and angiotensinogen genes were associated. Such sex-specific association may still show up in a traditional, sex-adjusted analysis. However, associations may also go in the opposite direction. Seda et al. [53] reported that in male French Canadians rs575121 GG homozygotes had the highest systolic BP (131 ± 4 mmHg) but the same allele in women was associated with the lowest BP (113 ± 4 mmHg). Different genetic variants may also contribute to hypertension relative to weight. Söber et al. [54] reanalyzed the discovery sample KORA S3 together with the replication samples stratified by weight. For the top 2 SNPs of this study, rs11195419 (ADRA2A) contributed to BP levels only among overweight subjects, whereas rs10889553 (LEPR) did only among normal-weight subjects. Some gene variants may contribute to increasing BP dependently or independently (e.g. *FTO*) of their effects on weight [55, 56].

Altogether, it appears that in order to truly understand the distinct genetic architectures contributing to the different hypertension phenotypes, existing GWAS should be re-analyzed after stratification by factors such as sex, body composition parameters and age. One may anticipate identification of distinct genotypes with likely large effect sizes. If future studies also include more precise assessment of actual BP levels, the cumulative effect of identified loci may explain a substantially larger part of the BP variance than the current 1–3%.

Further improvements will likely emerge when other environmental/lifestyle factors are incorporated, such as extent of alcohol intake, stress, salt intake, physical activity.

**Genes and Salt-Dependent Hypertension**

Among the many variables affecting BP, high sodium intake has a significant influence on BP and the increase in BP with age. Findings from the European Project on Genes in Hypertension (EPOGH) indicate that the phenotype-genotype
relations strongly depend on host factors such as salt intake [57]. Indeed, in a Chinese population (n=1,906) the estimate for heritability of systolic BP was 0.31 on regular dietary salt intake, and increased to 0.49 under low sodium intake [58]. Salt-sensitivity of BP has not yet been used in a genome scan to identify variants contributing to higher BP on high salt intake.

Salt and BP: In both INTERSALT and WHO-CARDIAC – two large multi-center cross-sectional studies, sodium intake was found to affect BP in most parts of the world. In INTERSALT [59] a significant linear relation was found between 24-h urinary sodium excretion and slope of systolic BP with age (0.3 mmHg/year/100 mmol Na) and diastolic BP (0.2 mmHg/year/100 mmol Na) across all populations in 52 centers (Fig. 3). When analyzed within individual centers, a significant positive coefficient for sodium and BP was found in 8 (3–8 mmHg systolic BP/100 mmol Na) and 3 (~4 mmHg diastolic BP/100 mmol Na) centers, while a negative coefficient was noted in 2 (~3 to ~6 mmHg systolic BP/100 mmol Na) and 3 (~3 to ~4 mmHg diastolic BP/100 mmol Na) centers. Similar differences were also present in populations in WHO-CARDIAC [60].

Assessment of salt-sensitivity: Several measures have been used to assess the BP response to salt – ranging from high versus low salt intake per se to volume/saline loading and salt depletion with diuretics. These methods do not address the same mechanisms and the latter can misclassify as many as 50% of individuals by activating the sympathetic nervous and renin angiotensin systems [61]. Office versus ambulatory BP also classifies different sets of patients as salt-sensitive (SS) or salt-resistant (SR) [62] with the latter giving better estimates [62–64]. Meta analyses of lifestyle interventions showed that decreasing sodium intake by 70–100 mmol/day decreases BP by about 3–4/2–3 mmHg in hypertensives and by only 1/0.5 mmHg in normotensives [e.g. 65]. INTERSALT and controlled trials of low versus high salt diet [59, 62, 66] indicate the presence of several phenotypes – SS, SR and counterregulators, but overall the BP response to salt appears to be normally distributed (Fig. 4). The correlation coefficient for difference in BP between periods of regular versus low salt intake in Whites ranged from ~0.25 (parent offspring), to ~0.50 (sib-sib) and ~0.7 (twin-twin) [Ref 67].

Age and SS: The BP response to salt intake can be seen at all ages but prevalence of SS increases in older age groups. Hurwitz et al. [68] demonstrated that there is an increment of 2.4 mmHg to the SS component of systolic BP for each decade of age. Analysis of data involving 47,000 individuals from 24 communities showed that the
decrease in both systolic BP and diastolic BP in response to sodium restriction is positively related to age. The decreases in BP with 100 mmol/day reduction in sodium intake increase from 5.0/1.8 mmHg at 10–19 years to 10.3/4.3 mmHg at 60–69 years [68]. Reductions in BP varied from 5.3 mmHg in individuals <45 years to 7.5 mmHg in those >45 years. The mechanisms responsible for this increase in salt sensitivity of BP with age have not yet been determined, but less reactive increase in renin release with ageing likely plays a major role.

Gender and SS: Both epidemiological observations and controlled trials show a clear gender difference in the BP response to salt [69]. In premenopausal females, estrogens appear to blunt the effect of high salt on BP [70]. Exacerbated hypertension develops in ovariectomized mRen [2] Lewis rats on high salt diet [71]. Otherwise healthy women previously classified as being SR were found to become SS following surgical menopause [72], whereas transdermal 17β-estradiol reduces salt sensitivity in post-menopausal women [73].

Candidate gene studies for salt-sensitive hypertension: Based on studies in animal models of SS hypertension and human monogenic forms, candidate genes have been studied for possible association with SS in case-control and sib-pair analyses. However, there is a paucity of such studies and most are limited in sample size. In larger samples from the general population, assessment of SS and the relationship to genotype has been limited to casual measurements of BP, urinary sodium (UNa), or questionnaires on average food intake. The lack of universal definition of SS and use of different strategies to classify individuals makes comparisons difficult. Short cross-over trials (<2 weeks on each diet) may also underestimate the actual effect [66]. In a 2004 review, Beeks et al. [74] found only 28 potential studies that assessed genetic contribution to SS by some intervention, of which only 23 provided sufficient details. Moreover, of these 23 only 15 assessed effects of dietary salt. A pooled analysis could not be performed due to heterogeneity in methods. Since this review, only 4 more studies were reported, 1 in Chinese, 1 in Japanese and 2 in Caucasian populations.

Studies of single variants and haplotypes: Among the studies reviewed by Beeks et al. [74] only the α-adducin G460W and HSD11B2 G534A and CA repeat variants showed reproducible positive association to SS in different populations. Homozygosity for the ADD1 WW was associated with ~two-fold greater response to sodium versus ADD1 G carriers. ADD1 WW is also associated with higher serum ouabain levels and higher increments of BP (2.2/1.4 mmHg per

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**Fig. 4** Difference in day systolic BP on 24-h ambulatory BP monitoring after 3 weeks of high (~300 mmol/day) or low (~100 mmol/day) salt diet in subjects with mild hypertension. Average decrease in systolic BP: −7.8±1.2 mmHg. Salt-sensitivity was defined as a decrease in systolic BP ≥5 mmHg on low vs high salt diet. (Leenen et al. unpublished data)
50 mmol increase in $U_{Na^+}$ in carriers taking a high salt diet [75]. Contrary to association studies in essential hypertension, both positive and negative associations with SS were noted for GNB3 C825T, ACE I/D and AGT M235T. In whites, mostly negative associations were found for AGTR1 A1166C, CYP11B2 C344T, and SCNN1G intron 2 conversion polymorphism. In Whites, the G protein-coupled receptor kinase 4 (GRK4) 486 V was associated with lower BP (~4.6 mmHg vs A486) [76]. In Japanese hypertensives (n = 83), the increase in BP with change in salt intake from ~50 to ~340 mmol/day varied according to the number of GRK4 variant alleles: 25 subjects with none were all salt-resistant whereas the 29 subjects with ≥3 alleles were all salt-sensitive [77]. In a cohort of 171 hypertensive and 48 normotensive White individuals the ADRB2 genotype was not associated with hypertension, but the difference in MAP between periods of low and high salt intake was higher for 46AA [16.5 mmHg] and 79CC [13.6 mmHg] homozygotes and individuals with the 46AA/79CC diplotype [16.5 mmHg] compared to 10.4 mmHg in the other genotypes [78]. WNK1 or KCNJ1 have not yet been studied.

Gene-gene interactions in salt-sensitive hypertension: Few studies assessed the simultaneous effect of several candidate genes on SS of BP. In a cohort of Spanish essential hypertensive patients, the II allele of ACE was found to be significantly associated with SS (odds ratio 2.23, 95% CI 1.34–3.71) and 9.8 mmHg increase in 24-h systolic BP on high salt in II vs 1.2 mmHg in DD [79]. However, no associations or interactions were found with AGTR1 and AGT. In contrast, clear interactions were reported for ADD1, WNK1 and NEDD4L variants and the change in systolic BP in response to a 2 h i.v. Na load in 341 Caucasian hypertensives, in a pattern opposite to the BP response to diuretic therapy [34]. It remains to be assessed whether the BP response to dietary salt follows this pattern.

In summary, little is known so far about the genes that are responsible for salt-sensitive hypertension. Most candidate genes associated with essential hypertension or changes in BP over time have shown little relation to SS or have not yet been studied for their effect on salt sensitivity. On the other hand, some genes that have not been found to be associated with essential hypertension might have significant impact on the SS phenotype.

Ottawa GWAS for BP Response to Salt

There have been no GWAS in humans assessing the genetic contribution to salt sensitivity of BP. Genes that are responsible for imparting salt resistance have so far been largely ignored. Given the fact that the genetic constitution of the body is evolutionarily programmed to preserve salt within a narrow physiological range (intake < 50–100 mmol/day) and high salt in the diet has been a relatively recent introduction [74], emergence of SR genes appears to be a more recent event that might be detected more easily than SS genes which are probably more ancient in origin. Indeed, it is likely that the five-fold greater salt intake in the average Western diet would have naturally predisposed everyone to chronic elevation of BP had it not been due to the emergence of yet to be identified salt resistant genes. In a few populations, such as the indigenous Kuna of Panama, there is primary evidence that the protective factor is genetic rather than environmental. In this population, the low prevalence of hypertension and age-related rise in BP do not change significantly following migration to an urban environment and an increased salt intake [80].

The aim of the Ottawa GWAS is to identify novel genes that might be responsible for BP response to salt by performing GWA studies of subjects with essential hypertension of Caucasian origin from Eastern Ontario and Western Quebec. This population is not genetically homogeneous, and is a fair representation of the diversity seen in many parts of Canada. In order to enrich for risk alleles, individuals from families with a history of early onset hypertension will be chosen. The subjects will be carefully phenotyped for BP responses to high vs. low salt intake using 24-h ABPM. We propose a two-phase GWAS using
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both the Affymetrix Genome-Wide Human SNP Array 6.0 and the Affymetrix Axiom Genome-Wide Human Array. The Affymetrix Genome-Wide Human SNP Array 6.0 presents advantages of featuring 1.8 million genetic markers, including more than 906,600 SNPs and more than 946,000 probes for the detection of CNVs. On the more recent Affimetrix Axiom array, genotyping accuracy, haplotype coverage, and interplate variation have been improved as compared to the 6.0 arrays and allow the exclusive genotyping of SNPs specific for the European ancestry (Caucasian) population. However, the Axiom arrays do not provide CNV information because of the nature of the reaction, a hybridization followed by single nucleotide chain termination at the polymorphism. In contrast, the 6.0 arrays rely on hybridization to generate the primary signal and have over 946,000 non-polymorphic CNV probes covering the entire genome. The two methods are only partially redundant (only ~150,000 SNPs are common to the two arrays).

The GWAS has two major goals:

1. To identify genetic variants associated with SS versus SR hypertension by performing the first GWAS for salt sensitivity/resistance of hypertensive subjects (Phase I).

2. To identify/confirm genetic variants associated with hypertension/salt sensitivity by performing the first GWAS of younger (< 60 years of age) hypertensives compared to older (> 65 years of age) normotensive controls, whose BP status is confirmed by 24-h Ambulatory BP Monitoring (Phase II).

For the phase I population, healthy Caucasian males and post-menopausal women between 20 and 60 years of age are eligible, if they have a family history for early onset hypertension defined as ≥1 family member who developed persistent hypertension before 60 years of age, and have a resting BP ≥ 130/85 and ≤ 160/100 mmHg after stopping antihypertensive drug therapy for 2 weeks. To exclude subjects with white-coat hypertension, (pre)–hypertension needs to be present on the day-time BP on 24-h ABPM. Eligible subjects are randomized to a 3-week period of either low or high salt intake, and then cross-over to the alternative level of salt intake. Current levels of salt intake are being assessed by food questionnaire and 24-h urine collection. For the low salt period, subjects receive personalized instructions how to lower their salt intake to ~100 mmol Na+/day over a 1–2 week run in period. At the end, a second 24-h urine collection is done to ensure that the target is reached. If still >130 mmol/day, then more instructions are given to further low salt intake, how salt diet is then maintained for 3 weeks. For the high salt period, subjects take a sodium supplement of up to 200 mmol/day to achieve a high salt diet of ~300 mmol/day. The supplement is gradually increased to diminish the chance of adverse GI side-effects. “Double-blinding” with placebo is of little value, since subjects will taste the salt. Instead, the study is single-blind, i.e. all measurements are done blinded for the level of salt intake.

At the end of each 3-week period specific assessment include: 24-h urine collection to confirm target levels of sodium intake, BP in the office and by 24-h ABPM and blood sampling for assessment of explanatory variables such as parameters of the circulatory RAAS, plasma catecholamines and endogenous Na+, K+-ATPase inhibitors, “ouabain” and marinobufagenin.

The sample size requirement for Phase 1 of the study was calculated based on the quantitative trait change of systolic BP. Following the principles of sample size recalculation within an ongoing clinical trial [81, 82], the sample size needed was updated based on the hypertensive patients recruited having a standard deviation of 9.6 for systolic BP in place of the general initial estimate of 11.5. Recalculating the sample size based on an additive genetic model, a minor allele frequency of 0.20, a minimal clinically important difference is the systolic BP change of 5.0 mmHg and the standard deviation of 9.6, a sample size of 230 patients will allow us to detect the systolic BP change of at least 5.0 mmHg with a level of significance of 0.001 and power of 90%.

SNPs identified to associate with salt sensitivity or resistance will be examined further in a phase II independent population of 1,500 hypertensive individuals compared to 1,500 older normotensive controls. Salt induced increases in BP occur in more than 50% of hypertensives, but in
fewer than 10% of normotensive controls [66, 68, 83, 84]. Thus, the phase II population using cohorts enriched for individuals sensitive to salt (hypertensive) versus those resistant to salt (normotensive) will permit replication of SNPs identified in the phase I population. The same inclusion criteria apply as described for the Phase I Population. For the purpose of this study, for the control population normotensive is defined as an average day BP $\leq 130/80$ mmHg on 24-h ABPM. Older (>65 years) normotensive subjects are collected as controls to minimize the possibility of phenotypic misclassification. After discontinuation of antihypertensive drug therapy for 1–2 weeks, subjects meeting the in- and exclusion criteria have the following measurements: level of salt intake by 24-h urine collection, office BP and 24-h ABPM.

We will repeat GWA using both arrays, seeking to replicate variations for salt-sensitive hypertension in the case control phase II population. In Phase II, only variations showing an association with a $p$ value of $<0.0001$ will be considered significant (after correction for false discovery rate). This Phase II population also provides an opportunity to replicate BP loci identified in previous GWAS.

For the second independent population of Phase II, which is used to ascertain replication of the initial loci showing associations with the systolic BP change in a case control study, a sample size of 1,500 cases and 1,500 controls is needed. This is based on an additive genetic model to detect an odds ratio of 1.5, for a minor allele frequency of 0.15, a level of significance of 0.00001 and power of 90%. The difference in genotype frequencies between cases and controls will be considered significant at $p<0.00001$ for each SNP.

**Conclusions**

From a public health perspective, high salt intake has a clear effect on BP and thereby cardiovascular morbidity and mortality. Lowering the amount of salt added to foods is an important public health strategy. From an individual perspective, the impact of high salt intake on his/her cardiovascular system can vary from minimal to substantial and appears to a large extent to be genetically determined. Genetic diagnosis would be an ideal method of choice to advice life-style interventions for a particular individual. Studies performing careful assessment of phenotype and genotypes are essential to achieve this goal and, in addition, will very likely provide important new insights into novel mechanisms contributing to salt sensitivity or resistance of BP.

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Gene–Environment Interactions: Their Role in Hypertension Development

Jaroslav Kunes, Michaela Kadlecova, and Josef Zicha

Abstract
Essential hypertension is a major risk factor for several cardiovascular diseases, the etiology of which is not yet completely understood. The problem is that blood pressure (BP) is a typical quantitative trait with multifactorial determination. The interactions of multiple genetic and environmental factors as well as gene–gene interactions cause modifications of various systems that adjust blood pressure to actual living conditions. Numerous environmental factors surrounding the organism could modify its development not only by the influence on the expression of genetic information but mainly by epigenetic mechanisms. However, despite considerable research effort, it is still difficult to identify all genes and/or other genetic determinants leading to essential hypertension and other cardiovascular diseases. This is mainly because these diseases usually become a medical problem in adulthood, although their roots might be traced back to earlier stages of ontogeny. The link between distinct developmental periods (e.g., birth and adulthood) should involve the changes in gene expression involving epigenetic phenomena. The purpose of the present paper is to bring some light on gene–environmental interactions potentially implicated in the pathogenesis of hypertension, with special attention to epigenetic inheritance.

Keywords
Cardiovascular system • Critical developmental periods • Dietary intervention • Epigenetics • Gene-environment interactions • Hypertension • Metabolic syndrome • Rat

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Introduction

The understanding of the mechanisms for long-term regulation of blood pressure (BP) is the first prerequisite to elucidate the pathophysiology of hypertension. This has important clinical significance mainly in regard to human essential hypertension, one of the major risk factors for cardiovascular diseases such as heart attack, congestive heart failure, stroke, and peripheral vascular disease. Epidemiological studies demonstrated that the number of people with hypertension is increasing in both developed and developing countries [1, 2]. Moreover, there is a big problem that this increase is significant even in children. As mentioned by Taylor and coworkers [3], the percentage of children with prehypertension rose in USA (between 1988 and 2002) from 7.7% to 10%, while the percentage of children with hypertension increased from 2.7% to 3.7%. This means that more than 400,000 additional children are newly diagnosed with prehypertension or essential hypertension.

Hypertension is a complex trait resulting from the interactions of multiple genetic and environmental determinants. There is a growing evidence that the complex of these interactions plays an important role in determining an individual’s risk of various common diseases including hypertension [4, 5]. Gibson [6] emphasized that gene–gene interactions and gene–environmental interactions must be ubiquitous, given the complexities of intermolecular interactions that are necessary to regulate gene expression and the hierarchical complexity of quantitative traits.

The interest in the genetic basis of human diseases has spanned centuries and discoveries of disease-related genes often suggest that tests to predict people at risk of future disease will soon be available. Progress in the battle against human diseases was accelerated by the availability of genomic information for humans, mice, and other organisms. The techniques of molecular biology and genetics as well as particular knowledge emerging from these genome projects have revolutionized the process of localizing and identifying genes involved in the disease. However, one should keep in mind that our knowledge could not always be translated into useful clinical applications. This is mainly true for common complex diseases. As our understanding of the role of genetics and the use of gene-based markers extend to complex multifactorial disease, physicians will have to learn how to recognize patients with increased risk.

It is true that not only genetic but also some environmental factors, for example, stress, dietary factors (salt, fat), ambient temperature, etc., could contribute to the development of a given disease [4, 5, 7], especially if they influence the organism in the “critical developmental periods” and/or “developmental windows.” [4, 8].

The major goal of this chapter should be to integrate information on the relationships among the genetic determinants of high BP, environmental influences, and epigenetics. We are still in the situation that we can ask the following questions – what should be the normal level of blood pressure in our population?, why we need higher BP?, what is the relationship between hypertension and other cardiovascular diseases?, is hypertension an adaptive trait?, etc. Several hypotheses were proposed mainly for the last question [9]. These are the “slavery hypothesis” [10] and the “thrifty genotype theory” [11]. In both hypotheses, environmental factors play a very important role for selection of such genes that helped our ancestry to survive stressful conditions at the price of higher BP. However, both hypotheses have weak points, so that we still need more information in order to understand the complexity of hypertension.

Animal Models for Experimental Hypertension

As mentioned many times, hypertension is a polygenic trait with unknown etiology. There is no doubt that the study of such complicated disease is much easier in experimental animals than in humans, but a simple comparison of two strains, one normotensive and one hypertensive, could not answer the question about the primary
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causes of hypertension development and about the secondary consequences that could be involved in its maintenance. The use of special models and modern genetic approaches with gene manipulations are great advantages for the experimental approach that should help to understand the etiology of human hypertension and its effective prevention.

To understand how important animal models for the study of hypertension are, one should search any reference databases. It is impressive how the amount of such studies is increasing with time. Many different rat and mouse models of genetic hypertension have been developed with different etiology of hypertension [12–15]. These different genetic models provide the opportunity to investigate the genetic determinants of different types of hypertension, but one must be careful to transfer simply the results obtained in animal models to human essential hypertension. The advantages of having appropriate models are numerous. Due to many-sided interventions in animals (ethically impossible in humans), such models can substantially increase our understanding of the neural, renal, endocrine, or other variables triggering and/or maintaining hypertension. Due to the shorter life span of experimental animals, the role of various risk factors can be studied, alone or in combination, across the life span, to determine whether critical periods of sensitivity exist and to discover which physiological and biochemical events are associated with hypertension induction. Numerous environmental factors that may produce established hypertension can be manipulated, and thus animal models could help to answer our questions about the mechanisms responsible for hypertension development. Such models allow us to ask whether the mechanisms of salt-induced hypertension differ from those of stress-induced or renal hypertension and to look for some common mechanisms. In principle, such knowledge could enhance our understanding of this pathophysiological process and thus improve prevention and treatment of human essential hypertension [8].

The spontaneously hypertensive rat (SHr) has been widely studied as an animal model for essential hypertension. However, numerous other genetic or induced models of hypertension exist in the rat (Table 2). BP of SHr rises most rapidly between the ages of 4 and 13 weeks [8], and this elevation can be partially suppressed by kidney transplantation [18], ACE inhibitors [19, 20], or gene therapy [21, 22]. The SHr develops many features of hypertensive end-organ damage [23], but they have no tendency to develop atherosclerosis or vascular thrombosis. Several other sub-strains of SHr were developed to study a variety of clinical features of essential hypertension such as stroke, myocardial infarction, obesity, etc. [24]. In addition to rat models, mainly mice are

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<td>Aorta coarctation</td>
<td>Ligature of aorta, ?2K2C Goldblatt</td>
</tr>
<tr>
<td>High salt intake</td>
<td>Salt-sensitive strains (Dahl, Sabra)</td>
</tr>
</tbody>
</table>

GH genetically hypertensive rats, SHR spontaneously hypertensive rats, DOCA deoxycorticosterone acetate, 2K1C two-kidney one-clip, 1K1C one-kidney one-clip, 2K2C two-kidney two-clip

same situation is known in monozygotic twins. The use of inbred strains is important for production of F₂ hybrids, recombinant inbred strains, backcross populations, etc., as a tool for the search for genetic determinants of hypertension and other cardiovascular diseases. In spite of the fact that any experimental model is inappropriate to human hypertension [16, 17], some similarities exist (Table 1).

The spontaneously hypertensive rat (SHr) has been widely studied as an animal model for essential hypertension. However, numerous other genetic or induced models of hypertension exist in the rat (Table 2). BP of SHr rises most rapidly between the ages of 4 and 13 weeks [8], and this elevation can be partially suppressed by kidney transplantation [18], ACE inhibitors [19, 20], or gene therapy [21, 22]. The SHr develops many features of hypertensive end-organ damage [23], but they have no tendency to develop atherosclerosis or vascular thrombosis. Several other sub-strains of SHr were developed to study a variety of clinical features of essential hypertension such as stroke, myocardial infarction, obesity, etc. [24]. In addition to rat models, mainly mice are
often used irrespective of difficulties with the determination of particular phenotypes such as hemodynamic parameters, including BP. Their advantage is easier genetic manipulation, such as gene knockout, gene knock-in, etc.

**Table 2** The list of basic models of the rat

**Genetic models**
- Genetically hypertensive rats – New Zealand (gH)
- Spontaneously hypertensive rats (SHR)
- Stroke-prone spontaneously hypertensive rats (SHRSP)
- Lyon hypertensive rats (LH)
- Münster spontaneously hypertensive rats (SHM)
- Prague hypertriglyceridemic hypertensive rats (HTG)
- Milan hypertensive strain (MHS)
- Prague hypertensive rats (PHR)
- Fawn hooded hypertensive rats (FHR)
- Dahl salt-sensitive rats (DS, SS/Jr)
- Sabra hypertension-prone rats (SBH)

**Induced models**
- Neurogenic hypertension
- Hypertension from sino-aortic denervation
- Renovascular (Goldblatt) hypertension – 1K1C, 2K1C, aorta coarctation, etc.
- Hypertension from renal mass reduction – subtotal nephrectomy, etc.
- Hypertension from dietary interventions – high-salt diet, high-fat diet, etc.
- Mineralocorticoid hypertension – DOCA-salt
- Insulin-resistant hypertension – high-fructose diet, etc.
- NO-deficient hypertension – L-NAME treatment
  - **DOCA** deoxycorticosterone acetate, **NO** nitric oxide, **L-NAME** N\(^\text{G}\)-nitro-L-arginine methyl ester

There is no doubt that gene–environment interaction is not a static process, but it plays a very significant role throughout the whole life of a particular individual. This interaction is important not only for the modification of phenotypes but also for the expression of genetic information [4, 8]. As demonstrated earlier, gene–environment interaction is more pronounced in specific developmental periods, called “critical developmental periods” or “developmental windows,” in which the organism is more sensitive to many environmental stimuli [8]. This is true even for humans where the intrauterine period is very important for programming basic physiological functions. The association between low birth weight and raised blood pressure in later life has been studied [25–28]. According to this “programming hypothesis,” an impairment of intrauterine environment modifies the fetus from its optimal development, leading to cardiovascular complications in later life. Although most clinical and experimental studies support this fetal programming hypothesis in relation to adult hypertension, there is still controversy regarding the cause and mechanisms underlying this phenomenon. Generally, the changes in maternal nutrition lead to abnormal fetal nutrition, which directly or indirectly influences growth and maturation of various organs. However, not all studies support this theory, suggesting that rather body size in early adolescence than birth weight is more important [29]. Eriksson et al. [28] have shown in a set of 2,003 people selected from the Helsinki birth cohort that two different paths of fetal, infant, and childhood growth precede the development of hypertension in adult life. In one path that was associated with more severe hypertension in people who tended to be obese, small body size at birth and during infancy was followed by rapid growth. In the other one, which was associated with a less severe hypertension, slow linear growth in utero and during infancy was followed by persisting small body size. More recently, animal and human studies support the hypothesis that congenital deficit in nephron number could be one of the possible common pathophysiological denominators between low birth weight and subsequent adult hypertension [30].

From the point of terminology one should keep in mind that the term **environment** means all stimuli that are not of genetic origin. This is important for understanding why there exists the variability of particular phenotypes in inbred strains or in monozygotic twins. Mainly monozygotic twins have been used to demonstrate the role of environmental factors in determining complex diseases and phenotypes but the true nature of the phenotypic discordance remains still poorly understood. It was demonstrated that the lighter twin has an elevated risk for adverse
changes in body composition and risk for diabetes and hypertension later in life [31, 32], demonstrating that genetic factors do not fully account for the adult disease risk. One possibility could be that the uterine environment may not be the same for both individuals. For example, the possible effects of sharing different proportions of a common placenta on fetal growth and development are well recognized, as are the effects of placental anastomoses leading to partially shared circulations, which may also have long-term consequences [33]. In experimental twin research, Gärtner [34] has demonstrated that only 20–30% of the range of the body weight in inbred mice was directly estimated to be of environmental origin. He suggested the rest of the variability was due to a “third component” (in addition to the genetic and environmental factors), which is effective at or before fertilization and may originate from ooplasmic differences.

However, the link among changes in particular periods of life (e.g., between the intrauterine period and adulthood) can be explained only partially by time-dependent different expression of particular genes. There are also changes involved in epigenetic phenomena. Thus, environmental changes occurring in the intrauterine period or shortly after birth may ultimately lead to altered gene expression via epigenetic mechanisms, resulting in an increased susceptibility to chronic disease in adulthood [35]. This epigenetic process of imprinting is thought to particularly affect many of the genes regulating fetal and placental growth [36].

Epigenetic Phenomena – The Way Environment Can Influence Genetic Information

Epigenetic inheritance is a complex process that depends on the participation of numerous components of epigenetic machinery. Four main types of epigenetic inheritance – DNA methylation, chromatin remodeling, genomic imprinting, and long-range control of chromatin structure – have been described [37]. It was even demonstrated that although the epigenetic pattern is not a part of DNA sequence, it is heritably transmitted during cell division [36, 38].

A typical example of epigenetic inheritance is the inheritance of the coat color gene agouti in the mouse [39, 40]. Several agouti viable yellow alleles (A<sup>hy</sup> or A<sup>hyIV</sup>) regulate the alternative production of black and yellow pigment in individual hair follicles. The coat color of mice with such an allele varies from yellow over mottled to wild-type agouti. Transcription of this gene occurs in the skin during a short period when the yellow subapical band is formed at the beginning of each hair growth cycle. When methylated, the agouti gene is expressed only in the hair follicles as in wild-type mice. On the other hand, unmethylated gene causes the yellow coat color. It was demonstrated that dietary supplementation of a/a dams with extra folic acid, vitamin B<sub>12</sub>, choline, and betaine alters the phenotype of their Ahvy/a offspring through a different degree of methylation [41]. Thus, Ahvy expression, already known to be modulated by imprinting, maternal epigenetic inheritance, and strain-specific modifications, might also be modulated by the maternal diet. These results also suggested that through maternal epigenetic inheritance, the diet might positively affect health and longevity of the offspring [39] because yellow mice become obese and develop a tumor with age.

It was demonstrated that the epigenetic changes might be different in different developmental periods. Persistent epigenetic changes are more pronounced in early exposure to harsh conditions. This was demonstrated in the so-called “Dutch Hunger” study [42]. It was evident that individuals who were exposed to famine in utero had very different methylation patterns of genes involved in growth and metabolic disease compared to controls. Methylation of several genes (IL-10, LEP, MEG3, etc.) was higher, whereas methylation of IGF2 was lower in these individuals [43]. This might be related to the fact that some mammalian genes are expressed preferentially from the paternal or maternal allele and are said to be genomically imprinted. Imprinted genes may regulate some of the crucial aspects of mammalian physiology connected with energy homeostasis,
reproduction, growth, etc. Most imprinted genes are found in clusters and the best-characterized imprinted domain comprises the insulin-like growth factor 2 (IGF2) gene. The maternally imprinted IGF2 gene on chromosome 11p15.5 is one of the best characterized epigenetically regulated loci. Recently, it has been demonstrated that substantial variation in DNA methylation of H19 and IGF2 region exists across individuals, suggesting that DNA methylation is a quantitative trait. Analysis of data in monozygotic and dizygotic twins revealed that a significant part of variation in DNA methylation could be attributed to heritable factors [44]. One of the most important findings of this study was the fact that the combined influence of environmental and stochastic factors did not increase from adolescence to middle age at the expense of heritability. On the contrary, Fraga et al. [45] observed age-related differences particularly in global DNA methylation, histone modification, and repetitive sequences. These authors speculated that the different results could be explained by heterogeneity in cell populations used for genomic DNA extraction. Moreover, we have demonstrated strong segregation of IGF2 genotype with plasma triacylglycerols in the population of F2 hybrids derived from Prague hypertriglyceridemic and Lewis progenitors [46]. There was also segregation between this genotype and blood pressure changes elicited by the blockade of either renin-angiotensin system or NO synthase. One could speculate that any epigenetic modification of the IGF2 gene might be involved in these regulatory processes.

Perspectives and Concluding Remarks

There is no doubt that not only genetic but also environmental factors are very important for the regulation and maintenance of blood pressure. There is growing evidence that complex interactions between multiple genes and multiple environmental factors play an important role in determining an individual’s risk of various common diseases including hypertension. Due to the complex trait it is still not simple to recognize individual genes, which should be the optimum target for genetic manipulation. Although some experimental results suggested certain possibilities, it is difficult to apply these techniques in humans. For example, gene therapy of 5-day-old SHR resulting in the long-term expression of AT1 receptor antisense transcript successfully prevented the development of this type of genetic hypertension [21, 47], but the effect of such treatment was only transient in adult animals [48].

Conclusions

Increased understanding of the mechanisms by which environmental factors can modify the development of hypertension and other cardiovascular diseases may lead to the development of new strategies not only in the therapy but also in the prevention of these diseases. Some preclinical and clinical studies have demonstrated that exposure to factors such as diet, stress, smoking, air pollution, etc. are able to modify epigenetic markers. However, more investigations are needed to determine whether the accumulation of epigenetic alterations can increase the incidence of hypertension.

To decrease the incidence of important cardiovascular diseases we should pay more attention to epigenetic changes rather than to “gene hunting.” This could be achieved by appropriate lifestyle modifications. At present, we can recommend the modification of lifestyle as a prevention of cardiovascular diseases including hypertension. Salt restriction to less than 6 g/day, maintenance of appropriate body weight, higher physical exercise, restriction of stress, augmented fruit and vegetable consumption, etc. should be a standard recommendation of physicians to their patients. Not only patients but the whole population must be motivated for lifestyle changes because most of the “civilization” diseases including hypertension have their roots in the earlier stages of ontogeny. Finally, it is evident that if a “sensitive” genome enters “toxic” environment during particular critical developmental period, there is a very high probability that disease will develop.
References

Abstract
Mutations in cardiac sodium channel gene SCN5A are responsible for a spectrum of hereditary arrhythmias including type-3 long QT syndrome (LQT3), Brugada syndrome (BrS), conduction disturbance, and sinus node dysfunction. These syndromes were originally regarded as independent entities with distinct clinical manifestations and biophysical properties. However, recent evidence shows considerable clinical overlap among these disorders, implicating a new disease entity referred to as an overlap syndrome of cardiac sodium channelopathy. Class IC sodium channel blockers often induce BrS phenotype in some patients with LQT3. Furthermore, recent genetic studies have revealed that E1784K is the most prevalent SCN5A mutation responsible not only for LQT3 but BrS, confirming the clinical and genetic overlap of LQT3 and BrS. Here I show evidence that the clinical manifestations of SCN5A mutations are most probably determined by the biophysical and pharmacological properties of the mutations. I also provide an overview of current knowledge on the clinical features, prevalence, and molecular and biophysical mechanisms underlying the overlap syndrome to gain more insight into this complex issue and generate better therapeutic strategies for patient management.

Keywords
Brugada syndrome • Flecaïnide • Long QT syndrome • Overlap syndrome • SCN5A

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**Introduction**

**Biochemistry and Biophysics of Cardiac Sodium Channels**

Voltage-gated sodium channel is responsible for the rapid upstroke of the action potential in most excitable tissues and plays a pivotal role in the initiation, propagation, and maintenance of normal cardiac rhythm in the heart. Cardiac sodium channel consists of the most prevalent pore-forming \( \alpha \)-subunit (Nav1.5) encoded by the gene \( SCN5A \) located on chromosome 3p21 and auxiliary \( \beta \)-subunits (Nav\( \beta \)1-Nav\( \beta \)4) encoded by the genes \( SCN1B-SCN4B \), respectively. The \( \alpha \)-subunits comprise a four-fold symmetry macromolecule consisting of structurally homologous domains (D1–D4), each containing six membrane-spanning segments (S1–S6) and a region (S5–S6 loop) controlling ion selectivity and permeation. The positively charged S4 segment of each domain functions as a voltage sensor [1, 2].

Na channels switch between three functional states (closed, open, inactivated), depending on the membrane potential. A membrane depolarization causes a rapid rise in local Na permeability due to the opening (activation) of Na channels from their resting closed state. Normally, activation of Na channels is transient owing to inactivation, another gating process mediated by structures located on the cytoplasmic face of the channel protein (mainly the D3–D4 linker). Na channels cannot reopen until the membrane is repolarized and they undergo recovery from inactivation. Membrane repolarization is achieved by fast inactivation of Na channels and is augmented by activation of voltage-gated K channels. Activation, inactivation, and recovery from inactivation occur within a few milliseconds. In addition to these rapid gating transitions, Na channels are also susceptible to slower inactivating processes (slow inactivation) if the membrane remains depolarized for a longer time. These slower events may contribute to determining the availability of active channels under various physiological conditions.

**Genetics of Cardiac Sodium Channelopathies**

Mutations of \( SCN5A \) are responsible for a spectrum of hereditary arrhythmias including type-3 congenital long QT syndrome (LQTS; LQT3) [3], acquired LQTS [4], Brugada syndrome (BrS; BrS1) [5], cardiac conduction disturbance (CCD) [6], congenital sick sinus syndrome (SSS) [7], atrial standstill [8–10], atrioventricular (AV) block [11], sudden infant death syndrome (SIDS) [12–14], and familial atrial fibrillation (FAF) [15–17] (Table 1). In addition to these primary electrical diseases which usually lack structural abnormalities, \( SCN5A \) mutations have also been reported in patients with dilated cardiomyopathy [15, 18]. Moreover, recent genetic studies have indicated that mutations in Na channel \( \beta \)-subunit genes, \( SCN1B \) and \( SCN4B \), were associated with type-5 BrS complicated with CCD (BrS5) [19] and type-10 LQTS (LQT10), respectively.

**Biophysical Properties of Cardiac Sodium Channel Channelopathies**

Congenital LQTS is characterized by the prolongation of the QT interval on surface electrocardiograms (ECGs) and an increased risk of potentially fatal ventricular arrhythmias, especially torsade de pointes [20]. QT interval is
determined by the cardiac action potential which is orchestrated by a fine balance between inward and outward currents expressed in myocardial cells. After the first identification of the \textit{SCN5A} mutation \textit{DKPQ}, comprising a deletion of three conserved amino acids 1505–1507 in the cytoplasmic D3-D4 linker in 1995 [21], more than 100 distinct \textit{SCN5A} mutations responsible for LQT3 have been reported. The common in vitro consequence of most of these mutations is a persistent Na current during the action potential plateau due to destabilized fast Na channel inactivation [3]. This failure of fast inactivation shifts the ionic balance during the plateau phase toward inward current (gain-of-function) and delays repolarization, thus increasing action potential duration and the corresponding QT interval. Na channel blockers, such as mexiletine (class IB) or flecainide (class IC), shorten QT in patients with LQT3 due to block of this persistent current [22–24], and therefore are theoretically useful in the management of affected patients.

\textit{BrS} is another primary electrical disorder without underlying structural heart diseases characterized by the coved-type ST elevation in the right precordial leads [25, 26]. It predisposes affected individuals to ventricular fibrillation (VF). Mutations in \textit{SCN5A} are identified in 20–30\% patients with \textit{BrS}, and most of the heterologously expressed mutant Na channels exhibit biophysical abnormalities resulting in reduced cardiac Na current (loss-of-function) [27]. Reduced Na current is thought to exaggerate differences in action potential duration between the inner (endocardium) and outer (epicardium) layers of ventricular muscle, thereby favoring substrate-promoting reentrant arrhythmias. Loss of function of cardiac Na channels is either owing to (a) haploinsufficiency due to nonfunctional mutations; (b) impaired altered channel gating properties including enhanced inactivation, disruption of activation, and impaired recovery from inactivation; or (c) impaired intracellular trafficking and decreased membrane surface expression of the channel molecules.

\textbf{Clinical Overlap of Cardiac Sodium Channel Channelopathies}

\textit{SCN5A} mutations with loss-of-function properties have also been identified in patients with CCD [6, 28], SSS [7], and atrial standstill [8], and numbers of reports have shown that the mutation carriers tend to exhibit overlapping clinical properties of these syndromes [29, 30] (Table 2). Importantly, such loss-of-function properties are apparently opposite to those described in LQT3 (gain-of-function), and different \textit{SCN5A}
mutations were initially linked to separate arrhythmia syndromes. Surprisingly, some LQT3 patients display ECG findings characteristic of BrS, suggesting that a single mutation can be associated with a wide spectrum of disease phenotypes. Such phenotypic overlap between LQT3 and BrS was first reported in a large multigenerational Dutch family with an insertion mutation 1795insD, in which the mutation carriers showed ECG features of both LQT3 and BrS, and sinus node dysfunction (SND) [38, 39]. Importantly, sodium channel block in the overlap phenotype shortens QT but exacerbates the ST segment elevation BrS phenotype, and thus enhances arrhythmia risk [39]. Biophysical studies demonstrated that the mutant channels displayed enhancement of both closed-state inactivation and slow inactivation which was thought to sensitize carriers to the BrS phenotype during flecainide therapy [61], in addition to the persistent Na current, a hallmark Na channel property of LQT3.

The overlap between the LQT3 and BrS phenotypes was also reported in other SCN5A mutations, such as ΔKPQ [31, 62], E1784K [31], and ΔK1500 [32]. Priori et al. showed the additional evidence for the elusive link between these two clinical syndromes by the fact that the class IC sodium channel blocker flecainide induced ST-segment elevation in the right precordial leads not only in patients with BrS but also with LQT3 [31]. In addition to the persistent Na current, a hallmark Na channel property of LQT3.

Clinical Phenotypes in 15 LQT3 Families with SCN5A-E1784K

We enrolled 44 genotyped LQT3 families with different ethnic backgrounds (Asian 20, Caucasian 24) ascertained in seven institutions of Japan, Italy, Germany, United Kingdom, and the United States. In 44 LQT3 families, E1784K was the most prevalent SCN5A mutation, identified in 15 families (34%). Two probands died suddenly, and 66 out of 93 surviving members underwent genetic testing. There were 41 mutation carriers and 25 noncarriers, and QTc was significantly prolonged in the carriers.

Spontaneous ST elevation in the right precordial leads was observed in 5/41 mutation carriers (shown with * in Fig. 1; coved-type: n=1, saddle-back type: n=4, Fig. 2a). Nine mutation carriers without diagnostic ST elevation at baseline underwent provocation with flecainide, ajmaline, or pilsicainide, and the test was positive (coved-type ST elevation, Fig. 2b) in five (shown with + in Fig. 1). Thus, the diagnosis of BrS was established in 9/41 mutation carriers (one individual, a; II:1, showed spontaneous saddle-back ST elevation which was converted to coved-type by ajmaline).

SND was common in the cohort, present in 16/41 mutation carriers (Fig. 2c), and 4 of these 16 carriers with SND also exhibited the BrS phenotype (Fig. 2b, d). Moreover, one carrier (a; III:5) showed SND without manifesting QT prolongation or ST elevation. Four patients received a permanent pacemaker and three received an implantable cardioverter defibrillator.
Fig. 1 Pedigrees of E1784K families. Pedigrees of 15 LQT3 families (a–o) carrying E1784K are shown. Probands are indicated by an arrow. Ten symptomatic mutation carriers, shown by the filled symbols, had episodes of syncope (n=9) and unexplained palpitations (n=1, b; II:2). Asymptomatic mutation carriers (n=31) are shown as symbols with a dot, and shaded symbols are the individuals with QT prolongation who declined genetic testing or sudden cardiac death victims (SCD; a; II:6 and e; III:1). Individuals exhibiting ST elevation in the right precordial leads are depicted with an asterisk. Values for QTc intervals are given beneath each symbol. The Na-channel provocation test was positive in individuals with + (a; II:1, a; III:8, a; III:9, e; II:3, k; II:1), and negative in the individuals with – (a; II:3, e; I:2, e; II:2, n; II:1). Individuals with a positive and negative Na-channel blocker provocation test are shown with + and −, respectively (Modified with permission from [41])
Fig. 2  ECG characteristics of E1784K mutation carriers. (a) QT prolongation (QTc = 470 ms) and spontaneous saddle-back type ST elevation observed in the right precordial leads in a carrier, a; II:1. (b) ECG recordings before and after the Na-channel blocker provocation test. Pilsicainide (left, patient k; II:1) induced coved-type ST elevation in V1 and the QTc was concomitantly shortened (QTc: control 495 ms, pilsicainide 459 ms). Ajmaline (right, patient A; III:9) also induced coved-type ST elevation in V1 and V2 and QTc shortening (control 501 ms, ajmaline 490 ms). (c) SND demonstrated by a 3.9 s sinus arrest in a carrier, a; I:1. (d) A Venn diagram representing electrophysiological manifestation of 41 SCN5A-E1784K mutation carriers. Thirty eight carriers exhibited an abnormally long QTc; three individuals had a normal QTc, and one exhibited SND (SND) only. SND and BrS were observed in 15 and 9 individuals, respectively, with 4 displaying both phenotypes (Modified with permission from [41])

Biophysical Properties and Membrane Trafficking of E1784K

Whole-cell patch clamp recording showed that E1784K has the following biophysical abnormalities: (a) significantly smaller peak current density, (b) persistent Na current, (c) significantly faster macroscopic current decay, (d) hyperpolarizing shift of the steady-state inactivation, (e) significant depolarizing shift in the voltage-dependent of activation, and (f) normal recovery from inactivation. Furthermore, using Na channel plasmid construct with an extracellular FLAG epitope, membrane trafficking of E1784K determined by a confocal laser scanning microscopy was comparable to the wild type. These observations (Fig. 3) provide strong evidence that the loss-of-function properties displayed by E1784K are most likely attributable to the aforementioned changes in gating properties rather than a change in channel density.

Molecular Mechanisms of Enhanced Flecainide Sensitivity

Class IC drug challenge test was positive in 56% patients with E1784K. We therefore investigated tonic block and use-dependent block by flecainide in WT and E1784K channels, and compared
them with those of T1304M, a mutation that did not show ST elevation during the flecainide challenge test [31]. Cells transfected with WT, E1784K, or T1304M were depolarized by 2 Hz pulse trains in the absence or presence of 10 μM flecainide. During exposure to flecainide, peak currents normalized to predrug baseline were progressively reduced by the repetitive pulses (Fig. 4a, b). There was a remarkable difference in the extent of first pulse (tonic) block that was only 4.5 ± 4.0% for WT, and 7.1 ± 2.7% for T1304M, compared to substantial tonic block in E1784K (43.7 ± 8.0%, p < 0.001). Conversely, use-dependent block, determined by the difference in peak current values between the 1st and 100th test pulses relative to the first pulse, was slightly attenuated in E1784K. The net effect of flecainide after a train of 100 pulses was significantly greater in E1784K than WT but not in T1304M. Moreover, dose–response curves for flecainide block measured at a holding potential of −120 mV and showed prominent tetrodotoxin (TTX)-sensitive late Na current (shown with arrows) and a faster decay in E1784K. (d) Steady-state availability for fast inactivation and the conductance-voltage relationship were measured with standard pulse protocols shown in the inset. Curves were fit with the Boltzmann equation. The voltage dependence of steady-state fast inactivation and activation was significantly shifted in the hyperpolarizing (−15.0 mV) and depolarizing (+12.5 mV) directions, respectively (Modified with permission from [41]).
To explore the functional determinants for the phenotypic overlap of BrS in LQT3 patients, we compared the biophysical and pharmacological properties of reported LQT3 mutations, and sought features commonly and specifically observed in those manifesting the BrS phenotype (Table 3). The overlapping phenotype (LQT3 and BrS) has been previously reported for 1795insD [38, 39], ΔKPQ [31, 62], ΔK1500 [32], and E1784K [31]. In contrast, a carrier of T1304M did not show ST elevation during a flecainide test [31]. Similarly, SND has been reported in carriers of the same SCN5A mutations, 1795insD [65], ΔKPQ [68], ΔK1500 [32], E1784K [40], and D1790G [23], but not in other SCN5A mutations, including T1304M. Thus, it is plausible to speculate that the biophysical characteristics common to these mutations but not found in T1304M are channel properties responsible for evoking mixed phenotypes of BrS and SND in patients with LQT3. To this end, Table 3 compares the functional properties of E1784K, and those reported for 1795insD, ΔKPQ, ΔK1500, E1784K, and T1304M [12]. Among the biophysical properties listed in Table 3, we found that both the negative shift in steady-state inactivation, and the enhanced tonic block by flecainide are common to 1795insD, ΔKPQ, ΔK1500, E1784K, and T1304M. This negative shift of inactivation will reduce the availability of the channels at the resting membrane potential, and increase the proportion...
of inactivated channels in both the open and closed state, reducing Na current and increasing the sensitivity to Na-channel blockers. A positive shift in activation is another “loss-of-function” property evident in all the mutants including T1304M, making it less likely that this specific channel property underlies mixed clinical phenotypes in LQT3. Other channel properties such as current decay, recovery from inactivation, slow inactivation, or use-dependent block were not common among 1795insD, ΔKPQ, ΔK1500, and E1784K.

A negative shift in inactivation is observed in E1784K, 1795insD, ΔKPQ, and ΔK1500, and may play a role in the overlap of the LQT3 clinical phenotype with BrS and SND in the mutation carriers, although the number of LQT3 mutations that have been evaluated in this detail is still small, biophysical and pharmacological properties presented in a cultured cell line may not necessarily reflect the situation in vivo, and the effects of the mutation may be different in ventricular myocytes versus sinus node cells. Further studies that combine clinical and in vitro phenotyping in LQT3 mutations with and without overlapping clinical phenotypes will be required to confirm the findings of the present study. Nevertheless, a negative shift in inactivation and enhanced tonic block are common biophysical properties observed among SCN5A mutations with the LQT3/BrS overlapping phenotype. These findings suggest that prophylactic class IC drugs should be avoided in LQT3 mutations displaying these biophysical properties in vitro.

**Conclusions**

E1784K is the most common LQT3 mutation. In patients with this and other LQT3 mutations, overlap with BrS and SND is relatively common. In vitro studies with E1784K and previous reports in LQT3 mutations with and without this clinical overlap syndrome implicate a negative shift in inactivation and enhanced tonic block by drugs as underlying mechanisms. These data suggest that patients with LQT3 mutations displaying these characteristics in vitro should not receive class IC drugs. Furthermore, the present findings reinforce the general concept that in vitro characterization of the function of ion channel variants is a key component in generating specific therapeutic strategies for patient management.

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29. Shirai N, Makita N, Sasaki K, et al. A mutant cardiac sodium channel with multiple biophysical defects associated with overlapping clinical features of


Abstract

Cardiac hypertrophy is the heart’s response to increased work, pressure, or volume overload. It begins with a compensatory phase that allows the heart to meet imposed demand through rapid expression of stress response genes. A decompensatory phase follows marked by additional adaptive stress response gene expression that with prolonged stress progressively turns maladaptive, leading the heart into failure. The transition from compensatory to decompensatory hypertrophy is likely to reflect changes in the transcription factors and regulatory molecules that control these programs in response to changes in stress stimuli and the status of cardiomyocytes throughout the hypertrophic process. Our laboratory has been studying the role of one such transcriptional regulatory molecule, CLP-1 (cardiac lineage protein-1), in the cellular response to hypertrophic stimuli. CLP-1, the mouse homolog of the human HEXIM1 gene, is an inhibitor of P-TEFb (transcription elongation factor b), a component of the transcriptional apparatus that controls RNA polymerase II activity and gene transcription. Knockout of the CLP-1 gene results in a severe form of hypertrophy in fetal mice suggesting that in the absence of the CLP-1 inhibitor, uninhibited P-TEFb activity may lead to unregulated expression of stress response genes and decompensatory hypertrophy. Because of its critical role in regulating the stress gene response to hypertrophic stimuli, we review our laboratory’s work on CLP-1, its control of P-TEFb under various hypertrophic conditions, and how it may play an important role in a novel gene control mechanism, called promoter proximal pausing, that ensures the rapid expression of stress response genes in response to hypertrophic stimuli.
Introduction

The prevalence of hypertension, diabetes, and valvular diseases among an increasingly aged adult population is leading to an increase in one of the clinical consequences of these diseases – enlargement of the heart or cardiac hypertrophy. Hypertrophy is essentially a response to maintain normal cardiac output in the face of stress-inducing physiological imbalances that make the heart work harder, be they abnormally high blood pressure, compromised renal function, or decreased cardiac output from heart valve insufficiencies. When the heart is challenged with increased work, pressure, or volume overload, it mounts an adaptive response to normalize heart function by increasing contractility and ventricular wall thickness. This adaptive or compensatory phase, called physiological hypertrophy, initially meets imposed demand, but with prolonged load imposition, the compensatory response deteriorates into a maladaptive or decompensatory phase that is marked by depleted metabolic reserves [1, 2], fibrosis [3–6], apoptosis of myocardial cells [7, 8], and eventually heart failure. The decompensatory phase, called pathological hypertrophy, is essentially irreversible and has proven refractory to therapies designed to restore the myocardium to a functional state.

Our laboratory has been investigating the molecules involved in the signaling and transcriptional events that underlie the compensatory and decompensatory phases of cardiac hypertrophy. We have focused our studies on one such molecule, CLP-1 (cardiac lineage protein-1), that we have shown to be an important mediator by which hypertrophic signals evoke a genomic stress response. In this context, we discuss the interaction of the Jak/STAT (Janus kinase/signal transducer and activator of transcription) signal transduction pathway, a well-documented hypertrophic signaling pathway, with CLP-1. Given their central role in transmitting hypertrophic signals to the genome, CLP-1 and the Jak/STAT signaling pathway are likely to interact with a number of other pathways involved in transmitting the hypertrophic stimulus. Since several excellent reviews on these other potential CLP-1-interacting pathways have been published [9–12], our focus will be on the Jak/STAT pathway in this review.

Compensatory and Decompensatory Hypertrophy as Programs of Stress Response

Many, if not all of the events contributing to compensatory and decompensatory hypertrophy are driven by underlying gene programs. In the compensatory phase, gene programs direct heart cells to take adaptive measures to meet imposed demand, while in the decompensatory phase, similarly adaptive programs provide short-term relief but turn maladaptive with continued imposition of work, pressure, or volume overload. The adaptive gene programs include re-expression of the fetal program of contractile protein gene expression [13] and a program of increased protein synthesis [14] that results in increased cell size and thickened ventricular walls to normalize intramural pressure. The maladaptive gene programs include differentiation of fibroblasts into myofibroblasts producing collagen, expression of glycolytic enzymes for glucose metabolism, and eventually programmed cell death of irreversibly malfunctioning cardiomyocytes. The compensatory and decompensatory
phases of hypertrophy can thus be viewed as genetic programs of stress response genes that direct cell behavior and ultimately heart function. Since the decompensatory phase leads to pathological hypertrophy, one way of controlling or managing cardiac hypertrophy clinically may be to prevent decompensatory hypertrophy by preventing expression of its underlying maladaptive gene programs.

As with hypertrophy, the maladaptive gene programs of decompensatory hypertrophy can be initially adaptive. For example, the cardiomyocyte adapts to increased energy demands by up-regulating the glycolytic program to make use of the more readily metabolized energy substrate, glucose [15]. While this works for a time, the continued demand for energy soon depletes the stores of glucose in adult heart cells, leaving the heart in metabolic distress and at risk of apoptosis. When the cells eventually die, a second compensatory program initiates to compensate for the loss of cardiomyocytes, which cannot be replaced by proliferation of healthy cardiomyocytes since these are post-mitotic. This compensatory program directs the expansion and differentiation of cardiac fibroblasts into myofibroblasts that produce and deposit increased amounts of extracellular collagen to compensate for the loss of cardiomyocytes, so-called replacement fibrosis [16]. As with glucose metabolism, this compensatory behavior is initially effective but soon turns maladaptive; continued collagen production leads to overproduction and increased stiffness of the myocardium or fibrosis, making contractility more labored and reducing, not increasing, heart function. From this it appears that the balance between glucose and fatty acid oxidation and fibroblast and myofibroblast differentiation and collagen deposition is critical to maintaining a compensatory versus decompensatory hypertrophy. Since these responses are driven by underlying gene programs, this balance is likely to be maintained by factors normally involved in the regulation of gene expression, in particular, regulatory molecules common to both processes and thus able to control the balance between compensatory and decompensatory gene expression. Since these processes involve expression of stress response gene programs, this regulatory molecule is likely to be a transcription factor controlling these programs throughout the hypertrophic process. Our laboratory has been studying the role of one such regulatory molecule, CLP-1, in the cellular response to hypertrophic stimuli [17–22]. CLP-1, the mouse homolog of the human HEXIM1 gene [23–25], is an inhibitor of P-TEFb (transcription elongation factor b), a component of the transcriptional apparatus that controls RNA polymerase II activity and gene transcription (see Fig. 1) [24, 26].
CLP-1 is Central to the Hypertrophic Response Via its Ability to Control the Transcriptional Status of Stress Response Genes

Two properties of CLP-1 make this transcription cofactor an excellent candidate for regulating hypertrophic stress response gene programs. The first is that it is a general transcription cofactor regulating RNA polymerase (pol) II function and hence the expression of most if not all pol II, protein-encoding genes. It is therefore likely to be involved in the transcription of compensatory as well as decompensatory gene programs. Second, as a regulator of P-TEFb, CLP-1 could be instrumental in controlling the expression of stress response genes, many of which comprise the compensatory and decompensatory gene programs. Recent studies on the function of P-TEFb have provided new insights into how this complex, formed by the association of cyclin-dependent kinase (cdk) 9 and cyclin T1, controls stress response gene expression. A new control mechanism called “promoter-proximal pausing” in which P-TEFb plays a central role has emerged as a way of poising stress response genes to respond rapidly to stress and other stimuli [26–29]. Such a mechanism could allow the rapid expression of stress gene programs in response to hypertrophic stimuli. Because of its central role in regulating stress genes and the fact that stress gene expression is critical to the hypertrophic response, CLP-1 has continued to be the focus of our laboratory’s research into the transcriptional mechanisms regulating the hypertrophic response. We review here our laboratory’s initial discovery, characterization, and investigation into the properties of CLP-1 that place it at the center of the genomic response to hypertrophic stimuli.

A cDNA clone encoding CLP-1 was first isolated by our laboratory using expression screening of a stage 6 chicken embryo cDNA library designed to isolate transcription factors by their binding to a specific sequence within the myosin light chain-2 gene promoter [17]. This screen yielded a cDNA clone that upon in situ hybridization analysis and immunocytochemical analysis using an antibody made to a CLP-1 fusion protein showed enhanced expression in regions of the embryo specific for early cardiogenic progenitors, for example, the cardiac crescent, giving us our first indication of a cardiogenic function for CLP-1, from which it derived its name: cardiac lineage protein-1 or CLP-1. At this time, CLP-1 was a novel protein with no known homologues, but sequence analysis showed a nuclear localization sequence in the open reading frame that suggested a nuclear function that was later corroborated by immunofluorescent localization to cardiomyocyte nuclei [13]. Together, these data suggested that CLP-1 could be a nuclear factor important to the specification and/or development of early cardiac progenitors.

To facilitate further analysis of CLP-1, our laboratory then cloned the mouse CLP-1 gene from a genomic library and a mouse cDNA from an embryonic heart cDNA library [18]. As with the chicken studies, Northern blot analysis showed an early expression pattern in mouse embryos beginning at embryonic day (E) 7. During this time, other laboratories reported the isolation of human CLP-1, called HEXIM-1, and sequence comparison confirmed the homology between chicken, mouse, and human clones. A wider survey of tissues for CLP-1 expression showed a much wider array of tissues in which CLP-1 is expressed, suggesting that despite its early cardiogenic expression, it may perform a more generalized function in all tissues. This was also confirmed by promoter analysis showing the CLP-1 gene promoter to be active in both muscle cells and the 10T1/2 fibroblast cell line. Up until this point, our preliminary data, while not directly assessing CLP-1 function, nonetheless suggested a positive function related to transcription. When we examined CLP-1 function by determining if it could up-regulate the MLC2v gene promoter, we found that either no up-regulation occurred (unchanged promoter activity with increasing amounts of CLP-1 added) or promoter activity went down with added CLP-1 (see Fig. 2). This latter result was obtained in transfected rat primary cardiac cells, suggesting that on at least one cardiogenic promoter in cardiac cells, CLP-1 was acting to suppress transcription. Since the function
Fig. 2 Effect of CLP-1 expression on MLC-2V promoter activity. Cells were transfected with a cardiac MLC-2V promoter-luciferase reporter construct along with increasing amounts of CLP-expression vector or empty vector alone. Promoter activity is reported as luciferase activity relative to internal control (constitutive promoter construct). Transfected cell types: (a) Rat primary cardiac cells; (b) H9C2 cells; (c) 10T1/2 cells; (d) primary rat cardiac cells transfected with a truncated MLC2V promoter driving the luciferase reporter. In rat primary cardiac cells, increasing CLP-1 input leads to decreasing transcription suggesting that CLP-1 may be exerting a negative effect on transcription (Taken from [18] with permission)

of HEXIM1/CLP-1 as an inhibitor of P-TEFb activity and RNA transcription had yet to be described, these results were among the first to indicate a negative rather than a positive regulatory role for CLP-1 in transcription.

Ablation of the CLP-1 Gene Leads to a Fetal Form of Cardiac Hypertrophy

To delve further into the function of CLP-1 in vivo, we ablated the CLP-1 gene in mice [20]. The CLP-1 gene is a single copy gene that lacks introns. Knockout of one CLP-1 allele gave heterozygous mice that were phenotypically normal and viable. However, when these mice were crossed to generate the null CLP-1 phenotype, genotype analysis of the first several litters showed no CLP-1−/− pups, indicating that the CLP-1 knockout was most likely embryonic lethal. When prenatal embryos and fetuses were genotyped, the expected 1:2:1 ratio for wild-type, heterozygote, and homozygote knockout genotype frequencies became skewed at E 17.5 resulting from a decrease in the number of knockout genotypes. This confirmed that the CLP-1 knockout was embryonic (or fetal) lethal beginning at approximately E17. Prior to E17, the overt appearance of embryos and fetuses showed all embryos to be similar in size and appearance; from E17 onward (until dead embryos were resorbed) the CLP-1 knockout fetuses began to exhibit decreased size and a much paler color than wild-type or heterozygous littermates (see Fig. 3). While an overall growth retardation phenotype
could not be ruled out, analysis of internal organs, particularly the heart, showed that it was hypertrophic with a thickened left ventricular wall and decreased left ventricular chamber (see Fig. 3). To further test the possibility of hypertrophy, we examined CLP-1−/− hearts for molecular markers indicative of the hypertrophic state, that is, increased expression of atrial natriuretic factor, β (beta)-myosin heavy chain, and α-skeletal muscle actin, and found that these markers were uniformly elevated in E16 CLP-1−/− hearts relative to wild-type or heterozygous hearts. These results were consistent with the CLP-1−/− hearts being hypertrophic. Ultrastructural analysis using electron microscopy showed cardiomyocytes from E15.5 to have normal nuclear morphology and sarcomeres with a normal banding pattern indicating a normal cell morphology and contractile apparatus. In contrast, E16.5 myocardium showed electron-dense chromatin masses coalesced near or abutting the nuclear envelope of cells and cytoplasmic mitochondria with far fewer cristae than in wild-type hearts. This morphology indicated that the E16.5 myocardium was undergoing apoptosis. In addition, the normal sarcomeric banding pattern seen in the myofibrils of E15.5 heart was completely destroyed in those of the E16.5 heart indicating a nonfunctioning contractile apparatus. Together, these data suggested that the E16.5 CLP-1 knockout hearts were hypertrophic and progressing to, if not already in, a stage of pathological hypertrophy and heart failure, the most likely cause of death for the CLP-1 knockout fetuses. In addition to its effect on heart function and hypertrophy, as a potential cardiogenic transcription factor or cofactor, we also wanted to examine how the absence of CLP-1 affected the expression of key cardiogenic genes such as Nkx2.5, eHAND, dHAND, and GATA4. Our results showed that dHAND was slightly decreased, but eHAND was completely absent in CLP-1−/− hearts as early as the E9.5 heart tube. Thattaliyath et al. have shown that in aorta-constricted mice with cardiac hypertrophy, eHAND expression is dramatically reduced [30]. In terms of eHAND expression in our CLP-1 knockout mice, this suggests that rather than being a transcription factor that is responsible for eHAND expression, a more likely explanation is that the absence of CLP-1 leads to cardiac hypertrophy, one consequence of which is a reduction in eHAND expression. Thus, this is another indication that the CLP-1 knockout leads to cardiac hypertrophy. Together, the results of our CLP-1 knockout studies indicate that eliminating CLP-1 results in a severe form of fetal cardiac hypertrophy. The developmental nature of the hypertrophy resulting from ablation of the CLP-1 gene could either indicate a requisite role for CLP-1 in critical developmental events or taken in a non-developmental context, a role in cardiac hypertrophy independent of developmental events and the developmental stage of the heart.

Fig. 3  (a) Overt appearance of wild-type, CLP-1 +/+ and CLP-1 −/− fetuses. The CLP-1 phenotype of smaller body size and paler color becomes apparent starting at E16.5 and continues until the embryos are fully resorbed. Some variability is noted in the severity of the phenotype; this reflects slightly different ages from litter to litter and also an effect on phenotype due to litter size with knockout fetuses being affected more in larger litters. (b) Hematoxylin and eosin stained coronal sections of E17.5 wild-type and CLP-1 −/− hearts. E17.5 CLP-1 (−/−) fetal hearts show a thickened left ventricular wall and decreased left ventricular chamber size. (Taken from [20] with permission)
Hypertrophic Signals Lead to Dissociation of CLP-1 from the P-TEFb Complex, Suggesting that Transcriptional Deregulation is Key to Development of Hypertrophy

During this time, a number of reports were published that provided further support for the notion that CLP-1 was involved in hypertrophy and, perhaps equally important, that this role involved transcriptional regulation. Sano and colleagues were among the first to implicate P-TEFb and its regulation in the response of cardiomyocytes to hypertrophic stimuli [31]. At the time of their studies, it had been shown that P-TEFb was negatively regulated by a small RNA molecule, called 7SK, that bound to cyclin T1 to reversibly inhibit P-TEFb cdk9 kinase activity [32, 33]. The release of 7SK RNA from cyclin T1 derepressed the cyclinT1-cdk9 complex to activate cdk9 to phosphorylate and thus activate RNA pol II and transcriptional elongation. Sano et al. showed that 7SK RNA release from P-TEFb could be induced by hypertrophic stimuli, suggesting that this might be one way in which hypertrophic cardiomyocytes increased total RNA and protein levels, a classic hallmark of hypertrophic cardiac cells. It was not until subsequent studies by Yik et al. and Michels et al. that this model of P-TEFb regulation under stress conditions became further refined [23, 25]. These laboratories were the first to show that the actual cdk9 inhibitor was HEXIM1, the human homologue of mouse CLP-1. The key finding linking CLP-1/HEXIM1 to the findings of Sano et al. was the demonstration that 7SK RNA actually played a support role for CLP-1/HEXIM1 binding to the P-TEFb complex: CLP-1/HEXIM1 could only bind to cyclin T1 via a supporting bridge formed by 7SK RNA [34]. Release of 7SK RNA was important to activating P-TEFb kinase activity only insofar as it mediated the release of CLP-1/HEXIM1, the actual cdk9 inhibitor (see Fig. 1). The findings of these laboratories were extremely important for guiding our subsequent studies on CLP-1’s role in cardiac hypertrophy.

With these insights into CLP-1’s molecular function and how that function might be involved in controlling the stress gene response to hypertrophic stimuli, we turned our attention to linking the two observations by confirming the association of CLP-1 with components of the P-TEFb complex in cardiomyocytes and examining the behavior of this association under the stress conditions imposed by hypertrophic stimuli [21]. Our studies showed that CLP-1, cdk9, and cyclin T1 all are expressed in embryonic, fetal, and postnatal heart, as determined by Western blot analysis. Co-immunoprecipitation studies showed that CLP-1 and cdk9 can be co-immunoprecipitated using antibodies to cyclin T1, suggesting an association of CLP-1 with the P-TEFb complex. Co-localization of CLP-1 with cdk9 and cyclin T1 using immunohistochemical and immunocytochemical techniques further confirmed the co-localization of these proteins in the nucleus of cardiomyocytes (see Fig. 4). Based on the work of Sano et al. [31], we examined the status of CLP-1’s association with the P-TEFb complex under normal versus hypertrophic conditions. Hypertrophy was induced in isolated cardiomyocytes using the well-documented technique of mechanically stretching cardiomyocytes by plating on expandable Flexcell 6-well plates and also by application of hypertrophic cytokines such as endothelin-1 and phenylephrine. Cell lysates were immunoprecipitated with antibodies to cyclin T1 and blotted with antibodies to either cdk9 or CLP-1. In all cases, hypertrophic cardiomyocytes showed P-TEFb complexes to be void of associated CLP-1 molecules indicating their release under hypertrophic conditions. We further tested this model by examining if CLP-1 can be released from P-TEFb complexes under conditions in which the hypertrophic response is abated by blocking a signal transduction pathway known to relay hypertrophic signals from membrane to nucleus, namely the jak/STAT pathway (discussed in further detail below). Cardiomyocytes were treated with the jak2 inhibitor AG490 prior to induction of hypertrophy by mechanical stretch. After 48 h of stretching, the cells were assayed by cyclin T1-CLP-1 co-immunoprecipitation assays. In cells treated with AG490, more CLP-1 was present in P-TEFb complexes than in cells left untreated, indicating that release of CLP-1 from P-TEFb occurs only under conditions in which
signal transduction pathways that transmit the hypertrophic signal are fully functional (see Fig. 4C). This suggests that the transduced Jak/STAT signal in some way controls the association and/or dissociation of CLP-1 with P-TEFb complexes, thereby controlling RNA pol II activity and transcriptional elongation. This work confirmed and extended the work of Sano et al. and placed CLP-1/HEXIM1 squarely in the center of the transcriptional response to hypertrophic stimuli. Despite the well-documented use of mechanical stretch and hypertrophic cytokines to induce hypertrophy, it still remained to be shown that CLP-1 regulation of P-TEFb activity is an integral part of the hypertrophic response in vivo in enlarged hearts.

**Lowering CLP-1 Levels and Increasing P-TEFb Activity Exacerbates the Hypertrophy Seen in Transgenic Models of Cardiac Hypertrophy**

To examine CLP-1 function in hypertrophic adult hearts, we resorted to the use of transgenic mice that develop hypertrophy from overexpression of specific genes in the heart [22]. We used two such mouse lines, a line of mice over-expressing calcineurin in the heart (MHC-CnA mice) [35] and a line of mice in which cyclin T1 is over-expressed in the heart (α-MHC-cyclin T1 mice) [31]. Calcineurin over-expressing mice exhibit...
compensatory hypertrophy during the first few postnatal months of life [36]. We determined CLP-1’s association with P-TEFb and its effect on RNA pol II phosphorylation during this time and found that CLP-1 was associated with P-TEFb to a lesser degree than in age-matched wild-type hearts, and that RNA pol II was phosphorylated to a greater extent than in wild-type hearts. This indicates that CLP-1 is predominantly dissociated from P-TEFb complexes in hypertrophic hearts in vivo resulting in activation of P-TEFb kinase activity and RNA pol II, a finding in accord with our in vitro studies. We next sought to test our model of CLP-1 function in hypertrophic hearts by reversing the experimental design: rather than using hypertrophic hearts to determine if CLP-1 is dissociated from P-TEFb, we generated mice having elevated levels of cyclin T1 and decreased levels of CLP-1 and asked if under these conditions the hypertrophy normally seen in the cyclin T1 over-expressors is exacerbated by the lowered CLP-1 levels and the resulting increase in P-TEFb and RNA pol II activity. This is based on the model of CLP-1-P-TEFb interaction in which association of CLP-1 with cyclin T1 inhibits cdk9 kinase activity: by increasing the concentration of cdk9 activator (cyclin T1) and decreasing its inhibitor (CLP-1), we hypothesized that this would lead to increased cdk9 kinase activity, increased RNA pol II transcriptional elongation, and given our results with the CLP-1 knockout mice, an exacerbated hypertrophic response leading to enlarged hearts greater than that seen in MHC-cyclin T1 mice. The results of these experiments did in fact bear out this hypothesis. Alpha-MHC-cyclin T1/CLP-1 +/− mice had much larger hearts than α (alpha)-MHC-cyclin T1 or CLP-1 +/− mice with the largest increase in heart-to-body weight ratio among these mice when compared to wild-type mice (see Fig. 5). Phosphorylation of serine 2 in the carboxy terminal domain of RNA pol II was increased by 50% in α (alpha)-MHC-cyclin T1/CLP-1 +/− hearts over that seen in α (alpha)-MHC-cyclin T1 hearts (Fig. 5). This particular phosphorylation is associated with the switch of RNA pol II from a transcriptional initiation state in which RNA synthesis is paused to one in which RNA pol II actively synthesizes the remaining RNA transcript. Thus, in α (alpha)-MHC-cyclin T1/CLP-1 +/− hearts, RNA pol II’s elongation activity is activated to a much greater extent than

Fig. 5 Exacerbated hypertrophic response in α (alpha)-MHC-cyclin T1/CLP-1 +/− mice. (a) Photographs of adult mice hearts of the indicated genotypes. The α (alpha)-MHC-cyclin T1/CLP-1 +/− heart appears to be the most enlarged relative to all other genotypes. It also exhibited the largest change in heart/body weight ratio of all genotypes relative to wild-type mice. (b) Quantitation of the relative changes in heart to body weight ratios of the hearts pictured in (a). (c) The second serine in the domain targeted by cdk9 in the carboxy terminal end of RNA pol II exhibits 50% greater phosphorylation than the α (alpha)-MHC-cyclin T1 mouse heart. This suggests that decreased CLP-1 levels lead to increased cdk9 activity directed to RNA pol II (Taken from [22] with permission)
that in α (alpha)-MHC-cyclin T1 or CLP-1 +/- hearts. This most likely reflects an increase in P-TEFb cdk9 kinase activity, a finding that also bears out our hypothesis.

CLP-1 and Promoter Proximal Pausing Act to Control the Responsiveness of Stress Response Genes to Hypertrophic Stimuli

As our studies on CLP-1’s control of P-TEFb in the hypertrophic response progressed, it became increasingly apparent from the work of other laboratories that P-TEFb and its regulatory molecules were at the center of a novel transcriptional control mechanism for the rapid up-regulation of stress response genes. These studies have provided new insights into gene transcription that have informed our understanding of how P-TEFb controls stress response gene expression, particularly as it relates to the hypertrophic stress response. A new control mechanism called “promoter-proximal pausing” has emerged as a way of poising stress response genes in a “ready state” to respond rapidly to stress and other stimuli [26–29, 37]. In this mechanism, the basal or unstimulated state of stress response genes is one in which RNA pol II has assembled into an initiation complex that initiates RNA synthesis and synthesizes a small nascent but incomplete RNA transcript of very short length whose synthesis has been “paused.” Stress signals act to “unpause” the RNA pol II to rapidly complete the transcript and allow for new RNA pol II enzymes to occupy the already assembled gene transcription apparatus and begin synthesizing more RNA (see Fig. 6). Controlling the paused state is P-TEFb. When CLP-1/HEXIM1 is bound to P-TEFb, its cdk9 kinase activity is repressed and its substrate, RNA pol II, is unphosphorylated and in a state of paused synthesis, having initiated synthesis of nascent transcripts. Upon receipt of the appropriate signals, CLP-1/HEXIM1 dissociates from P-TEFb derepressing cyclin T1 and activating cdk9 to phosphorylate the paused RNA pol II and its associated pause factors, NELF (negative elongation factor) [38] and DSIF (DRB sensitivity-inducing factor) [39]. Release of NELF allows RNA pol II to complete synthesis of nascent RNA transcripts into full-length mRNAs for translation into protein. By pre-assembling a transcriptional complex capable of immediately resuming

![Fig. 6 Activation of P-TEFb by hypertrophic signals. CLP-1/HEXIM1 binds to the P-TEFb complex and inhibits cdk9 kinase activity, causing RNA pol II to remain in an abortive elongation stage (initiation complex) aided by presence of negative transcriptional elongation factors NELF and DSIF. Hypertrophic signals release CLP-1/HEXIM1, NELF, and DSIF to promote RNA pol II activation by cdk9 and productive RNA chain elongation (Adapted from [47] with permission)]
transcription upon receipt of stress signals, the genomic stress response can be launched much more quickly than if all the various components of the transcriptional apparatus had to be assembled de novo. Given our and others’ observations on the behavior of P-TEFb and its regulatory components in the response of cardiomyocytes to hypertrophic stimuli, promoter-proximal pausing is likely to be an important transcriptional mechanism controlling the responsiveness of hypertrophic stress response genes to hypertrophic stimuli.

Transduction of Hypertrophic Signals Via the Jak/STAT Pathway Could Directly Affect CLP-1’s Control of P-TEFb

While the transcriptional components involved in promoter-proximal pausing have been detailed, little is known about how these components interact with or respond to signals that promote “unpausing” of RNA synthesis and gene transcription. Since the hypertrophic genomic response is driven by hypertrophic signals, how these signals interact with P-TEFb to “unpause” hypertrophic stress response genes remains an essential aspect of this process that has yet to be fully elucidated. Some indication that signaling pathways can interact with P-TEFb has come from studies of stressed cells in which Ca^{2+}-based signaling, PI3K/Akt signaling, and MEK1 signaling pathways were shown to control P-TEFb activity [40–42]. None of these pathways, however, were examined in hypertrophic heart or cardiomyocytes. While such studies will no doubt prove informative, our laboratory has focused on the Jak/STAT signaling pathway as a potential regulator of CLP-1 dissociation from P-TEFb. Our laboratory has a long-standing interest in the Jak/STAT signal transduction pathway, which has been shown to be a major transducer of hypertrophic signals in hypertrophic hearts and cardiomyocytes (reviewed in [43]). The involvement of Jak/STAT in the stress gene response is also well documented with a number of studies showing STAT3 to be essential for inducing cardioprotective genes, a class of stress response genes promoting cardiomyocyte survival in hypertrophic hearts and mechanically stretched cardiomyocytes (reviewed in [44]). Given that both Jak/STAT and P-TEFb are involved in up-regulating stress response genes, we asked if the STAT signaling pathway might be involved in regulating P-TEFb activity under hypertrophic conditions. Since CLP-1 appears to be fully or near fully dissociated from the P-TEFb complex during hypertrophy induced in cardiomyocytes and in our transgenic models of hypertrophy, we wanted to determine if blocking the Jak/STAT pathway under hypertrophic conditions would lead to CLP-1 being retained by P-TEFb complexes. To do this, we repeated studies in our laboratory in which cardiomyocytes pretreated with the Jak2 kinase inhibitor AG490 and then stimulated with hypertrophic agents failed to develop hypertrophy [45]. When we examined the interaction of CLP-1 with P-TEFb in these cells, we found that despite stimulation with hypertrophic agents, CLP-1 remained bound to P-TEFb [21] (see Fig. 4). Since inhibition of Jak2 prevents STAT activation and mobilization to the nucleus, these results suggested that activated STAT dimers may in some way interact with P-TEFb to prevent CLP-1 binding. For the Jak/STAT pathway to interact with P-TEFb during hypertrophy, Jak2 kinase must first phosphorylate and activate STAT dimers to translocate to the nucleus. Since STATs are not kinases, they most likely activate P-TEFb by competitively preventing or blocking CLP-1 binding. Hou et al. have shown in IL-6-treated HepG2 hepatocarcinoma cells that activated nuclear STAT3 binds to the cdk9/cyclin T complex prior to DNA interaction, suggesting that STAT3 may prevent CLP-1 binding and directly bring the active form of P-TEFb to STAT3-responsive genes where they phosphorylate and switch RNA pol II from its “paused” to elongation state. Such a mechanism could up-regulate compensatory hypertrophic as well as cardioprotective genes in hypertrophic cardiomyocytes. Thus, in addition to promoter-based transcriptional
activation, activated STAT dimers may also up-regulate gene transcription by promoting transcriptional elongation. The Jak/STAT pathway and its involvement in the hypertrophic response is an excellent example of how signaling transcription factors like the STATs might interact with P-TEFb to rapidly induce stress response genes. Because they bind specific promoter sequences, STATs could impart sequence specificity to the “unpausing” of stress response genes that P-TEFb would otherwise be unable to do. Documenting this will provide novel information on the role of STATs and CLP-1 in signal-dependent regulation of hypertrophic stress response genes.

Conclusions

In cardiac hypertrophy, work, volume, or pressure overload trigger signal transduction pathways to activate specific genetic programs that allow the heart to increase its output while maintaining normal hemodynamic function. Many of these genetic programs involve stress response genes that are poised to respond immediately to hypertrophic stress signals. This poised state is regulated by a mechanism known as promoter proximal pausing that in turn, is controlled by the transcriptional regulator CLP-1. To trigger the release of stress response genes from this paused state, hypertrophic stress signals must cause the release of CLP-1 from the promoter proximal pausing complex to allow for transcription of response genes. By acting as a signal-dependent switch regulating the transition from poised to active transcription, CLP-1 can control the expression of genes that comprise the genomic compensatory stress response to hypertrophic stimuli.

References


Molecular Mechanisms of Subcellular Remodeling in Congestive Heart Failure

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Abstract
Cardiac dysfunction in congestive heart failure (CHF) is generally associated with changes in the biochemical activity of different subcellular organelles including the sarcolemma (SL), sarcoplasmic reticulum (SR), and myofibrils (MF). Extensive research has been carried out by employing a wide variety of experimental models as well as myocardial tissue from patients with CHF to examine if changes in the activities of subcellular organelles are due to corresponding changes in cardiac protein content and gene expression. Varying degrees of alterations in mRNA levels and protein content for SL, SR, and MF have been observed in the failing heart. Furthermore, improvement in cardiac function in CHF by different pharmacologic interventions has been associated with attenuation of changes in gene and protein expression for SL, SR, and MF. These observations are consistent with the view that subcellular remodeling and subsequent cardiac dysfunction in CHF may be a consequence of alterations in cardiac gene expression.

Keywords
Ca²⁺ transport • Ca²⁺-pump ATPase • Cardiac dysfunction • Cardiac remodeling • Congestive heart failure • Myofibrillar ATPase • Myofibrils • Na⁺-Ca²⁺ exchange • Na⁺-K⁺ ATPase • Sarcolemma • Sarcoplasmic reticulum • Subcellular remodeling

Introduction
It is generally accepted that cardiac dysfunction in congestive heart failure (CHF) is due to changes in the size and shape of the heart (cardiac remodeling) [1–4]. However, there are several experimental and clinical situations where cardiac dysfunction has been observed without any
evidence of cardiac remodeling and vice versa [5–8]. In fact, cardiac remodeling at initial stages of myocardial hypertrophy is accompanied by no changes or hyperfunction of the heart [9]. Accordingly, it has been proposed that cardiac dysfunction in CHF is associated with remodeling of subcellular organelles including sarcolemma (SL), sarcoplasmic reticulum (SR), myofibrils (MF), and mitochondria (MIT), as well as extracellular matrix (ECM) [5–8]. It should be noted that subcellular remodeling refers to changes in molecular and physical structure as well as the chemical composition of different organelles in the myocardium. Such defects may occur in one or more organelles depending upon the stage of CHF development as well as the type of pathophysiologic stimulus for heart disease. Although different mechanisms concerned with the synthesis and degradation of subcellular organelles are considered to result in subcellular remodeling [5–8], the present article is intended to focus on changes in some of the gene and protein expressions which have been reported to induce subcellular defects for the occurrence of cardiac dysfunction in CHF. In particular, it is planned to highlight the significance of alterations in the expression of some proteins for the remodeling of SL, SR, and MF, which are known to play a major role in determining the subcellular activities and thereby the status of heart function. It is pointed out that it is not our intention to undermine the importance of MIT or ECM remodeling in inducing cardiac dysfunction during the development of CHF as these mechanisms of heart disease are known to play a major role in energy production and maintenance of the shape and size of the heart, respectively.

**Remodeling of SL Membrane in CHF**

Although SL membrane contains several receptors, exchangers, transporters, and enzymes that play an important role in the regulation and maintenance of cardiac function, it is planned to limit the discussion to the Na⁺-K⁺ ATPase and Na⁺-Ca²⁺ exchanger to illustrate their remodeling and modification of their function in CHF. The Na⁺-K⁺ ATPase is a prominent member of the SL protein family [10], which exists in virtually all animal tissues, as well as the human myocardium [11]. This enzyme is responsible for the active transport of Na⁺ ions out of the cell, while simultaneously importing K⁺ ions [10], and hence functions to maintain cell volume, establish ionic gradients, and preserve membrane potential of the cardiomyocyte [12]. Consequently, a defect in the function of this enzyme was found to be related to abnormalities in cardiac performance and has been outlined as a salient feature of the subcellular basis of CHF [11–15]. A wide variety of changes in the mRNA level, protein content, and activities of Na⁺-K⁺ ATPase as well as Na⁺-Ca²⁺ exchanger were observed in different studies, as shown in Table 1 [16–22].

It is noteworthy that Na⁺-K⁺ ATPase is composed of different subunits such as α₁, α₂, α₃, β₁, β₂, and β₃, and several investigators have examined alterations in these subunits in heart disease. Charlemagne et al. [13] reported that there was a reduction in the α₂ mRNA and protein levels in mild and severe stages of hypertrophy, while the compensated stage of hypertrophy revealed no alterations in the α₁ and β₁ mRNA and protein levels. Furthermore, the α₃ mRNA and protein levels were increased at 5 days and 30–50 post-stenosis of the abdominal aorta, respectively. In another study, Semb et al. [15] observed that 6 wks post-MI showed a reduction in the α₂ mRNA and protein levels, with no alterations in the expression of the α₁ and β₁ subunits; however an increase in the α₃ subunit at the transcriptional level was noted. This was supported by Book et al. [23], who indicated that at 8 wks post-stenosis of the left renal artery, there was a decrease in the α₂ mRNA and protein levels with unchanged α₁ expression, and a reduction in the β₁ protein levels. A unique experimental animal model of heart failure, UM-X7.1 cardiomyopathic hamsters, was studied by Kato et al. [14], who reported a decrease in the α₁ mRNA, protein and α₃ protein levels with enhanced α₁ and β₁ mRNA and protein levels, with contrasting undetected levels of α₃ mRNA.

In terms of the functional alterations in the Na⁺-K⁺ ATPase, Dixon et al. [24] monitored its
activity at 4, 8, and 16 wks post-MI, and discovered that at 4 wks, the activity was unchanged, but at 8 and 16 wks the activity was significantly decreased. This finding suggested that the activity of the Na⁺−K⁺ ATPase may conceivably play a part in the adaptive mechanism of the heart that occurs during the development of CHF. Auxiliary investigations by Shao et al. [17] demonstrated that reduced activity of this SL enzyme was coupled with the reduction in the expression of the α₂, α₃, and β₁ mRNA and protein levels, in addition to an elevation in the expression of the α₃ subunit. Shao et al. [17] further demonstrated that when imidapril (an angiotensin converting enzyme inhibitor (ACE)) was administered in animals with CHF, the Na⁺−K⁺ ATPase activity improved and the changes in the gene expression of the SL proteins were attenuated as a consequence of the blockade of the renin-angiotensin system (RAS). Further studies by Ren et al. [18] revealed that after 37 wks of treatment of 3 wks post-MI with imidapril attenuated the reduction in the activity of the Na⁺−K⁺ ATPase, with paralleled trends in the expression of both the mRNA and protein content.

<table>
<thead>
<tr>
<th>Table 1 Modifications in SL membrane protein and gene expressions post myocardial infarction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal model/time point</td>
</tr>
<tr>
<td>Male SD rat, CL/8 wks</td>
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<tr>
<td>Male SD rat, CL/8 wks</td>
</tr>
<tr>
<td>Male SD rat, CL/37 wks</td>
</tr>
<tr>
<td>Human end stage heart failure</td>
</tr>
<tr>
<td>Male Wistar rat, CL/110 days</td>
</tr>
<tr>
<td>Male Wistar rat, CL/4 and 12 wks</td>
</tr>
<tr>
<td>Male Wistar rat, CL/1 wk, 3 wks, 3 months</td>
</tr>
</tbody>
</table>

SD Sprague Dawley, CL coronary ligation, SHR spontaneously hypertensive rats, NCX Na⁺/Ca²⁺ exchanger, Na⁺/K⁺ Na⁺/K⁺ ATPase, wks weeks, NC no change observed

prominent member of the SL proteins and is situated in the T-tubules closest to the sites of Ca²⁺ release from the SR [25]. The fully mature form of the Na⁺−Ca²⁺ exchanger exists as 120 kDa and is responsible for maintaining Ca²⁺ homeostasis in the cell [26]. Since its discovery, the kinetic parameters of this enzyme suggest that this system can encompass rapid Ca²⁺ transport in and out of the myocardial cell during the cardiac contractile cycle [27]. Though its significance in cardiac excitation-contraction coupling in the normal heart has been well established, its role in the failing myocardium has yet to be fully characterized. Some of the studies [17–22] indicating alterations in SL Na⁺−Ca²⁺ exchanger activity, as well as gene and protein expressions, are shown in Table 1. In a clinical study on dilated cardiomyopathy (DCM) and coronary artery disease (CAD), Studer et al. [28] evaluated the expression of the Na⁺−Ca²⁺ exchanger together with the SR Ca²⁺-pump ATPase (SERCA). Among the two patient groups employed, the mRNA and protein levels of the Na⁺−Ca²⁺ exchanger were elevated in contrast to the reduction in the mRNA and protein levels of SERCA [28]; this observation gave rise to the idea that the increased expression of the Na⁺−Ca²⁺ exchanger somewhat
compensated for the diminished function of the SR to remove Ca\(^{2+}\) from the cytosol during relaxation. Furthermore, these findings were supplemented by Hasenfuss et al. [29] who affirmed that the decreased levels of SERCA in concert with unaltered levels of the Na\(^+-\)Ca\(^{2+}\) exchange, accounted for the disorder in diastolic dysfunction. On the other hand, early stages of CHF due to MI have shown a reduction in the activity, mRNA, and protein expression of Na\(^+-\)Ca\(^{2+}\) exchange [17, 30]. Schillinger et al. [19] illustrated, in the hours preceding cardiac transplantation, that the increase in the Na\(^+-\)Ca\(^{2+}\) exchanger, collectively with the reduction in SERCA, provided a substantial association amongst changes in neurohormonal levels of epinephrine and SL activity of Na\(^+-\)Ca\(^{2+}\) exchange. They further projected that during CHF, the activation of the sympathetic nervous system conceivably amplified the expression of the Na\(^+-\)Ca\(^{2+}\) exchanger, which may potentially have a role in the onset of malignant ventricular arrhythmias. The intensifying concern of progressing arrhythmias in CHF was previously examined by Reinecke et al. [31]. It was indicated that the enhanced activity of the Na\(^+-\)Ca\(^{2+}\) exchange in end-stage HF was a result of its increased protein levels, and that this increase provided an augmented influx of Na\(^+\), which was further associated with potential membrane depolarizations to create amplified arrhythmogenesis. Nonetheless, alterations in the activation as well as mRNA and protein levels for both Na\(^+-\)Ca\(^{2+}\) exchanger and Na\(^+-\)K\(^+\) ATPase suggest the occurrence of SL remodeling during the development of CHF.

Remodeling of SR Membrane in CHF

During cardiac relaxation, Ca\(^{2+}\) is pumped from the cytosol into the SR through the 105 kDa SR Ca\(^{2+}\)-pump ATPase [32, 33]. As this enzyme is responsible for the diastolic phase of the cardiac cycle, any impairments in this process of Ca\(^{2+}\) sequestration could possibly contribute to the pathophysiology of CHF. Supporting evidence for this postulation regarding cardiac contractile dysfunction in the failing heart include (a) an increased abundance of SERCA with contrasting decreased levels of PLB in response to prolonged exposure to thyroxine [34, 35]; (b) an abnormal force–frequency relationship, whereby increased frequency of stimulation gives a decreased developed tension [36]; and (c) a down-regulated β-adrenergic receptor to give rise to reduced levels of cAMP, which further affects the regulation of PLB and, in turn, disrupts its negative inhibitory regulation of SERCA2a [37, 38]. Extensive research has been focused on elucidating the mechanisms involved in the development of CHF in various experimental models. Clinical studies performed on the isolated human myocardium have shown an overall decrease in SERCA mRNA [39–41] and protein levels, in addition to a reduction in the abnormal handling of Ca\(^{2+}\) by SERCA itself [42, 43]. To assess the validity of these applications, different investigators have attempted to compare the findings in animal models of CHF with the failing human myocardium. In a report by Movsesian et al. [44], findings showed a reduction in the Ca\(^{2+}\) sequestration of the failing human myocardium, consistent with those of the animal models, and can be attributed to decreased levels of SERCA mRNA produced during gene transcription. Further studies supporting this decrease in SERCA expression and function in the failing heart include models of the pressure-overloaded rat [33], the tachycardia-induced mongrel dog [45], the volume-overloaded rat [46], and the transgenically engineered hypertensive rat [47]. Though this offers insight into the molecular pathogenesis of CHF, the data accumulated thus far raises more questions than have been answered. If in fact the protein level of SERCA remains unchanged in the failing heart, the answer may possibly lie in the complex mechanisms concerning transcription, translation, and protein degradation of the SR membrane [44]. Varying degrees of alterations in different SR protein content and gene expressions indicating remodeling SR membrane in CHF due to MI are shown in Table 2 [17–19, 21, 28, 29, 48–60].

The Ca\(^{2+}\) uptake process of SERCA is intimately modulated through another SR protein, PLB, which is composed of five equal monomers. This 30 kDa protein [61] inhibits SERCA through
Table 2  Modifications in SR protein and gene expressions post myocardial infarction

<table>
<thead>
<tr>
<th>Animal model/time point</th>
<th>Genes of interest</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male SD rat, CL/5 wks</td>
<td>SERCA2a/PLB/RyR</td>
<td>↓ SERCA2a/PLB/RyR mRNA ↓ SERCA2a/PLB/RyR protein</td>
<td>Sanganalmath et al. [48]</td>
</tr>
<tr>
<td>Male SD rat, CL/1 day, 2 wks, 4 wks</td>
<td>SERCA2a</td>
<td>↓ SERCA2a mRNA day 1 ↓ SERCA2a protein at 4 wks</td>
<td>Sallinen et al. [49]</td>
</tr>
<tr>
<td>Male Wistar rat, CL/12 wks</td>
<td>SERCA2a</td>
<td>↓ SERCA2a mRNA levels</td>
<td>Prunier et al. [50]</td>
</tr>
<tr>
<td>Male SD rat, CL/7 wks</td>
<td>SERCA2a/PLB/RyR</td>
<td>↓ SERCA2a/PLB/RyR mRNA levels</td>
<td>Shao et al. [17]</td>
</tr>
<tr>
<td>Male SD rat, CL/37 wks</td>
<td>SERCA2a/PLB/RyR/CQS</td>
<td>↓ SERCA2a/RyR/CQS mRNA ↓ PLB mRNA levels</td>
<td>Ren et al. [18]</td>
</tr>
<tr>
<td>Male SD rat, CL/16 wks</td>
<td>SERCA2a/PLB/RyR</td>
<td>↓ SERCA2a, PLB mRNA levels ↑ RyR mRNA levels</td>
<td>Xu et al. [51]</td>
</tr>
<tr>
<td>Male SD rat, CL/7 wks</td>
<td>SERCA2a/PLB/RyR/CQS</td>
<td>↓ SERCA2a/PLB/RyR/CQS mRNA ↓ SERCA2a/PLB/RyR/CQS protein</td>
<td>Guo et al. [52]</td>
</tr>
<tr>
<td>Human end stage heart failure</td>
<td>SERCA2a</td>
<td>↓ SERCA2a protein</td>
<td>Schillinger et al. [19]</td>
</tr>
<tr>
<td>Male SD rat, CL/12 wks</td>
<td>SERCA2a/RYR</td>
<td>↓ SERCA2a/RYR mRNA levels</td>
<td>Sakai et al. [53]</td>
</tr>
<tr>
<td>Male SD rat, CL/6 wks</td>
<td>SERCA2a/PLB</td>
<td>NC</td>
<td>Ambrose et al. [54]</td>
</tr>
<tr>
<td>Human heart DCM, ICM</td>
<td>SERCA2a</td>
<td>↓ SERCA2a protein levels</td>
<td>Hasenfuss et al. [29]</td>
</tr>
<tr>
<td>Male SD rat, CL/8 wks</td>
<td>SERCA2a/PLB</td>
<td>↓ SERCA2a/PLB mRNA &amp; protein</td>
<td>Shao et al. [55]</td>
</tr>
<tr>
<td>Male SD rat, CL/3 wks</td>
<td>SERCA2a</td>
<td>↓ SERCA2a protein levels</td>
<td>Zhang et al. [56]</td>
</tr>
<tr>
<td>Male SD rat, CL/4, 12 wks</td>
<td>SERCA2a</td>
<td>↓ SERCA2a mRNA 12 wks</td>
<td>Hanatani et al. [21]</td>
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<tr>
<td>Male SD rat, CL/4 wks</td>
<td>SERCA2a</td>
<td>↓ SERCA2a mRNA levels</td>
<td>Iijima et al. [57]</td>
</tr>
<tr>
<td>Human idiopathic DCM</td>
<td>SERCA2a/PLB</td>
<td>NC</td>
<td>Munch et al. [58]</td>
</tr>
<tr>
<td>Male SD rat CL/1 day, 1 wk and 6 wks</td>
<td>SERCA2a/PLB</td>
<td>NC in SERCA2a mRNA or protein ↓ PLB mRNA transiently on day1</td>
<td>Yue et al. [59]</td>
</tr>
<tr>
<td>Male SD rat, CL/4, 8 and 16 wks</td>
<td>SERCA2a</td>
<td>↓ SERCA2a mRNA at 4, 8, 16 wks ↓ SERCA2a protein at 8 and 16 wks</td>
<td>Zarain-Herzberg et al. [60]</td>
</tr>
<tr>
<td>Human DCM, CAD</td>
<td>SERCA2a</td>
<td>↓ SERCA2a mRNA and protein</td>
<td>Studer et al. [28]</td>
</tr>
</tbody>
</table>

SD Sprague Dawley, CL coronary ligation, SERCA2a sarc(o)plasmic reticulum Ca2+ ATPase, PLB phospholamban, RyR ryanodine receptor, CQS calsequestrin, DCM dilated cardiomyopathy, ICM idiopathic cardiomyopathy, CAD coronary artery disease, NC no change observed, wks weeks

direct interaction, which depresses the transport of Ca2+ into the SR [62]. As phosphorylation of PLB relieves this inhibition and allows for Ca2+ uptake to occur, it is thought that this resulting de-inhibition through kinase activity is the principal molecular mechanism responsible for the inotropic effects involved in the β-adrenergic receptor system [44]. Compelling evidence to substantiate this phenomenon was seen in a study involving the isoproterenol myocardial response in mice deficient in the PLB gene in comparison to their control [63]. This investigation has demonstrated that the PLB-deficient mice exhibited high rates of contraction and relaxation in the absence of isoproterenol with no increase in response in its presence, which correlated well with the observation that the control mice had low rates of contraction and relaxation in the absence of isoproterenol, but showed increased rates with progressive exposure to isoproterenol. In accordance, these results reflect the mechanism by which the phosphorylation of PLB removes the inhibitory effect on SERCA to permit the transport of Ca2+ into the SR.

Ca2+ release from the SR is achieved through the RyR, which is composed of four monomers of ~560,000 kDa [64, 65]. Evidently, the cardiac RyR mRNA is unique to the myocardium and is not expressed in fast- or slow-twitch skeletal muscle [66, 67]. Although the status of Ca2+ pumping into the SR has been extensively studied in CHF of humans and animal models, relatively fewer studies are available on the Ca2+ release process in the failing heart. When SR membrane
vesicles were incorporated into artificial planar phospholipid bilayers with the activity of single channels subsequently recorded using voltage clamp conditions, unaltered characteristic behavior of these channels was observed in CHF from ischemic cardiomyopathy, DCM, congenital disease, or valvular disease as compared to normal hearts [68, 69]. Yet contradictory to these results, another study revealed that the threshold of Ca\(^{2+}\) release, as induced by caffeine, was remarkably increased in the hearts of patients with DCM as opposed to normal hearts, thus suggesting an impaired gating mechanism in the Ca\(^{2+}\)-release channel [70]. While comparing the Ca\(^{2+}\)-release activity in both pressure-overloaded and volume-overloaded rats, Hisamatsu et al. [46] have documented enhanced Ca\(^{2+}\) release in the compensated left ventricular hypertrophy stage of the pressure-overloaded model, with a contrasting decrease in Ca\(^{2+}\)-release activity and number of RyR in the volume-overloaded model. Cory et al. [71] have observed that both the density of the SR terminal cisternae and the activity of RyR were reduced in the Doberman Pinscher dog CHF model, as well as during rapid ventricular pacing in mongrel dogs. Additionally, Arai et al. [41] documented a reduction in RyR mRNA in patients suffering from end-stage CHF from primary pulmonary hypertension, DCM, or ischemic heart disease. Furthermore, Pennock et al. [72] described that after ligation of the circumflex artery in New Zealand white rabbits, the administration of 3,5-diiodothyropropionic acid (DITPA) prevented the reduction in the protein density of RyR, with no measurable changes at the gene mRNA level, thereby improving SR function in the infarcted rabbit heart. In view of these studies, it offers that changes in the expression of mRNA and protein content for RyR may depend upon the stage of SR remodeling during the development of CHF.

**Remodeling of MF in CHF**

The structural contractile unit of the myocardium controls the transition of the diastolic state to the systolic state through various intricate steric, allosteric, and cooperative mechanisms of the MF thick and thin filaments [73]. Upon cardiac distress, the MF, which represent more than 50% of the cell volume, are considered to adapt by increasing in size, number, and overall expression [74]. One of the major components of the cardiac contractile apparatus is the myosin motor of the thick filament protein system, which acts as the cross-bridge for interacting with the thin filament system to produce force via ATP hydrolysis [75]. As shown in Table 3 [76–83], various studies have revealed a significant reduction in MF ATPase activity in the failing heart [84], including CHF due to mitral valve insufficiency, pressure overload, idiopathic cardiomyopathy, and CAD [85–89]. Composed of two heavy chains, each associated with two different light chains [90, 91], myosin is involved in the imperative process that influences cardiac systolic and diastolic functions. The myosin light chains (MLC) are categorized into special groups and are called essential MLC (MLC-1) and regulatory MLC (MLC-2). In contrast to the thick filament family, the troponin network is a collection of proteins that encompasses the thin filament regulatory elements [92] and plays a crucial role in Ca\(^{2+}\) sensitivity on the MF and regulating MF ATPase activity [93]. Currently, it is believed that the binding and removal of Ca\(^{2+}\) from troponin transmits conformational changes to tropomyosin, which, in due course, activates the contractile elements for triggering the cross-bridge interaction between the actin and myosin [94–98]. Since the MF are considered as the contractile machinery in the cell, any structural or functional modifications to myosin, actin, troponin, and tropomyosin may contribute to MF remodeling in heart disease.

There are two genes of particular interest located in tandem on chromosome 14 that encode the cardiac myosin heavy chain (MHC), and are termed α-MHC and β-MHC [99, 100]. Given that the α-MHC isoform results in a high-power, low economy ATPase activity, whereas the β-MHC isoform gives rise to a low-power, high economy ATPase myofilament activity, the events associated with cardiac stress promote a shift in expression toward the β-MHC for a more efficient performance [101–106]. In a study by
Swoap et al. [107], both systemic hypertension and caloric restriction resulted in the enhanced expression of β-MHC protein and mRNA levels due to increased transcription activity, in concert with a reduction in the expression of α-MHC protein and mRNA levels. Eble et al. [108] conducted another molecular study in the failing hearts of rabbits, and reported an increase in the MHC synthesis in left ventricular dysfunction due to chronic ventricular tachycardia that could be explained by an increased MHC translational efficiency. Such a change was further supported by Imamura et al. [109], who reported an elevated synthesis in MHC in dogs subjected to pressure overload. Furthermore, in the rat model of pressure-overload hypertrophy, Toffolo et al. [110] described an augmentation in cell size followed by a change in the expression of myosin, to produce the slow migrating, economic V3 isoform, while exhibiting an increased number of myofibril units during the adaptive process of the myocardium. These observations are consistent with the view that remodeling of MF occurs with respect to its protein content in CHF and such a defect seems to be due to alterations in the expression of genes for different MF proteins.

### Conclusions

From the foregoing discussion, it is evident that cardiac dysfunction in CHF is associated with a wide variety of alterations in the activities as well as the expressions of genes and proteins for SL, SR, and MF. It should be noted that changes in cardiac gene expressions can be seen to result in alterations in the content of SL, SR, and MF proteins and thus may form the molecular basis for changes in the chemical composition of the subcellular organelles in the failing heart. Such a mechanism based on changes in cardiac gene expression does not rule out the participation of other mechanisms such as depression in the activity of different subcellular proteins due to changes in myocardial metabolisms or cation homeostasis in the cell. In addition, proteolysis of SL, SR, and MF

### Table 3 Modifications in MF protein and gene expressions post myocardial infarction

<table>
<thead>
<tr>
<th>Animal model/time point</th>
<th>Genes of interest</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male SD rat, CL/4 wks</td>
<td>α- and β-MHC</td>
<td>↓α-MHC mRNA; ↑β-MHC mRNA</td>
<td>Hart et al. [76]</td>
</tr>
<tr>
<td>Male SD rat, AB (PO)/15 wks</td>
<td>α- and β-MHC</td>
<td>↓α-MHC mRNA; ↑β-MHC mRNA</td>
<td>Schwarzer et al. [77]</td>
</tr>
<tr>
<td>Male SD rat, CL/5 wks</td>
<td>α- and β-MHC</td>
<td>↓α-MHC mRNA; ↑β-MHC mRNA</td>
<td>Sanganalmath et al. [48]</td>
</tr>
<tr>
<td>Male SD rat, ACS (VO)/4,10 wks</td>
<td>α-β-MHC/α-cardiac/α-SK</td>
<td>↑β-MHC mRNA at 4 and 10 wks NC α-MHC/α-cardiac/α-SK 4 wks ↓α-MHC mRNA at 10 wks NC α-cardiac/α-SK at 10 wks</td>
<td>Freire et al. [78]</td>
</tr>
<tr>
<td>Male SD rat, CL/8 wks</td>
<td>α- and β-MHC</td>
<td>↓α-MHC mRNA; ↑β-MHC mRNA</td>
<td>Wang et al. [79]</td>
</tr>
<tr>
<td>Male SD rat, CL/7 wks</td>
<td>α- and β-MHC</td>
<td>↓α-MHC mRNA; ↑β-MHC mRNA</td>
<td>Wang et al. [80]</td>
</tr>
<tr>
<td>Male Wistar rat, AB (PO)/18 wks</td>
<td>α- and β-MHC</td>
<td>↓α-MHC mRNA; ↑β-MHC mRNA</td>
<td>Huang et al. [81]</td>
</tr>
<tr>
<td>Male SD rat/1 day, 1 wk, 6 wks</td>
<td>β-MHC/α-SK</td>
<td>↑β-MHC mRNA 1 day/1 wk/6 wks ↑α-SK mRNA 1 day/1 wk/6 wks</td>
<td>Yue et al. [59]</td>
</tr>
<tr>
<td>Male SD rat, AB (PO)/22 wks</td>
<td>α- and β-MHC</td>
<td>↓α-MHC protein; ↑β-MHC protein</td>
<td>Chang et al. [82]</td>
</tr>
<tr>
<td>Male Wistar rat, CL/1 wk, 3 wks and 3 months</td>
<td>α-β-MHC/α-cardiac/α-SK</td>
<td>↓α-MHC mRNA levels ↑β-MHC/α-SK mRNA levels NC α-cardiac mRNA levels</td>
<td>Yoshiyama et al. [22]</td>
</tr>
<tr>
<td>Male SD rat, CL/20 wks</td>
<td>α-SK</td>
<td>↓α-SK mRNA levels</td>
<td>Simonini et al. [83]</td>
</tr>
</tbody>
</table>

SD Sprague Dawley, CL coronary ligation, MHC myosin heavy chain, a-cardiac α-actin cardiac, α-SK α-actin skeleton, ACS aortocaval shunt, AB aortic banding, PO pressure overload, SHR spontaneously hypertensive rat, VO volume overload, NC no change observed, wks weeks
proteins by the activation of various proteolytic enzymes has also been indicated to account for subcellular remodeling and cardiac dysfunction during the development of CHF [5–8]. Furthermore, remodeling of one or more subcellular organelles may depend on the type and stage of CHF and some of the subcellular changes at any given time-point may be adaptive or pathogenetic in nature. Various signal transduction mechanisms, which are altered during the development of CHF, may also affect cardiac gene and protein expressions, as well as the subcellular activities in the myocardium. Thus, a great deal of caution should be exercised while interpreting the data on subcellular remodeling and its consequence in the development of cardiac dysfunction in CHF. Nonetheless, prevention of subcellular remodeling in the failing heart represents a real challenge for the development of novel therapies and the improvement of cardiac function in CHF.

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68. Holmberg SR, Williams AJ. The calcium-release channel from cardiac sarcoplasmic reticulum: function in the failing and acutely ischemic heart. Basic Res Cardiol. 1992;87(suppl 1):255–68.


Molecular Mechanisms of Subcellular Remodeling in Congestive Heart Failure

Cardiomyopathy is caused by functional abnormalities of cardiac muscle, which include both extrinsic and intrinsic factors. The intrinsic factor involves mutations in genes playing roles in performance, regulation, or maintenance of cardiac function. Cardiomyopathy caused by the intrinsic factor is called idiopathic or primary cardiomyopathy, and there are several clinical types of primary cardiomyopathy including hypertrophic cardiomyopathy and dilated cardiomyopathy. Linkage studies and candidate gene approaches have deciphered the disease genes for hereditary primary cardiomyopathy: mutations in genes for components of sarcomere, sarcolemma, Z-disk, proteins of I band region, nuclear membrane, and transcriptional machinery. The most interesting findings are that mutations in different disease genes can be found in the same clinical types of cardiomyopathy and that mutations in the same disease gene can be found in different clinical types of cardiomyopathy. Functional analyses of disease-related mutations have revealed that characteristic functional alterations are associated with each clinical type of cardiomyopathy. In this review I focus on the cardiomyopathy-associated mutations found in genes for sarcomere and Z-disk elements and their functional relevance in the pathogenesis of primary cardiomyopathy.

Keywords
Calcium sensitivity • Cardiomyopathy • Mutation • Stress response • Stretch response • Z-disk
Introduction

Cardiomyopathy is a heterogeneous disease caused by functional abnormality of cardiac muscle and classified into primary cardiomyopathy and secondary cardiomyopathy [1]. Secondary cardiomyopathy is caused by extrinsic factors including infection, ischemia, hypertension, and metabolic disorders. On the other hand, diagnosis of primary cardiomyopathy is based on the exclusion of secondary cardiomyopathy and there are several different clinical types [2, 3]. Hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) are two major clinical types of primary cardiomyopathy. HCM, a major cause of sudden death in young and heart failure, is characterized by left ventricular hypertrophy, often asymmetric, accompanied by myofibrillar disarrays and reduced compliance (diastolic dysfunction) of cardiac ventricles. In contrast, DCM is characterized by dilated ventricular cavity with systolic dysfunction. Clinical symptom of DCM is heart failure and is often associated with sudden death. Other clinical types of cardiomyopathy include restrictive cardiomyopathy (RCM) and arrhythmogenic right ventricular cardiomyopathy (ARVC). RCM is accompanied by increased stiffness of the myocardium with diastolic dysfunction without significant hypertrophy, while ARVC is characterized by a dilated dysfunctional right ventricle (RV), ventricular arrhythmias, and fibrofatty replacement of the RV. Another cardiomyopathy is the left ventricular noncompaction (LVNC) characterized by trabeculations in the left ventricle (LV) accompanied by LV hypertrophy and/or dilatation.

During the last two decades, the etiology of primary cardiomyopathy has been unraveled to be associated with genetic abnormalities at least in part of the patients [4]. More than half of HCM patients have family history of the disease consistent with autosomal dominant genetic trait. In the case of DCM, about 20–35% patients had family history of the disease, mainly consistent with the autosomal dominant inheritance, although some familial cases can be explained by autosomal recessive or X-linked recessive trait. Familial occurrence is also noted in RCM, ARVC, and LVNC. Because the presence of family history suggested the genetic etiology of the disease, linkage studies in multiplex families and subsequent candidate gene approaches have been successful in unraveling novel disease genes. As shown in Table 1 many different disease genes were identified. It should be noted here that each patient or family usually carries only one mutation in the disease gene, albeit that exceptional cases harbor mutations in two or more disease genes, demonstrating the genetic heterogeneity of cardiomyopathy. Another noteworthy issue is the overlapping of disease genes for different clinical types. The disease genes can be classified into several categories; mutations in genes for sarcomere components, Z-disk elements, sarcoplasmic proteins, sarcolemma proteins, nuclear lamina, and others. The majority of disease genes encode sarcomere components, but a considerable number of disease-associated mutations can be found in Z-disk elements (Fig. 1).

Sarcomere Mutations in Cardiomyopathy: Sarcomeropathy

The first report of the disease gene for HCM was the identification of a disease-linked missense mutation in cardiac β-myosin heavy chain gene (MYH7) found in a large multiplex family [5]. Subsequently, MYH7 was analyzed for mutations in HCM patients and many different missense mutations were identified. However, frequency of MYH7 mutations in the patients was less than half, and linkage studies in non-MYH7-linked HCM families have revealed disease-linked mutations in α-tropomyosin gene (TPM1), cardiac troponin T gene (TNNT2), and cardiac myosin binding protein-C gene (MYBP3). Because these genes encode components of sarcomere involved in muscle contraction, genes for other sarcomere components were analyzed and led to the identification of HCM-associated mutations in ventricular myosin essential light chain gene (MYL3), ventricular myosin regulatory light chain gene (MYL2), cardiac troponin I gene (TNNI3), cardiac...
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AD autosomal dominant, AR autosomal recessive, XR X-linked recessive
α-actin (CACT), and cardiac troponin C (TNNC1). Therefore, mutations in any components of sarcomere can result in HCM [4], suggesting that a part of cardiomyopathy can be sarcomeropathy.

Initial analysis of functional changes caused by the HCM-associated MYH7 mutations demonstrated decreased power generation by the mutant myosin heavy chains [6], and the identification of HCM-related mutations in sarcomere components, troponin T, and α-tropomyosin led to a hypothesis that HCM is the disease of sarcomere and that cardiac hypertrophy was a compensation of decreased power generation [7]. However, we found HCM-associated TNNI3 mutations at the contraction inhibitory domain [8], which implied that the decreased power was not a common functional change caused by the sarcomere mutations. Indeed, subsequent functional analyses of mutations in genes for other sarcomere components than MYH7 have revealed that contractile performance was not decreased by the mutations and most HCM-associated sarcomere mutations resulted in increased Ca²⁺ sensitivity of muscle contraction [9–14]. Because a MYH7 mutation that caused HCM in transgenic mice also increased Ca²⁺ sensitivity at the muscle fiber level [15], a common functional alteration caused by HCM-related sarcomere mutations may be the increased Ca²⁺ sensitivity. Muscle contraction is regulated by the concentration of intracellular Ca²⁺ that is released from sarcoplasmic reticulum (SR) via ryanodine receptor (RyR2) and reuptaken to SR via sarcoplasmic reticulum Ca²⁺–ATPase (SERCA). When the concentration of Ca²⁺ is increased or decreased, muscle is contracted or relaxed, respectively. Increased Ca²⁺ sensitivity is a leftward shift of Ca²⁺-tension curve; more tension is generated by mutant sarcomere than normal sarcomere at the same Ca²⁺ concentration (hypercontraction) or muscle with mutant sarcomere is under less relaxed states (diastolic dysfunction) than normal sarcomere. This is consistent with the finding that characteristic features of HCM are hypercontraction and diastolic dysfunction.

On the other hand, mutations in the sarcomeric genes have also been found in patients with DCM.  

**Fig. 1** Schematic representation of sarcomere components. Half sarcomere is schematically shown. Components in which cardiomyopathy-associated mutations are found are underlined.
The first demonstration of DCM-linked sarcomeric mutation was the identification of cardiac α-actin gene (*CACT*) mutations in multiplex families with autosomal dominant DCM [16]. As described above, *CACT* mutation can be found in HCM [17], which demonstrates that sarcomere mutations cause both HCM and DCM, i.e., overlapping disease genes for different cardiomyopathy. Molecular basis of different phenotypes caused by *CACT* mutations was suggested by the fact that DCM-associated mutations were found at the α-actinin interacting domain [16], while HCM-associated mutations were at the interacting domain to myosin heavy chain [17]. In addition, recent data have suggested that there is a difference in folding property between the DCM-associated mutant actin and the HCM-associated mutant actin [18]. Another example of overlapping disease gene was the identification of *TNNT2* mutation in DCM [19]. Functional study of *TNNT2* mutations clearly demonstrated the difference between DCM-associated mutation and HCM-associated mutation, i.e., the DCM-associated *TNNT2* mutation decreased Ca²⁺ sensitivity of muscle contraction, which is in clear contrast to the increased sensitivity caused by the HCM-associated mutation [20]. Therefore, sarcomere mutations can be found in both HCM and DCM, but differences in the functional alterations may determine the different phenotypes.

**Z-Disk Element Mutations in Cardiomyopathy: Z-diskopathy**

Mutations in the sarcomere components could be found in only less than half of patients with hereditary cardiomyopathy, and a considerable part of the patients carried disease-associated mutations in Z-disk elements. Identification of a HCM-associated mutation in titin gene (*TTN*) was the first example of disease gene other than the sarcomere components [21], and the functional alteration due to the *TTN* mutation was an increased binding to α-actinin (Fig. 2). In addition, we demonstrated that the HCM-associated Tcap gene (*TCAP*) mutations increased the binding of Tcap to titin and calsarcin-1 [22] (Fig. 3), leading to a hypothesis that Z-disk mutations in HCM may result in increased binding of Z-disk components and hence “stiff sarcomere” (Fig. 4). “Stiff sarcomere” would increase passive tension upon stretch of the sarcomere. Because the increased passive tension was associated with the increased Ca²⁺ sensitivity [23–25], we have speculated that HCM-associated abnormality in both Z-disk components and sarcomere components causes the increased Ca²⁺ sensitivity.

In clear contrast, several DCM-associated Z-disk protein gene mutations could be identified in *TTN*, *CSRP3*, *TCAP*, and Cypher/ZASP gene (*LDB3*) [4]. Functional analysis of the DCM-associated *TTN* mutation at the actinin-binding domain revealed a decreased binding to actinin [26] but the opposite functional change as was found with the HCM-associated *TTN* mutation [21] (Fig. 2). In addition, another DCM-associated *TTN* mutation at the Tcap binding domain showed decreased binding to Tcap. Furthermore, *TCAP* mutations found in DCM patients showed opposite functional alterations to that caused by the HCM-associated mutations, i.e., DCM-associated mutations decreased binding of Tcap to titin, calsarcin-1, and MLP [22] (Fig. 3). These observations led us to hypothesize that DCM was the disease of “loose sarcomere” (Fig. 4). The loose sarcomere is evident in an animal model of DCM, *CSRP3* (MLP) knockout mouse, in which the Z-disk was wide and stretch response was impaired [27]. Since the stretch response is a hypertrophic response of cardiomyocytes against passive tension and Z-disk element is suggested to be a stretch sensor of cardiomyocytes, abnormality in Z-disk elements may alter the regulation of stretch response. It should be noted here that a possible controversy exists, i.e., HCM-associated MLP gene (*CSRP3*) mutations were reported to decrease the binding to α-actinin and N-RAP [28, 29]. However, because DCM-associated mutations were found in *CSRP3* and α-actinin gene (*ACTN2*), these mutations decreased binding to each other [30]. Therefore, the decreased binding between MLP and α-actinin was associated with both HCM and DCM. This discrepancy should be resolved by further studies.
Fig. 2 Schematic representation of TTN mutations found in the Z-disk domain and their functional alterations. Titin in the Z-disk domain is encoded by 17 exons. One HCM-associated mutation was found in exon 14, while two DCM-associated mutations were found in exon 3 and exon 14. Functional analyses showed altered binding of titin to Tcap or actinin.

Cypher/ZASP is a Z-disk element connecting calsarcin and actinin [31]. Calsarcin binds calcineurin [32], a Ser/Thr phosphatase involved in the process of hypertrophic program of cardiomyocytes [33, 34]. The functional significance of calcineurin anchorage to the Z-disk is not fully understood but it may be involved in the stress-induced calcineurin-NFAT activation, because heterozygous MLP knockout mice showed reduction in NFAT activation along with dislocation of calcineurin from Z-disk [35]. In addition, Cypher/ZASP is known to bind protein kinase C (PKC), [31] and a DCM-associated LDB3 mutation in the PKC binding domain was found to increase the binding [36]; it was suggested that phosphorylation/dephosphorylation of Z-disk elements might be involved in the stretch response. The identification of target protein(s) for phosphorylation (by PKC)/dephosphorylation (by calcineurin) will unravel the molecular mechanism(s) of stretch response and/or signaling molecule(s) of cardiac hypertrophy.

Several other LDB3 mutations not in the PKC interacting domain were reported in DCM or LVNC [37]. Because the functional changes caused by these mutations had not been demonstrated, we have searched for the binding protein to Cypher/ZASP by using yeast two-hybrid method, and found that phosphoglucomutase-1 (PGM1) is a novel binding protein [38]. PGM1 is an enzyme involved in the conversion between glucose-6-phosphate and glucose-1-phosphate, which is involved in the glucose/glycogen metabolism. Functional significance of the binding between PGM1 and the Z-disk element Cypher/ZASP was not known, but the DCM-associated mutations showed decreased binding to PGM1 [38] (Fig. 3). In addition, PGM1 was demonstrated to be localized at the Z-disk under the stressed culture conditions, low serum and low
**Fig. 3** Schematic representation of TCAP mutations and their functional alterations. Tcap is encoded by 2 exons. Two HCM-associated mutations and two DCM-associated mutations were found in exon 2. Domain structure of Tcap is schematically indicated below the exon–intron structure. Functional analyses showed altered binding of Tcap to titin, MLP, and calsarcin.

**Fig. 4** Schematic representation of functional alterations caused by Z-disk mutations. Functional alterations caused by the HCM-associated mutations (red stars) are shown in the upper panel, while the lower panel indicated the functional changes caused by the DCM-associated mutations (blue stars). Broken arrows show the altered interactions caused by the mutations. CN; calcineurin, CS1; calsarcin-1.
glucose, suggesting the role of PGM1 in the energy metabolism at the Z-disk [38]. These observations suggested that the decreased stress response due to the abnormality in Z-disk elements might be involved in the pathogenesis of DCM.

There are other DCM-associated mutations found in genes for other Z-line-associated proteins desmin (DES) and metavinculin (VCL) [4]. The VCL mutation was shown to impair the binding to actin [39], while the DES mutations resulted in a subtle change in the cytoplasmic desmin network [40]. In addition, mutations in the myopalladin gene (MYPN) have recently been reported in DCM. Although the molecular mechanisms of MYPN mutations leading to DCM remained unclear, the DCM-associated mutations impaired the myofibrinogenesis [41]. Because myopalladin binds a transcriptional cofactor, CARP [42], and CARP is involved in the regulation of gene expression associated with stretch response, cardiac remodeling, and myofibrinogenesis [43], Z-disk might be in part involved in the cardiac remodeling process related with pathogenesis of cardiomyopathy. In this regard, it is noteworthy that HCM-associated CARP mutations were reported to increase binding to myopalladin [44], although the significance of functional alterations remains to be resolved further.

Another Z-disk element, the mutation of which was associated with cardiomyopathy, is BMP10. BMP10 is a member of TGF family and is specifically expressed in the cardiomyocytes especially in fetal heart [45]. It was reported that knockout of BMP10 gene in mice led to hypoplastic heart with less trabeculation [46]. We have previously reported that expression of BMP10 is increased in the course of cardiac remodeling associated with hypertensive heart disease and found a BMP10 variant, Thr326Ile, in two DCM patients accompanied by hypertension [47]. This variant was a rare polymorphism but is a significant risk factor of DCM in the presence of hypertension. It should be noted here that this variant was also found in the father of a DCM patient and he also suffered from hypertensive heart disease. Because another TGF family, myostatin, which is specifically expressed in the skeletal muscle, is reported to bind Tcap [48], we have investigated the binding of BMP10 with Tcap and the effect of BMP10 variant on the binding, if any. It was found that BMP10 indeed bound Tcap and the variant decreased the binding, which in turn resulted in the increased secretion of BMP10 [47]. We also demonstrated that BMP10 facilitated hypertrophy and maturation of rat cardiomyocytes in primary culture [47]. These observations suggested a pivotal role of the Z-disk in cardiac remodeling through release of cardiac-specific growth factor, BMP10, and its impairment was involved in the pathogenesis of cardiomyopathy (Fig. 4).

Conclusions

In this review, cardiomyopathy-associated mutations in genes for sarcomere and Z-disk elements are summarized. Cardiomyopathy caused by sarcomere mutations may be considered as sarcomeropathy, and the functional alterations associated with sarcomeropathy can be an altered Ca2+ sensitivity. On the other hand, cardiomyopathy due to mutations in Z-disk element can be classified as Z-diskopathy, but the functional alterations in the Z-diskopathy may be heterogeneous abnormalities in stretch response, stress response, myofibrinogenesis, and/or cardiac remodeling. There are many other disease genes by which different functional alterations were elicited to ultimately lead to cardiomyopathy. Even though many disease genes have been deciphered, there is still a considerable proportion of patients whose disease genes remain to be unraveled. Nevertheless, each family or patient should have usually only one disease-causing mutation, and cardiomyopathy is both clinically and etiologically heterogeneous even in a specific clinical type such as HCM or DCM. Because different causes result in the same phenotype, there may be several common pathways in the pathogenesis. Intervention of these common pathways will be a therapeutic or preventive strategy for cardiomyopathy caused by different mutations.

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References


Left ventricular assist devices (LVADs) are currently used to either “bridge” patients with terminal congestive heart failure (CHF) to cardiac transplantation or optionally for patients with contraindications for transplantation (“destination therapy”). Mechanical support is associated with a marked decrease of cardiac dilation and hypertrophy as well as numerous cellular and molecular changes (“reverse cardiac remodeling”), which can be accompanied by improved cardiac function (“bridge to recovery”) in a relatively small subset of patients. In these patients, heart transplantation is no longer necessary even after removal of the device (“weaning”). In the recent past, novel pharmacological strategies have been developed and are combined with mechanical support, which has increased the percentage of patients with improved clinical status and cardiac performance. Gene expression profiles have demonstrated that individuals who recover after LVADs show different gene expression compared to individuals who do not respond to unloading. This chapter focuses on signal transduction, transcriptional regulation, and aspects of neurohormonal activation in the failing human heart before and after ventricular unloading.

Keywords
Chromogranin A • Heart failure • Hypertrophy • Myocytes • Natriuretic peptides • Remodeling
**Introduction**

Chronic heart failure is a major cause of morbidity and mortality in both western industrialized and developing nations and causes considerable economic burden to the medical systems. The average mortality from the time of diagnosis of heart failure is greater than 60% at 5 years and much higher when patients reach the most advanced stages [1]. Patients typically become progressively less responsive to medical therapy with the consequence of impaired heart function as well as quality of life [2].

Transplantation is currently the most established treatment for refractory heart failure [1]. However, this option is only available to fewer than 2,300 patients per year, with a relatively constant donor organ supply. As a result, the use of left ventricular assist devices (LVADs) in treating patients with end-stage heart failure has increased significantly in recent years [3]. LVADs are electrically powered either pulsatile or non-pulsatile pumps or turbines, which can be installed extracorporally or intrathoracically parallel to the circulation, i.e., they transport blood from the left ventricle to the ascending aorta and thereby provide profound volume and pressure reduction and restore systemic blood pressure and flow to near normal levels.

Regardless of the cause of myocardial injury, a clinical phenotype is recognized that is referred to as remodeling, which includes eccentric dilatation of the ventricular chamber, reduction in contractile function, and an increase in cardiac filling pressures and wall stress. These clinical phenotypic changes of heart failure are paralleled by significant histological, cellular, and molecular changes in most structural and functional components of the myocyte including alterations in myocyte geometry and size, progressive interstitial fibrosis, upregulation of cytokines and inflammation, as well as reductions in myocardial energetics, beta receptor density, and calcium-handling proteins. There is now compelling evidence that prolonged near-total unloading of the left ventricle in CHF is associated with numerous morphological and molecular changes in the myocardium (“reverse remodeling”) [4, 5], which can be accompanied by functional improvement and decreased cardiac dilatation [6]. Despite encouraging data on morphological and molecular changes of the affected heart, clinical cardiac recovery sufficient to allow device removal (“weaning”) is reported to occur only in a small subset of individuals [7, 8]. In general, it is accepted that cellular and molecular improvement is greater than clinical cardiac recovery [9]. The present paper mainly focuses on the major morphological and molecular changes that occur in the heart during “reverse cardiac remodeling” by ventricular unloading.

**Functional Improvements in Myocytes in Response to the LVAD Linked to Molecular and Gene Expression Changes**

There is evidence of early mechanical improvements in failing hearts treated with VADs [9–12]. These early findings are important in showing that contractile performance of myocytes was partially recovered in response to therapy with a VAD. Myocyte contractile performance was significantly greater in isolated myocytes of failing human hearts post VAD support compared to failing human hearts without VAD support [11]. Improvements in the magnitude of shortening were seen in response to beta-adrenergic agonists, and basal relaxation was also improved in myocytes following VAD support. This was confirmed in isolated trabecular preparations in a separate study [13].

A novel combination therapy consisting of a left ventricular assist device (LVAD) combined with pharmacological therapy including the selective beta-2-agonist clenbuterol has shown promise in restoring ventricular function in patients with heart failure [14]. Using microarray analysis of six paired human heart samples harvested at the time of LVAD implant and at the time of LVAD explant for recovery of ventricular function, the
authors identified different pathways between heart failure and recovery. Significant changes in genes of the beta-adrenergic signaling pathway were observed including Rap guanine nucleotide exchange factor 4, (EPAC2), protein kinase, regulatory type I alpha (PKAr), phosphodiesterase 1A (PDE1A), phosphodiesterase 3B (PDE3B), and calcineurin A (PPP3CA/PP2B) [15].

miRNA

miRNAs constitute one of the more abundant classes of molecules regulating genes in animals. miRNAs are small, endogenous noncoding RNAs [16]. Many of these miRNAs have been shown to inhibit posttranscriptional processing [16, 17]. At least 80% of human miRNAs are conserved in fish [17]. This high degree of conservation suggests an important regulatory role for miRNA. Recent work by Matkovich and colleagues suggests that adding miRNA profiling to mRNA profiling enhances the ability of mRNA profiles to categorize the clinical status of heart failure before and after biomechanical unloading [18]. The results of this study confirmed three earlier identified miRNAs associated with heart failure (miR-24, miR-125b, and miR-195) [18, 19]. In addition, this study extended earlier work in mouse models [20] showing that miR-21, miR-23a, and miR-199a-3p were also regulated in human heart failure [18]. One important point of this study was that these miRNAs were reversible after LVAD support.

Natriuretic Peptides and Chromogranin A

Despite divergent etiologies, heart failure is characterized by activation of neurohormonal systems: catecholamines, natriuretic peptides (NP), and components of the renin-angiotensin axis are increased and were found to have pathophysiological and prognostic implications. Some of these molecules exert local paracrine activation but their plasma levels were demonstrated to be markers of clinical outcome. Increased cardiac ANP and BNP expression in CHF patients is associated with increased expression of the NP metabolizing NPR-C receptors and blunted responsiveness of GC-A to ANP by reduced cGMP synthesis.

“Reverse remodeling” after unloading reverses these changes and reestablishes the local responsiveness of GC-A to ANP. Cardiac expression of ANP, BNP, and NPR-C mRNA correlated significantly with cardiomyocyte diameters. In contrast to the latter, the levels of the natriuretic peptides are fully reversed to the level of the controls, indicating that their expression is partly independent of cardiac hypertrophy and regulated by CHF-associated factors, such as cardiomyocyte stretch [21].

Chromogranin A is an acidic calcium-binding protein and is the major soluble constituent in secretory vesicles throughout the neuroendocrine system. Chromogranin A was found to be significantly up-regulated during CHF and is co-stored with catecholamines and NP. Chromogranin A is secreted into the circulation with a long-term plasma half-life of approximately 18 min. BNP and chromogranin A are co-stored in the myocardium of patients with dilated cardiomyopathy, whereas this co-localization was not found in healthy controls [22]. We investigated the expression of natriuretic peptides and chromogranin A by immunohistochemistry and morphometric quantification before and after VAD. In a different set of patients, chromogranin A was evaluated in the plasma. We demonstrated that in-line with ANP and BNP, chromogranin A is significantly increased in CHF compared to healthy controls and decreased by ventricular support. Moreover, sarcoplasmic co-localization of BNP and chromogranin A is diminished after unloading. However, due to its low expression the negative regulation of chromogranin A is not reflected by plasma levels; thus, chromogranin A does not appear to be an appropriate biomarker for the monitoring of “reverse cardiac remodeling” after unloading [23] (see Fig. 1).
Transduction Pathways

Among others, three major signal transduction pathways have been shown to be involved in the pathogenesis of cardiac hypertrophy and “remodeling”: (1) the mitogen-activated protein kinases (MAPK) with the extracellular signal-related kinases (Erks), c-jun N-terminal protein kinases (JNKs), and p38 MAPK subfamilies [24]; (2) the Ca²⁺/calmodulin activated protein kinase (CaM kinase) and phosphatase (calcineurin) [25]; (3) the protein kinase B/Akt and its downstream target glycogen synthase kinase 3β (GSK3β) [26]. All three pathways become activated during CHF in humans. Whereas the Erks are activated by hypertrophic stimuli such as phenylephrine, angiotensin II (ATII), and endothelin-1 [24], the JNK and p38 MAPK are activated by cellular stress and seem to be involved in apoptotic cell death [27]. Apart from acting as an anti-apoptotic factor, Akt is a major repressor of cardiac hypertrophy by inhibitory phosphorylation of GSK3β [28]. The phosphatidylinositol-3-OH kinase (PI3K) is a signaling system that acts through Akt and p70S6 kinase, which is a key factor in angiotensin II (ATII) receptor-type-2-mediated cardiac hypertrophy [29]. We investigated the activity of the mitogen-activated protein kinases (MEKs), Erks, Akt, GSK3β, p70S6 kinase, JNKs, and p38 in terminal CHF before and after unloading [30]. Western blot analysis revealed a dramatic decrease in dually phosphorylated active forms of Erk-1 and Erk-2 after mechanical support. Also the Erk-activating kinases (MEK-1/2) were shown to be significantly less phosphorylated after LVAD. After unloading, Akt is inactivated, whereas GSK3β becomes activated. Besides Akt, another kinase involved in the PI3K/Akt pathway, p70S6 kinase showed a dramatic decrease in its phosphorylation in a subset of patients. Despite the fact that p70S6 kinase and its isoform p85S6 are associated with cardiomyocyte hypertrophy mediated by angiotensin II (ATII) receptor type 2, there was no correlation between cardiomyocyte diameter reduction and phosphorylation of p70S6 kinase. In contrast, neither the JNK nor the p38-mediated signaling cascades were altered with LVAD support in this study, suggesting specific regulation of kinase signaling after mechanical support in humans in vivo. In summary, our findings underscore the emerging evidence of MEK/Erks and Akt/GSK3β in the pathogenesis and regulation of cardiac hypertrophy. The inactivation of MEK/Erks and the activation of GSK3β after LVAD are in-line with the opposing effects of these two signaling cascades with regard to cardiac “reverse remodeling” [30].
Conclusions

Numerous noxious stimuli, such as chronic ischemia, inflammation, or genetic alterations, may affect the myocardium and induce rather nonspecific compensatory and adaptive changes including cardiomyocyte hypertrophy. Although salutary at the beginning, these adaptive mechanisms may become maladaptive and deleterious over time and eventually lead to impaired cardiac function. The increased cardiac wall stress and local ischemia may be the mechanisms that activate numerous molecular and cellular responses. Protective mechanisms are overrun and the myocardium cannot further adapt to increased biomechanical stress. Neurohormonal activation, inflammatory mediators, alterations in β-adrenergic signal transduction and Ca²⁺ metabolism, and interstitial fibrosis further impair cardiac function. Despite improving medical strategies, until now cardiac transplantation remains the only curative therapeutic approach. Due to the shortage of donor organs, LVADs are currently used to maintain cardiac function in patients with terminal CHF until a donor organ is available or optionally used as a permanent therapy for patients with contraindications for transplantation. As outlined above, the use of unloading is associated with changes at cellular, molecular, and genetic levels. Although these results are encouraging, there is agreement that the clinical cardiac improvement and performance are less pronounced in the majority of cases and only a small subset of patients can be weaned from the device and live without transplantation. The approach described by Birks and coworkers consisting of combined use of mechanical support and medical treatment including clenbuterol shows promising results for the future, as the reported percentage of patients who can be weaned is considerably higher. Another important problem in this field is the absence of a suitable serum/plasma biomarker that accurately indicates cardiac recovery under LVAD therapy and possibly predicts the clinical outcome and the changes for successful weaning from the device. ANP and BNP levels are widely used but these molecules are mainly stretch-induced and their levels decrease under support because of volume and pressure reduction, but not necessarily indicate “reverse remodeling” or clinical recovery. We could not show chromogranin A serum levels to be a usable biomarker for this group of patients. Gene expression profiles, as performed by Birks et al., might serve as a possible readout frame for the future helping to identify patients who can be weaned from the device. However, the master molecular switches orchestrating the process of “reverse remodeling” are still unknown. The heterogeneity of patients with regard to the etiology of CHF, the duration of support, the type of LVAD implanted, and medication causes further difficulties, although we never found these factors to be influential on the parameters investigated. In conclusion, despite the limited clinical improvement occurring only in a subset of patients, a better understanding of the underlying biological mechanisms of cardiac “reverse remodeling” is crucial for the development of future therapeutic strategies in this still intriguing scientific field.

References


Wilfried Briest and Mark I. Talan

Abstract

The vascular form of the Ehlers–Danlos syndrome (vEDS) is a rare inherited connective tissue disorder. Patients have a reduced life span (under 50) due to spontaneous and often fatal rupture of blood vessels and hollow organs. Until very recently no evidence-based treatment had been available. VEDS results from mutations in the COL3A1 gene that encodes the chains of collagen type III and alters the sequence in the triple-helical domain. A mouse model of vEDS created by inactivation of the Col3a1 gene has been of limited use as only 5% of homozygous animals survived to adulthood.

The haploinsufficiency for one COL3A1 allele is one of the genotypes resulting in vEDS. In this review we provide evidence that haploinsufficiency for Col3a1 in mice recapitulates features of vEDS in humans and might be used as an experimental model. There was a reduced level of aortic collagen and correspondingly reduced aortic wall strength. A spectrum of lesions was detected in the aorta similar to those observed in human patients. Lesions increased in number and age and were more common in male than in female mice.

Furthermore, potential treatment strategies are discussed including the already tested β-adrenergic receptor (AR)-blocker therapy, the inhibition of extracellular matrix degrading enzymes, and the only causative approach of selective silencing of the mutant form of COL3A1 by allele-specific RNA interference.

Keywords

Aorta • Aortic wall strength • Beta-blocker therapy • Collagen • Connective tissue • Ehlers–Danlos syndrome • Extracellular matrix • MMP inhibition • Treatment strategies
Introduction

The Ehlers–Danlos syndrome (EDS) is a heterogeneous group of inherited connective tissue disorders characterized by joint hypermobility, skin hyperextensibility, and tissue fragility [1–3]. The syndrome is named after Ehlers and Danlos, Danish and French dermatologists, respectively, who published their observations independently in the first decade of the twentieth century [2]. However, the pathology was first described by Tschernogobow in 1892 [2].

EDS type IV, the vascular type (vEDS, also known as Sack–Barabas syndrome, OMIM 130050), is a dominantly inherited disorder that results from mutations in the COL3A1 gene, which encodes the constituent \( \alpha_1 \) chains of type III procollagen [4–7]. VEDS is a rare disease (prevalence 1–2:100,000) [5]. In addition to complications in the cardiovascular system like arterial dissections and ruptures, manifestations of vEDS involve the skin, joints, and hollow organs, resulting in thin/translucent skin, extensive bruising, characteristic facial appearance, and intestinal/uterine rupture [3]. The causative gene for vEDS is COL3A1. Currently, nearly 200 mutations in the COL3A1 gene leading to synthesis of an abnormal type III procollagen protein or haploinsufficiency have been identified [8]. Most of the mutations are single-nucleotide substitutions for glycine residues in the triple-helical domain of the pro\( \alpha_1(III) \) chain, resulting in a regular quantity of abnormal collagen III. Haploinsufficiency of collagen III (reduced quantity of normal collagen III) is mostly the result of degradation of mutant transcripts due to nonsense-mediated mRNA decay. Therefore, this haploinsufficiency is a functional haploinsufficiency, not based on a complete deletion of the COL3A1 gene on one allele. This true haploinsufficiency had been reported only once: a complete loss of one allele through hemizygous deletion of COL3A1 and flanking genes [9].

Type III collagen is the second most abundant collagen in human tissues and occurs particularly in tissues exhibiting elastic properties, such as skin, blood vessels, and various internal organs. Several studies concerning the relative amounts of type I and III collagen have been published, all giving more or less different results, depending mainly on the tissue extraction methods used to solubilize the insoluble collagen. For example, the initial work on arterial collagens suggested a predominance of type III collagen over type I [10], but later results have suggested that type I collagen accounts for 55–88% of the total amount of these two [11, 12]. It has been suggested and this seems to be true still today that type III collagen predominates in tissues requiring higher levels of compliance, while type I, which forms larger fibers, predominates in denser connective tissues that are less distensible [13].

The quantitative defects or qualitative deficit of structurally normal collagen III in vEDS and corresponding changes in the wall structure are responsible for major complications observed in afflicted individual: arterial, bowel, and uterine rupture. Because of these dramatic complications, life expectancy is shortened to a mean of <50 years [6, 14]. In approximately 50% affected individuals the heredity of disease cannot be established and spontaneous mutations are implicated. Those cases are especially deadly, because the life-threatening complication is often the first presentation of disease. Until very recently, there was no evidence-based treatment or preventive strategy available. However, in the just completed first multicenter randomized trial, almost 4 years (47 months) treatment with celiprolol – a long-acting \( \beta_1 \) antagonist with partial \( \beta_2 \) agonist properties used for treatment of hypertension – decreased the incidence of arterial ruptures in patients with clinical diagnosis of vEDS [15]. However, positive interpretation of the results was not universally accepted.

Research directed to understanding of the pathophysiology and prevention of major complications is hindered by the lack of an appropriate animal model. The Col3a1 KO mouse (Col3a1 tm1Jae ) was constructed in 1997 and proposed to serve as animal model for the vEDS [16]. However, this model was difficult to exploit because heterozygous mice, according to the authors, were
phenotypically normal, while the phenotype of homozygotes was too severe. Homozygotes had an average survival rate of 5% at weaning, most dying within 48 hours of birth. Few surviving mice had a much shorter life span compared with wild-type mice. The major cause of death of mutant mice was the rupture of the major blood vessels, similar to patients with vEDS. This homozygous knockout mouse is not a suitable model for two reasons: first, human homozygosity for COL3A1 null is statistically improbable and only a single case has ever been reported [17]. This individual did not present a typical vEDS phenotype [17]. Second, the homozygous mice have an extreme preweaning mortality, in comparison with a disease that typically manifests itself clinically in the third or fourth decade in humans. Therefore, we performed a deep evaluation of the heterozygous knockout mouse as a potential model for vEDS [18]. The results of this study will be summarized in this review and potential treatment strategies will be discussed.

Because vascular complications of large vessels are the most serious cause of morbidity and mortality in this disease, the phenotypic strategy included first of all a detailed histological assessment of pathology in the aorta across the life span in both sexes, including characterization of lesions observed. Complementary functional and biomechanical studies of the heart and large elastic (aorta) and medium muscular (gracilis) arteries were undertaken to assist in the evaluation of the vascular phenotype as well. Finally, anatomical, biomechanical, and transcriptional studies of the colon, a second major site of complications, were performed.

**Histopathology of the Aorta**

Different lesions were observed in the aorta of Col3a1tm1Lae heterozygous mice. Grade 1 lesions appeared as a small break in the internal elastic lamina with no significant spindle cell proliferation (not shown). The broken ends of the laminae frequently curled under into the media. In grade 2 lesions, the distance between the fragmented ends of the internal elastic lamina was greater and the intervening space was filled with a moderate proliferation of spindle cells and the accumulation of an abundant collagen-rich extracellular matrix (Fig. 1b). Grade 3 lesions were larger with more florid medial spindle cell proliferation and often fragmentation of one to two medial elastic laminae (Fig. 1c). In grade 4 lesions, there was a marked and abrupt attenuation of the wall thickness with abundant fibrosis and more severe fragmentation of several elastic laminae (Fig. 1d). A lack of infiltrating leukocytes as well as a relative sparing of the aortic root clearly distinguished the lesions in these mice from the recently described aortitis of BALB/c mice [19]. Grade 1 lesions were seen in 55% of analyzed aortas (93 of 170) with almost no discrimination between wild type and heterozygous mice. Because of their mild nature, as well as a high frequency in both sexes and genotypes, grade 1 lesions were excluded from statistical analysis.

Lesions were not found in female and younger male wild-type mice; however lesions were observed in a small number of older (9 months and older) male wild-type mice (Fig. 2). The number of lesions increased in number and severity with age. While there were no lesions found in 2–5-month-old mice, in older animals between 9 and 14 months of age, there were lesions found in 47% female and 88% of male Col3a1tm1Lae heterozygous mice [18]. The lesions are similar to those reported in vEDS patients [6, 20–22]. These lesions in mice were focal to multifocal and apparently stochastic in distribution. Additionally, the nature of the lesions in haploinsufficient Col3a1 mice overlaps with the morphologically similar, but usually milder, lesions noted in older wild-type mice, indicating that the observed lesions are themselves nonspecific. Rather, heterozygotes exhibit both a higher number and cumulative severity of lesions, suggesting that the differences between genotypes are quantitative rather than qualitative [18]. Similar quantitative rather than qualitative differences in diseased and “normal” control aortas have been noted previously in humans with vEDS as well as for other genetic and acquired aortic diseases [23–26].
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Quantification of Extracellular Matrix in the Aorta and Functional Testing

Picro-sirius red staining of the aorta sections shows diminished total collagen in heterozygotes relative to wild-type mice (Fig. 3). There was no significant difference in total collagen content of the ascending or transverse aorta between genotypes (Fig. 4). However, there was a nonsignificant decrease in total collagen in descending thoracic aorta, and a significant decrease in total collagen in the abdominal aorta of heterozygotes (Fig. 4). This could be explained by the relative higher amount of collagen and less elastin in the abdominal aorta [26, 27]. These data were in agreement with less collagen III protein
detected by Western blotting only in the abdominal aorta of heterozygotes [18]. In the descending thoracic aorta, no difference was detectable [18]. Furthermore, the data are consistent with the paradigm of a vascular wall that is structurally weakened while still apparently normal at the gross and microscopic levels of resolution.

Functional testing of the strength of the aortic wall supports this conclusion. The maximum pressure (rupture pressure) in the abdominal aorta was similar in wild-type and heterozygote mice at 14 months but significantly lower in heterozygotes at 21 months (Fig. 5). No change was detected in the distensibility of aorta of 10-month-old heterozygous animals, while higher compliance was detected in small arteries from heterozygous mice compared with the wild type [18].
Furthermore, a reduced strength and increased compliance was found in colons of heterozygous mice [18], and increased compliance was found in bladder tissue of heterozygotes as well [28]. Therefore, these findings, taken together, indicate that haploinsufficiency for \( \text{Col3a1} \) in mice recapitulates features of vEDS in humans and might be used as an experimental model. However, this mouse model has a number of important limitations, including the lack of spontaneous clinical signs or endpoints, which necessitate the development of a new vEDS mouse model with a knock-in of a mutation. A glycine substitution mutation would be favored since it is the most common one. Unfortunately, nobody reported a creation of this model yet.

**Potential Treatments for vEDS**

Clinical studies have previously addressed phenotypic characteristics and genetic features of vEDS but no randomized treatment trial has been done until the celiprolol trial [15]. Patients were often treated empirically with drugs such as \( \beta \)-blockers [29] or renin-angiotensin-aldosterone blockers [30] that have protective effects in Marfan’s syndrome. However, the pathophysiology of Marfan’s syndrome is different from that of vEDS. Marfan’s syndrome is defined by a deficiency in fibrillin-1 and abnormal elastin synthesis that leads to changes in the elastic properties of the aortic wall. The ascending aorta becomes especially stiffer, which results in a higher pulse wave velocity. In contrast, vEDS is characterized by a deficiency of synthesis or structure of type III collagen. A dominant negative mechanism of pathogenesis has been inferred [31]. In this paradigm, 50% of pro-collagen chains are structurally abnormal, meaning that only 1 in 8 mature collagen homotrimers is structurally sound [2]. The abnormal collagen or the decreased concentration of collagen III in cases of haploinsufficiency affects the entire arterial tree, together with skin and intestine. Ultrastructural analysis of tissue from \( \text{Col3a1} \) knockout mice revealed that type III collagen is essential for normal collagen I fibrillogenesis in the cardiovascular system and other organs [16]. Abnormalities in the vEDS patients’ vessel wall consist of decreased intima-media thickness associated with increased mechanical stress of fragile tissues [32]. These features are not present in patients with Marfan’s syndrome in which dilatation of stiffened aorta predominates [33, 34].

**Beta-Blocker Therapy**

The rationale for treatment by \( \beta_1 \)-AR blocker was mainly mechanical – to reduce the arterial wall stress by controlling the rate of increase of
pressure over time in the pulse wave (dP/dt). The celiprolol, however, used in the successful clinical trial was not a typical $\beta_1$-AR blocker. It combines $\beta_1$-AR inhibition with some properties of $\beta_2$-AR stimulation and was proven beneficial for vEDS patients without affecting hemodynamic variables [15]. The heart rate or systolic and diastolic pressure was not decreased and pulse pressure was even elevated in patients on celiprolol. This prompts reconsideration of alternative mechanisms of celiprolol’s protective effect in vEDS [35]. Celiprolol may act on molecular pathways within the arterial wall that are independent of hemodynamic stress. The key might be the transforming growth factor $\beta$ (TGF$\beta$) signaling pathway which could be activated by $\beta_2$-AR stimulation [36, 37]. It was shown that in Marfan’s syndrome, one key factor in the pathogenesis of arterial lesions is an increased bioavailability of TGF$\beta$ in response to the defect in its chelation by abnormal fibrillin. The role of TGF$\beta$ in vEDS is not clear. Elevated TGF$\beta$ levels were detected in some vEDS patients as well [38], but the pathogenic relations were not shown. Raised TGF$\beta$ concentrations might be the consequence of repeated skin or arterial healing. It was proposed that the beneficial effect of losartan therapy in Marfan patients was based on TGF$\beta$ antagonism [39]. However, the causal relation between activation of TGF$\beta$ and arterial lesions might be more complex [15]. TGF$\beta$ is also an important growth factor in wound healing. TGF$\beta$1 and 2 are necessary for collagen synthesis and TGF$\beta$3 for organization of scar tissue [40]. Local delivery of TGF$\beta$1 was associated with stabilization of experimental aortic aneurysms in rats [41]. On the other hand, TGF$\beta$ inhibition is not always accompanied by vascular protection: mice with angiotensin II infusion developed fatal aortic aneurysms despite exposure to neutralizing TGF$\beta$ antibodies [42]. Taken together, these studies highlight the complexity and the context-dependent roles of TGF$\beta$ in vascular disease [43].

The increased pulse pressure among the patients with celiprolol suggesting a possible increase in arterial stiffness might be a sign of induced TGF$\beta$ signaling, although the effect on TGF$\beta$ signaling was not further analyzed [15]. There are strong associations between $\beta$-adrenergic receptors and TGF$\beta$ pathways. TGF$\beta2$ is activated by $\beta_1$-adrenergic receptor stimulation [36, 37]. Chronic $\beta_2$ stimulation might enhance collagen synthesis through increased expression of TGF$\beta$. This would be an opposite effect of the proposed losartan mechanism in Marfan’s syndrome, based on TGF$\beta$ antagonism. Indeed, $\beta_2$ stimulation by clenbuterol in rats boosted mRNA expression of TGF$\beta$1, 2, and 3 [36]. TGF$\beta$ could enhance the production of type I and III collagen and lead to fibrosis [44, 45]. Thus in response to celiprolol, an up-regulation of collagen synthesis might have strengthened the arterial wall, reducing its susceptibility to rupture [15, 35]. The hypothesis of a beneficial effect of $\beta_2$ stimulation through TGF$\beta$ stimulation and consequently collagen accumulation should be analyzed in the vEDS mouse model, the already existing mouse model of haploinsufficiency of Col3a1, or better in knock-in mouse model with a glycine mutation that is yet to be developed.

With respect to using $\beta$-AR blockers for patients with vEDS, celiprolol, while successful in the recent clinical trial, is not a universally available drug. For instance, it is not approved for use in the USA at this time. Moreover, the idea of salutary effects of $\beta_2$-AR agonist in vEDS is only a speculation, until it is proven that effectiveness of celiprolol exceeds that of other, more common, $\beta_1$-AR blockers.

**MMP Inhibition**

Collagen fibrils are part of the extracellular matrix. The homeostasis of extracellular matrix is equilibrated by synthesis and degradation of their components. Therefore, down-regulation of collagen-degrading enzymes would be another strategy to strengthen the arterial wall. Collagen is degraded by matrix metalloproteases (MMPs) [46].

It has been reported that in the third stage of aneurysm development, rapid expansion and increased risk of rupture are associated with accelerated degradation of collagen [47].
Doxycycline, a tetracycline antibiotic and broad spectrum MMP inhibitor, has been successfully tested in pilot clinical trials for the treatment of abdominal aortic aneurysms [48–51]. Doxycycline, given in a subantimicrobial dose, is also the only MMP inhibitor approved by the FDA. It is currently used for the treatment of periodontal disease [52, 53] and rosacea [54]. We hypothesized that MMP inhibition in the mouse experimental model of vEDS would shift the balance between collagen degradation and synthesis in the vascular wall and protect against vascular damage.

We tested this hypothesis in the Col3a1tm1Jae haploinsufficient mouse model [55]. Following a 3-month treatment with doxycycline, 9-month-old heterozygote mice were subjected to a surgical stressing of the aorta. While untreated heterozygote showed increased MMP-9 activity in the carotid artery and decreased collagen content in the aorta, there was normalization to wild-type levels in doxycycline-treated animals. A threefold increase in stress-induced aortic lesions found in untreated heterozygotes was fully prevented in the doxycycline dose group [55]. Since the efficacy of proposed doxycycline therapy was tested only in the haploinsufficient mouse model, it can be so far proposed as a potential treatment for only a small subset of vEDS patients with haploinsufficiency for COL3A1. Whether this treatment can be effective in the rest of vEDS patients shall be tested in the appropriate genetic mouse model yet to be developed. However, a convincing argument for doxycycline treatment was its effectiveness in the Marfan mouse model with a mutation in fibrillin-1 (Fbn1C1039Y) [56–58]. Doxycycline delayed the aneurysm rupture [56], and was effective in secondary prevention of thoracic aortic aneurysm in combination with losartan [58].

A defective gene cannot be replaced in dominant inherited disease such as vEDS. However, the recently developed RNA interference (RNAi) technology, which enables gene posttranscriptional expression silencing, may be applicable. Research into the treatment approach for the dominantly inherited diseases, for which cures have not yet been developed, is advancing [59–61].

Majority of the patients are heterozygous for a mutation in one copy of the COL3A1 gene. Patients that are haploinsufficient for COL3A1 have a reduced amount of non-mutated COL3A1, and seem to exhibit less severe symptoms and less subsequent complications [9, 17, 62]. Therefore, one approach to a targeted treatment of vEDS is the elimination of the mutated form of the COL3A1 gene to transform the more severe phenotype to the less severe haploinsufficient type. This would be possible by selective silencing the mutated form of the COL3A1 gene without affecting the wild-type allele of COL3A1, and thus by restoring the structural integrity of affected extracellular matrix. Allele-specific silencing of several mutant targets has been studied for diseases including osteogenesis imperfecta [63], sickle cell anemia [64], primary retinal degeneration [65], spinocerebellar ataxia [66], pachyonychia congenita [67], Huntington’s disease [68], Alzheimer’s disease [69], SCCMS [70], and sialuria [71].

We tested the feasibility of allele-specific knockdown of mutated COL3A1 in fibroblasts from a vEDS patient heterozygous for COL3A1 allele encoding a glycine substitution [72]. We identified one siRNA with a centered position of the mutation silencing more than 90% of the mutant allele without affecting the wild-type allele. Transmission and immunogold electron microscopy of extracellular matrices from fibroblasts of the vEDS patient revealed structurally abnormal fibrils. After siRNA treatment collagen fibers were not distinguishable from fibroblasts of normal donors [72]. Thus, a personalized siRNA therapy to replace mutated collagen condition with the condition when collagen is normal but reduced opens the possibility for consequent pharmacological stimulation of total collagen production and inhibition of its degradation.

Conclusions and Potential Treatment

Gene therapy for permanent correction of genetic mutation in COL3A1 would be an ultimate goal, but this idea is reserved for the distant future.
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Multi-scale, Multi-physics Heart Simulator as a Tool to Link Bench and Bedside

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Abstract

Advances in molecular and cell biology have enabled us to identify the genes responsible for the origin of various heart diseases but, in most cases, the detailed mechanisms by which such genetic defects lead to the signs and symptoms observed at the bedside remain to be elucidated. In an attempt to investigate such problems, we have developed a multi-scale, multi-physics heart simulator, in which normal and abnormal functioning of the heart is reproduced based on the molecular mechanisms of the cardiac excitation-contraction (E-C) coupling process. This simulator, based on the finite element method, consists of solid elements representing the myocardium and fluid elements representing the blood in the heart chamber. Each solid element is implemented with a molecular model of E-C coupling and thus behaves as a virtual cardiomyocyte. Because the governing equations for the solid and fluid parts are solved by the strong coupling method, we can obtain detailed information on the blood flow as well as the electrical and mechanical states of every myocyte during the cardiac cycle. Accordingly, this simulator can be used as a tool to see whether any specific molecular abnormality would lead to the development of macroscopic findings, thus making it applicable to various fields of cardiovascular research.

Keywords

Body surface voltage map • Cardiac resynchronization therapy (CRT) • Cross-bridge kinetics • Excitation-contraction coupling • Heart 3D model • Implantable cardioverter defibrillator (ICD) • Magnetic resonance imaging (MRI) • Mathematical modeling • Pacemaker • Ultrasonic cardiogram (UCG) • Ventricular fibrillation • Ventriculoplasty • Voxel model • Windkessel

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Introduction

As in other areas of science, current cardiovascular research is facing an explosion of knowledge evidenced by the huge number of scientific journals published daily. Furthermore, since even in this limited area the research covers various subjects ranging from molecular biology to macroscopic organ physiology, it is virtually impossible for an individual doctor or researcher to understand all the subjects and memorize them in an organized manner. Quite naturally, the use of digital archives or a database has been considered, but for such databases to be useful they need to meet the specific requirements in cardiology.

Cardiac function is based on electromechanical phenomena driven by the biochemical reactions in each cell, and thus most of the clinical tests evaluate these parameters. On the other hand, knowledge at the molecular level is indispensable for the development of causal treatment. In this regard, the database required in cardiology must collect and integrate knowledge on multi-physics phenomena functioning cooperatively over multi-scale levels to facilitate our understanding of the functioning of the heart and human body under both normal and diseased conditions. This idea is now widely shared in scientific society and computer simulation is considered to be a powerful tool for its realization [1, 2].

We have developed a multi-scale, multi-physics heart simulator by applying cutting-edge techniques in computational sciences. In this simulator the propagation of excitation and contraction and relaxation of the tissue, and the resulting pressure development and blood flow, are reproduced, based on the molecular mechanisms of the cardiac excitation-contraction coupling process implemented in each element of the model representing a myocyte. This dynamic model is a functional database of the heart, retaining the 3D structure from which we can retrieve functional information of the heart from any aspect and at any level of interest. The application of the model is not limited to knowledge management or education; it can also be used as a tool for the development of novel diagnostic or therapeutic devices and strategies.

Outline of Multi-scale, Multi-physics Heart Simulator

As stated above, in the post-genome era, the integration of molecular and cellular findings to understand the functioning of organs or individuals is recognized as an important field of science [1, 2]. However, even with the advancement in computational science, this is not an easy task to accomplish because, in addition to the integration of events across the scale, description of each event involves integration of various disciplines, such as electricity, physical chemistry, solid mechanics, and fluid dynamics (in other words, a multi-physics simulation). Indeed, the simulation studies reported so far deal only with the electrophysiology [3–7] or the mechanics at either the cross-bridge kinetics [8–10] or macroscopic model [11–14]. Our heart simulator, called UT-Heart, is the only model that includes all the important physics and integrates them over the scale to reproduce clinically useful macroscopic behavior of the heart.

Framework of the Model

Cardiomyocyte Model

Since the development of the Purkinje cell model by Noble [15], studies on mathematical modeling of cardiac excitation have been reported for myocytes from various species. In this study, we adopted the human ventricular myocyte model by Ten Tusscher et al. [16], the atrial myocyte model by Courtemanche et al. [17], and the Purkinje cell model by DiFrancesco et al. [18]. All these models formulate the charge and discharge of the cell membrane as a capacitor using ion currents through channels, pumps, and transporters, while the model by Ten Tusscher includes 12 ion currents [16]. Such cell models are coupled to form the myocardial tissue in which excitation propagates through the assumed intercalated discs. We used both bi-domain and mono-domain formulations depending on the purpose of study.

In each myocyte, excitation of the cell membrane (depolarization) activates the calcium (Ca)
current through an L-type Ca-channel which, in turn, triggers the Ca release from the sarcoplasmic reticulum to produce the transient rise in cytosolic Ca concentration. Cytosolic Ca binds to troponin C on a thin filament to initiate the force generation by actomyosin interaction. This mechanism of the cardiac excitation-contraction (E-C) coupling process has also been modeled by researchers and we modified the one by Rice et al. [10] to include the co-operativity in force development. By coupling these two models for excitation and E-C coupling through the Ca signal, we were able to create the virtual myocyte model.

Heart Model

Our heart simulator is based on the finite element method and its morphology on the human multi-detector CT data. The model consists of a structural part representing the myocardium (664,334 elements) and a fluid part representing the blood in the heart chamber (435,227 elements), with the mechanics of both solved simultaneously using the strong coupling methods developed by Zhang and Hisada [19]. In the myocardial part we have reproduced the fiber and sheet structures and the conduction system has also been created as a distinct structure (Fig. 1). In addition, we have developed a voxel model of the heart with a finer mesh (20 million elements) for the electrophysiological analysis (the two models are solved simultaneously). Currently our model includes both atria and ventricles with a short segment of aortic arch, while the windkessel model of circulation with appropriate parameter values is connected to each inlet and outlet to allow physiological simulation (Fig. 2). The small dots on the heart surface correspond to the finite elements, in each of which a molecular model of the excitation-contraction coupling process is implemented. Such virtual myocytes are connected mechanically and electrically exactly as in real tissue. All the program codes were written in our laboratory and the computations were

**Fig. 1** Heart model. Morphology of the heart model is based on the multi-detector CT data and consists of solid and fluid parts
Simulation Results

Simulation of a normal heartbeat is shown as the time-lapsed images in Fig. 3. Upon stimulation applied to the sinoatrial node (pacemaker site), the excitation of the virtual myocyte propagates to the adjacent ones to spread through the atria and ventricles via the conduction system to induce the synchronous contraction of the heart (Fig. 3b). Contraction of the heart generates pressure in the atrial and ventricular cavities to drive the ejection and filling of blood (Fig. 3c). The color at the open ends of the heart models indicates the flow velocity. For simulation videos please visit our website at http://www.sml.k.u-tokyo.ac.jp/. During this process, subcellular variables, such as membrane potential, ion currents, intracellular Ca concentration, cross-bridge states, and the developed force, are calculated in each element (Fig. 3a) at time intervals of 500 μsec. Simultaneously, regional stress/strain in the myocardial tissue and the pressure and flow distributions in the fluid (blood) part are also calculated. In other words, multi-physics phenomena including mechanics, electrical events, and fluid dynamics are solved at multi-scale levels to realize a dynamic 3D database of cardiac function.

In Silico Diagnosis

To further examine and validate our simulation results, we compared them with clinical data. As stated repeatedly, because we calculate various parameters involved in the cardiac function, we can diagnose our in silico heart by extracting and presenting the relevant data in the form of diagnostic modalities, such as an electrocardiogram (ECG) or ultrasonic cardiogram (UCG). First, we checked the ECG of our in silico heart, and for this purpose,
we also created a torso model with the major organs in the thorax and the specific conductivity of each. By placing our heart in this torso model and calculating the electrical field using bi-domain formulation, we can obtain the body surface voltage map during the cardiac cycle (Figs. 4a, b). As the excitation of myocardial tissue propagates from the atria to ventricles (Fig. 4a), the high voltage area on the chest wall shifts to the rear side. We can find similar observations for human subjects in the literature [20]. Furthermore, if we calculate the difference between two appropriate points on the chest, we can obtain the surface ECG as shown in Fig. 4c. In this case we show only the standard limb lead II data; data in other leads including the pre-cordial leads are easily obtained in a similar way. We can apply this technique to the study of arrhythmias and have confirmed the characteristic ECGs for ventricular fibrillation, ventricular tachycardia, and bundle branch block (data not shown).

Motion of the in silico heart wall and/or valves in the appropriate 2D plane fixed in space corresponds to the B-mode echocardiogram and matches the clinical observations. In particular, regional strains (radial, circumferential, longitudinal) were compared with experimental data obtained by either maker technique or diffusion tensor magnetic resonance imaging (MRI) to confirm the validity of our model. In addition, because our simulator calculates the flow in 3D space at every time step (Fig. 5a), temporal changes in flow velocity at any point in the heart chambers are readily available in the form of a Doppler echocardiogram. An example of this is shown in Fig. 5b. Mitral flow (red) and aortic flow (green) reproduce the characteristic patterns recorded in human subjects.

Fig. 3  Simulation of a normal heart beat: (a) representative ion currents in myocyte model, (b) propagation of excitation, (c) contraction and blood flow. Sequence is indicated by the arrow
Fig. 4 ECG simulation: (a) propagation of excitation, (b) body surface voltage map, (c) torso model and the ECG

Fig. 5 Simulation of Doppler UCG: (a) flow distribution in the heart chamber during the cardiac cycle (upper panels show a magnified view of the aortic root), (b) flow patterns across the mitral valve (red) and aortic valve (green)
**In Silico Treatment**

This simulator is also applicable to the simulation of various types of treatments. Figure 6 shows the *in silico* treatment of ventricular fibrillation using an implantable cardioverter defibrillator (ICD) with a right ventricular electrode and generator (Fig. 6a). In this simulation, only the electrical activity in ventricles was calculated to reduce the computational cost. After ventricular fibrillation was induced by cross-field stimulation, an electrical pulse was applied at the timing indicated by the yellow thunderbolt sign to successfully terminate the spiral waves (Fig. 6b). We also found that cardioversion was successful only with the electrical power output above a certain threshold. This finding indicates that the simulator can be used to judge the efficacy of therapeutic strategies or devices.

Cardiac surgery is another area of application since reoperations should be avoided or are, in most cases, not possible. We have already attempted the simulation of ventriculoplasty for cases of intractable heart failure. Aortic root replacement surgery is another potential example [21].

**Conclusions**

As discussed above, the heart simulator can be used to determine both the indication and treatment strategies. Of these, development of a cardiac resynchronization therapy (CRT) device is a suitable field of application, in which the multi-physics nature of our simulator would certainly produce an innovative design with high efficiency. Finally, the application of the heart simulator is not limited to the optimization and development of therapeutic devices. The multi-scale, multi-physics
characteristics thereof allow us to use it as a tool in drug discovery and for the development of novel treatments that rely on various physics.

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