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New Cellular and Molecular Approaches for the Treatment of Cardiac Disease

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Similar to the kidney in uremia, end-stage cardiac failure is an outcome common to many disparate disease processes including hypertension, various inflammatory pathologies, as well as ischemic loss of tissue. In regard to the heart, cellular and molecular mechanisms responsible for heart failure have been investigated with renewed intensity over the past several years with newer techniques of molecular genetics, genomic analysis, and cell biology. Although this article reviews some recent advances made in our understanding of molecular and cellular events in the heart leading to heart failure and explores possible new targets for therapeutics, the main point is to stress the importance of investigative interactions between organ physiologists and molecular and cellular biologists. These interactions between organ physiologists and molecular geneticists is stressed and supported as a mechanism for rapid advancement for both understanding the underlying pathophysiology of human disease and the development of therapeutic strategies.
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The sciences of cardiac organ physiology and of molecular genetics and genetics emerged rapidly in the latter half of the past century. It seems amazing from the current perspective of the postgenomic era that less than 20 years ago most cardiovascular researchers had little interest in what the tools of molecular biology and genetics had to offer. Indeed many had to be dragged, kicking and screaming, to the realization that each discipline had major benefits to offer the other for synergistic understanding of cardiovascular physiology and pathophysiology. Too often the notion prevailed of one organ and 2 sciences: the idea that study of the organ physiology of the heart could be distinct from study of the cell biology and molecular physiology of cardiomyocytes. The lessons of the past few decades have happily put to rest that division and this article provides several examples that indelibly link basic pathophysiology of the cardiovascular system with basic cell and molecular biology. Perhaps a better title might be "How the Basic Scientists and the Cardiac Physiologists Learned to Love One Another."

How the Genetic Program in Cardiomyocytes Senses Events in the Heart's Contractile Apparatus

Cardiac myofibrils are exquisite structures made up of several scores of distinct proteins whose contractile properties are engendered by the culmination of extracellular signals triggering the mechanical dislocation of myosin-based thick filaments relative to the actin-based thin filaments. It is the accumulation and synchrony of myriads of such filamentous cytoskeletal actions that provides a myocardiocyte with contractile force. Regional synchronized contractions of myocardiocytes are, of course, what gives rise to the contractions of a beating heart.

Any events that lead to myofibrillar degeneration can be the basis for serious clinical problems. Changes in the myo-

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cardial cytoskeleton and loss of myofilaments are hallmarks of chronic dilated and progressive cardiomyopathy including the sequelae of myocardial infarction. In addition, the cardiomyopathy associated with antineoplastic drugs, such as Adriamycin (Pharmacia Italia, Milan, Italy) routinely administered for chemotherapeutic treatment of certain cancers, also is associated with myofibrillar degeneration.[1]

Each of the many myofibrillar cytoskeletal proteins is encoded in the genome by a separate gene. Drugs such as Adriamycin selectively and severely effect the ability of those genes to make the messenger RNA required for the synthesis of the myofibrillar proteins such as the α-actins, myosin light chain, the cardiac troponins, and even the important cardiac-specific metabolic enzymes.[2] Indeed, if one assays the messenger RNAs for almost any of the contractile protein genes, they disappear from the treated cells within a matter of hours. We now know that this happens because the initial event after Adriamycin is the triggered destruction of the mechanisms that lead to the production of the specialized transcription factors, such as MEF2 and GATA4, that enable the cytoskeletal protein genes to be activated in the first place.[3] Loss of myofilament proteins rapidly leads to myofibrillar degeneration and to the dilated cardiomyopathy associated with cardiotoxic doses of Adriamycin.

Studies of the genetics of inherited familial cardiomyopathies have uncovered families carrying, in the protein coding information of these same genes, mutations that alter the amino acid sequence, usually by just a single residue. In most of these cases, the mutation is located in a critical functional site that affects the ability of the protein to do its job. Although it is easy to understand why wholesale depletion of contractile proteins, as in Adriamycin-induced changes, can lead to a dilated cardiomyopathy, the effects of amino acid changes in the contractile protein genes almost always lead to hypertrophic cardiomyopathy. How does the failure of myofibrils to contract normally (caused by the mutation) trigger the complex array of signals that engender cardiac hypertrophy? One possible clue is that in both the drug-induced and the hypertrophic cardiomyopathy there is disarray of myofibrils.

Recent work from our group, in particular the work of Kong, has provided insights as to how signaling might be performed between disordered myofibrils and an altered genetic program in the cells. This story revolves around previously undiscovered functions in the myofibrillar protein, tropomodulin. Tropomodulin is known to cap the free (pointed) end of the actin-based thin filaments of the cardiac sarcomere,[4,5] most likely as 2 molecules at each filament end, 1 for each of the 2 intertwined actin polymers. The actin thin filaments are bound by encircling tropomyosin molecules that stabilize the actin polymers. Tropomodulin actually binds to the last 2 tropomyosins on each filament, thus stabilizing the filament and preventing further accretion of additional actins or tropomyosins.[6]

Several years ago Sussman et al[7] found that perturbation of tropomodulin levels in cardiomyocytes had profound effects on the stability and length of the actin filaments. Increased levels of tropomodulin in cells led to much shorter filaments whereas depletion of tropomodulin led to runaway elongation of thin filaments. We concluded that the equilibrium between tropomodulin concentration and actin thin filament length was critically important to normal filament homeostasis and filament physiologic alignment. With the disassembly of myofibrils, such as after severe cardiac ischemia, comes an increase in the soluble pool of the myofibrillar proteins, including tropomodulin.[7]

What Kong and Keddes[8] found was that tropomodulin protein molecules, when not bound in thin filament structures, migrate to the nucleus of the cell. Kong discovered that tropomodulin carries within its structure distinct 3-dimensional signals that allow both nuclear import and export.[9-11] Furthermore, under normal circumstances, a tiny fraction of tropomodulin is always cycling through the nuclear compartment. Indeed, when one mutates the tropomodulin molecule so as to disrupt the nuclear export signal, tropomodulin accrues in the nucleus. When the pool of free tropomodulin is increased by thin filament disruption, more tropomodulin enters the nucleus. Build-up of nuclear tropomodulin specifically inhibits transcription of genes encoding proteins for sarcomere assembly—myosin heavy chain, tropomodulin, and others—thus, leading to further disruption of the filament structure. We do not yet know exactly how the presence of excess tropomodulin in nuclei effects gene expression. However, clearly we now do know that there is a feedback signaling mechanism that links the status of sarcomere structure to the regulation of production of sarcomeric proteins. Thus, by starting to understand in myocardiocytes the molecular genetic dynamics linking their genetic program with their structural organization and physiology, we are beginning to understand the events that lead to specific and profound clinical scenarios such as myofibrillar degeneration.

Deciding Cell Fate in Building a Heart and a Vascular System

How cells and their progeny are forced to follow a specific fate remains the subject of intense research efforts dating back to the experiments of Hans Spemann and Hilde Mangold in the early 20th century. The heart itself is formed predominantly from cells in the embryo that begin in the neural crest, migrate ventrally, and coalesce in a primitive heart tube. In the heart, as in most organs, a small number of cells give rise to many highly specialized tissues including the coronary arterial and venous circulations. Indeed, a small number of genes seems responsible for initiating the events that decide that certain endothelial cells will migrate and coalesce to form tube-like structures and then differentiate into either venous or arterial precursor structures. Being able to control those events is an important goal, with implications for regulation of abnormal blood vessel growth (eg, in neoplasia), or for engendering neovascularization in an ischemic myocardium.

The clue that there may be a relatively small number of genes involved in vascular fate decisions has had its strongest

support from observations made in the developing embryos of that tropical aquarium favorite, the zebra fish (*Danio rario*). The zebra fish embryo is transparent. One can watch the development of a circulatory system under a low-powered microscope and watch the flow of blood through the primitive vessels. This is an incredible advantage in selecting for abnormal developmental events. Thanks to the phylogenetic conservation of pathways and genetic functions, what is true for the zebra fish is often true for mankind. These genes that lead to abnormal fate decisions in blood vessel or cardiac chamber formation likely have similar if not identical roles in mammalian development.

One mutant zebra fish line that caught the attention of Mark Fishman and his colleagues[12,13] had a serious defect in aortic great vessel formation. The mutation was in a gene they named *gridlock*, because the blood had nowhere to flow. *Gridlock* is a target of an intercellular signaling pathway called Notch. The gridlock protein represses other genes and prevents cells from maturing. If you lose the functions of *gridlock*, cells and organs stay in their default state: vascular tubes remain like veins and myocardium remains atrialized. In mammals there are at least 3 *gridlock*-related genes called the Herp family.[14,15]

The Notch protein is a cellular transmembrane protein that senses the presence in adjacent cells of specific ligands. When Notch interacts with its specific ligand, an intracellular tail of the Notch protein is cleaved from it. This Notch intracellular domain is targeted to enter the nucleus and act there as a specific positive regulator of expression of yet another set of genes that encode the Herp proteins. These proteins are made in many organ systems and abnormalities in their expression or mutations that prevent expression lead to abnormalities in the nervous system, skeletal muscle, the vascular system, the eye, and elsewhere. What the Notch pathway seems to be controlling in most cases is the fate of differentiation of specific cell types. This seems to work in the main by one group of cells that express the Notch ligands, inhibiting the differentiation of adjacent cells that express Notch. The inhibited cells remain undifferentiated or take a different developmental pathway.

What Tatsuya Iso, Yasuo Hamamori, and I found when we discovered the Herps (at about the same time as did several other groups[13,16-20] is that they each are capable of suppressing expression from other genes. In other words, Notch signaling leads to shutting off of genes by the Herps. To prove this in cells, we first showed that the Notch intracellular domain was involved in direct stimulation and activation of the Herp genes, giving rise to Herp messenger RNA and Herp protein production. We also were able to show that when Herp protein was brought to a site in the nucleus where there was active gene expression, it immediately shut down the expression. Clearly, Herp is a target and an effector of the Notch pathway and acts to shut off (repress) the activity of specific genes.[21]

One major example of this comes from our observation that 2 of the Herps, Herp1 and Herp2, have nonoverlapping expression patterns in the atrium and the ventricle.[15] Only Herp 1 is expressed in the ventricle and only Herp 2 is expressed in the atria. This is distinct from the pattern of expression of other transcription regulation proteins important in heart chamber development. Factors such as GATA4, MEF2C, and NKX2.5 are equally well expressed and active in both chambers. Why is Herp expression so different? Might HERP1 down-regulate atrial chamber–specific genes in the ventricles while HERP2 down-regulates ventricular chamber–specific genes in the atria?

Fortunately, we were able to test this in one case of a cell line, HL1, which retains characteristics of atrial cardiomyocytes. Unlike ventricular cardiomyocytes, HL1 cells express atrial-specific myosin light chain 2a (mlc2a) as well as the atrial natriuretic protein (ANP). Importantly, HL1 cells also express Herp2 but not Herp1. If our idea that the expression of Herp 1 in the ventricle suppresses atrial-specific genes is correct, then we reasoned that forced expression of Herp 1 in HL1 cells might selectively shut off expression of MLC2a, ANP, and Herp2. To perform this test we genetically engineered nonreplicating adenoviruses to carry a working copy of the Herp1 gene. The adenovirus readily infects nearly all cells in a culture dish and brings with it any genes it encodes. When we infected HL1 cells with a normal adenovirus or with an adenovirus that expressed Herp2 there was no effect on the cells or on the expression of MLC2a, ANP, or endogenous Herp2. However, when we infected the cells with the HERP1-bearing adenovirus, expression of MLC2a, ANP, and, remarkably, Herp1, were all but completely shut down.[14]

We interpret such experiments to mean that determination of cell fate in the developing heart extends to atrialization and ventricularization. Although those 2 sets of organs share many characteristics, they have distinct physiologies and protein constituents even though they arise embryologically from an intermixed small number of precursor cells. Thus, an organ fate decision is part of the developmental process and it is not surprising that a mechanism to allow distinct pathways of differentiation to arise has been selected by evolution. Although yet unproven in the heart, our findings suggest that Notch ligands in cells destined to become atria stimulate adjacent cells, and in those cells Herp1 is activated. Herp1 in turn suppresses Herp2 and also suppresses atrial-specific genes, thus turning the cell into a ventricular myocyte.

Building New Blood Vessels

Such research has allowed us and many other investigators to understand that developmental decisions can be triggered by cell-cell interactions. Subsequent intracellular signaling molecules turn on genes that repress specific cell fates.

In the case of blood vessel formation these same events are part of neovascularization for collateral vessel formation or for the angiogenesis associated with neoplasia. HERPs may regulate expression of an arterial phenotype by repressing genes involved in vessel formation.[15] One such gene known to effect an arterial cell fate is vascular endothelial growth factor (VEGF). If VEGF is repressed normally, we wondered whether its forced overexpression might lead to new vessel formation even in an ischemic myocardium. Once again, the interface between the molecular biologists and the cardiac

physiologists proved to be synergistic for us as we began a collaboration with the laboratory of Robert Kloner to study this question.[22,23] We used a rat myocardial infarction model to test the hypothesis that overexpression of VEGF would lead to new arterial vessel formation.

A month after a left coronary artery ligation, we injected the site of the infarct in 24 rat hearts with a recombinant DNA construct expressing the gene for VEGF. A month later we examined these hearts and compared them with a control group of 16 infarcted hearts that had been injected with normal saline. The saline-injected hearts showed no differences from noninjected infarcted hearts: a consistent infarct scar was readily visible macroscopically that also looked quite typical on microscopic section. The VEGF-injected hearts were remarkably different. Of the VEGF-treated hearts, 21 of 24 showed neovascularization but with angioma-like formation, not true angiogenesis. Microscopically there was increased vessel density in these angioma-like entities compared with the vessel density in the saline-injected controls. Most importantly, the regional blood flow, measured by injection into the coronary arteries of radioactive microbeads, was unable to detect any differences in regional myocardial blood flow between VEGF- and saline-treated rats. Thus, these angioma-like tumors on the surfaces of the infarcted hearts provided no vascular delivery to the heart. Although the results in terms of potential therapeutics for myocardial infarction were disappointing, the experiments did show that recombinant genes delivered to an infarcted tissue could deliver gene product and effect cellular growth and vascular processes. Perhaps it is not surprising in retrospect that VEGF was not a cure-all because it has become clear that angiogenesis is a cell fate decision, perhaps triggered at least in part by HERP gene expression, but that VEGF and many other gene targets are involved in blood vessel formation. Such work has set the stage for the development of truly therapeutic approaches to revascularization of an infracted organ.

The interactions between organ physiologists and molecular biologists seem especially useful in the current scientific era. The interface between disciplines is always the locus of the most innovative and exciting science. If the examples in this article encourage nephrologists and those dealing with whole-organ failure to develop interactions with molecular geneticists and biologists, then it will have served its purpose.

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