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Converging Pathways and Principles in Heart Development and Disease: CV@CSH

Meeting Review

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"One country, two systems."
—Deng Xiaoping, referring to the reunification of Hong Kong and China.
New York Times, January 2, 1985

As noted by a former Chairman, the term "one country-two systems" was initially coined to describe a new experimental paradigm for normalized relations between Hong Kong and China, two territories that had long been united, but had evolved along separate paths during the Twentieth Century. Over the past 50 years, studies of the cardiovascular system have also evolved along distinct pathways, largely based upon the territorial boundaries of modern developmental biology and clinical physiology. The developmental viewpoint has been underpinned by genetics, and a reductionist approach toward mechanism, with molecular biology as its primary tool, and the smallest biological unit being individual cell types. The clinical cardiology viewpoint has been based on physiology, and an integrative approach toward mechanism, with interventional/device/imaging technologies as the primary tools, and the smallest biological unit being the intact organ. In short, studies of the cardiovascular system have traditionally been approached by citizens of "two" very different scientific cultures and "countries," each with their own societies, uniforms, icons, and customs. If the recent 67th Quantitative Symposium of Cold Spring Harbor Laboratory on "The Cardiovascular System" is any guide, this era of "one system-two countries" is coming to a close. During this symposium, converging pathways and perspectives of cardiovascular development and physiology were presented, driven by post-genomic tools and technology, a new generation of scientists and physicians trained to work at the interface of developmental biology, genetics, physiology, and human disease, and an integrative approach to biology. This review highlights some of the central themes presented by delegates of both "countries" that provided new insights into developmental principles of human cardiovascular physiology and disease.

Zebrafish Genetics: An Experimental System Linking Cardiovascular Development and Disease

Zebrafish genetics is beginning to uncover genes and pathways for cardiac development at a breathtaking pace, forging unsuspected connections with human cardiovascular physiology and disease. The translucent nature of the organism has facilitated large scale mutagenesis screens for diverse cardiovascular phenotypes (for a review, see Shin and Fishman, 2002), while the density of the zebrafish genetic map and ongoing genome project have led to an ability to clone the corresponding genes within a relatively short time span (3–6 months). The system appears to be particularly valuable for identifying new genes and pathways in vasculogenesis. *gridlock,* a gene that encodes a member of the Hairy/Enhancer of Split family of related bHLH proteins, acts as a molecular switch for the distinct identity of arteries and veins by repressing the venous cell fate in pre-angioblasts, which is thereby permissive for artery formation. The zebrafish mutant phenotype resembles aspects of human aortic coarctation (Zhong et al., 2001). The ability of gridlock to govern the decision between arterial and venous cell fates is a reflection of its activity as a transcriptional target of the Notch signaling pathway, long known to dictate mutually exclusive cell fates in diverse cell types. Surprisingly, knockout mice for the gridlock ortholog Hey2 do not show aortic coarctation, but frequently succumb to severe neonatal cardiomyopathy. A combined loss of Hey1 and Hey2, however, leads to vascular defects similar to gridlock, suggesting that these genes have undergone partial sub- or neofunctionalization during vertebrate evolution (M. Gessler, University of Wuerzburg).

Two new genes that lie in an identical pathway for the endothelial-myocardial driven cues for valvulogenesis (Walsh and Stainier, 2001) (D. Stainier, UCSF) were presented, as well as a new transgenic strain harboring a *fli*-1 GFP construct that allows in vivo visualization of the entire vasculature. This strain should facilitate subsequent modifier screens utilizing existing mutants with interesting vascular phenotypes (Weinstein, NIH). In this regard, over 700 mutants that affect discrete steps of zebrafish vessel formation and/or maturation have been isolated (H. Habeck, Exelixis, Germany). Mark Fishman (MGH) presented an elegant case for the zebrafish as a system to unravel the "logic" of disease pathogenesis. Toward this end, a compelling argument can be made that a parallel effort on physiological characterization of this model organism is now warranted to allow direct connections between novel genes and clinically relevant functional endpoints. Clearly, studies in zebrafish are demonstrating that the border between developmental biology and disease is dissolving as shared pathways for prenatal morphogenic steps and postnatal physiological events continue to be uncovered.

Vascular Lineages and Therapeutic Angiogenesis

In 1994, the first report of therapeutic angiogenesis based on the single administration of one angiogenic factor, VEGF, appeared (Takeshita et al., 1994). This result pointed to the promise of therapeutic angiogenesis as a new molecular therapy for chronic coronary and peripheral arterial disease. Although therapeutic an-

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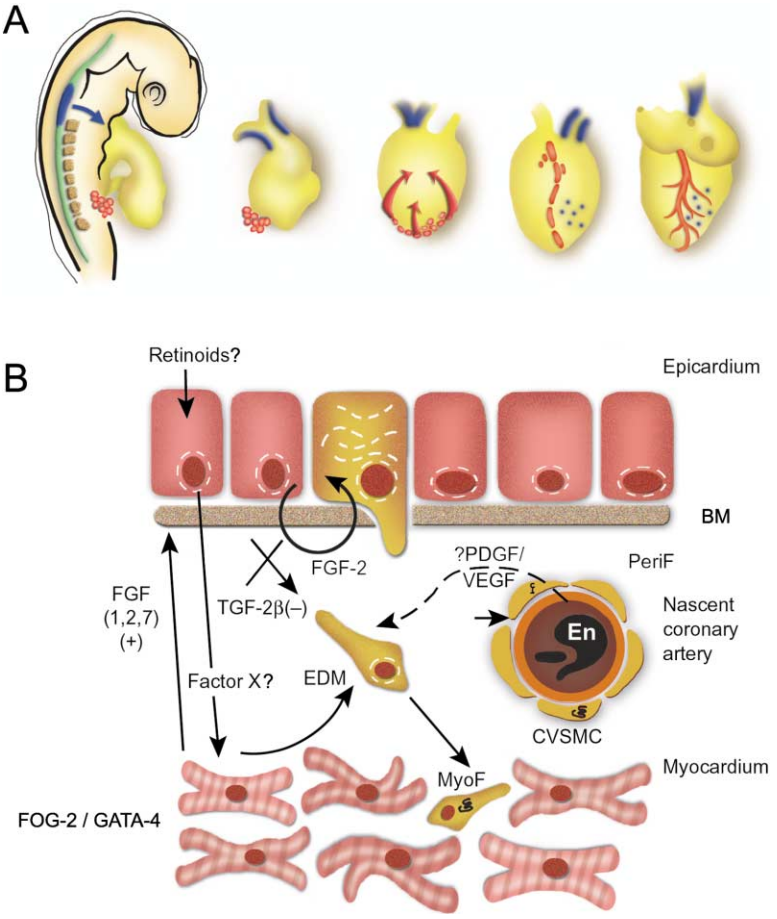

Figure 1. Multiple Lineages and Pathways for Formation of the Heart Vasculature

(A) Migratory cell lineages of the heart vasculature. A subpopulation of neural crest cell lineages migrates to the cardiac region (blue), and is required for the reorganization of the outflow tract in the developing heart. Neural-crest-derived cells surround the pharyngeal arch arteries and populate the aorticopulmonary septum and conotruncal cushion. A patch of neural-crest derived cells is also consistently observed in the ventricles of mouse embryos. Their physiological significance is currently unknown, but may be involved with the development of the coronary circulation. Coronary vasculogenesis depends on a selected population of precursors from the liver primordia, the pro-epicardial organ (in red). Pro-epicardial cells migrate onto the surface of the fetal heart at the region of the atrioventricular groove; they migrate ventrally and cephalically until they completely cover the heart. Coronary arteries and veins are assembled from endothelial, coronary vascular smooth muscle, and pericyte precursor cells within the subepicardial matrix.

(B) Molecular pathways for the formation of the coronary vasculature. A GATA4/Fog2 pathway mediates the myocardial secretion of FGF factors that stimulate the epicardium to transform into mesenchyme, while TGF-β antagonizes this transition. Activated epicardial derived mesenchymal cells (EDM) migrate through the basement membrane (BM) to the subepicardial membrane where signals from endothelial (EN) cells and/or the myocardium recruit EDM cells to form nascent vessels. As the mesenchymal cells are incorporated into nascent vessels, they differentiate into coronary vascular smooth muscle cells (CVSMC), pericytes (Perif), and intermyocardial fibroblasts (Myof) (modified from Morabito et al., 2002).

giogenesis continues to be a laudable goal, its experimental validation remains an open question, and the clinical efficacy of therapeutic angiogenesis remains unclear. If the recent papers presented at CSH are any measure, it appears as if early studies examining the role of single angiogenic factors as therapeutic strategies may have severely underestimated the biological complexity of vasculogenesis. One of the obligatory triggers for angiogenesis is VEGF, a factor that has been shown to be both necessary and sufficient for angiogenesis in vivo. However, recent studies of a host of additional angiogenic factors, including angiopoietins-1 and -2 (Yancopoulos et al., 2000) (Regeneron), suggest that additional components may be required to form fully mature vessels that contain pericytes, smooth muscle cells, and distinct endothelial subtypes. Further, VEGF can specifically induce expression of the synergistic factor angiopoietin-2 in muscle cells by an intracellular pathway, but not when administered exogenously. This suggests that VEGF gene and protein delivery might elicit different biological effects as therapeutic angiogenesis strategies (H. Blau, Stanford).

Intriguingly, it now appears that there may be organ-specific angiogenic programs that are based on tissue-specific angiogenic factors, exemplified by EG-VEGF, an angiogenic factor that is expressed in steroidogenic glands such as ovary, adrenal, and testes, and acts selectively on endothelial cells of such endocrine organs through a distinct class of G-protein-coupled receptor pathways (N. Ferrara, Genentech) (LeCouter et al., 2001). Accordingly, it may be over-simplistic to expect that the administration of a single gene would be sufficient to promote therapeutic coronary or peripheral arteriogenesis, without first identifying the unique combination of core and tissue-specific angiogenic programs required in the target organ of interest. This may be particularly important for therapeutic coronary arteriogenesis, as the formation of the coronary arterial system in the fetal heart is now known to require concerted developmental cues derived from four discrete lineages: endothelial, myocardial, pro-epicardial, and neural crest lineages (Figure 1). Growing muscular coronary arteries that are built to withstand the intrinsic biomechanical stress of each heartbeat may first require a better definition of the developmental cues and secreted factors required for normal coronary arteriogenesis. The ability to isolate

primed coronary arterial progenitor cells could represent a valid alternative strategy that could complement existing approaches, particularly if these can be effectively isolated from adult stem cell populations, and the factors that are required for their recruitment and self-renewal identified.

Direct validation of anti-angiogenic strategies as independent chemotherapeutic regimens for broad classes of human cancers has also been elusive. As judged by the recent data presented at CSH, there not only appear to be distinct molecular pathways for vasculogenesis in specific organ systems, but the existence of multiple, parallel pathways for tumor angiogenesis (R. Hynes, MIT; R. Benezra, Sloan-Kettering), supporting the view that the elimination of a single angiogenic factor may not independently lead to the loss of solid tissue tumors. Recent studies of spontaneous tumors suggest that tumors can escape the blockade in single pathways in part via selection for alternative pathways (Benezra, 2001). It may become possible to tailor the anti-angiogenic strategy for specific tumor types based upon tissue and tumor-specific angiogenic pathways, as well as the synergistic effects of current chemotherapeutic regimens. In this regard, kinase inhibitor treatment of a genetically engineered mouse model of pancreatic islet cancer implicated PDGF receptor signaling in tumor vessel-associated pericytes as crucial for maintenance and continuing angiogenesis of tumor vasculature (Bergers, UCSF). Additional results from that model demonstrated the benefits of combinatorial anti-angiogenic therapies utilizing protease inhibitors and low dose, "metronomic" chemotherapy (Bergers).

Neural Crest Lineages and Congenital Heart Disease

The clinical link between neural crest lineages and cardiac development was established by the characterization of children harboring a characteristic subset of congenital heart defects that were invariably accompanied by neural-crest-related defects. These children harbor microdeletions in chromosome 22 and a minimal region has been established by careful genotype-phenotype correlations in large global patient populations. Attempts to refine the disease interval and to identify the specific gene(s) responsible for the defects have been elusive at the clinical level, but recent studies in mice have shown that a single gene, TBX 1, is likely to be the most important gene within this region to account for a subset of the neural crest-related cardiac congenital defects (Lindsay et al., 2001; Merscher et al., 2001; Jerome and Papaioannou, 2001). However, to date, no patients with TBX1 mutations have been found that display the full spectrum of cardiac neural crest defects, suggesting that other genes in this minimal region may contribute to the human phenotype. In this regard, TBX1-deficient mice only display a subset of the neural crest defects found in DiGeorge children (aortic arch anomalies), again providing support for DiGeorge as a contiguous gene syndrome. Given the number of genes in the minimal critical region of chromosome 22, and the existence of non-overlapping deletions that result in the same phenotype, it is likely that positional effects and long-range chromatin interactions contribute to the difficulty in pin-pointing a single gene as the culprit in all aspects of this disease.

The question arises as to whether the requirement for TBX1 actually is localized within neural crest lineages per se, or whether it reflects non-cell-autonomous effects. Surprisingly, a growing body of evidence now supports the concept that the TBX1 requirement is located outside of neural crest lineages. Thus, the cardiac morphogenic defects associated with DiGeorge syndrome do not reflect a primary effect on neural crest formation, proliferation, or survival, but rather result from the loss of guidance cues from the pharyngeal endoderm that are required for neural crest migration into the aortic arch region (A. Baldini, Baylor). This view has been independently supported by studies of the regulatory programs that control TBX1 expression in the mouse (D. Srivastava, UTSW). Fgf8 may mediate the Tbx1 non-cell-autonomous effects on neural crest cells, as heterozygous deletion of the Fgf8 gene enhances the Tbx1 haploinsufficiency phenotype in mice (Vitelli et al., 2002), a point supported by two other independent laboratories (Frank et al., 2002; Abu-Issa et al., 2002). This secondary TBX1 effect on neural crest migration might imply that an additional "hit" within neural crest lineages acts as a modifier to enhance the phenotype, which might reflect actions of other genes within or adjacent to the DiGeorge minimal region. Interestingly, 90% of individuals with neural crest-related defects display hemizygous deletions of CDC 45, a cell cycle regulator which falls within the DiGeorge minimal region, although mice that are heterozygous for a CDC 45 null allele do not display cardiac defects (Yoshida et al., 2001). Accordingly, it will be of interest to generate compound mutant mice which harbor a complete deficiency of TBX1 and partial deficiencies in other contiguous genes, such as CDC 45, that lie within the DiGeorge minimal region. Such an approach has the potential to identify key modifiers of cardiac congenital heart defects.

Renal Cell Lineages and Hypertension

Hypertension is a complex trait that is influenced by multiple organs, including the brain, heart, kidney, adrenals, and vascular endothelium. Recent studies of rare forms of familial hypertension are beginning to reveal the first genetic pathways for rare familial forms of human hypertension. By collecting families from around the globe which have severe, early onset hypertension, mutations in a number of new human hypertensive genes including those encoding the epithelial sodium channel (ENAC), the mineralocorticoid receptor, and a renal WNK kinase have been identified (Lifton et al., 2001). Remarkably, in addition to these hypertensive genes, another set of human mutations has been found that lowers blood pressure. All of these genes have their primary effect in renal cell lineages, pointing to the central role of the kidney in the onset of human hypertension. As noted by Rick Lifton (Yale), while it is clear that blood pressure can be controlled by a number of different agents that work outside of these renal pathways, the question arises as to whether the design of specific antagonists to these specific renal targets might have long-term beneficial effects that go beyond effects on the control of blood pressure per se, but extend to chronic effects of hypertension on end-organ diseases of the heart, brain, or the kidney itself. The recent unsus-

pected major therapeutic benefit of mineralocorticoid antagonists in heart failure raises interesting questions about the potential role of this nuclear hormone receptor in heart muscle cells. Accordingly, it will be interesting to cross mice that harbor cardiac-restricted mutations of this gene into well-characterized mouse models of cardiomyopathy and heart failure. In short, the discovery of these new pathways responsible for rare forms of human hypertension is beginning to provide novel insights and therapeutic targets for common forms of the disease. The identification of a number of quantitative trait loci (QTLs) in spontaneous in-bred hypertensive rat strains (Jacob and Kwitek, 2002), coupled with the ongoing advances in the rat genome project, suggest that additional insight into genetic pathways for the control of hypertension will be forthcoming from experimental model systems, which should allow a direct comparison in studies of more common forms of hypertension in human populations.

Transcriptional Control of Ventricular Growth and Development

Since the discovery of master regulators of skeletal muscle development, such as MyoD and myogenin, there has been a push to identify corresponding regulators of cardiac cell fate. Such factors would have obvious potential in converting non-cardiac cells to the cardiac lineage as an approach for repair and regeneration of the damaged adult myocardium. However, it appears that forming a cardiac muscle cell is not so simple. While several cardiac-restricted transcription factors have been identified, none have been found to possess the ability to confer cardiac identity on their own. However, combinations of factors such as the zinc finger protein GATA4, the homodomain proteins Nkx2.5 and Tbx5 and the MADS box protein serum response factor (SRF) are able to activate some cardiac genes in transfected cells. Tim Mohun (NIMR, UK) reported that ectopic expression of GATA4 in injected frog animal caps was capable of inducing the formation of beating cardiomyocytes. Similar findings have been made with GATA5 in zebrafish (D. Stainier, UCSF) (Reiter et al., 1999). Since these GATA factors are not restricted to the heart, and alone cannot activate endogenous cardiac genes in transfection assays, they must act in conjunction with other transcription factors and signaling systems in the context of the frog or fish embryo to initiate cardiogenesis in a subset of cells. The concept that specific sets of extracellular signals must be interpreted in a cell-specific manner to generate the cardiac phenotype is consistent with studies in *Drosophila*, in which overlapping gradients of cardiogenic signals act in conjunction with cell autonomous transcription factors to specify the identity of cardiac cells (M. Frasch, Mt. Sinai).

SRF is required for the opposing processes of cell proliferation and myogenesis. Myocardin, a novel cardiac-restricted transcription factor, has recently been shown to stimulate transcription of SRF-dependent muscle genes through its association with SRF (Wang et al., 2001). Injection of myocardin mRNA into frog embryos was reported to be sufficient to induce ectopic cardiac gene expression (E. Olson, UTSW, and P. Krieg, University of Arizona). Conversely, dominant negative mutants of myocardin can prevent heart formation in frog embryos. It will be of interest to identify additional cofactors for myocardin and to investigate its potential roles in cardiac gene expression during later stages of development and disease. In addition, the growing database of putative cardiac transcriptional regulators suggests new insights in the combinatorial pathways that control the gene program may be on the horizon (J. Epstein, University of Pennsylvania, and E. Olson, UTSW).

A zebrafish mutant, *liebeskummer*, with an excess of cardiomyocytes was also reported (M. Fishman, MGH). The mutation was shown to activate reptin, an ATP-dependent helicase that acts through an as yet unknown mechanism to control growth of the ventricles. Ventricular growth in the embryo is dependent on signals from the overlying epicardium. Andrew Lassar (Harvard) presented evidence that epicardial signaling requires Epo and retinoic acid, which act through parallel pathways to stimulate myocardial growth by inducing a cardiac myocyte mitogen in the epicardial cells. Neuregulin signaling from the endocardium to the myocardium was also shown by Richard Harvey (UNSW, AU) to play a critical role in embryonic growth and trabeculation of the ventricular chambers.

Signaling Pathways for Cardiac Hypertrophy and Remodeling

The adult myocardium responds to extrinsic forms of stress such as hypertension, myocardial infarction, and pressure-overload by a hypertrophic growth response (for a review, see Chien, 1999). Inherited mutations in components of the sarcomere and cytoskeleton also result in hypertrophic cardiomyopathy. Hypertrophy in response to pathologic stimuli is accompanied by activation of a fetal gene program, which results in maladaptive changes in contractility and calcium handling (Figure 2). Growth of the heart during normal postnatal development and in response to exercise also occurs through hypertrophy. A key issue in the field is to decipher the pathways that control pathologic and physiologic hypertrophy, such that the former might be inhibited and the latter augmented pharmacologically. Changes in intracellular calcium handling have been implicated as a trigger for cardiac hypertrophy, and numerous calcium-dependent and calcium-independent (Gq, RAS, PI3K, p38) (for review, see Hoshijima and Chien, 2002) signaling systems have been shown to be necessary and sufficient to drive multiple features of cardiac growth.

The notion that aberrant calcium handling plays a central role in the hypertrophic program has been supported by several observations: (1) hypertrophic cardiomyocytes exhibit alterations in calcium sensitivity and handling, some of which can be corrected by enhanced activity of the SR Ca-ATPase through inhibition of its inhibitor phospholamban (Minamisawa et al., 1999; Hoshijima et al., 2002); (2) numerous calcium-sensitive signaling pathways are activated in hypertrophic cardiomyocytes (Molkentin et al., 1998) (for a review, see Frey et al., 2000); (3) forced activation of calcium-sensitive signaling pathways is sufficient to induce myocyte hypertrophy in vivo and in vitro (for review, see Leinwand, 2001). Thus, it is reasonable to assume that calcium

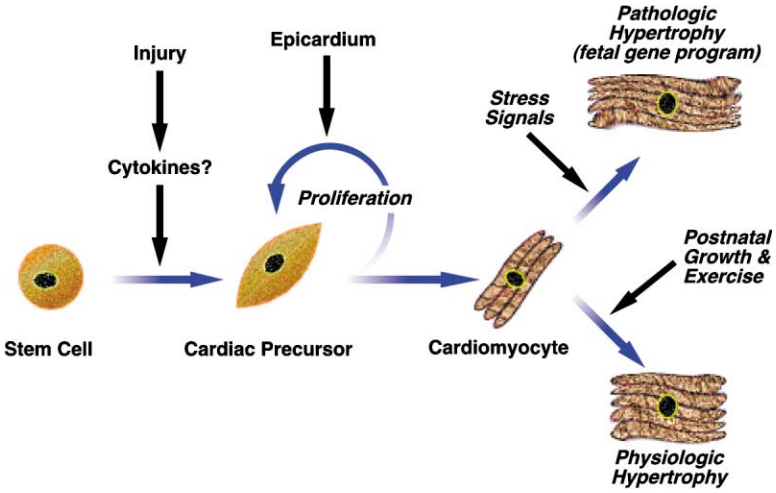

Figure 2. Cellular Pathways for Cardiac Development and Remodeling

Cytokines may attract circulating hemotopoietic stem cells to sites of cardiac injury. These cells then adopt a cardiac fate through mechanisms that remain unclear. During development, the epicardium provides cues for cardiac precursor proliferation. Cardiomyocytes withdraw irreversibly from the cell cycle after birth, but adult myocardial cells can respond to stress signals to enter the program for pathological hypertrophy or can respond to normal growth signals for physiological hypertrophy.

signaling plays a key role in many forms of hypertrophy, but several other calcium-independent pathways are also likely to mediate key aspects of the hypertrophic response, as well (for a review, see Sugden, 2001).

The involvement of calcium in hypertrophic signaling raises many obvious questions. For example, where does the calcium come from? Given the extreme fluctuations in intracellular calcium levels that accompany each cycle of contraction and relaxation, is there a specific pool that controls hypertrophy? If so, how is it compartmentalized or insulated from other calcium in the sarcoplasm? While calcineurin, CaMK, and MAPK have each been shown to be sufficient, and in some cases necessary, for hypertrophy, each of these enzymes is activated by different concentrations and waveforms of calcium. Do each of these calcium-sensitive effectors get activated by different hypertrophic signals? How are hypertrophic signaling pathways interconnected, and how do they intersect with the calcium cycling machinery?

Mice engineered to express a mutant form of α-MHC with a codon 403 mutation that mimics a common human mutation develop hypertrophic cardiomyopathy and exhibit abnormal calcium homeostasis, such that the mutant sarcomere requires a greater amount of calcium than the wild-type sarcomere to generate the same level of force (Fatkin et al., 2000). SR calcium levels are also reduced in these mutants, suggesting that the mutant sarcomere sequesters a higher level of calcium than wild-type, with resulting perturbation in intracellular calcium homeostasis. This notion is supported by the finding that the L-type calcium channel blocker diltiazem restores SR calcium levels and normal contractility and prevents hypertrophy in the mutant heart. Jon Seidman (Harvard) presented evidence suggesting that sarcomere mutations may activate different hypertrophic signaling pathways from pressure overload. However, the molecular mechanisms whereby sarcomere mutations result in hypertrophic cardiomyopathy remain unknown.

Another major question in the field is to determine how hypertrophic signals are transmitted to the nucleus, with resulting reprogramming of cardiac gene expression. The MEF2 transcription factor appears to be a critical target for hypertrophic signals, as revealed with a transgenic mouse line that harbors a lacZ reporter under control of MEF2 binding sites (Naya et al., 1999). Using this mouse, Olson and coworkers showed that the transcriptional activity of MEF2 is activated through a post-translational mechanism in response to hypertrophic signals. Activation of MEF2 appears to be mediated by the signal-dependent dissociation of class II histone deacetylases (HDACs), which are normally tethered to MEF2, resulting in repression of MEF2 target genes (McKinsey et al., 2000). Stress signals lead to the phosphorylation of HDACs by an as yet unidentified kinase. Consistent with this model, signal-resistant mutants of HDACs act as irreversible repressors of hypertrophy, and HDAC knockout mice are sensitized to hypertrophic signals.

By studying the effects of exercise on expression of the MEF2-lacZ transgene, Leslie Leinwand (University of Colorado) found that physiologic signals do not stimulate MEF2 activity in the heart. Intriguingly, cardiac MEF2 activity is dramatically induced in male, but not female, mice that misexpress mutant MHC. Paradoxically, removal of estrogens from the diet restores cardiac function in these male mice. These findings suggest that MEF2 is a specific endpoint for pathologic stimuli that lead to hypertrophy, and that there is a gender-specific influence on this stress-response pathway. Thus, approaches that specifically interfere with upstream signaling pathways that lead to MEF2 activation might have clinical benefit as anti-hypertrophic agents.

Mechanical Signaling

In addition to these recent advances in dissecting the signaling cascades and transcriptional mediators of hypertrophy, genomic databases are also uncovering a growing number of novel cytoskeletal genes whose products play pivotal roles in mechanosignaling and stress-responsiveness (for a review see Chien, 2000; Clarke et al., 2002). An expanding number of these novel, muscle-specific cytoskeletal components are localized in the cardiac Z disc, thereby implying that the Z disc may play a specialized function beyond a purely structural role (Figure 3). At the Z disc, α-actinins are organized into a series of anti-parallel dimers, which cross-link polymerized actin filaments (thin filaments). The Z

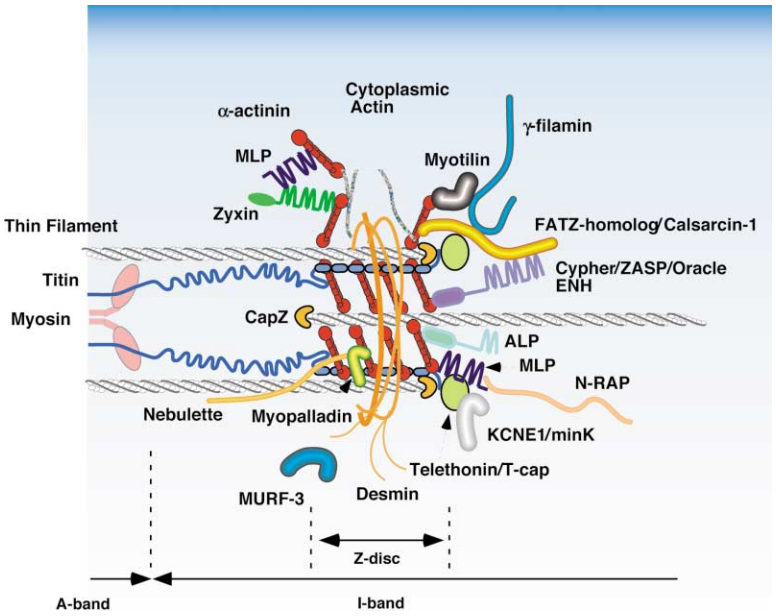

Figure 3. Cytoskeletal Proteins Localized in the Cardiac Z Disc

α-actinin is a major cross-linking component of actin at the Z disc and the C-terminal domain of titin is anchored at the Z disc. Several muscle-restricted LIM domain proteins are also localized to this region. MLP, muscle-specific LIM protein; ALP, α-actinin associated LIM protein; ZASP, Z-band alternatively spliced PDZ motif protein; ENH, enigma homolog; FATZ, filamin-, actinin-, and telethonin binding protein of the Z disc; N-RAP, nublulin-related anchoring protein; MURF-3, muscle-specific RING-finger protein-3.

disc also anchors titin, a 2-3 MDa muscle-specific protein. Titin contains two α-actinin binding domains at its N terminus and spans the length of each half-sarcomere, reaching the M line in the middle of A-band. T-cap, a 19 kDa muscle-specific protein was isolated as a titin binding protein at the Z disc. In cardiac myocytes, Z disc proteins and other components of this cytoskeletal protein network play diverse roles in sarcomeric organization, force transduction, and force transmission. Recent studies indicate that there may be an additional specific role in biomechanical stretch responses and associated downstream signaling events (K.R.C., UCSD). In addition, experimental studies in gene-targeted animal models and familial forms of the human disease are beginning to point to an important, previously unsuspected role of the cardiac myocyte cytoskeleton in the pathogenesis of dilated cardiomyopathy (Chien, 2000; Seidman and Seidman, 2001). It will be interesting to determine whether Z disc proteins also participate directly in the localization of signaling molecules involved in transmission of mechanical signals, as suggested by the localization of the PKC binding of the PDZ-LIM domain protein Cypher (E. Zhou et al., 1999, 2001) and the calcineurin binding protein, calsarcins (E. Olson), to the Z disk.

The mechanistic pathways that link cytoskeletal defects with cardiomyopathy and associated heart failure are currently unclear, but hints are being derived from experimental model systems. Studies of a mouse model of the delta-sarcoglycan related cardiomyopathy now suggest that the cardiac lesions appear secondary to a primary requirement in the coronary arterial smooth muscle, which develops chronic vasospasm, thereby accounting for the focal, perivascular nature of the phenotype (E. McNally, University of Chicago).

Mutations in titin have now been directly implicated in familial forms of dilated cardiomyopathy in humans (Gerull et al., 2002) and studies in a zebrafish model system suggest that the defects most likely reflect effects on sarcomeric assembly (Xu et al., 2002). Knockouts of three genes encoding members of the muscle-restricted Z disc cytoskeletal LIM domain family (MLP, ALP, and Cypher, see Figure 3) result in distinct forms of right ventricular cardiomyopathy, neonatal cardiomyopathy, and classical adult onset dilated cardiomyopathy, suggesting specific roles for these proteins at discrete stages of pre- and postnatal cardiac growth and development (Arber et al., 1997; Pashmforoush et al., 2001; Zhou et al., 2001). The precise molecular mechanisms that link defects in the Z disc or other cytoskeletal protein mutations with the onset of DCM and the progression of heart failure remain largely unknown. In this regard, genetic complementation studies in MLP deficient mice have shown that the loss of Z disc proteins does not inherently lead to cardiac structural and functional defects (Minamisawa et al., 1999), thereby indicating that the disruption of sarcomeric assembly, force generation, and sarcolemmal integrity in end-stage DCM in this model system may reflect secondary, versus primary effects of this class of cytoskeletal mutation. The generation of mice harboring a double knockout of MLP and the muscle specific inhibitor of sarcoplasmic reticulum (SR) Ca^{2+}ATPase, phospholamban, display complete rescue from the onset of dilated cardiomyopathy and heart failure, while somatic gene transfer of a phospholamban inhibitory peptide in the setting of delta-sarcoglycan deficiency prevents the onset of end-stage heart failure (Hoshijima et al., 2002), pointing to a critical role of SR calcium cycling in heart failure progression. The critical issue is whether these beneficial effects of enhanced calcium cycling represent primary effects on Z disc proteins and DCM, or whether this reflects a non-cell-autonomous effect secondary to relieving wall stress. If there are distinct mechanistic pathways by which each of these diverse cytoskeletal mutations lead to dilated cardiomyopathy, careful study of these disease pathways may offer an opportunity to identify the precise functions of these genes in the control of in vivo cardiac myocyte function.

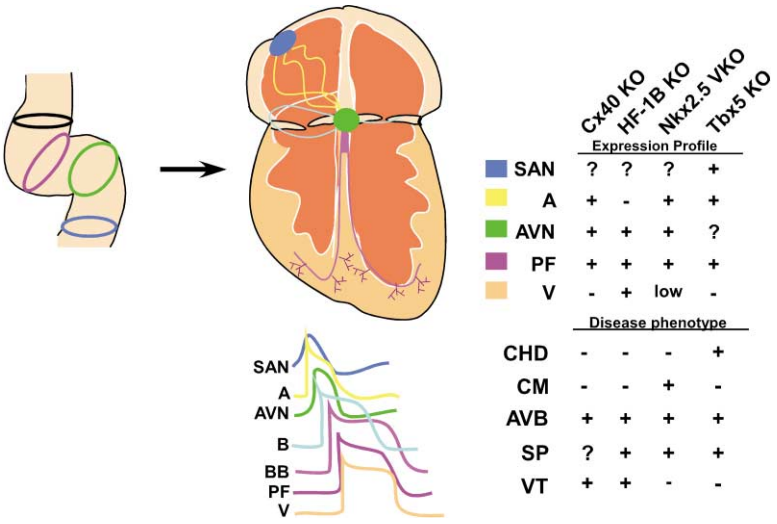

Figure 4. Developmental Pathways for the Formation of the Cardiac Conduction System

The interzonal myocardial rings from the linear heart tube to the four chambered heart correspond, in part, to components of the specialized cardiac conduction system including the SAN, AVN, and Bundle of His and PF (rings and components of the conduction system are color coded). Looping of the immature heart tube brings these rings to their approximate positions in the mature heart. Normal electrophysiological function of the heart depends on proper development of all lineages within the cardiac conduction system, as illustrated by the distinct electrophysiological profile of each individual component. Disruption of any one of these components is not lethal, but can lead to numerous electrophysiological defects. A number of genes have been identified in various components of the conduction system including, but not exclusive to, *connexin-40*, *HF1b*, *nkx2.5,* and *tbx5*. Mouse knockout models for each of these genes have been studied revealing numerous electrophysiological defects related to abnormalities in various components of the conduction system. VKO: ventricular restricted knock out; SAN sinus node; A, atria; AVN, atrioventricular node; PF, Purkinje fiber; V, ventricle; B, common bundle; BB, bundle branches; CHD, congenital heart defects; CM, cardiomyopathy; AVB, AV block; SP, sinus pause; VT, ventricular tachycardia.

Conduction Lineages and Arrhythmogenesis

The orderly propagation of cardiac electrical impulses is mediated by a spatially restricted network of specialized conduction system cell lineages (Figure 4). Until recently, the precise identification of these lineages, their origins, and their precise role in specific forms of cardiac disease, have been a mystery. An important step forward was the demonstration that a major portion of the conduction system cells in the ventricular chamber, Purkinje cells, were actually derived from myogenic precursors, indicating that these represent extensively modified cardiac myocytes that acquire specialized electrophysiological properties of spontaneous pacemaker function and other distinct regulatory pathways that are not shared with their neighboring ventricular muscle cell lineages (for review, see Gourdie et al., 1999). Elegant studies by Glenn Fishman (NYU) have led to the development of a mouse model that expresses LacZ selectively in the cardiac conduction system in the heart (Rentschler et al., 2001). This mouse has allowed a detailed view of the maturing conductive system network and together with optical mapping studies correlating morphology of the conduction system with functionality, these studies provide a basis for understanding the regulatory circuitry guiding conduction system specialization (see Figure 4). Utilizing this model, a direct role for neuregulin pathways in triggering the formation of the conduction system has been found (Rentschler et al., 2002), which may represent one of the subendocardial cues that has been suspected to contribute to this transition. Since each of the components of the conduction system has distinct electrophysiological properties (SA node, AV node, His bundle, Purkinje; see Figure 4), there must be additional factors that lead to the formation of specific conduction system lineages. New models that will allow the sorting of conduction system cell lineages, and genomic databases could eventually be instructive in identifying these molecular pathways.

Defects in the developmental pathways that control conduction system formation have now been directly implicated in cardiac sudden death and associated arrhythmias in both experimental model systems and rare forms of human diseases (Figure 4). Previous studies in genetically engineered animal models suggest that defects in the transition between ventricular and conduction system cells (Purkinje cells) can lead to the anatomic substrate for sudden death, including decreased expression and mislocalization of the conduction system-restricted marker, connexin 40 (Nguyen-Tran et al., 2000). HF1b, a member of the SP-1 family of transcription factors, displays a restricted pattern of expression in conduction system lineages, and HF1b$^{-/-}$ mice display normal cardiac structure and contractile function, but display defects in Purkinje fiber formation, manifested as a confused electrophysiological identity in Purkinje and ventricular cell lineages, resulting in cardiac sudden death and marked tachy and brady arrhythmias. Since HF1b is expressed in ventricular myocardium as well as the neural tube, which gives rise to the neural crest, the question arises as to where the requirement for HF1b is located with respect to the defects in conduction system lineages. Neural crest lineages are important for the formation of the autonomic nervous system that innervates the AV node, and also are critical for the formation of the coronary arterial system, which has been shown to generate cues for Purkinje fiber formation. As a number of neural-restricted genes are expressed within conduction system lineages, it will become of interest to define more precisely the contribution of neural crest lineages to conduction system development (K.R.C., UCSD).

Recent studies of patients with familial forms of con-

genital heart disease also support the concept that defects in transcriptional factors can lead to cardiac arrhythmogenesis via effects on both ventricular and conduction system lineages. Patients that harbor mutations in the transcription factor TBX-5 display large atrial septal defects and a wide spectrum of other cardiac malformations that can be associated with severe conduction defects (Bruneau et al., 2001). The question has arisen as to whether the conduction system defects are simply secondary to the structural defects in the heart and associated distortion of conduction system development. Recent studies in TBX-5-deficient mice support the concept that TBX-5 may have dual effects on the disruption of the normal spatial configuration of the developing conduction system, as well as cell-autonomous effects on conduction lineages, as TBX5 appears to be a direct transcriptional regulator of the Connexin-40 gene (C. Seidman, Harvard). Recent clinical studies have also shown that mutations in the cardiac transcription factor Nkx-2.5 can lead to cardiac congenital heart defects that are accompanied by arrhythmogenesis, complete heart block, and sudden death (Schott et al., 1998) (Kasahara et al., 2000). Conventional Nkx-2.5 knockout mice that harbor a complete ablation of the Nkx-2.5 gene display early embryonic lethality and cardiac looping defects, consistent with the known role of this cardiac homeobox gene in the earliest stages of cardiogenesis (Lyons et al., 1995). It will become of interest to determine if this association with NKX2.5 and cardiac arrhythmias represents a direct effect on conduction system cell lineages, thereby reflecting an additional role for this homeobox gene at later stages of cardiac maturation, including the postnatal myocardium (K.R.C., UCSD). The list of genetic pathways for cardiac arrythmogenesis that arise from developmental defects in conduction system lineages is likely to grow as the pathways that control conduction system development continue to be unraveled by multiple laboratories.

Stem Cell Lineages and CV Disease

The major causes of heart failure are related to the onset of cardiac injury by a variety of pathological stimuli, particularly after the irreversible damage that arises from myocardial infarction. The longstanding axiom has been that the myocardium has a limited capacity for self repair or regeneration, and the irreversible loss of muscle sets into play a series of events that ultimately leads to increased wall stress, chamber dilation, and progressive heart failure. The loss of myocyte survival cues is associated with diverse pathways for heart failure (Chien, 1999), underscoring the importance of maintaining the number of viable heart muscle cells during heart failure progression. This axiom has recently been challenged by the discovery that bone-marrow-derived stem cells can be transduced into cardiac myocyte lineages following their in vivo injection into the scar of post-infarcted hearts (Orlic et al., 2001). A recent report (Murry, University of Washington) at this meeting now suggests that the ability of such stem cells to effectively improve long-term cardiac function may be more limited than initially reported. Studies employing LacZ-marked hematopoietic stem cells have provided little evidence of the regeneration of the infarcted mouse heart (C. Murry). In addition, studies by two independent groups also support a negligible level of transduction of host cells to cardiac myocytes in transplanted human hearts (C. Murry, J. Epstein) (Glaser et al., 2002). While the hope remains that cardiac homing of circulating hematopoietic stem cells might participate in repair of the injured myocardium, it is also apparent that the endogenous repair process is inadequate to improve cardiac function following damage. Thus, future efforts should be directed toward the identification of signals involved in attraction, proliferation, and cardiogenic conversion of circulating stem cells.

One of the fundamental differences between cardiac and skeletal muscle is that skeletal muscle is able to regenerate in response to damage by the awakening of quiescent precursor cells, known as satellite cells, which lie between the basal lamina and the muscle fiber. Cardiac satellite cells have not been identified, and if they do exist, must be present in amounts too low for efficient cardiac repair. Others have reported the presence of stem cells in skeletal muscle that can be coaxed into cardiac lineages in the in vitro context (N. Epstein, NHLBI), suggesting that indeed there may be merit in studying the pathways that lead to the renewal and recruitment of muscle stem cells into cardiac lineages in the in vivo context. The nature of the paracrine pathways that might enhance these steps in stem cell commitment is largely unknown, but a recent report suggests that IGF-1 might serve as a priming stimulus to recruit and commit circulating stem cells to the myogenic lineage in damaged or degenerating muscle (N. Rosenthal, EMBL), suggesting that there might be a similar effect for cardiac lineages as well. Establishing high throughput assay systems that eventually will allow the expression cloning and/or biochemical identification of the external factors and cues required for cardiac stem cell renewal and recruitment should now be possible. Microarray and bioinformatics approaches to identify possible cytokines and other extracellular factors involved in stem cell attraction and propagation in the heart also represent potentially powerful approaches to this problem. Currently, the realistic prospects for stem cell therapy for cardiac repair appear to be distant, based on the need to purify and to deliver in vivo a sufficient quantity of the cells to improve global cardiac function, and the potential for electrophysiological heterogeneity that could represent the substrate for arrhythmogenesis. A longer term, but perhaps more feasible strategy would be to identify the factors required for self renewal and recruitment of cardiac stem cells into the injured myocardium.

Future Perspectives

As witnessed by CV@CSH, a new interdisciplinary approach to cardiovascular biology is underway, with the traditional boundaries between the fields for cardiovascular development and disease rapidly vanishing. Given the current arsenal of post-genome technology, databases, and multiple model organisms, there has never been a more opportune time to tackle complex questions in integrative physiology in general, and cardiovascular physiology in particular. Higher throughput physiological assay systems and computational biology are

likely to play increasingly important roles. Insights from human population studies are on the horizon, but will require large numbers, longitudinal follow-up, and validation in experimental model systems to move beyond association studies and toward mechanistic disease pathways. In an era when complex human biology is the object of our desire, the CV system has the inherent advantage that the in vivo integrated functional endpoints are readily quantifiable, largely conserved across species, and can be directly linked to human biology and disease. Given the growing burden of cardiovascular disease world-wide and the opportunities presented by the numerous in vivo quantitative endpoints in the CV system, a renaissance in integrative physiology may be in the offing. In the post genome era, the heart is well-positioned to move from being simply a disease-based area of inquiry to a model for understanding complex human biology and systems at an integrated molecular level. To accomplish this task, it may be necessary to forge global CV integrative biology networks. In short, cross strait relations have never looked more auspicious. "One country-one system" appears to be the evolutionary path ahead for the field.

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