Review

Recent advances in cardiovascular development: promise for the future

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1. Introduction

Nearly three years ago, an extra issue of \textit{Cardiovascular Research} was published which spotlighted the developing cardiovascular system (\textit{Cardiovascular Research}, Vol. 31, February, 1996). The spotlight issue was accompanied by an editorial suggesting that the field of developmental cardiology was entering a period analogous to adolescence [1]. In retrospect, that seems to have been an accurate characterization. The past three years have seen enormous growth, paradigm shifts and exciting new discoveries in the field of cardiovascular development. Applications of new molecular techniques and model systems have provided insights into many of the very basic questions relevant to the control of cardiac morphogenesis. For the first time, the promise of understanding the molecular basis of many forms of congenital heart defects seems within our grasp.

In this review, we will attempt to cover some of the major new advances that have occurred during the past three years in this rapidly expanding field. We were given the task of relating the recent developments to the reviews and papers that were contained in the original spotlight issue. Accordingly, this review will cover the same general subjects presented in the February, 1996 issue. In further keeping with our goal, each contributor to that issue was contacted and given the opportunity to provide a recent update related to their original topic (over half of the authors responded) and we appreciate those who took the time to answer. Because the field is advancing so rapidly, it is impossible to detail all the significant progress that has been made. We apologize to all whose work could not be discussed herein because of space limitations (and our own short-sightedness). Several recent reviews offer more comprehensive coverage of selected aspects of cardiac development which should provide a valuable supplement to this article [2–5].

2. Gene expression and cardiac morphogenesis

2.1. Cardiac looping and asymmetry

Looping of the heart is thought to represent one of the first asymmetric events in the developing vertebrate embryo and thus factors regulating left–right pattern formation have always been of primary interest to cardiac developmental biologists. Likewise, disruptions of left–right patterning can result in catastrophic cardiovascular defects that demand the attention of clinicians. Over the last few years, there has been an abundance of new molecules identified that show asymmetric expression in the early embryo and are potentially involved in defining the left–right body plan (reviewed in [6,7]). However, one of the most exciting new discoveries has been the recent demonstration that the \textit{iv} (inverted viscerum) mutation in mice results from a mutation in a gene encoding an axonemal dynein heavy chain [8]. This mutation causes left–right inversion, or situs inversus, in half of live-born homozygotes, thus resulting in randomization of left–right orientation. Having previously localized the \textit{iv} gene to the distal region of mouse chromosome 12 [9], Brueckner and colleagues utilized a positional cloning strategy to compare regions mutated in the \textit{iv} mouse to those altered in the legless mouse, a transgenic insertional mutant mouse that

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also displayed randomization of left–right sidedness [10]. They identified mutations in a gene they called left–right dynein (lrd) and then went on to demonstrate expression in the day 3.5 blastocyst by PCR and subsequent restricted expression of the gene to the ventral cells of the node during gastrulation using in situ hybridization. Interestingly, lrd expression was detected well before other markers of asymmetry and was not detected in the heart at any stage, thus suggesting it is involved in the very earliest stages of left–right determination. While the exact mechanisms of lrd-defined asymmetry is unknown, it is clearly a critical component in the most fundamental pathways defining the body plan.

Another major advancement in the understanding of how left–right asymmetry is established in vertebrates is the recent identification of Pitx-2 as the first transcription factor in the regulatory pathway controlling ‘handedness’ in vertebrates [11,12]. This homeodomain protein is first expressed in the left lateral plate mesoderm in Xenopus, chicken and mouse shortly after the expression the TGFβ superfamily members Sonic hedge hog (Shh) and Nodal. Shh and Nodal are known to be involved in directing establishment of the left embryonic axis and Pitx-2 can be induced by ectopic expression of these growth factors. In addition, Pitx-2 continues to be expressed asymmetrically in several organs including the left side of the heart. In iv/iv mice, Pitx-2 expression is randomized. In inv/– mice, which in contrast to iv/iv mice, demonstrate situs inversus in virtually 100% of fetuses as a result of a transgenic inactivation of a yet to be identified gene, Pitx-2 expression is restricted to the right lateral mesoderm. Finally, ectopic expression of Pitx-2 in the right lateral plate mesoderm results in isomerism or reversed looping of the heart in chicken and Xenopus. While the exact role of Pitx-2 in human congenital heart disease has yet to be determined, Pitx-2 is likely to be a critical link in the molecular pathway defining left-sided cardiac and vascular structures.

### 2.2. Chamber specification

Taking a very different approach Srivastiva and colleagues [13] have identified and characterized two complementary basic helix–loop–helix transcription factors, dHAND and eHAND, that are restricted in expression to the right and left ventricles, respectively. In addition, dHAND is expressed in neural crest derived structures. Targeted disruption of dHAND resulted in embryonic lethality by day 10.5 and embryos demonstrated a marked attenuation of the primitive right ventricle and right ventricular outflow tract. This initial set of experiments suggested that these transcription factors might play a critical role in determining left–right asymmetry in the developing embryo. To further clarify the role of the HAND genes, this group of investigators [14] crossed both the dHAND and eHAND mutant mice with the inv/inv situs inversus mice [15]. The analysis of these genetic crosses showed that dHAND was expressed in the pulmonary ventricle and eHAND in the systemic ventricle whether the ventricles were in their normal or reversed orientation. In addition, double mutant (dHAND–/–;inv–/–) embryos failed to develop a pulmonary ventricle situated to the left of a normal systemic ventricle. Thus, dHAND and eHAND do not define left and right developmental fields but rather are the first factors identified that regulate chamber specific gene transcription. In addition, these transcription factors, at least in the case of dHAND, are required for specific chamber development. While the downstream targets of HAND gene activation have yet to be identified, they provide a unique opportunity to begin the molecular characterization of ventricular specification.

### 2.3. Myocyte growth

As described above and in the original spotlight issue, investigations into regulation of myocardial transcription resulted in fundamental observations that have significantly advanced our understanding of cardiac development. In addition, these efforts have recently presented novel and unexpected findings that could be quite important in unraveling the mystery of cardiac hypertrophy after birth. In an effort to isolate cofactors that might interact with the zinc finger GATA-4 transcription factor during cardiac development (reviewed in [16]), Molkentin and colleagues [17] screened a 10.5 day embryonic heart expression library utilizing the yeast two-hybrid strategy to detect protein:protein interactions. They identified a member of the Nuclear Factor of Activated T cells family of transcription factors, NFAT3 (see below) as a potential partner, and went on to demonstrate specific interactions between NFAT3 and GATA 4 that lead to a 20-fold increase in GATA4 activity. Like all members of the NFAT family (Fig. 1), NFAT3 resides in the cytoplasm in an inactive phosphorylated state. After calcium-mediated activation of the phosphatase calcineurin, NFAT3 is dephosphorylated and translocates to the nucleus where it can participate in transcriptional activation. By developing mice that constitutively express an activated form of calcineurin or NFAT3, these investigators were able to produce cardiac hypertrophy and heart failure that mimicked the human condition. In addition, they were able to demonstrate that pharmacological inhibition of calcineurin activity inhibited the development of hypertrophy in tissue culture and in vivo. These experiments define a unique pathway regulating cardiac hypertrophy and may offer a novel strategy for the pharmacological treatment of heart failure. As described below for NFAT2 (NFATc) and endocardial differentiation, this role for NFAT3 outside the mature immune system was completely unanticipated and provides an entirely new direction for future cardiovascular research.
conjecture. However, utilizing embryonic stem cell-derived embryoid bodies, fluorescent cell sorting analysis, and differential plating strategies, several groups have recently identified such a precursor population [18–20]. These new findings provide an exciting new avenue of investigation into factors that regulate differentiation of critical components of both the blood and vascular wall.

Interestingly, the search for vascular progenitor cells has uncovered the presence of a population of circulating endothelial precursors in peripheral human blood [21]. These primitive cells were able to differentiate into ‘mature’ endothelial cells and were capable of incorporating into sites of active angiogenesis in several models of in vitro and in vivo angiogenesis to augment vessel formation. The possible utilization of these ‘roving’ angioblasts in supporting collateral vessel growth and as vehicles for delivery of angiogenic agents is quite exciting and highlights the potential benefit of harnessing developmental mechanisms to alter later disease processes.

3.1. Endothelial-pericyte interactions

Some of the most significant advances in the past 2–3 years have come as a result of efforts to decipher mechanisms of early endothelial differentiation, vascular assembly, and remodeling [22–24]. Much of this progress has been an unexpected benefit of the work of cancer biologists investigating basic paradigms of tumor neovascularization and angiogenesis. The remarkable parallels between tumor neovascularization and early vascular development in the embryo have been quite striking. Previous work in the field emphasized the interactions of vascular endothelial growth factor (VEGF) and its tyrosine kinase receptors Flk1 and Flt-1 in initial endothelial differentiation, proliferation and formation of a primary vascular plexus. More recent work has focused on critical steps in the remodeling of the primary vascular plexus and the maturation and stabilization of blood vessels (Fig. 2). To this end, the ability of vessels to recruit perivascular and smooth muscle cells has proven to be an essential process for normal vascular ontogeny and involves an intricate molecular cross-talk between developing endothelial cells and the surrounding mesenchyme (reviewed in [25,26]). In some instances, these interactions may result in a ‘transdifferentiation’ of aortic endothelial cells into smooth muscle cells, though this process is poorly understood [27,28]. However, as will be discussed later, it has become quite clear that the interaction of smooth muscle cells, particularly those of neural crest origin, with pharyngeal arch endothelial cells is a critical component in the remodeling of the immature aorta to form mature vascular structures.

A critical step in understanding the interplay between endothelial cells and pericytes was the recent cloning and initial characterization of angiopoietin-1 (Ang1), a new ligand for the receptor tyrosine kinase Tie2 (or TEK). Previously, the endothelial-specific receptor tyrosine ki-
Fig. 2. Schematic representation of the endothelial growth factors and receptors identified as important in vessel formation. Initial endothelial differentiation from the mesoderm and angioblast proliferation are mediated by VEGF and the primary VEGF receptors flk-1, flt-1. Differentiation of angioblasts into arterial or venous endothelial cells is at least partially regulated by expression of the EphrinB2 ligand and EphB4 receptor, respectively. Development of mature vessels requires recruitment of smooth muscle cells and pericytes. Ang-1 is expressed by these perivascular cells and serves as a ligand for the Tie-2 receptor, initiating endothelial expression of other growth factors that act as chemo-attractants and or mitogens for the perivascular population. Ang-2, structurally similar to Ang-1, acts as a competitive inhibitor modulating Ang-1/Tie-2 interactions. In addition, endothelin-1 (ET-1) production by endothelial cells of the aortic arches and cardiac outflow tract serves as the ligand for the endothelin receptor-A (ET_A). This receptor–ligand interaction is essential for remodeling and normal aortic arch vascular patterning.

nases, Tie1 and Tie2 were known as ‘orphan’ receptors in that their ligands had not been identified. Knock-out experiments had clearly defined a role for these receptors in angiogenesis and remodeling subsequent to the action of VEGF, as null mutant animals initiated vascular development but were unable to form mature vessels. Davis and colleagues [29] utilized a unique secretion-trap expression cloning strategy to isolate a 70-kD growth factor-like glycoprotein that specifically bound the Tie2 receptor. Although this glycoprotein was localized to areas of forming vessels, in vitro studies suggested it was not involved in endothelial proliferation or tube formation. This in vitro work was subsequently confirmed by studies performed in mice carrying a null mutation in the angiopoietin-1 gene [30]. In these mice, initial vasculogenesis and angiogenesis occurred in a normal fashion, but defects in smooth muscle and pericyte investment of developing vessels prevented remodeling and led to intrauterine vascular insufficiency at later stages of development.

Interestingly, the cardiac phenotype seen in the angiopoietin-1 knock-out was that of a poorly developed endocardium, suggesting a defective interaction with the underlying myocardium. Simultaneously with the discovery of Ang1 was a report of two families carrying a mis-sense mutation in Tie2 that resulted in venous malformations with deficient smooth muscle development [31], further supporting the pivotal role of angiopoietin-1 in endothelial cell–smooth muscle cell interactions.

Defects observed in the Ang1 knock-out mice closely resembled those previously described in the Tie2 knock-out mice, confirming the role of Ang1 as the major Tie2 ligand. All of these experiments highlight the critical role of endothelial–pericyte/smooth muscle cell interactions in directing vessel maturation. The Tie2–Ang1 signalling cascade appears to be particularly important in later stages of vessel development which do not directly involve VEGF. However, recent evidence suggests that this pleiotropic growth factor may play a role in initial pericyte recruitment, prior to the interactions of the Tie2–Ang1 system. The initial observation that VEGF was required for maintenance of newly formed vessels was made several years ago [32], but Benjamin and colleagues, using a novel model of retinal neovascularization, have recently demonstrated that VEGF facilitates this role by promoting the initial coverage of vessels by smooth muscle cells. Smooth muscle investment, in turn, leads to persistence of the vessel during subsequent remodeling and this process may be mediated by platelet-derived growth factor (PDGF) [33].

An additional layer of complexity in the regulation of angiogenesis was added with the isolation and characterization of both murine and human forms of angiopoietin-2 (Ang2), a naturally occurring antagonist of Ang1 action [34]. Human and mouse Ang2 are 80% identical to each other and 60% identical to their Ang1 homologues. Ang2 can bind Tie2 with the same affinity as Ang1, but Ang2–Tie2 interactions do not induce activation (autophosphorylation) of the Tie2 receptor, as does Ang1. Thus, Ang2 can act as competitive antagonist of Ang1. Of interest, Ang2 could induce activation of Tie2 transfected fibroblasts, suggesting there is some unique component of endothelial cells that interacts to inhibit Ang2-induced phosphorylation. Unlike Ang1, Ang2 expression is limited to the smooth muscle cell layers of the dorsal aorta and major aortic arch vessels and is not expressed in the developing heart. In the adult animal, Ang2 expression was only detectable in sites of active vascular remodeling such as the ovary, placenta, and uterus. Transgenic overexpression of Ang2 resulted in embryonic lethality during later stages of vascular ontogeny, giving a similar phenotype to the Tie2 or Ang1 knock-outs. Taken together, these experiments suggest that Ang2 can act to modulate the activity of Ang1 by inhibiting pericyte recruitment and thus create an environment conducive to endothelial proliferation and new vessel formation. These studies emphasize the delicate balance between angiostatic and angiogenic factors that must be carefully orchestrated to allow normal vessel ontogeny [35].
3.2. Not all endothelial cells are created equal

It has long been postulated that endothelial cells form very distinct populations with unique characteristics. However, the molecular confirmation of functional distinctions has been quite limited. An exciting development in the progress toward understanding endothelial heterogeneity during vascular development has recently been described in a report that the Eph-B4 receptor and its obligate ligand, the membrane bound ephrin-B2, distinguish developing veins and arteries, respectively (reviewed in Ref. [36]) from the onset of endothelial differentiation (Fig. 2). The Eph group of receptors is the largest family of receptor tyrosine kinases described and they bind ephrin ligands. Unlike other ligands for receptor tyrosine kinases, the ephrins must be membrane bound to activate their receptors and cannot, therefore, serve as diffusible signals. In addition, some of the ephrin ligands not only activate their receptor, but in turn undergo autophosphorylation during receptor binding to provide a mechanism for bidirectional signalling during cell–cell interactions. These receptor–ligand interactions have been extensively characterized as regulators of axonal guidance in the nervous system [37] but more recently, Anderson and colleagues provided convincing evidence that they are important in vascular patterning during mammalian development [38]. Utilizing inactivation of the ephrin-B2 locus by a LacZ ‘knock-in’ targeting vector, these investigators documented the expression of ephrin-B2 in both extraembryonic yolk sac and embryonic arterial endothelial cells from the initial point of endothelial differentiation from the mesoderm in heterozygote embryos. They then compared this to Eph-B4 receptor expression by in situ hybridization experiments and clearly demonstrated a non-overlapping pattern of expression restricted to developing veins. Surprisingly, when heterozygote animals were bred to homzygocity from the primary mesoderm (E7.5) but null mutations did not result in embryonic demise until E14.5. Close examination of mutant embryos revealed complete absence of aortic and pulmonary valve formation as well as ventricular septal defects which resulted in congestive heart failure and in utero death. Interestingly, the process of endothelial: mesenchymal transformation in the inflow and outflow endocardial cushions was completely normal and only minimal abnormalities were observed in the tricuspid and mitral valves (Fig. 3). These results were quite unexpected, as the expression of NFATc in the mature animal is primarily restricted to lymphocytes where it plays a critical role in calcium-mediated, calcineurin-dependent cytokine expression [42]. In addition to providing the first evidence of an endocardial specific transcription cascade, these experiments suggest that semilunar and AV formation involve distinct mechanisms. While the embryonic stimuli for activation of NFATc and subsequent downstream targets have not been identified, further elucidation of major components in this signalling cascade will provide important insights into endocardial differentiation and semilunar valve formation.

Although the role of endocardial transformation to mesenchyme during A–V valve formation has been extensively studied [43], other primary roles for the endocardium during cardiac development have not been previously appreciated. An unexpected but essential role for the endocardium in orchestrating cardiac development was ‘serendipitously’ elucidated by studies of the neuregulin growth factor (Fig. 4). This epidermal growth factor-like molecule signals through a family of protein tyrosine kinases of the EGFR family, Erb2, Erb3, Erb4 (reviewed in Ref. [44,45]) that form heterodimers (ErbB2/ErbB3 or ErbB2/ErbB4) at the cell surface. Previous studies on neuregulin signaling have primarily focused on its role in neural development as well as oncogenic transformation. The discovery that mice deficient for neuregulin develop a relatively normal early heart but fail to undergo ventricular trabeculation documented an essential role for this receptor and it’s ligands in cardiac development [46,47]. Interestingly, neuregulin is expressed by the endocardium of the heart and the ErbB2/ErbB4 complex is expressed in a reciprocal pattern by the underlying myocardium. In contrast, the ErbB2/ErbB3 complex is expressed by the mesenchymal cells adjacent to the endocardium of the endocardial cushions in the atrioventricular canal and outflow tract. Deletion of either ErbB2 [48] or ErbB4 [49] results in absent trabeculation of the embryonic ventricle,
Fig. 3. Abnormal semilunar valve formation in NFAT3(c) null mutant mice. Histological cross sections through the outflow tract of embryonic day 14.5 mice. Low power magnification (A) and high power magnification (B) of normal pulmonary valve (pv) formation compared to the absence of valve leaflet formation in knock-out (−/−) mice (C). Similar low power (E) and high power (F) magnification of normal aortic valve (av) formation in wild type embryos compared to abnormal leaflet formation in the mutant embryos (G). Normal AV valve (mv=mitral valve, tv=tricuspid valve) formation in wild type (D) and mutant embryos (H) at E14.5. (ao=aorta, rap=right atrial appendage, la=left atrium, lap=left atrial appendage, rv=right ventricle, lv=left ventricle, ivs=interventricular septum, cv=coronary vessels).

These data firmly establish the neuregulin signaling pathway between endocardium and myocardium as a very specific and essential step in ventricular morphogenesis. Likewise, targeted null mutations in the ErbB3 receptor [50,51] result in abnormal endocardial cushion development and defective valve formation resulting in congestive heart failure at E13.5. Thus, endocardial signaling via neuregulin is critical for several critical events intrinsic to myocardial development. In addition, neuregulin signaling may play a critical role in extrinsic processes directly effecting cardiac ontogeny. Recent evidence suggests that neuregulin signaling through ErbB2/Erb3 receptor heterodimers is essential for sympathetic neural crest migration from the neural tube to the dorsal aorta [52,53]. Because deleted embryos die early, the predictable spectrum of conotruncal malformations that result from neural crest ablation can not be evaluated. However, as conditional mutations are developed, it should not be surprising to find an increased incidence of conotruncal malformations in ErbB2, Erb3, and neuregulin defective embryos.

4. Contractile proteins

It is well known that expression of several contractile protein isoforms is developmentally regulated (reviewed in [54]), but the functional significance of some of these changes is unclear. In particular, it has been proposed that the expression of the slow skeletal troponin I isoform (sTnI) during fetal cardiac development is largely responsible for the lower threshold for Ca$^{2+}$-activated tension and the greater resistance to acidosis-induced desensitiza-
tion of the contractile proteins compared to adults. However, these assumptions were largely based on indirect evidence. Recently, Westfall et al. [55] used an adenovirus gene transfer approach to replace the adult cardiac TnI isoform (cTnI) with fetal ssTnI in adult rat ventricular myocytes. This isoform replacement resulted in a change to the fetal phenotype. Adult ventricular myocytes expressing ssTnI exhibited a reduced threshold for Ca$^{2+}$-activated tension and a leftward shift in the Ca$^{2+}$-tension relationship. Tension development was observed over a pCa range of 7.0–6.5 in ssTnI expressing cells, whereas cells expressing the adult isoform (cTnI) were fully relaxed over this same range of Ca$^{2+}$. Furthermore, the acidosis-induced decrease in myofilament Ca$^{2+}$ sensitivity was markedly reduced in cells expressing ssTnI compared to those expressing cTnI. Thus, these studies provide direct evidence for the role of ssTnI in conferring greater Ca$^{2+}$ sensitivity to fetal myocytes and for protecting the fetal heart from the negative inotropic effects of acidosis. In addition, this report demonstrates the utility of adenovirus-mediated gene transfer for remodeling myofilament structure and function in adult cardiac myocytes.

5. Electrophysiology

In order to function effectively as a pump, the developing embryonic heart must initiate and sustain rhythmic contractions. As the myocardial mass increases and chamber differentiation proceeds, a specialized system for conducting electrical impulses becomes necessary. Although much remains to be learned, considerable progress has been made in efforts to define the mechanisms involved in the formation of the cardiac conduction system. The embryology of the conduction system has been recently reviewed in detail by Moorman et al. [56].

Other factors regulating normal development of the conduction have recently been identified (reviewed in Ref. [57]). Most recently Schott and colleagues [58] documented that mutations in the cardiac transcription factor NKX2-5 are associated with atrial septal defects and conduction abnormalities involving the AV node. It has been known for some time that NKX2-5 is required for normal myocyte differentiation as targeted disruption of the gene in mice resulted in arrest of cardiac development at the straight heart tube stage and therefore, embryonic lethality [59]. However, these new observations are the first to implicate this, or any other transcription factor, in A–V nodal development. While the exact mechanism by which NKX2-5 regulates these critical events in cardiac morphogenesis has yet to be delineated, these data provide compelling molecular evidence for the close association of processes linking atrial septation with ontogeny of the conduction system.

For many years, a number of investigators have sought to characterize developmental differences in expression and activity of various ion channels involved in cardiac depolarization and repolarization [60]. Baruscotti and colleagues successfully isolated sino-atrial node (SAN) cells from newborn rabbits and compared their sodium current characteristics to those of adult SAN cells [61,62]. Newborn cells were found to be highly sensitive to the sodium channel blocker, tetrodotoxin (TTX) and in contrast to adult cells, exhibited significant reductions in the rate and duration of spontaneous action potentials in the presence of TTX. These developmental differences appear to be due to age-related differences in sodium channel expression. Newborn SAN cells express a neuronal type I-like isoform that was not observed in adult cells. During postnatal maturation, the expression and activity of this channel isoform declines and SAN cells assume a mature phenotype. Thus, fundamental differences in sodium channel activity may contribute to developmental changes in mechanisms involved in SAN depolarization and cardiac rhythmicity.

Another important current involved in the generation of spontaneous action potentials is the hyperpolarization-activated pacemaker current, known as $I_{f}$ [63,64]. Despite considerable investigation, the molecular basis and functional role of this current during mammalian cardiac development remains somewhat obscure. It has previously been thought that $I_{f}$ was expressed in the embryonic and fetal ventricle, but that expression was turned off during subsequent maturation. Recently, however, Robinson et al. [65] demonstrated that ventricular myocytes from newborn rats exhibit $I_{f}$ in high density and that the threshold voltage was relatively positive ($\sim 70 \text{ mV}$; well within the physiological range of resting membrane potential). In contrast, $I_{f}$ was present in equal density in adult cells, but the threshold for activation was much more negative ($\sim 113 \text{ mV}$). The implication is that $I_{f}$ expression is maintained during postnatal maturation, but the voltage dependence shifts outside the physiological range.

Ludwig et al. recently reported the molecular cloning and functional expression of genes encoding three related hyperpolarization-activated cation channels, one of which (HAC1) is present in the heart [66]. Although HAC1 (or a related gene product) is an attractive candidate, the molecular components of $I_{f}$ have yet to be clearly defined. However, we can anticipate rapid advances in this area as a result of the recent cloning of this family of ion channels.

6. Excitation–contraction coupling

6.1. Functional role of the sarcoplasmic reticulum

A number of earlier studies, although largely indirect, suggested that the sarcoplasmic reticulum (SR) plays a limited role during normal excitation–contraction (EC) coupling in immature ventricular myocytes (reviewed in [67]). However, an unanticipated recent finding is that the
SR in ventricular myocytes isolated from newborn rabbits is Ca\(^{2+}\) loaded and capable of releasing large amounts of Ca\(^{2+}\) in response to caffeine [68,69]. Comparison of the magnitude of steady-state twitches with those induced by the rapid application of caffeine (caffeine contracture) indicated that the fractional release of SR Ca\(^{2+}\) is lower in newborns than in adults, despite comparable SR Ca\(^{2+}\) load [68]. Miller et al. demonstrated that even when the SR was depleted with caffeine, contractions in newborn rabbit myocytes were largely unaffected [69]. Furthermore, the role of SR reuptake during relaxation appears to be diminished in neonatal cells, with a greater reliance on sarcolemmal Na\(^{-}\)–Ca\(^{2+}\) exchange for Ca\(^{2+}\) extrusion [68].

The conclusion from both of these studies was that although the SR at birth in rabbits appears to be fully capable of Ca\(^{2+}\) loading and release (in response to caffeine), the SR is somehow functionally isolated from triggered Ca\(^{2+}\) release during normal EC coupling. It is likely that the lack of triggered SR Ca\(^{2+}\) release is attributable to a deficiency in the cellular components involved in coupling extracellular Ca\(^{2+}\) entry to SR Ca\(^{2+}\) release (Ca\(^{2+}\)-induced Ca\(^{2+}\) release). However, much remains to be learned regarding the mechanisms of EC coupling in the fetus and embryo and the factors involved in the transition from an immature SR-independent pattern of EC coupling to the mature phenotype. Lastly, it is apparent that species differences exist in EC coupling mechanisms and ontogeny [70] which must be considered when comparing studies among different species or in attempting to extrapolate to human cardiac development.

6.2. Functional role of sarcolemmal sodium–calcium exchange

In view of the diminished role of triggered release (and subsequent reuptake) of SR Ca\(^{2+}\) during normal EC coupling in immature myocytes, it is reasonable to consider that the sarcolemmal Na\(^{-}\)–Ca\(^{2+}\) exchanger may provide the predominant source of Ca\(^{2+}\) for activation of contractions (reviewed in Ref. [67]). During the past few years, additional evidence has been reported in support of this concept. Haddock et al. measured Na\(^{-}\)–Ca\(^{2+}\) current density (I\(_{\text{NaCa}}\)) in ventricular myocytes from newborn and adult rabbits under nominally chloride-free conditions (to allow more accurate quantification of I\(_{\text{NaCa}}\)) [71]. These data confirmed greater I\(_{\text{NaCa}}\) density at birth and demonstrated that Na\(^{-}\)–Ca\(^{2+}\) exchange alone is sufficient for generating robust contractions in neonatal, but not adult myocytes. Using a different experimental approach, Chin et al. [72] demonstrated a dominant role for the Na\(^{-}\)–Ca\(^{2+}\) exchanger at birth in rabbits, but the relative magnitude of the contribution of Na\(^{-}\)–Ca\(^{2+}\) exchange to contractions was somewhat less than that reported by others.

Since Ca\(^{2+}\) transport by the Na\(^{-}\)–Ca\(^{2+}\) exchanger is dependent upon the duration of membrane depolarization (see for example [71]), it is important to take into account the effects of a shorter action potential at birth on the magnitude of Ca\(^{2+}\) transport by the exchanger. Previous voltage clamp experiments were performed using the same duration of membrane depolarization for neonates and adults, which might artificially magnify age-related changes in the functional contribution of I\(_{\text{NaCa}}\) to Ca\(^{2+}\) influx. In other words, a shorter action potential at birth might negate the impact of greater I\(_{\text{NaCa}}\) density, since there would be less time for Ca\(^{2+}\) entry under normal physiological conditions. For these reasons, Haddock et al. used the action potential voltage clamp technique to compare I\(_{\text{NaCa}}\) and contractions generated by Na\(^{-}\)–Ca\(^{2+}\) exchange in the setting of an age-appropriate action potential waveform in newborn and adult rabbit ventricular myocytes [73]. These experiments demonstrated that even when measured using a relatively shorter action potential in neonates, I\(_{\text{NaCa}}\) density was greater at birth and was sufficient to generate contractions when all other major Ca\(^{2+}\) transport pathways were blocked. In contrast, Na\(^{-}\)–Ca\(^{2+}\) exchange alone was not capable of producing contractions under these conditions in adult myocytes. Thus, despite a much shorter action potential, Ca\(^{2+}\) transport by the Na\(^{-}\)–Ca\(^{2+}\) exchanger appears to play a major role in EC coupling under physiological conditions in neonatal rabbits.

Taken together, these new results emphasize the importance of transsarcolemmal Ca\(^{2+}\) fluxes as the predominant source of activator Ca\(^{2+}\) for contractions and as the dominant pathway for Ca\(^{2+}\) extrusion during relaxation in the immature rabbit heart. What is lacking however, is the relevance of these data to other species, especially humans. Furthermore, the mechanisms of Ca\(^{2+}\) regulation in the early embryonic and fetal mammalian heart remain to be defined.

6.3. EC coupling in neural crest defects

It has generally been assumed that cardiac pump dysfunction observed in humans with congenital heart defects or in animal models of congenital heart disease are secondary to the abnormal hemodynamics engendered by the structural anomalies. More recently however, fundamental abnormalities in myocellular Ca\(^{2+}\) regulation have been demonstrated in animal models of neural crest-related cardiac malformations [74,75]. Nosek et al. physically ablated the cardiac neural crest in embryonic chicken to generate embryos with persistent truncus arteriosus [74]. Calcium regulation and EC coupling were compared in normal and neural crest ablated cardiac preparations. Markedly reduced cardiac Ca\(^{2+}\) transients were observed in the experimental animals that appeared to be due to impaired Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the SR and diminished SR reuptake of Ca\(^{2+}\). Thus, cardiac neural crest ablation affected EC coupling in chicken embryos with persistent truncus arteriosus, but the mecha-
isms responsible for this observation remain unclear at present.

Because important differences exist in the mechanisms of EC coupling in birds and mammals, it is illuminating to characterize Ca\(^{2+}\) regulation in mammalian models, as well. Accordingly, Conway et al. [75] used the homozygous splotch (Sp\(^{2H}\)) mutant mouse to characterize EC coupling in a mammalian model of abnormal neural crest-related conotruncal abnormalities. Myocytes from embryonic hearts with persistent truncus arteriosus exhibited very small Ca\(^{2+}\) transients and significantly diminished L-type Ca\(^{2+}\) current. It was speculated that these abnormalities in Ca\(^{2+}\) regulation are likely to be responsible for the embryonic heart failure and in utero lethality associated with this mutation.

In summary, two distinct models of neural crest-related cardiac malformations exhibit fundamental abnormalities in myocardial EC coupling and Ca\(^{2+}\) regulation. Although it is intriguing to speculate on the mechanism(s) and implications of these findings for human cardiac anomalies, it is clear that additional work will be necessary.

7. Clinical correlations

7.1. Neural crest and conotruncal defects

Since the initial observation almost 15 years ago by Kirby and colleagues that mechanical ablation of the cranial neural crest results in conotruncal and aortic arch anomalies [76], extensive efforts have been focused at defining the molecular cues that directly cardiac crest migration (reviewed in [77]). However, until recently, these efforts have met with very limited success. The initial observation that mice deficient for endothelin-1 demonstrated aortic arch and craniofacial anomalies along with ventricular septal defects provided one of the first cues that the endothelin signaling cascade might be involved in defining neural crest migration to the heart and pharyngeal arches [78,79]. A more recent detailed examination of the developmental expression of endothelins 1 (ET-1) and 3 (ET-3) as well as the endothelin receptors A (ET\(_A\)) and B (ET\(_B\)) during cranial and cardiac development in the human embryo has uncovered a very complicated pattern of expression [80]. While both ET\(_A\) and ET\(_B\) receptors are expressed by neural crest cells as they leave the neural tube, ET\(_A\) expression is rapidly down-regulated and subsequently restricted to a small cluster of cells within pharyngeal arches 1 and 2, and thus is unlikely to play a major role in aortic arch development. However, ET\(_A\) expression remains high in the peripheral mesoderm of pharyngeal arches 3–5, the site of active aortic arch remodeling. ET-1 expression is also high in the endothelial cells, smooth muscle cells, and both neural crest as well as non-neural crest mesoderm of the pharyngeal arches. Some perivascular cells appeared to express both ET-1 and ET\(_A\). These observations raise the interesting possibility that ET-1 mediates both autocrine (activation of receptor on the same cell that produces ET-1) and paracrine (activation of ET\(_A\) receptor on distant cell) functions in organization of the pharyngeal arches during aortic arch selection. It is therefore not surprising that defects in ET-1 lead to such dramatic cardiovascular defects.

The role of these ET-1/ET\(_A\) interactions during cardiovascular development has been clarified by recent reports of targeted null mutations in the ET\(_A\) receptor in mice [81]. In these studies, Yanagisawa and colleagues confirmed murine expression of the ET\(_A\) receptor by neural crest-derived ectomesenchymal cells in the pharyngeal arches and cardiac outflow tissues, with reciprocal ET-1 ligand expression by epithelium, paraxial mesoderm and arch vessel endothelium. Mice carrying a null mutation demonstrated severe craniofacial, aortic arch, and conotruncal cardiac defects very similar to those described in humans with DiGeorge/velocardiofacial syndrome. Virtually identical defects are detected in mice with a homozygous null mutation in endothelin-converting enzyme-1 (ECE-1), the enzyme which catalyzes the proteolytic activation of big endothelin-1 to endothelin-1 [82]. Further analysis of these mice utilizing neural crest, endothelial and smooth muscle specific markers convincingly documented that the defects observed were not a result of alterations in neural crest migration or initial vessel formation. Rather, they demonstrated that ET-1/ET\(_A\) interactions are important for post-migratory neural crest development into smooth muscle and that disruption of this signaling cascade results in asymmetrical patterning or remodeling of the aortic arch, leading to abnormal regression and persistence of specific arch arteries [83].

An additional clinically relevant cue to the molecular pathways that might result in abnormal neural crest function and subsequent aortic arch defects was recently described in mice with mutations in the Mesenchyme Fork Head-1 (MFH-1) gene [84]. MFH-1 is a forkhead (also called winged helix) transcription factor known to be diffusely expressed in cephalic neural-crest and cephalic mesoderm-derived mesenchyme in the early embryo with subsequent restriction of expression to the smooth muscle cell and endothelial cell layers of the developing dorsal aortas. Interestingly, a targeted null mutation in this gene resulted in interrupted aortic arch Type B, coarctation of the aortic arch, and occasionally, aortic arch atresia and small ventricular septal defects in newborn homozygote pups. Close examination of homozygous embryos in utero documented normal formation of the left fourth aortic arch but abnormal regression of this structure during remodeling of the aortic arch. Unlike endothelin-1 knock-out mice, in which diffuse aortic arch anomalies were seen, the MFH-1 knock-outs showed more restricted defects that are much more characteristic of defects seen in humans. In fact, one could argue that the MFH-1 knock-out provides...
one of the first single gene mutations in mice that almost exactly mirrors cardiovascular defects seen in human infants. Regardless, both ET-1 and MFH-1 mutant embryos highlight the requirement for carefully orchestrated interactions between endothelial cells, surrounding smooth muscle cells, and pericytes (neural crest and non-neural crest derived) in defining the final vascular pattern of the mature embryo. In addition, results from these recent molecular studies confirm previous morphological observations describing the importance of neural crest-derived cells in defining the mature vascular pattern [85].

Because ET_{A}, ECE-1 and MFH-1 mutant mice demonstrate craniofacial abnormalities and aortic arch defects, it is tempting to speculate that these genes might be involved in DiGeorge syndrome. DiGeorge syndrome is a haploinsufficiency syndrome characterized by aplasia or hypoplasia of the thymus and parathyroid gland, conotruncal cardiac defects, aortic arch defects, cleft palate and dysmorphic facial features (reviewed in Ref. [86]). It has been postulated that this syndrome is the result of developmental field defect involving cranial neural crest [87]. While the defects seen in the ET_{A}, ECE-1 and MFH-1 mutant embryos overlap with those seen in DiGeorge, there are no thymic or parathyroid defects noted and no abnormalities were detected in heterozygotes. In addition, MFH-1 has been mapped to chromosome 16 [88] and ET_{A} to chromosome 4q28 [82], while DiGeorge patients show a consistent microdeletion of chromosome 22q11 [89]. Therefore, it is unlikely that these genes are candidates to directly explain the DiGeorge phenotype. However, while considerable progress has been made in defining the genes within the minimal DiGeorge critical region in the human [90] and syntenic region in the mouse [91], no clear candidate genes to explain the entire phenotype have emerged. Therefore, it is possible that ET_{A}, ECE-1 and MFH-1 could interact with genes in the DiGeorge region to modify the variable phenotype seen in this syndrome.

7.2. Gap junctions and cardiac morphogenesis

While our understanding of the molecular regulation of cardiac neural crest behavior has been quite limited, initial insights into factors regulating delamination from the neural tube and migration to the conotruncal region of the heart are starting to appear. Once again, a gene critical for a developmental process was found as an ‘unanticipated’ developmental consequence of gene mutation studies. In an effort to study the role of gap junction proteins, Reaume and colleagues created a targeted null mutation of the connexin 43 gene [92]. Connexins, which are membrane channels that allow the diffusion of small molecules between cells, had been thought to be primarily involved in cell–cell conduction of electrical impulses within the heart. However, mice carrying a homozygous null mutation died at birth, not from a primary conduction abnormality, but from severe pulmonary outflow tract obstruction and right ventricular hypertrophy. In addition, transgenic mice overexpressing Cx43 in the cardiac neural crest cell population also developed conotruncal defects involving the right ventricular outflow tract [93]. These data strongly suggest that the level (either too little or too much) of Cx43-mediated intercellular communication is critical for normal cardiac neural crest migration to the outflow tract of the heart and thus is essential for normal conotruncal septation.

A subsequent study using high resolution magnetic resonance microscopy and in utero ultrasound further detailed the abnormalities in conotruncal development that result from altered levels of Cx43 expression [94] and confirmed a pivotal role for this protein in outflow tract ontogeny. This study also emphasizes the need for more technologically sophisticated non-invasive approaches to the analysis of cardiovascular defects in living murine embryos [95]. Such approaches will become critical to assist in the evaluation of the exponentially increasing number of primary in utero defects of heart and vessels that result from various experimental genetic manipulations.

8. Future directions

We have attempted to provide an overview of selected key issues in developmental cardiology which have appeared during the past 2–3 years. In so doing, it is clear that considerable progress has been made in our efforts to understand the incredibly complex processes involved in the formation of the cardiovascular system. Despite these recent advances, however, much remains to be learned. Every new answer seems to generate numerous new questions. The rational and directed application of the ever-expanding array of molecular and genetic tools is almost certain to provide the means to answer these new questions. Furthermore, as illustrated above, the integration of studies from other scientific disciplines (e.g. cancer biology, angiogenesis, CNS development, etc.) is an important source of information relevant to the study of cardiac development. It is apparent that cardiac biologists must not limit their view only to the cardiovascular system.

As alluded to in the introduction of this review, we are presently poised on the brink of a revolution in cardiac developmental biology and genetics. There now exists real potential for defining the intricacies of both normal and abnormal cardiovascular development during the coming years. The implications for this type of work are profound and include the ability to perform precise molecular diagnoses, early detection of cardiovascular abnormalities, more rational and specific therapeutic interventions, genetic approaches to treatment and ultimately, prevention of many forms of congenital and familial cardiovascular diseases. When we consider the phenomenal progress
during the past few years and the seemingly exponential rate of growth in this field, it is clear that a promising future lies ahead.

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