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Making a heart: advances in understanding the mechanisms of cardiac development

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Abstract

Purpose of review—The study of cardiac development is critical to inform management strategies for congenital and acquired heart disease. This review serves to highlight some of the advances in this field over the past year.

Recent findings—Three main areas of study are included that have been particularly innovative and progressive. These include more precise gene targeting in animal models of disease and in moving from animal models to human disease, more precise in-vitro models including three-dimensional structuring and inclusion of hemodynamic components, and expanding the concepts of genetic regulation of heart development and disease.

Summary—Targeted genetics in animal models are able to make use of tissue and time-specific promoters that drive gene expression or knockout with high specificity. In-vitro models can recreate flow patterns in blood vessels and across cardiac valves. Noncoding RNAs, once thought to be of no consequence to gene transcription and translation, prove to be key regulators of genetic function in health and disease.

Keywords

animal models; cardiac development; copy number variation; hemodynamic models; noncoding RNA

INTRODUCTION

The current review highlights some of the advances in the field of cardiac development within the past year. As this topic cannot be covered in its entirety here, we have chosen to focus on three areas of particular progress – first, tools allowing greater precision in making and interpreting animal models of congenital heart disease; second, models that incorporate hemodynamics and three-dimensional structure into in-vitro study of heart development; and third, advances in the understanding of genetics of congenital heart disease.

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Conflicts of interest

There are no conflicts of interest.

REFINING ANIMAL MODELS: TISSUE AND TIME-SPECIFIC GENE EXPRESSION

Determining the precise temporal and spatial expression pattern of cardiac genes is a cornerstone of the study of heart development. RNA labeling and lineage tracing studies (in which specific groups of cells, e.g. neural crest, can be followed over time) have demonstrated networks of overlapping and in some cases mutually exclusive genes expressed in the developing heart at different stages. Knockout experiments, in which a gene of interest is deleted from the genome of an animal model, are another cornerstone of the field. Unfortunately, knockout of key regulatory genes often results in global effects, often embryonic lethality at early stages, such that subtle or later stage roles of the gene cannot be examined. Recent studies are addressing this problem by using time-conditional and tissue-specific gene knockout, based on the expression of certain key genes in key places and times during development. A recent example used *Msx1* (a gene expressed in neural crest cells of the heart and brain) under the control of CreERT2 (a promotor activated only in the presence of the drug Tamoxifen; AstraZeneca, London, UK). This gene expression construct allows for tamoxifen-induced knockin of any gene of interest, only in neural crest cells as they migrate and incorporate into the developing heart. Using a reporter gene expressed by *Msx1CreERT2*, the authors verify recapitulation of *Msx* expression *in vivo*, which included the atrioventricular canal endocardium and endocardial cushion during early valve development [1■]. This will be an extremely useful tool to add to the growing list of tissue and time-restricted drivers of gene expression. This list already includes constructs that allow gene targeting to the endothelium (*Tie2cre*) [2], the endocardium (*Nfatc1 Cre*) [3], the neural crest (*Wnt1Cre*) [4], the second heart field/neural crest (*Islet1 Cre*) [5,6], and others. Combining these tissue-specific expression studies is also proving useful to tease out specific contributions of genes expressed in specific tissues. One group looked at the gene *Noggin*, an antagonist of bone morphogenic protein (BMP) signaling. First, they showed that endocardial-specific (*Nfatc1 Cre*) *Noggin* overexpression resulted in early embryonic lethality (E10.5) with small poorly contractile hearts. This study highlights the importance of interactions between endocardium and BMP during myocardial development [7]. Overexpression of *Noggin* in an even earlier time period, using *Nkx 2.5-Cre*, arrested heart development in the linear heart tube with poor development of contractile elements and of sodium and potassium channels necessary for membrane depolarization [8]. These findings extend the list of roles of BMP signaling in the developing heart to include structural elements within cardiac muscle. Another study used a complex combination of mutations and partial rescue to determine that BMP signaling in the endocardium (using *Tie2Cre*), not in neural crest (using *Wnt1Cre*), is responsible for the outflow tract septation defects observed in a previously described model of truncus arteriosus [9]. Combination strategies such as this allow a more precise understanding of cardiac structural phenotypes that have been described, but not fully explained, using more global knockout strategies.

Novel mouse models of human disease continue to be created and studied. Arrhythmogenic right ventricular (RV) cardiomyopathy was reported in a cardiac-restricted (*SM22a*) knockdown of the gene Rho-kinase. These mice developed desmosomal (cell–cell junction) abnormalities, myocardial fibrofatty changes, and ventricular arrhythmias similar to those found in humans [10■]. A cell polarity gene, *Vangl2*, was linked to cardiac outflow tract defects when knocked out specifically within the secondary heart field [responsible for

development of the RV and the outflow tracts (OFT)] in the mouse. Without Vangl2, cells within the OFT were shown to be disorganized, leading to a thickened, rather than elongated outflow tract [11]. Thus, cell polarity, a basic property of cell biology affecting how cells align with one another, plays an important role in the development of the outflow tract and will likely be found to be altered in other disease models. For example, in a comprehensive study from human patients to animal models and back, Durst *et al.* [12] recently showed that another cell polarity gene, DCHS1, caused mitral valve prolapse. These investigators identified a familial mutation in the *DCHS1* gene in a single multigenerational family with nonsyndromic mitral valve prolapse. Two subsequent families were later identified with different mutations in the same gene. Zebrafish knockdown and mouse knockout studies confirmed mitral valve thickening and prolapse in a mouse model. Importantly, heterozygous mice were affected, as would be expected for autosomal dominant conditions; this can be a discrepancy between humans and mouse models. Even with a well defined genetic cause in a single family, there can be significant phenotypic variation, likely the effect of so-called modifier genes. In one study examining modifying gene effects, the Ts65Dn mouse model of human trisomy 21 was crossed to an allele of *Tbx5*, a well studied regulator of cardiac development. Mice with trisomy and the *TBX5* null allele had a higher incidence of congenital heart defects than expected, specifically more conotruncal defects [13]. This is interesting in that conotruncal defects including tetralogy of Fallot (TOF) and TOF with atrioventricular septal defects are seen in human trisomy 21 patients.

EXPANDING IN-VITRO MODELS: INCLUSION OF THREE-DIMENSIONAL STRUCTURE AND HEMODYNAMICS

Cardiac stem cells have provided a powerful in-vitro model of cardiomyocyte development, from primary cell cultures, to immortalized cell lines, to induced pluripotent cells from adult tissues of any source. Stem cell differentiation protocols have been perfected to 'push' cells into various lineages, including cardiomyocytes and endothelial cells. For example, one recently reported protocol used a stirred suspension bioreactor in combination with a series of growth factors to generate cardiomyocyte spheroids with 90% purity over a course of 10 days [14]. Numerous studies have worked toward providing a three-dimensional framework to model the best geometry and mechanical stress to promote differentiation into 'mature cardiomyocytes'. Human induced pluripotent stem cells can be grown on polyethylene glycol patterned substrates coated with Matrigel (Corning, Corning, New York, USA) to form microchambers of beating cardiomyocytes [15] as an inroad to modeling cardiac chambers. As such approaches become more sophisticated and physiologic, it will be possible to model normal and abnormal hemodynamic states *in vitro*.

The effects of hemodynamic forces can be detected at the level of gene expression, and this is being appreciated in a growing number of settings. Bressan *et al.* showed enrichment of connexin 40 and Nav1.5, markers of rapid conduction in the ventricular Purkinje network, in atrial muscle bundles subjected to mechanical loading or stretch. The cell cycle marker cyclin D1 was induced in this system, implying increased proliferation of atrial myocytes under such conditions [16]. These findings have implication for atrial conduction patterning during development and potentially in the development of atrial arrhythmias in cardiac disease states. Several recent reviews highlight studies of hemodynamic effects on gene

expression in heart valves, endothelial cells, and blood vessels using high-resolution imaging techniques [17–19]. Jahnsen *et al.* showed that ablation of flow in a mouse model resulted in failure to activate Notch, a key determinant of arterial vs. venous phenotypes, in endothelial cells. Some, but not all, downstream genes of Notch were affected. This suggests that multiple factors related to mechanical stress may regulate gene expression in the endothelium [20]. Gene defects known to cause abnormal vascular patterning have been reexamined using optical techniques/three-dimensional imaging. Using three-dimensional imaging by optical projection tomography in several knockout models with vascular abnormalities, Anderson *et al.* [21] were able to distinguish primary defects in vessel patterning from defects secondary to abnormal heart development.

Complex miniaturized systems are now being utilized to assess and model hemodynamic flow *in vivo*. Pharyngeal arch flow in developing chick hearts was examined under conditions of altered flow using a combination of computational fluid dynamic simulations and in-vivo assessment of aortic flow velocity by Lindsey *et al.* Interestingly, the fourth pharyngeal arch, which forms the eventual aortic arch, was found to be the most sensitive to flow [22]. Boselli and Vermot [23] similarly used computational fluid dynamic simulations and live confocal microscopy in developing zebrafish hearts and to calculate shear stress on the myocardium. Using an alternate technique, optical coherence tomography, Midgett *et al.* [24,25] obtained both structural and Doppler flow imaging of chick hearts during cardiac looping. This technique, used widely in both ocular and intravascular imaging in humans, allows assessments of cardiac stroke volumes and wall shear stress during elongation of the cardiac outflow tract concomitant with cardiac looping. The chick embryo is an ideal model for such technology given the transparency of embryos and their ability to survive culturing at this stage. An alternate technique was reported by Ghaffari *et al.* [26,27,28] using injected endothelial-specific dye for vascular imaging and injected fluorescent microspheres imaged with a high-speed camera. This technique allows imaging of a labeled embryo over 10–16h to examine changes in flow dynamics and shear stress. Similar studies in avian blood vessels assessed hemodynamic conditions in vessel conducive to new vessel sprouting. Another interesting new technique used a gradient-index optical probe miniaturized to fit inside a pressurized rat artery to measure calcium transients with subcellular resolution within the vessel wall. Using this technique, the investigators were able to detect changes in cell shape at different pressures, showing that cell flattening in the region of IP3 receptors appeared to be to alter calcium signaling in a rat model [29]. Further refinement of techniques like these to study hemodynamics of the developing embryo likely will be of great importance in human fetuses one day.

THE NEW GENETICS: INCORPORATING NONCODING RNA AND COPY NUMBER VARIATION

Finally, it is clear that genetics is much more complicated than Mendelian inheritance. Genetic sequencing, once a complex, expensive, and timeconsuming task, is becoming more routine and feasible at the individual level. Wilson *et al.* [30] reported ‘a rapid, high-quality cost-effective comprehensive and expandable targeted next-generation sequencing assay for inheritable heart disease’ with a total run time of 3 days and cost of \$100 per sample. The

expandability of the assay allows for new probes to be added as they are discovered for cardiomyopathies and perhaps one day for congenital heart defects.

Noncoding RNA sequences have been increasingly recognized to be important in genetic regulation of congenital and acquired heart diseases. Such noncoding RNAs were previously thought to be restricted to ribosomal and tRNAs, with structural roles in DNA and RNA replication and transcription. Now it is well understood that noncoding RNAs of various sizes and complexity are key regulators of gene expression. Excellent reviews are provided by Devaux *et al.* [31], Homsy *et al.* [32], and Gelb *et al.* [33]. Generally, RNAs longer than 200 base pairs are considered long noncoding (lnc) RNAs, whereas those shorter than 200 base pairs are small, micro, or 'piwi-interacting'. MicroRNAs (miRs) tend to mediate their effects by inducing RNA degradation. Recent studies defining specific roles of miRs included stimulation of cardiomyocyte proliferation (miR-410 and miR-495) [34], linkage of BMP signaling to regulate early cardiac specification (miR130) [35], and inhibition of epithelial to mesenchymal transition in the avian atrioventricular canal (miRs 23b, 199a, and 15a) [36]. Other studies of miRs have focused on expression patterns in specific disease states, looking at patterns of expression and beginning to correlate these to function. Bittel *et al.* found 61 miRs to have altered expression in TOF myocardium compared with control, using primary cell culture derived from RV tissue removed at surgery. From these, they reported an upregulation of miR-421 in TOF patients, which has been linked in other studies to cell proliferation and outflow tract differentiation [37]. Sucharov *et al.* similarly looked at the miR profile of the RV in patients with hypoplastic left heart syndrome, using explanted hearts at transplantation compared with explanted hearts with dilated cardiomyopathy. Differences due to developmental gene defects vs. physiologic (volume and pressure loading in systemic RV) remain to be understood [38]. The same group also reported miR profiles from the serum of pediatric patients with cardiomyopathy being evaluated for transplant and found differential expression of four miRs between patients who recovered (5/34) vs. those who went on to die or require transplantation. This could represent an important biomarker to predict outcomes in such patients at the time of presentation [39].

More diverse than miRs, lncRNAs can come from intergenic, intronic, promoter, or enhancer sequences and be sense or antisense in direction. Their functions also vary, and lncRNAs can include direct repression or activation of gene transcription or of translation [31].

Enhancers in particular have been described in cardiac development; two such enhancers were reported in 2013, Fendrr in mouse heart [40] and Braveheart in mouse embryonic stem cells [41]. Using embryonic mouse hearts, Ounzain *et al.* [42] identified seven putative cardiac-specific enhancers, four of which were conserved in humans and two of which were also shown to be reactivated in adult hearts in an injury model. This same group recently reported an enhancer lncRNA in human cardiac precursor cells, termed cardiac mesoderm enhancer-associated noncoding RNA. This was shown by knockdown studies in differentiation assays to be upstream of specification of cardiac mesoderm and in injury models to be reactivated in both mouse and human hearts [43].

Single nucleotide polymorphisms (SNPs) and copy number variation (CNV) are increasingly recognized to play a role in normal genetic variability and in disease states, including congenital heart disease. SNPs have been recognized as an important contributor to genetic

variation, and maps of SNPs have been useful in comparative genetics. CNVs are sections of the genome that are repeated (or deleted) a variable number of times in an individual. Thus, the concept that every individual has exactly two alleles of each gene is actually too restrictive; individuals may have multiple copies of any given allele, and this variability may contribute to both health and disease. Like SNPs, CNVs have 'hot' spots in the genome. Recently, a comprehensive map of CNVs in the human genome was published, identifying the distribution of gains and losses of DNA segments throughout the genome using a meta-analysis approach, combining CNVs from 55 published studies using a variety of sequencing methods, and SNP-based microarrays [44■■■]. This analysis identified between 11000 and 24000 (depending on stringency of definition) CNV regions. The CNV regions identified included more areas of loss than gain and had an uneven distribution throughout the genome. This map will be a very useful reference in future studies of genetic causes of congenital heart disease. A role for CNVs in specific congenital heart lesions has been found in TOF, left ventricular outflow tract obstructive lesions, and heterotaxy but not for atrioventricular septal defects (with or without trisomy 21) [33]. Costain *et al.* [45] recently added transposition of the great arteries to this list, identifying a 2.3-fold increase in rare CNVs in these patients relative to controls and identifying two regions (chromosome 10q26 and 13q13) as critical regions containing multiple candidate genes. Glessner *et al.* examined the role of *de novo* CNVs in patients with congenital heart defects of various types with no known or suspected genetic cause, with parental DNA as comparison (congenital heart disease trios). Using a combined approach with SNP arrays and whole exome sequencing, they found 63 *de novo* CNVs in 51 patients, about 10% of their original cohort of 538 trios [46]. Some CNVs were rare and others recurrent in the population; several candidate genes were identified within the CNV regions.

CONCLUSION

Creative, integrative approaches continue to be developed and applied to the study of heart development. More specificity in gene expression and knockout studies, models that can approach not only cell function but tissue and organ function, and expanded concepts and techniques in genetics are areas of important progress. With these advances, we will continue to progress in our understanding of both congenital and acquired heart disease.

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KEY POINTS

- Tissue-specific promoters provide unprecedented precision in gene-targeting strategies in animal models of cardiac development and congenital heart disease.
- Defects in cell polarity genes have been shown to play a role in genetic cardiac abnormalities, including familial mitral valve prolapse.
- In-vitro modeling of flow and shear stress can be coupled with high-resolution imaging and with genetic expression studies, for simultaneous studies of both cardiac development and function.
- Genetic regulation of heart disease is more complex than previously understood and includes noncoding RNAs and variation in gene copy number among individuals.
- Genetic sequencing, once a complex, expensive, and time-consuming task, is becoming more routine and feasible at the individual level.