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Heart Fields and Cardiac Morphogenesis

Robert G. Kelly[1], Margaret E. Buckingham[2], and Antoon F. Moorman[3]

[1]Aix Marseille University, CNRS, IBDM UMR 7288, 13288 Marseilles, France

[2]Department of Developmental and Stem Cell Biology, URA CNRS 2578, Pasteur Institute, 75015 Paris, France

[3]Department of Anatomy, Embryology & Physiology, Academic Medical Centre, 1105 AZ Amsterdam, The Netherlands

Correspondence: robert.kelly@univ-amu.fr

In this review, we focus on two important steps in the formation of the embryonic heart: (i) the progressive addition of late differentiating progenitor cells from the second heart field that drives heart tube extension during looping morphogenesis, and (ii) the emergence of patterned proliferation within the embryonic myocardium that generates distinct cardiac chambers. During the transition between these steps, the major site of proliferation switches from progenitor cells outside the early heart to proliferation within the embryonic myocardium. The second heart field and ballooning morphogenesis concepts have major repercussions on our understanding of human heart development and disease. In particular, they provide a framework to dissect the origin of congenital heart defects and the regulation of myocardial proliferation and differentiation of relevance for cardiac repair.

In this review, we consider the origin of cardiac progenitor cells in the early embryo and show how progressive specification, differentiation, and morphogenetic events lead to formation of the embryonic heart. We will focus on two conceptually important steps: (i) the regulation of late differentiating progenitor cells (the second heart field) from pharyngeal mesoderm that drives progressive heart tube extension during looping morphogenesis, and (ii) the emergence of patterned proliferation within the embryonic myocardium that generates distinct cardiac chambers. During the transition between these steps, there is a switch from proliferation of progenitor cells outside the early heart as the heart tube elongates to myocardial proliferation within the heart to promote atrial and ventricular chamber morphogenesis. Dissection of the genetic and cellular regulation and lineage relationships implicit in the second heart field and ballooning morphogenesis models are a major focus of ongoing research. Although emphasis will be placed on heart development in the early mouse embryo, with additional insights from avian and fish models, the second heart field and ballooning morphogenesis concepts have major biomedical repercussions on our understanding of human heart development and disease. We illustrate how they provide a framework to dissect the etiology of congenital heart defects, in addition to insights into the regulation of myocardial proliferation and differentiation of relevance for cellular and paracrine approaches to cardiac repair.

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CARDIAC SPECIFICATION AND EARLY HEART TUBE DEVELOPMENT

Cells that give rise to the early heart tube are specified and differentiate in lateral anterior splanchnic mesoderm as a result of combinatorial signals from surrounding tissues. Cranial mesoderm is derived from progenitor cells that activate the bHLH transcription factor MESP1 in the primitive streak, under control of the T-box factor Eomesodermin (Saga et al. 2000; Costello et al. 2011). The pattern of inductive signals from adjacent endoderm and overlying ectoderm together with inhibitory signals from the embryonic midline and posterior region of the embryo refine the sites in which the cardiomyogenic transcriptional program is first activated (Marvin et al. 2001; Harvey 2002; Lopez-Sanchez and Garcia-Martinez 2011). These signals, including bone morphogenetic protein (BMP), fibroblast growth factor (FGF), and WNT signals, in addition to short range signaling including fibronectin mediated cascades, result in the activation of key upstream transcriptional regulators of the cardiac phenotype including genes encoding the transcription factors NKX2-5, GATA4, and TBX5, and chromatin remodeling protein SMARCD3 (BAF60c) (Lopez-Sanchez and Garcia-Martinez 2011; Cheng et al. 2013). Combinatorial transcription factor activity in turn activates the panoply of genes encoding sarcomeric components and the enzymatic machinery that define the differentiated myocardial phenotype (Bruneau 2002). Ectopic activation of SMARCD3, GATA4, and TBX5 has been shown to be sufficient to drive cardiomyogenesis in noncardiogenic regions of the embryo (Takeuchi and Bruneau 2009). Convergence of left and right precardiac regions in the embryonic anterior ventral midline results in the formation of the cardiac crescent and linear heart tube (Fig. 1). These early events in heart morphogenesis must be considered in the context of broad embryonic morphogenetic events including embryonic coelom formation and ventral folding of the embryo associated with foregut closure.

MYOCARDIAL PROGENITOR CELLS IN SUBPHARYNGEAL MESODERM: THE SECOND HEART FIELD

The early myocardium, in which cardiac contractions commence, is initially a trough open dorsally onto the ventral foregut endoderm and contiguous along its entire length with more medial splanchnic mesoderm that forms the dorsal coelomic wall of the pericardial cavity (Fig. 1A). Myocardial specification and differentiation are ongoing processes that continue in splanchnic mesoderm for a 3 d period in the mouse, throughout the time of heart tube elongation and cardiac looping. Thus initial growth

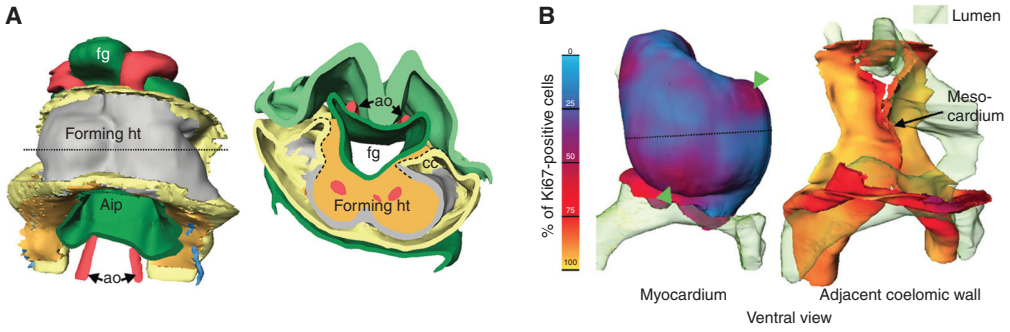

Figure 1. Early events in heart tube formation. (*A*) The forming human heart in a ventral view and transverse section at stage 9 showing the heart tube open dorsally to foregut endoderm. (*B*) Quantitative 3D reconstruction of Ki67-positive cells at stage 10 showing the paucity of proliferating cells in the myocardium compared with the dorsal pericardial wall. Note the local increase in proliferation (arrowheads) representing the initiation of ballooning morphogenesis. (From Sizarov et al. 2011; reproduced, with permission.)

of the heart tube is driven by cells that are added along its entire length. Subsequently, the dorsal mesocardium, by which the cardiac trough is suspended in the pericardial cavity, breaks down dorsally, isolating the ventral heart tube from initially contiguous splanchnic mesodermal cells in the dorsal pericardial wall (Kelly and Buckingham 2002). These cells maintain continuity with the early heart tube at the venous and arterial poles of the heart, positioned posteriorly and anteriorly, respectively. Fluorescent dye labeling and proliferation studies have revealed that these cells divide rapidly (Fig. 1B) and are displaced toward the poles of the heart tube where they progressively differentiate as the linear dimensions of the heart increase rapidly during looping morphogenesis (Tirosh-Finkel et al. 2006; Abu-Issa and Kirby 2008; van den Berg et al. 2009). Addition of progenitor cells to the heart may in fact be a major driver of rightward looping, the regulation of which is as yet poorly understood at the molecular and cellular levels. The rate of displacement of these cells toward the poles of the heart tube has been estimated at 70 μm/h in avian embryos (van den Berg et al. 2009). These subpharyngeal cells are termed the second heart field and have been shown by genetic tracing and retrospective lineage analysis in the mouse to contribute to a large part of the definitive heart including atrial and inflow myocardium at the venous pole and right ventricular and outflow tract myocardium at the arterial pole (Buckingham et al. 2005; Kelly 2012). In contrast, early differentiating cardiac cells forming the cardiac crescent are termed the first heart field and give rise to part of the left ventricle. The embryonic heart is thus comprised of cardiomyocytes derived from the cardiac crescent and linear heart as well as those derived from second heart field progenitor cells in pharyngeal mesoderm (Fig. 2). Retrospective lineage analysis and genetic tracing using Cre recombinase support a two lineage model of heart development corresponding to the contributions of the first and second heart fields (Cai et al. 2003; Meilhac et al. 2004). Furthermore, a population of late differentiating cardiomyocytes has been found to add to the poles of the frog and fish heart suggesting that this mechanism for heart tube elongation is evolutionarily conserved across vertebrate species (de Pater et al. 2009; Gessert and Kuhl 2009; Hami et al. 2011; Lazic and Scott 2011; Zhou et al. 2011). The potential of the zebrafish model for forward genetic screens suggests that this will be an informative system to probe the genetic mechanisms underlying heart tube elongation.

REGULATING PROLIFERATION AND DIFFERENTIATION IN SUBPHARYNGEAL CARDIAC PROGENITOR CELLS

Second heart field cells in subpharyngeal mesoderm are characterized by a number of core properties. First, the continued proliferation of these cells allows separation of the sites of proliferation and differentiation during early heart tube formation, potentially facilitating early cardiac function. Proliferation in subpharyngeal mesoderm is highest in the posterior re-

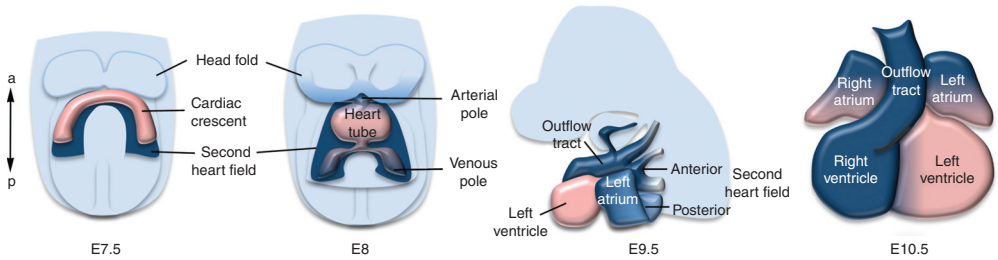

Figure 2. The contribution of the second heart field to heart tube extension. Cartoon showing the progressive addition of second heart field progenitor cells (dark blue) to the elongating heart tube between 7.5 and 9.5 d of mouse development. In the midgestation heart (*right*), second heart field–derived parts of the heart are indicated in blue. (From Kelly 2012; reproduced, with permission.)

gion of the dorsal pericardial wall in avian embryos although high rates of proliferation are observed throughout the dorsal pericardial wall in the mouse embryo (van den Berg et al. 2009; de Boer et al. 2012). Progenitor cell proliferation in pharyngeal mesoderm is regulated by canonical WNT, FGF, and Hedgehog signaling pathways (Dyer and Kirby 2009; Rochais et al. 2009). Differentiation is tightly orchestrated and triggered as cells approach the ends of the heart tube. The intercellular signaling events associated with heart tube elongation involve complex exchange between pharyngeal mesoderm and surrounding cell types, in particular pharyngeal endoderm and neural crest cells. At the arterial pole of the heart tube BMP signaling antagonizes the pro-proliferative role of FGF signaling and promotes progressive myocardial differentiation (Hutson et al. 2010; Tirosh-Finkel et al. 2010). Neural crest cells play an important role in mediating the BMP inhibition of FGF signaling during second heart field differentiation. Absence of neural crest derived mesenchyme in the pharyngeal region results in increased progenitor cell proliferation and impacts negatively on heart tube elongation. This role of neural crest derived cells in the pharyngeal region precedes their requirement within the outflow tract for arterial pole septation. Notch and noncanonical WNT signaling also regulate differentiation during second heart field deployment (High et al. 2009; Rochais et al. 2009). At the venous pole of the heart, WNT, Hedgehog, and BMP signaling have been shown to play important roles in regulating progenitor cell deployment and atrial septation (Xie et al. 2012; Briggs et al. 2013).

Gene regulatory networks controlling proliferation and differentiation in the second heart field have been identified, including regulatory nodes controlled by key transcription factors such as ISL1, NKX2-5, or TBX1 (Cai et al. 2003; Liao et al. 2008; Chen et al. 2009). TBX1, for example, promotes differentiation delay through multiple mechanisms, including direct interference with intracellular components of the BMP signaling cascade and negative regulation of *Mef2c* transcript and SRF protein levels (Chen et al. 2009; Fulcoli et al. 2009; Pane et al. 2012). Myocardial differentiation at the arterial pole of the heart tube is reinforced by BMP driven microRNA repression of *Isl1* and *Tbx1* (Wang et al. 2010). These transcription factors thus intersect with signaling pathway activity to control second heart field development. Analysis of *cis*-regulatory elements has provided insights into the wiring of transcriptional networks operative in the second heart field. Examples include two enhancers regulating *Mef2C* expression in the second heart field, one activated by ISL1 and GATA4 (Dodou et al. 2004), and one by NKX2-5 and FOXH1 (von Both et al. 2004), and an *Isl1* enhancer activated in the second heart field by FOXC2 and GATA4 (Kang et al. 2009; Kappen and Salbaum 2009). Recently, the enhancer responsible for *Fgf10* expression in the SHF has been shown to be regulated by TBX1, ISL1, and NKX2-5, with competition between ISL1 and NKX2-5 for similar homeodeomain binding sites (Watanabe et al. 2012). This is functionally important because NKX2-5 can act as a repressor in the heart tube leading to down-regulation of both *Fgf10* and *Isl1* on differentiation (Prall et al. 2002). In the case of NKX2-5, transcriptome analysis of mutant versus normal cells revealed many candidate targets in the SHF where NKX2-5 mainly functions as an activator (Prall et al. 2002). Despite these advances, systematic dissection of SHF regulatory networks remains to be performed. There is also little information about transcriptional cofactors in the second heart field or how genomic context, through transcription factors bound to adjacent sites, modulates transcriptional output. Furthermore, heterogeneity in the progenitor cell population complicates the definition of gene regulatory networks that should ideally be analyzed at the single cell level, with the challenge of relating information based on dissociated cells back to localization in the second heart field.

ENCODING DIVERSITY IN THE SECOND HEART FIELD

The progressive contribution of pharyngeal mesoderm to different regions of the elongating heart tube suggests that future cardiac regions

are prepatterned in the progenitor cell population. For example, a specific myocardial region surrounding the base of the pulmonary trunk appears to derive from a *Tbx1* dependent subpopulation of progenitor cells (Théveniau-Ruissy et al. 2008). How and when different regions of the definitive heart are prepatterned in the progenitor cell population is poorly understood. However, the importance of anterior-posterior patterning within the second heart field is illustrated by the expression profile of anterior *Hox* genes in progenitor cells in the posterior region of the second heart field that give rise to both the venous pole of the heart and the *Tbx1*-dependent subpulmonary myocardial domain at the arterial pole (Bertrand et al. 2011). Consistent with these observations, retrospective clonal analysis has revealed a clonal relationship between cardiomyocytes in the outflow tract and venous pole of the heart (Lescroart et al. 2012). Retinoic acid signaling has been shown to operate upstream of *Hox* gene expression in defining the posterior boundary of the second heart field as well as playing a later role in promoting distal outflow tract development (Ryckebusch et al. 2008; Sirbu et al. 2008). In addition to anterior–posterior patterning, intersection between second heart field regulators and the downstream laterality gene *Pitx2* plays a role in conferring left identity to structures at the arterial and venous poles of the heart tube (Liu et al. 2002).

Analysis of populations of embryonic stem cell derived cells expressing *Isl1* and *Tbx1* has shown that these genes are expressed in multipotent cardiovascular progenitor cells that can give rise to endothelial and smooth muscle cells in addition to cardiac myocytes (Laugwitz et al. 2005; Chen et al. 2009). The time at which these different cell types diverge in vivo and how this multipotency is encoded in subpharyngeal mesoderm during heart tube extension is currently unclear, although clonal analysis has identified multipotent progenitor cells in the avian second heart field (Hutson et al. 2010). Multipotency in pharyngeal mesoderm extends to a skeletal muscle fate. *Isl1*, *Tbx1*, and *Fgf10* are expressed in the progenitor cells of a subset of craniofacial skeletal muscles termed branchiomeric muscles involved in mastication, facial expression, and laryngeal and pharyngeal function (Kelly 2010). Branchiomeric skeletal muscles activate the *MyoD* family of myogenic determination genes in the core mesoderm of each pharyngeal or branchial arch. Retrospective clonal analysis has shown that branchiomeric skeletal muscles share a clonal relationship with second heart field derived parts of the heart, but not with the left ventricle, demonstrating that common cardiac and skeletal muscle progenitor cells exist in pharyngeal mesoderm after the split between the first and second cardiac lineages (Lescroart et al. 2010). Furthermore, clonally distinct populations of mesoderm exist at the level of different pharyngeal arches: First arch derived skeletal muscles involved in jaw closure share a lineage relationship with the right ventricle and second arch derived muscles involved in facial expression share a lineage relationship with outflow tract myocardium (Lescroart et al. 2010). These common lineages reflect the fact that the linear heart tube forms from anterior cranial mesoderm at the level of the future face and progressively moves in a posterior direction in the embryo as pharyngeal arch and arch artery morphogenesis proceeds. In the protochordate Ciona intestinalis, *Isl1* expressing cells adjacent to cardiac progenitor cells have been shown to have a skeletal rather than cardiac muscle fate, suggesting an evolutionary origin for the vertebrate second heart field by which a population of *Isl1* positive cells may have adopted a cardiac progenitor cell fate during vertebrate radiation (Stolfi et al. 2010). Recently, Wang and colleagues have shown that antagonism between the ascidian NKX2-5 and TBX1 homologs regulates cardiac versus skeletal muscle fate in the ascidian second heart field (Wang et al. 2013).

THE BIOMEDICAL IMPACT OF THE SECOND HEART FIELD MODEL

The outflow tract and venous pole of the heart are hotspots of congenital heart defects in human patients. Dissecting the mechanisms involved in the morphogenesis of these parts of the heart is thus an essential step toward understanding the etiology of human disease. Under-

development of the *Tbx1*-dependent myocardial domain at the base of the pulmonary trunk, for example, is considered to be the primary defect in hearts with tetralogy of Fallot, characterized by pulmonary atresia, a ventricular septal defect, overriding aorta, and right ventricular hypertrophy (van Praagh 2009). DiGeorge syndrome patients, haploinsufficient for a multigene deletion on chromosome 22 that includes *TBX1*, display a range of conotruncal congenital heart defects, including tetralogy of Fallot. The contribution of progenitor cells in the posterior region of the second heart field to the venous pole of the heart has also been associated with congenital heart defects including atrial and atrioventricular septal defects. These anomalies result from failure of development of a structure called the dorsal mesenchymal protrusion that plays a critical role in atrial and atrioventricular septation. The dorsal mesenchymal protrusion is derived from *Isl1* and *Tbx5* expressing progenitor cells in the posterior second heart field and requires Hedgehog and BMP signaling for its correct development (Snarr et al. 2007; Hoffmann et al. 2009; Briggs et al. 2013). It is important to note that perturbations of the final stages of heart tube extension have a greater likelihood of being encountered in human congenital heart defects than more severe earlier perturbations. Investigation of the mechanisms of the terminal stages of second heart field addition to the heart tube is thus likely to yield clinically relevant insights into the etiology of common human congenital heart defects. In addition to providing insights into normal and pathological heart development, identification of the signals and transcription factors regulating cardiac progenitor cell fate and triggering differentiation in the early embryo will contribute to cellular and paracrine approaches to myocardial repair.

PATTERNS OF INTRACARDIAC PROLIFERATION AND THE INITIATION OF CHAMBER MORPHOGENESIS

From midgestation in the mouse, or the fifth week of human gestation, addition of pharyngeal mesoderm to the poles of the heart is complete and there is a shift from proliferation in extracardiac progenitor cell populations to intracaradiac myocardial proliferation as the main driver of cardiac growth. The emergence of patterned gene expression domains in the embryonic heart drives chamber morphogenesis and initiates septation and conduction system differentiation. Ventricular and atrial working chamber myocardium develops on the outer curvature of the embryonic heart while cardiac cushions develop from endocardium underlying atrioventricular canal and outflow tract myocardium (Fig. 3A). BMP and Notch signaling play upstream roles in restricting the expression patterns of transcription factors of the T-box gene family to different regions of the developing heart. Overlapping expression patterns of T-box containing transcription factor

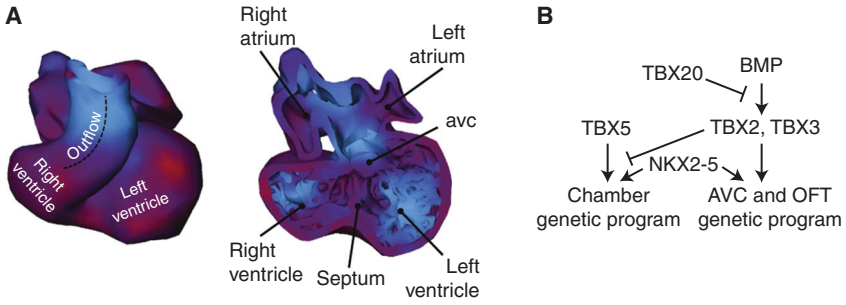

Figure 3. The emergence of patterned proliferation in the embryonic heart. (*A*) Reconstructions of the myocardium and lumen of a mouse heart at day 11 of development in ventral views showing elevated BrdU incorporation in forming camber myocardium at the outer curvature of the heart tube. (*B*) Patterned proliferation in the heart is regulated by transcription factors including T-box family members and NKX2-5 (*right*). (Panel *A* reproduced, with permission, from de Boer et al. 2012.)

encoding genes plays a central role in the emergence of cardiac form and early establishment of the cardiac conduction system (Fig. 3B) (Hoogaars et al. 2007; Greulich et al. 2011). The transcriptional repressors TBX2 and TBX3 play a role in induction of cardiac cushions and in repressing the working myocardial program in atrioventricular myocardium (Habets et al. 2002). The T-box transcriptional activators TBX20 and TBX5 restrict *Tbx2* and *Tbx3* expression to the atrioventricular canal region and compete with these factors for interaction with core myocardial transcription factors such as NKX2-5, respectively (Habets et al. 2002; Singh et al. 2009). An important outcome of these patterning events is the emergence of chamber-specific transcriptional programs and proliferative centers on the outer curvature of the embryonic heart, resulting in the development of right and left atrial and ventricular chambers through a process termed ballooning morphogenesis (Christoffels et al. 2000). In addition to patterned proliferation, cellular mechanisms contribute to ballooning morphogenesis. DiI labeling and Cre genetic tracing experiments have shown that descendants of *Tbx2* expressing cells in the outflow tract and atrioventricular canal adopt a working myocardial phenotype and contribute to growth of right and left ventricular free walls (Rana et al. 2007; Aanhaanen et al. 2009).

Growth of the ventricular wall is accompanied by the development of distinct trabeculated and compact myocardial layers. Analysis of patterns of proliferation during formation of the four-chambered mouse heart has revealed that proliferative centers at the base of the trabecules contribute to growth of the compact myocardial layer (de Boer et al. 2012). Growth of ventricular myocardium occurs in response to Notch and Neuregulin signaling from endocardium at the base of the trabecular pits and FGF signals from the epicardium and endocardium (Grego-Bessa et al. 2007; Peshkovsky et al. 2011). The regulation of ventricular growth through signals from adjacent endocardium in response to hemodynamic and flow related forces highlights the interplay between form and function in cardiac morphogenesis. Dissecting how extrinsic functional parameters such as blood flow intersect with intrinsic genetic regulation to pattern growth of the ventricular myocardium is a major challenge.

THE BIOMEDICAL IMPACT OF BALLOONING MORPHOGENESIS

Understanding the mechanisms underlying the shift to intracardiac proliferation at the onset of chamber morphogenesis and cardiac septation is of major biomedical importance. Indeed, failure of myocardial proliferation, frequently associated with altered flow patterns in the fetal heart, results in ventricular hypoplasia. Recent evidence has identified the regulation of myocardial proliferation as a key step in regenerative therapies for myocardial repair. In the zebrafish model, regeneration of damaged myocardium has been shown to primarily involve myocyte proliferation rather than de novo differentiation of resident stem cells (Kikuchi and Poss 2012). Similarly in the mouse, continued proliferation of myocytes in the first week after birth is associated with the ability of myocardial cells to repair cardiac damage by additional cycles of proliferation (Porrello et al. 2011, 2013). Dissecting the mechanisms regulating the onset of myocardial proliferation in the midgestation mouse heart will thus uncover mechanisms that may be used to trigger cell cycle reentry of postnatal myocytes.

CONCLUDING REMARKS

Understanding the origins of cardiac cells in the early embryo and the mechanisms regulating the orchestrated development of diverse lineages during cardiogenesis is an essential step toward the goals of identifying the etiology of common congenital heart defects and successfully regenerating myocardium after cardiac damage. Here we have discussed two major stages in the construction of the embryonic heart: heart tube extension by addition of progenitor cells from subpharyngeal mesoderm, and the onset of chamber development. The transition between these phases is marked by a shift in proliferation from progenitor cells outside the early heart tube to differentiated cardiomyocytes. Gaps in

our current knowledge have been highlighted, including the mechanisms by which diversity is encoded in subpharyngeal mesoderm, and how functional parameters impact on patterned proliferation in the embryonic heart. Additional insights obtained in response to these and other questions from animal models including zebrafish, mouse, and chick, will undoubtedly contribute to identifying new mechanisms regulating heart development and pathology in man.

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