Molecular mechanisms controlling the coupled development of myocardium and coronary vasculature

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ABSTRACT

Cardiac failure affects 1.5% of the adult population and is predominantly caused by myocardial dysfunction secondary to coronary vascular insufficiency. Current therapeutic strategies improve prognosis only modestly, as the primary cause – loss of normally functioning cardiac myocytes – is not being corrected. Adult cardiac myocytes are unable to divide and regenerate to any significant extent following injury. New cardiac myocytes are, however, created during embryogenesis from progenitor cells and then by cell division from existing cardiac myocytes. This process is intimately linked to the development of coronary vasculature from progenitors originating in the endothelium, the proepicardial organ and neural crest. In this review, we systematically evaluate approx. 90 mouse mutations that impair heart muscle growth during development. These studies provide genetic evidence for interactions between myocytes, endothelium and cells derived from the proepicardial organ and the neural crest that co-ordinate myocardial and coronary vascular development. Conditional knockout and transgenic rescue experiments indicate that Vegfa, Bmpr1a (ALK3), Fgfr1/2, Mapk14 (p38), Hand1, Hand2, Gata4, Zfpm2 (FOG2), Srf and Tnnd2 in cardiac myocytes, Rixa and Wt1 in the proepicardial organ, EfnB2, Tek, Mapk7, Pten, Nf1 and Casp8 in the endothelium, and Bmpr1a and Pax3 in neural crest cells are key molecules controlling myocardial development. Coupling of myocardial and coronary development is mediated by BMP (bone morphogenetic protein), FGF (fibroblast growth factor) and VEGFA (vascular endothelial growth factor A) signalling, and also probably involves hypoxia. Pharmacological targeting of these molecules and pathways could, in principle, be used to recreate the embryonic state and achieve coupled myocardial and coronary vascular regeneration in failing hearts.

LIMITATIONS OF CURRENT THERAPEUTIC APPROACHES IN CARDIAC FAILURE

Approx. 1.5% of the population of the UK – over 878,000 patients – suffer with heart failure, at an annual cost to the NHS of over £625 million a year ([1] and http://www.heartstats.org). Of patients with a diagnosis of heart failure, 30–40% will die within a year of diagnosis, and 60–70% will die within 5 years (reviewed in [2]). A major cause of heart failure is cardiac myocyte damage secondary to vascular insufficiency and ischaemia. This creates a permanent deficiency of normally functioning cardiac myocytes and results in increased myocardial wall stress, changes in cardiac size, shape and function (remodelling), neurohormonal activation and further myocyte loss (reviewed in [3]). Current
therapeutic strategies designed to reduce wall stress, remodelling and neurohormonal activation improve prognosis only modestly, as the primary cause – loss of normally functioning cardiac myocytes – is not being corrected. For instance, in the SOLVD (Studies Of Left Ventricular Dysfunction) trial, the ACE (angiotensin-converting enzyme) inhibitor enalapril improved mortality from 40 to 35% over 3.5 years; in the COPERNICUS (Carvedilol Prospective Randomized Cumulative Survival) trial, the β-blocker carvedilol improved mortality from 17 to 11.8% per year; and in the RALES (Randomized Aldactone Evaluation Study) trial, the aldosterone antagonist spironolactone improved mortality from 46 to 35% over 2 years (reviewed in [4]). As a result, there is a growing interest in myocardial replacement as definitive therapy for heart failure (reviewed in [5]).

WHEN AND WHERE ARE NEW CARDIAC MYOCYTES CREATED?

Although traditional views have indicated that the adult mammalian myocardium has limited regenerative capacity (reviewed in [6]), a number of recent studies have suggested that new cardiac myocytes can be created in the adult, either from progenitor cells, or by cardiac myocyte division. These studies have been extensively reviewed elsewhere [7–9] and are controversial [10–13]. It suffices to say that, even if they are true, the regenerative capacity of the adult myocardium is so limited that it is unable to cope with significant injury. This is in contrast with organs such as the bone marrow, intestinal epithelium or liver, which have enormous regenerative capacity throughout the lifetime of the organism.

There is relatively little controversy, however, regarding the creation of new cardiac myocytes during mammalian embryonic development. The myocardium is initially formed from cardiomyocyte progenitor cells in primary and secondary heart fields (reviewed in [14–16]). The primary heart field – recognizable as the cardiac crescent at E7.75 (embryonic day 7.75) in the mouse – thickens by E10.5. The epicardial myocardium begins to thicken by E11.5 to give rise to the compact zone (reviewed in [22]). This is associated with a high DNA synthesis rate, approaching 45% of all myocytes (reviewed in [13]). Lineage tracing experiments, where a single cell can be marked and then followed, have shown that embryonic cardiomyocytes divide to give rise to clonal cell populations of new cardiomyocytes [23,24]. Lineage analysis also shows that the compact and trabecular zones are clonally related, with wedge-shaped clones, wider at the epicardial surface, extending to the endocardial surface [24].

CORONARY VASCULAR DEVELOPMENT

The epicardium, coronary vasculature and interstitial cardiac fibroblasts arise from an outgrowth of the septum transversum called the proepicardial organ (reviewed in [25,26]). Lineage tracing in the mouse embryo shows that epithelial cells from this structure envelop the heart between E9.5 and E10.5 to form the epicardium [27,28]. Some of this epithelium turns into mesenchymal cells by E11.5–12.5. These cells migrate into the underlying subepicardial space and the myocardium, and give rise to the smooth muscle cells of the coronary vasculature and a subset of intermyocardial fibroblasts [28]. In contrast with the chick, the coronary endothelium in the mouse does not appear to be derived from the proepicardial organ, and instead originates by invagination from the endocardium [28–30]. The coronary arteries finally connect to the aorta by E13, joining the systemic circulation [30]. The development of the coronary system is initiated at the time the compact zone begins to thicken, suggesting that the two processes are biologically coupled.

APPROACHES TO MYOCARDIAL REPLACEMENT THERAPY

Based on the above concepts, one approach to myocardial replacement therapy is the use of myocardial progenitor cells, and this is currently a very active area of research (reviewed in [31,32]). Another approach is to understand the molecular pathways involved in the coupling of myocardial and coronary development. This could, in principle, provide targets for the development of novel pharmacological therapies that enhance the formation of new cardiac myocytes and vasculature in the adult heart. The purpose of this review is to systematically identify genetic evidence for pathways and mechanisms that control the formation of new cardiac myocytes and its coupling with the formation of coronary vasculature during embryogenesis in the mouse. We have focussed on genetic evidence (i.e. gene deletion or mutation), as it integrates information over the life-time of the organism.
Molecular mechanisms controlling coronary development

Table 1  Mutations associated with abnormal myocardial development

<table>
<thead>
<tr>
<th>Established factor</th>
<th>Mutated gene</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Receptor binding molecules</td>
<td>Adm*, Angpt1, Bmp10, D04*, Efnb2-Tek-cre*, Epor†, Fgf9, Fgfr1, Notch2, Nog, Wgfl, Myf2-cre†.</td>
<td>[50,52,55,66,81,93–104]</td>
</tr>
<tr>
<td>Receptors</td>
<td>Adh1b1*, Avell1*, Bmp10 × Mhca-cre, Bmp10 × Wnt1-cre, Cadd†, Epor†, Erbb2, Erbb3, Erbb4, Fgfr1 × Fgfr2 × Myf2-cre, Htr2b, Id3, Jag1 × Notch2, Pdgfra, Tek and Tgfbr3.</td>
<td>[51,52,55,57,68,69,72,100,101,105–115]</td>
</tr>
<tr>
<td>Intracellular signalling molecules</td>
<td>Fkbp1a, Gna11, Gnaq, Kras, Mapk7 × Tek-cre†, Mapk14 × Myl7-cre, Mt1, Mt1 × Tek-cre, Pak4, Pten × Tek-cre† and Ptpn11.</td>
<td>[58,59,63–65,116–124]</td>
</tr>
<tr>
<td>Transcription factors</td>
<td>Ep300*, Evi1†, Fosc1 × Fosc2, FoxH1, FoxM1, Fosq1, Gata4†, Gata4 × Mhca-cre &amp; × Nkx2-5-cre†, Hand1 × Flax × TyNkk3-5-cre × Hand2, Hdac5 × Hdac9, Hey1 × Hey2*, Hif1α*, Hoxa1, Kdrl, Mef2c*, Men1, Mt1a2, MorH11, Mycn*, Ncoa6*, Nfat4 × Nfat5-cre, Nkx2-5*, Nr2f2, Pta3, Pth1, Phd2, Ptp4, Ptp2, Rara, Rbl2, Rbx1 × Gata4-cre†, Smarc2, Smyd1, Srf × Myh7-cre, Tbx20*, Tead1*, Tead4*, Tief1, Wnt1, Wnt1 and Zlpn2†.</td>
<td>[18,27,28,36,43,53,61,62,71,73,78,79,125–168]</td>
</tr>
<tr>
<td>Cell cycle and apoptosis</td>
<td>Casp8*, Casp9 × Tiel-Cre, Cond1 × Cond2 × Cond3, Clar and Lats2.</td>
<td>[67,169–172]</td>
</tr>
<tr>
<td>Structural proteins</td>
<td>Actc1, Bcrl1, Bin1, Calα*, Igav*, Klf1a, Mesp1, Myh10, Pdil1, Pkp2, Ptp4* and Vcam1†.</td>
<td>[44,60,173–184]</td>
</tr>
<tr>
<td>Energy metabolism</td>
<td>Gys1, Mβ, Tnn1 and Tnn2 × Myl2-Cre.</td>
<td>[54,185–187]</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Adh1a2*, Ate1, Gja1†, Has2* and Met*.</td>
<td>[70,188–191]</td>
</tr>
</tbody>
</table>

and is a powerful predictor of potential drug targets [33]. We have also focussed on the mouse as, of all genetically tractable organisms, it is closest to man in terms of cardiac anatomy, physiology, development and evolution.

SYSTEMATIC IDENTIFICATION OF MOUSE MUTATIONS WITH ABNORMAL MYOCARDIAL AND CORONARY VASCULAR DEVELOPMENT

Failure of cardiomyogenesis would be predicted to give rise to visible abnormalities in ventricular myocardial development. We used the MGI (Mouse Genome Informatics) database (http://www.informatics.jax.org/) to search for the following phenotype ontology terms: abnormal myocardial trabeculae morphology (MP:0005374) to focus on those pathways that were associated with abnormal myocardial development are indicated in Table 1. These included receptor binding, receptor, intracellular signalling, transcription factor, cell cycle and apoptosis, structural factors and energy metabolism regulation (Table 1).

Importance of deconstructing heart development

A limitation of standard gene-knockout techniques is that loss of the gene in the zygote results in its loss in all tissues, both intra-embryonic and extra-embryonic. When multiple phenotypic effects are produced it becomes difficult to identify the primary function or locus of action of the gene. This is particularly important in the heart where multiple progenitor cell types (e.g. myocardial, endothelial, epicardial and neural crest) create the finished organ. A more powerful approach is to delete the gene specifically in the tissue of interest, for instance in cardiac myocytes. This uses a conditional ('floxed') allele which, combined with a tissue-restricted cre-transgene, creates a tissue-specific gene deletion (reviewed in [34]). Conditional targeting of genes in cardiac myocytes is typically achieved by using Nkx2-5-cre (cardiac progenitor cells), Myl2-cre (MLC2v-cre; ventricular myocytes), Myl7-cre (MLC2a-cre; atrial and ventricular myocytes), Myh7-cre (βMHC-cre; ventricular and atrial myocytes) and Mybca-cre (αMHC-cre; atrial and ventricular myocytes) mice [35–40]. Conditional targeted alleles that affect myocardial development are indicated in Table 1.
Role of extra-embryonic tissues in myocardial development

Of the 89 genes affecting myocardial development, 30 affected extra-embryonic tissue (i.e. allantois, chorion, amnion, extra-embryonic endoderm, trophoectoderm, umbilical cord, yolk sac and the placenta) morphology (Table 1). This suggests that abnormal extra-embryonic morphology is associated with abnormal myocardial development. Does abnormal extra-embryonic morphology or function cause abnormal myocardial development or are they linked by a common factor? Abnormalities in extra-embryonic morphology can be rescued by tetraploid complementation, which contributes wild-type cells to the trophoblast but not to the embryo. In one instance (the transcription factor Pparg), tetraploid complementation also rescued myocardial thinning, suggesting that normal extra-embryonic/placental development is essential for normal myocardial development [43]. On the other hand, the MGI database has 301 gene mutations affecting extra-embryonic morphology and most are not associated with abnormal myocardial development. Of the 60 gene mutations reported that affected placental development, only 13 also affected cardiac development. Thus further work using tissue-specific knockout is required to support the idea that normal extra-embryonic/placental development is required for normal myocardial development. Importantly, abnormal myocardial development, resulting in impaired circulation, may also affect intra- and extra-embryonic vascular development, as suggested by the Ttn (Titin) shrunken-head mutation [44].

Role of the cardiac myocyte in myocardial development

Cardiac-myocyte-restricted deletions of Erbb2, Rxra, Il6st, Gja1 and Gnaq/Gna11 do not give rise to an embryonic myocardial phenotype observed in corresponding global knockouts, indicating that their locus of action must be elsewhere [45–49]. However, embryonic myocardial development phenotypes are observed with myocardial-specific knockouts of the receptor-binding signalling molecule Vegfa, the receptors Bmpr1a and Fgfr1/Fgfr2, the transcription factors Gata4, Hand1/Hand2 and Srf, and the mitochondrial oxidoreductase Tnxrd2 [36,39,50–54]. These genes are therefore required within cardiac myocytes for normal myocardial development.

Abnormal myocardial development is frequently associated with reduced cardiac myocyte proliferation. For instance, cardiac myocyte proliferation is decreased in global knockouts of Epo, Fgf9, Gata4, Htr2b, Kras, Map2k5, Myh10, Nfatc3/Nfatc4, Tbx20 and Tnxrd2 [52,54–62]. Decreased cardiac myocyte proliferation and abnormal myocardial development observed in mice with cardiac-myocyte-specific deletion of Fgfr1/Fgfr2 and global deletion of its ligand Fgf9, indicates a specific role for FGF (fibroblast growth factor) signalling in cardiac myocyte proliferation [52]. Myocyte-restricted deletion of Mapk14 results in increased cardiac myocyte proliferation [63]. Cardiac Mapk14 activity increases spontaneously in later developmental stages and, taken together, these observations indicate a key role for Mapk14 in the normal decline in cardiac proliferation as development progresses [63].

Role of the coronary vasculature in myocardial development

A number of gene mutations that affect myocardial development also affect coronary artery development (Table 1), suggesting that the two processes are coupled. Genetic evidence indicates that normal endothelial function is necessary for myocardial development. Endothelium-restricted knockouts of Nf1, Mapk7, Efnb2 and Casp8, and of the endothelial-specific receptor tyrosine kinase Tek, result in abnormal embryonic myocardial development [64–69]. Interpretation of these experiments is of course complicated by the abnormal extra-embryonic development that occurs in endothelium-restricted Mapk7, Efnb2 and Tek knockout mice (Table 1). The most compelling genetic evidence supporting the idea that normal myocardial development is dependent on normal coronary vascular and epicardial development, however, comes from a proepicardial-organ-specific knockout of Rxra, which results in abnormal coronary vasculature and a thin myocardial wall [28]. In support of this idea, epicardial cells have been shown to secrete retinoic-acid inducible trophic protein factors that promote the proliferation of cardiac myocytes [21]. FGF2, FGF9 and WNT9b are likely candidates for these retinoic-acid-inducible trophic factors [28,52]. As discussed above, genetic evidence (Fgf9 deletion and cardiac-myocyte-restricted Fgfr1/Fgfr2 deletion) indicates that FGF signalling in cardiomyocytes is necessary for proliferation [52]. The role of retinoic acid in cardiac myocyte development is also suggested by the myocardial defects observed in Aldh1a2 (retinaldehyde dehydrogenase) knockout mice [70]. Further supporting evidence for molecular coupling of coronary and myocardial development comes from the global deletion of the transcription factor Wt1, where defective coronary vasculature and thin myocardium are rescued by transgenic Wt1 [27].

The interactions between coronary vasculature and myocardium are bidirectional. The dependence of coronary development on the myocardium is demonstrated by the ZfpM2 knockout. Here, the myocardial specific expression of ZfpM2 rescued both abnormal coronary and myocardial development [71]. Also, as discussed above, cardiomyocyte-restricted knockout of Vegfa results in coronary vascular deficiency and myocardial thinning [50]. These results indicate that initiation of myocardial development...
compact zone thickening and the development of coronary vasculature, two processes that begin at E11–12 in the mouse, occur in an interdependent and coupled manner.

**Role of the neural crest in myocardial development**

The cardiac neural crest is a population of cells that originates from the neural tube. Lineage tracing shows that these cells migrate down the branchial arches to reach the heart. Here they contribute to the formation of the aorticopulmonary septum, conotruncal cushions and smooth muscle cells in the walls of proximal coronary arteries [41]. They also contribute to a small extent to the epicardium, from where they invade the underlying myocardium like other epicardial cells [72]. Genetic evidence indicates that the neural crest is necessary for normal myocardial development. Deletion of Bmpr1a specifically in the neural crest results in defective myocardium formation [72]. In addition, mutation of Pax3, a gene expressed in the neural crest, also results in a thin myocardium, and this is rescued by transgenically expressing Pax3 [73].

**Coupled development of myocardium and coronary vasculature**

Based on conditional tissue-specific gene deletions and global knockouts that have been transgenically rescued, we have drawn a map of the cellular and molecular mechanisms that control myocardial and coronary vascular development during mouse embryogenesis (Figure 1). These experimental approaches indicate that Vegfa, Bmpr1a, Fgfr1/2, Mapk14, Hand1, Hand2, Gata4, Zfpm2, Srf and Txnr2 are essential in cardiac myocytes
for normal myocardial development. They also identify Bmpr1a and Pax3 in neural crest cells, Rxra and Wt1 in the proepicardial organ, and Efnb2, Tek, Mapk7, Pten, Nf1 and Casp8 in the endothelium as being essential for normal myocardial development. Using known protein interactions we can speculate add further molecules, such as Fgf9 and Bmpr10, which, as global knockouts, also affect myocardial development, to this map (Table 2 and Figure 1). Fgf9 is expressed in endocardium and epicardial cells and binds its receptor FGFR (FGF receptor) 1/2 on cardiac myocytes [52,74], whereas Bmpr10 is expressed in the myocardium and binds its receptor BMPR1A [BMP (bone morphogenetic protein) receptor type 1A], which is expressed on neural crest cells and on cardiac myocytes [75,76]. Taken together, these results indicate that the development of myocardium and coronary vasculature is coupled at a molecular level, and requires interactions between cardiac myocytes, endothelium and cells originating in the proepicardial organ and the neural crest. The molecular mechanisms that regulate this coupled development include BMP, FGF and VEGFA (vascular endothelial growth factor A) signalling (Figure 1) and also probably involve hypoxia in a manner analogous to the development of tumour angiogenesis [77]. In support of this idea, deletion of the hypoxia-induced factor Hif1a, dominant-negative inhibition of Hif1a in endothelial cells and deletion of the hypoxia-inducible genes Adm, Epo, Vegfa and Tek result in abnormal myocardial development [50,55,68,69,78–81].

### PROSPECTS FOR MAGIC BULLETS

In principle, it should be possible to therapeutically modulate molecular pathways in the adult heart to recreate the embryonic state and achieve coupled myocardial and coronary vascular regeneration. This will probably require simultaneous modulation of multiple pathways using combinations of small molecules and growth factors. Proof-of-concept for this idea has recently been established by experiments showing that a combination of FGF1 and the Mapk14 inhibitor SB203580 acts synergistically to induce cell division in adult cardiac myocytes [63]. What other druggable targets and pathways can be identified using information from mouse knockouts? Approx. 25 % of all marketed and experimental drugs act on GPCRs (G-protein-coupled receptors), 10 % act on kinases and 3 % on nuclear hormone receptors, indicating that these molecules are highly druggable [82]. GPCRs that control embryonic myocardial development include Adrbk1 and Htr2b. Candidate kinases and phosphatases include Epor, Bmpr1a, Erbb2, Erbb3, Erbb4, Fgfr1, Fgfr2, Il6st, Pdgfra, Tek, Tgfbr3, Map2k5, Map3k3, Mapk7, Pak4, Pten and Ptpn11. Enzymes that regulate Hif1a function, such as prolyl and asparaginyl hydroxylases (Egln1–3 and Hif1an), could also be potential targets (reviewed in [83]). Nuclear hormone receptor candidates include Rxrb, Rara and Rarg. Small molecules, such as serotonin and retinoic acid, and growth factors, such as FGFs, neuregulins, VEGF and erythropoietin, that act via these mechanisms have all been shown to induce cardiac myocyte DNA synthesis or proliferation [52,63,84–89]. High-throughput screening methods for
small molecules and growth factors that promote coupled cardiac myocyte proliferation and coronary vasculogenesis will be needed to identify optimal combinations that may be therapeutically useful. In vitro screening will probably involve the use of cell-based approaches that combine adult cardiac myocytes co-cultured with epicardial/coronary progenitor and endothelial cells grown on three-dimensional scaffolds [90–92] to mimic the cellular interactions that occur in vivo.

CONCLUSIONS

Conditional gene deletion studies in the mouse have allowed us to build a mechanistic framework that explains some of the molecular mechanisms controlling the coupled development of the myocardium and coronary vasculature during embryogenesis. Only a few of the potential mechanisms identified by global knockouts can be definitively incorporated into this framework. More extensive use of the conditional knockout approach, and the identification of protein interactions and pathways in which these genes function, will lead to more powerful and detailed models. Pharmacological targeting of these molecules and pathways could, in principle, be used to recreate the embryonic state and achieve coupled myocardial and coronary vascular regeneration in failing hearts.

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