How to make a cardiomyocyte
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ABSTRACT
During development, cardiogenesis is orchestrated by a family of heart progenitors that build distinct regions of the heart. Each region contains diverse cell types that assemble to form the complex structures of the individual cardiac compartments. Cardiomyocytes are the main cell type found in the heart and ensure contraction of the chambers and efficient blood flow throughout the body. Injury to the cardiac muscle often leads to heart failure due to the loss of a large number of cardiomyocytes and its limited intrinsic capacity to regenerate the damaged tissue, making it one of the leading causes of morbidity and mortality worldwide. In this Primer we discuss how insights into the molecular and cellular framework underlying cardiac development can be used to guide the in vitro specification of cardiomyocytes, whether by directed differentiation of pluripotent stem cells or via direct lineage conversion. Additional strategies to generate cardiomyocytes in situ, such as reactivation of endogenous cardiac progenitors and induction of cardiomyocyte proliferation, will also be discussed.

KEY WORDS: Cardiac development, Cardiomyocyte, Heart, Progenitor, Stem cell

Introduction
The heart is one of the first organs formed in the embryo, highlighting its vital function in supplying oxygen and nutrients to the various tissues of the organism both during development and in later life. In humans the primordial heart begins to beat at around 22 days after fertilization, whereas in mice this occurs around day 8. The early beating of the heart is due to the presence of cardiomyocytes, which are coordinated to contract en masse in order to pump blood and nutrients around the developing embryo. The crucial role of the cardiomyocytes in ensuring proper heart function is maintained throughout life. Thus, manipulating cardiomyocyte number and function has emerged as a focus for cardiac disease prevention and treatment.

Given the crucial role of the heart, and of the cardiomyocytes in particular, it is not surprising that diseases or injuries compromising heart function are often severe or fatal (see Box 1). Acquired heart diseases, such as myocardial infarction, often result in a massive loss of cardiomyocytes due to an ischemic event. Even though the heart has a low intrinsic cardiomyocyte turnover rate (Ali et al., 2014; Berghman et al., 2009; Kajstun et al., 2010a,b; Senyo et al., 2013), it is clearly inadequate to reverse the decreased cardiac function following injury, spurring research into alternative modes of heart repair. There are multiple different approaches for increasing cardiomyocyte numbers in the adult heart, including stimulation of proliferation in existing cardiomyocyte populations, direct lineage conversion of fibroblasts into cardiomyocytes, activation of endogenous cardiac progenitor cells, or transplantation of in vitro derived cardiomyocytes into the injured area. Over the past decade, further efforts have been made to enhance cardiac function following injury, particularly via cell therapy using non-cardiac and cardiac adult stem cell sources. However, the benefits from such treatments have remained controversial and, generally, at best modest (reviewed by Garbern and Lee, 2013; Hansson et al., 2009; Nowbar et al., 2014). Given the staggering amount of cells that are lost during a myocardial infarction (~109 (Laflamme and Murry, 2005)), it is clear that a very large number of cardiomyocytes will be required to achieve a clinically meaningful regenerative response in the heart.

In this Primer, we review the fundamental principles that govern cardiogenesis and, in particular, the important processes involved in cardiomyocyte specification and proliferation. We then provide an update on current progress in generating cardiomyocytes de novo, both in vitro and in vivo, which depends on an in-depth understanding of cardiomyocyte development and homeostasis.

Cardiac specification and development
The formation of the mammalian heart is regulated by the interplay between major developmental signaling pathways, an
Increasingly cardiac-specific array of transcription factors, as well as other transcriptional regulators. The four-chambered heart consists of the left and right atria, left and right ventricles (the myocardial components), the epicardium (the outer epithelial layer of the heart) and the endocardium (inner endothelial layer of the heart) (Fig. 1). The major cell types of the heart include cardiomyocytes, cardiac conduction cells, cardiac fibroblasts and vascular smooth muscle cells (predominantly within the myocardium) and endothelial cells (predominantly within the myocardium and endocardium) (Fig. 2). The cardiomyocyte lineage is highly specialized, consisting of several different subtypes defined both by their location and function (see Box 2). The first step in understanding how to generate functional and mature cardiomyocytes in vitro is to unravel the complexity that is intrinsic to their formation in vivo.

**Specification of the cardiac mesoderm**

Most of the cardiac cell lineages arise from the mesoderm, which is one of three distinct germ layers specified from the inner cell mass following gastrulation at embryonic day (E) 6 during mouse development (Fig. 1) (reviewed by Abu-Issa and Kirby, 2007; Rossant and Tam, 2009). The induction of mesoderm formation and its further specification into cardiac mesoderm and distinct populations of cardiac progenitor cells is primarily controlled by three families of extracellular signaling molecules: wingless-integrated (Wnt), fibroblast growth factor (FGF) and transforming growth factor-beta (TGFβ) superfamily ligands, which include Wnt3a, bone morphogenetic protein 4 (BMP4), Nodal and activin A (Fig. 2) (reviewed by Noseda et al., 2011). These ligands are expressed in gradients, thereby patterning the developing embryo and sending either activating or inhibitory instructive cues to the underlying cells, depending on the spatiotemporal context. The future cardiac field is specified in response to this combination of signaling factors and downstream transcriptional events, which eventually lead to the expression of cardiac-specific factors in a defined population of mesodermal cells (reviewed by Noseda et al., 2011).

**The next step: cardiac tissue formation**

Several cardiac progenitor pools contribute to the different cell types and structures of the nascent heart, primarily derivatives of mesoderm posterior I (Mesp1) mesodermal cells identified as the first (FHF) and second (SHF) heart fields (Figs 1 and 2) (Brade et al., 2013; Vincent and Buckingham, 2010). Of these, the FHF acts as the initiator of heart formation and is exclusively committed to a cardiomyogenic cell fate (Liang et al., 2013; Später et al., 2013). The FHF forms via the migration of Mesp1 mesodermal cells towards the anterior of the embryo where they coalesce to form a crescent-shaped structure that constitutes the first morphological sign of heart development (Fig. 1). The FHF will develop into the primitive heart tube that rapidly begins to contract in order to pump blood and nutrients throughout the embryo. The SHF progenitors are located posteriorly and medially to the FHF-derived cardiac crescent and later, behind the primitive and looping heart tube in an area also known as the pharyngeal mesoderm. From there, SHF progenitors migrate towards the primitive and looping heart tube, which serves as a scaffold for the arriving cells, allowing subsequent growth of the heart (Fig. 1).

The fine tuning of canonical Wnt signaling in a spatiotemporal manner is essential for these early steps in cardiac development. Active Wnt/β-catenin signaling is required for primitive streak and pre-cardiac mesoderm formation (Barrow et al., 2007; Haegel et al., 1995; Liu et al., 1999; Rivera-Pérez and Magnuson, 2005), whereas its inhibition via Crescent [also known as frizzled-related protein 2 (FRZB2)] and Dickkopf 1 (DKK1), expressed in the adjacent endoderm, is required to allow further specification into cardiac progenitor cells (Foley and Mercola, 2005; Schneider and Mercola, 2001). At later stages, active Wnt signaling is important for the specification and expansion of SHF progenitors; the inactivation of β-catenin in vivo results in a partial loss of SHF progenitors, whereas its constitutive activation leads to their expansion (Ai et al., 2007; Bu et al., 2009; Cohen et al., 2007; Klaus et al., 2007; Kwon et al., 2007, 2009; Lin et al., 2007; Qyang et al., 2007). FGF, BMP, Hedgehog (Hh), Notch and non-canonical Wnt signaling are also involved at various stages during FHF and SHF progenitor specification, expansion and differentiation (Cohen et al., 2008; High et al., 2009; Hoffmann et al., 2009; Klaus et al., 2007; Nagy et al., 2010;
The cardiomyocyte lineage

The mesoderm-derived FHF and SHF are the predominant sources of cardiomyocytes in the heart, with a minor contribution from the proepicardium. In vivo and in vitro lineage-tracing experiments and clonal analysis using an Islet1 (Isl1)-based reporter system have suggested that Isl1+ SHF progenitors in mouse and human can contribute to various cell lineages within the heart, such as cardiomyocytes of the RV and atria as well as vascular smooth muscle and endothelial cells (Fig. 2) (Bu et al., 2009; Moretti et al., 2006). In vivo lineage tracing and single-cell clonal analysis have demonstrated that, unlike SHF progenitors, FHF cells are marked by the expression of the ion channel hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) and are committed very early on towards a cardiomyogenic cell lineage. These cells contribute to left ventricular cardiomyocytes, as well as to cardiomyocytes of the atria (Fig. 2, Box 2) (Liang et al., 2013; Späther et al., 2013). There is a minor mutual contribution of FHF cells to the RV and of SHF cells to the left ventricle (LV) and they also both contribute to distinct parts of the cardiac conduction system (CCS), which is thought to arise through maturation of immature cardiomyocytes (Box 2) (Mikawa and Hurtado, 2007; Miquerol et al., 2011). Although under discussion, recent studies have suggested that cells of the epicardium, which undergo an epithelial-to-mesenchymal transition (EMT) and migrate into the myocardium as epicardium-derived cells (EPDCs), also contribute to a small number of cardiomyocytes (Fig. 2) (Cai et al., 2008; Christoffers et al., 2009; Katz et al., 2012; Zhou et al., 2008). The PEO has been shown to be compartmentalized into subpopulations, marked either by Wilms tumor 1/T-box18 (Wt1/Tbx18), or semaphorin 3D (Sema3d) and scleraxis (Scx) (Katz et al., 2012). Lineage-tracing studies in mouse revealed a predominant contribution of both PEO populations to vascular smooth muscle cells and fibroblasts, whereas the Sema3d/Scx-positive populations also contributed to endothelial cells (Cai et al., 2008; Katz et al., 2012; Zhou et al., 2008).

Following cardiomyocyte lineage specification, newly formed cardiomyocytes must undergo proliferation and terminal differentiation in order to produce sufficient numbers of fully functional cells. Paracrine signals from the epicardium and endocardium play a crucial role in orchestrating these processes. Secreted insulin growth factor (IGF) from the epicardium as well as canonical Wnt signaling are involved in controlling cardiomyocyte
Box 2. Cardiac myocyte subtypes within the heart
Cardiac myocytes of the adult mammalian heart display significant heterogeneity in their function with regard to anatomical location. Specialized cardiac myocyte subtypes include atrial and ventricular cardiomyocytes, and cardiac conduction cells such as those of the sinoatrial node (SAN), the atrioventricular node (AVN), the HIS bundle, the left and right bundle branches (LBB and RBB), and Purkinje fibers. The frequency at which the heart contracts under normal circumstances is determined by the pacemaker cells of the SAN that generate spontaneous action potentials leading to the depolarization and contraction of the atria. The electrical impulses converge on the AVN and spread through the ventricular conduction system (LBB, RBB, Purkinje cells), leading to a depolarization of the syncytium of cardiomyocytes, thereby ensuring orchestrated contraction of the chambers. Nodal, atrial and ventricular cardiomyocytes can be defined based on their location as well as their functional, molecular and electrophysiological properties (see table); the characteristic action potentials (APs) of cardiac myocyte subtypes are illustrated (Atkinson et al., 2011; Bird et al., 2003; Bootman et al., 2011; Chandler et al., 2009; Gourdie et al., 1998; Greener et al., 2011; Hoogaars et al., 2007; Mikawa and Hurtado, 2007; Miquerol et al., 2011; Ng et al., 2010).

<table>
<thead>
<tr>
<th>Cardiac myocyte</th>
<th>Location</th>
<th>Morphology</th>
<th>Molecular markers</th>
<th>Electrophysiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrial</td>
<td>Right and left atria</td>
<td>Central body, flat, radial projections, rod-shaped, prominent contractile apparatus, sarcomeric striation, myofibrils, lack of transverse tubules (T-tubules)</td>
<td>cMyBP-C, Mlc-2a, Cx40, Sarcolipin, Nppa, Cx43, Smpx, Hrt1</td>
<td>No distinct plateau during repolarization, see AP #1</td>
</tr>
<tr>
<td>Ventricular</td>
<td>Right and left ventricle</td>
<td>Central body, flat, radial projections, rod-shaped, prominent contractile apparatus, sarcomeric striation, distinct myofibrils, T-tubules</td>
<td>cMyBP-C, Mlc-2v, Irx4, Cx43, Hrt2, Serca</td>
<td>Distinct plateau during repolarization, see AP #2</td>
</tr>
<tr>
<td>SA nodal cell</td>
<td>Right atrial wall, entrance of superior vena cava</td>
<td>Spindle-like cells, primitive contractile apparatus, no T-tubules</td>
<td>Hcn4, Tbx18, Tbx3, Shox2, Lbh</td>
<td>‘Funny’ (Ia) or ‘pacemaker’ current, automaticity (spontaneous depolarization), see AP #3</td>
</tr>
<tr>
<td>AV nodal cell</td>
<td>Base of interatrial septum, right side</td>
<td>Primitive contractile apparatus, spindle-like or rod-shaped, no T-tubules</td>
<td>Hcn4, Cx30.2, Tbx3</td>
<td>Slow conduction velocity, see AP #4</td>
</tr>
<tr>
<td>HIS bundle, LBB, RBB</td>
<td>Top, left and right of ventricular septum</td>
<td>Primitive contractile apparatus, no T-tubules</td>
<td>Hcn4, Cx40, Tbx3 (proximal part of LBB+RBB)</td>
<td>Fast conduction velocity</td>
</tr>
<tr>
<td>Purkinje fiber cell</td>
<td>Subendocardial, ventricular wall</td>
<td>Primitive contractile apparatus, no T-tubules, dendritic or neuronal-like shape</td>
<td>Hcn4, Cx40, sMHC, Nav1.1, NF-M, Nppa, cMyBP-C</td>
<td>Fast conduction velocity</td>
</tr>
</tbody>
</table>

Molecular markers are cross-species. For some markers, the expression is enriched but not specific. cMyBP-C, cardiac myosin binding protein C; Mlc-2a, myosin light chain 2a; Cx40/30.2/43, connexin 40/30.2/43; Nppa, natriuretic peptide A; Smpx, small muscle protein, X-linked; Hrt1/2, Hairy-related transcription factor 1/2; Mlc-2v, myosin light chain 2v; Irx4, Iroquois homeobox gene 4; Serca, sarco/endoplasmic reticulum Ca2+ ATPase; Hcn4, hyperpolarization-activated cyclic nucleotide-gated channel 4; Tbx18/3, T-box transcription factor 18/3; Shox2, short stature homeobox 2; Lbh, limb bud and heart development transcription factor; sMHC, slow tonic myosin heavy chain; Nav1.1, voltage-gated sodium channel; NF-M, neurofilament-M.

numbers and thereby also the size of the mouse embryonic and postnatal heart (Buikema et al., 2013; Kerkela et al., 2008; Li et al., 2011). Interestingly, both Wnt and IGF pathways synergistically act via glycogen synthase kinase 3 beta (GSK3b) to stabilize β-catenin and are connected to the Hippo signaling pathway via the transcriptional co-activator Yes-associated protein (YAP), an important mediator of cardiac growth and size (Heallen et al., 2013; von Gise et al., 2012; Xin et al., 2011). FGFs and BMPs were important mediator of cardiac growth and size (Heallen et al., 2011). Interestingly, both Wnt and IGF pathways synergistically act via glycogen synthase kinase 3 beta (GSK3b) to stabilize β-catenin and are connected to the Hippo signaling pathway via the transcriptional co-activator Yes-associated protein (YAP), an important mediator of cardiac growth and size (Heallen et al., 2013; von Gise et al., 2012; Xin et al., 2011). For many years it was believed that cardiomyocytes undergo terminal differentiation and permanently exit the cell cycle shortly after birth. Subsequent growth of the heart, which occurs in humans until the age of 20, is thought to be mostly achieved by hypertrophy of cardiomyocytes. However, a recent study discovered a preadolescent proliferation burst at postnatal day (P) 15 in mouse, initiated by a surge in the concentration of thyroid hormone (T3), which increased the cardiomyocyte number by ~40% or 500,000 cells through the activation of the IGF1/IGF1R/AKT pathway, before subsequent growth is achieved via hypertrophy (Naqvi et al., 2014).
Establishing a cardiomyocyte gene regulatory network

The specification of the cardiomyogenic lineage is a complex process governed by extracellular signaling pathways and a network of transcription factors. Most of the heart is of mesodermal origin, as characterized by the expression of the T-box transcription factor brachyury (Bry), a direct target of canonical Wnt signaling (Yamaguchi et al., 1999). Bry+ cells will give rise to Bry+/Flk1+ [fetal liver kinase 1; also known as vascular endothelial growth factor receptor 2 (VEGFR2) and kinase insert domain receptor (KDR)] subpopulations, which further develop into hematopoietic and vascular progenitors, as well as cardiovascular progenitor cells, (Huber et al., 2004; Kattman et al., 2006; Kouskoff et al., 2005; Yang et al., 2008). Downregulation of Bry coincides with the upregulation of the T-box transcription factor eomesoderm [Eomes; also known as T-box brain protein (Tbr2)] and the long noncoding RNA Braveheart (Bvht), which in turn induce expression of the transcription factor Mesp1 along the primitive streak (Costello et al., 2011; Klattenhoff et al., 2013). Bvht further mediates epigenetic changes that facilitate cardiac lineage specification. Mesp1 is not specific to the cardiogenic mesoderm, as Mesp1-Cre lineage tracing also demonstrated its contribution to other mesodermal-derived populations in the embryo, but it is crucial for cardiac development (Chan et al., 2013). An Mesp1 mutation results in cardiac biffida and Mesp1 and Mesp2 double-mutant cells fail to contribute to the forming heart (Kitajima et al., 2000). Further, Mesp1 was shown to act upstream of a set of key cardiac-specific transcription factors, including GATA binding protein 4 (Gata4), Is11, myocyte enhancer factor 2c (Mef2c) and NK2 homeobox 5 (Nkx2.5), which are indispensable for cardiac development (Bondu et al., 2008; Kitajima et al., 2000; Saga et al., 1999).

In Drosophila, genome-wide ChiP-seq experiments revealed a transcription factor collective composed of Pannier (Pnr; a member of the GATA transcription factor family), Tinman (Tin; the homolog of vertebrate Nkx2.5) and Dorsocross1 (Doc; a T-box transcription factor), which, together with DNA-binding effectors of the Wingless (Wnt) and Decapentaplegic (Dpp; also known as BMP) signaling pathways, cooperatively bind downstream target genes that define the genetic program for cardiac differentiation (Junion et al., 2012). Experiments in mammalian systems support the requirement for these factors in cardiogenesis, and work over past decades has established a complex network of interactions between growth factors and transcriptional regulators involved in the development of the heart fields and their specification into the various cell lineages (reviewed by Vincent and Buckingham, 2010). Transcription factors, such as the zinc finger factors Gata4 and Gata5, the homeodomain factor Nkx2.5, T-box factors such as Tbx5, the MADS-box factor Mef2c, and the basic helix-loop-helix factors heart and neural crest derivatives expressed 1 (Hand1) and Hand2, are expressed at the cardiac crescent stage, when myocardial differentiation is initiated. In addition, mutations in GATA4, Nkx2.5 and in Tbx1, Tbx5 and Tbx20 all cause congenital heart disease in humans, further emphasizing their crucial role in human cardiogenesis (Bruneau, 2008; McCulley and Black, 2012; Srivastava, 2006).

Recent findings in mouse identified chromatin remodeling as a key event responsible for the onset of the cardiac master program. It was shown that, in the presence of the chromatin remodeling component BRG1-associated factor 60c [Baf60c; also known as SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3 (Smarcd3)], Gata4 and Tbx5 are sufficient to induce beating myocardial tissue when ectopically expressed in mesoderm (Takeuchi and Bruneau, 2009). Gata4 and Baf60c also trigger Nkx2.5 expression, which acts with Gata4 to initiate the cardiac program, whereas Tbx5 is required for further differentiation. Gata4 and Nkx2.5 also interact with the MADS-box transcription factor serum response factor (Srf), which, together with myocardin (Myocd), plays an important role in establishing and maintaining the contractile apparatus of cardiomyocytes (Balza and Misra, 2006; Niu et al., 2007; further reviewed by Evans et al., 2010; Noseda et al., 2011).

De novo cardiomyocyte generation in vivo

For many years, the prevailing dogma of cardiac biology was that there is no renewal of cardiomyocytes during adult life. However, this has been challenged in recent years, and today there is a growing consensus that the mammalian heart exhibits a capacity, albeit limited, to generate new cardiomyocytes (Bergmann et al., 2009, 2012; Kajstura et al., 2010a,b; Senyo et al., 2013). Huge variations in cardiomyocyte turnover rates varying from 0.5% to 40% have been reported: carbon-14 birth-dating studies suggested that fewer than 50% of cardiomyocytes are replaced over an entire life span (Bergmann et al., 2009), whereas iododeoxyuridine labeling, as used in cancer therapy, has suggested that the human heart replaces its entire myocyte population several times during life (Kajstura et al., 2010a). Although it is difficult to reconcile this variation between studies, evidence continues to mount for a low (~1% per annum) turnover rate in the adult mammalian heart, which declines with age (Ali et al., 2014; Garbern and Lee, 2013; Malliaras et al., 2013; Mollova et al., 2013; Senyo et al., 2013; Walsh et al., 2010). Given the fact that de novo cardiomyocyte generation has been observed in the adult mammalian heart, it is tempting to postulate that this phenomenon could be harnessed in order to stimulate de novo cardiomyocyte generation following cardiac injury or in disease.

Considerable debate exists as to the source of the newly generated cardiomyocytes: some studies attribute cardiomyocyte turnover mainly to proliferation, whereas others suggest a requirement for an endogenous cardiac stem or progenitor population. Importantly, these two possibilities are not mutually exclusive, and both represent possible avenues for increasing de novo cardiomyocyte generation for cardiac regenerative therapies.

Cardiomyocyte proliferation as a strategy to enhance regeneration

During early postnatal stages, the mouse heart possesses a transient robust regenerative capacity in response to injury (Naqvi et al., 2014; Porrello et al., 2011; Strungs et al., 2013) that is comparable to that of certain fish and amphibians, which can regenerate their heart throughout life (Garbern et al., 2013; Gonzalez-Rosa et al., 2011; Jopling et al., 2011; Wang et al., 2011). Unfortunately, as shown using several different injury methods, this regenerative capacity is rapidly lost (Mahmoud et al., 2013; Porrello et al., 2011; Strungs et al., 2013). During the regenerative process, the highly organized sarcomeric structure disassembles in order to allow cell division. Although postnatal regeneration has been reported by several labs (Sadek et al., 2014), a recent study questioned the neonatal regenerative capacity of mice (Andersen et al., 2014a,b). In this study, injured hearts healed after apex resection, but regeneration was incomplete and accompanied by scar formation. Further follow-up studies are required in order to determine the reason for the differences seen in cardiac regenerative capacity of postnatal mice. Nevertheless, it is important to note that in all studies neomyogenesis occurred in neonatal mice, whether complete or not, suggesting that this model constitutes a valuable tool to provide further important insights in the search for repair strategies for the adult mammalian heart (Kotlikoff et al., 2014).
Many research groups have focused their efforts on identifying the mechanisms that govern the eventual postnatal loss of cardiomyocyte proliferation, with the aim of manipulating these pathways to reactivate cardiomyocyte proliferation in the injured adult mammalian heart. The mitogen-activated protein kinase (MAPK) p38α (Mapk14) signaling pathway is a key target for such studies (Engel et al., 2005, 2006; Jopling et al., 2012). In neonatal mice, the miR-15 family and the transcription factor Meis homeobox 1 (MEIS1) play a role in the postnatal loss of cardiomyocytes upon myocardial injury, suggesting a contribution from a stem cell or precursor pool (Hsieh et al., 2007) (Table 1). The postnatal metabolic changes, from a glycolytic to an oxidative metabolism, and a coinciding DNA damage response have also been uncovered as a major mechanism to mediate permanent cell cycle exit in the majority of cardiomyocytes. Interference with these processes has been shown to prolong the postnatal proliferative window (Puente et al., 2014).

### Endogenous cardiac progenitor cells as a source for new cardiomyocytes
To date, numerous different cardiac stem or progenitor cell populations have been reported as a source of new cardiomyocytes in the adult heart. Hsieh and colleagues used an inducible lineage-tracing system permitting irreversible labeling of cardiomyocytes to trace the origin of newly formed cardiomyocytes after myocardial infarction (Fig. 3) (Bersell et al., 2009; Engel et al., 2006; Kühn et al., 2007). Postnatal metabolic changes, from a glycolytic to an oxidative metabolism, and a coinciding DNA damage response have also been uncovered as a major mechanism to mediate permanent cell cycle exit in the majority of cardiomyocytes. Interference with these processes has been shown to prolong the postnatal proliferative window (Puente et al., 2014).

### Table 1. Strategies to generate cardiomyocytes in vivo

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Method</th>
<th>Route/agent</th>
<th>Strength</th>
<th>Weakness</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell therapy</td>
<td>Transplantation of in vitro derived CMs</td>
<td>Intramyocardial</td>
<td>Large-scale human ESC-derived CMs; integration to host tissue; potential use of autologous iPSC-derived CMs/no immunogenicity</td>
<td>Arrhythmias due to immature and heterogeneous cardiomyogenic cells (atrial, ventricular, cardiac conduction cells); purification of CMs and removal of non-cardiac ESC-derived cells; risk of teratoma formation; in vivo delivery; potential need for immunosuppressive therapy</td>
<td>(Chong et al., 2014; Shiba et al., 2012)</td>
</tr>
<tr>
<td>In vivo reprogramming</td>
<td>Direct lineage conversion of cardiac fibroblasts to CMs</td>
<td>Intramyocardial retroviral delivery of transcription factors</td>
<td>Potential replacement of fibrotic scar with CMs</td>
<td>Low efficiency; immature and heterogeneous cardiomyogenic cells; in vivo delivery; cell-specific targeting; risks associated with viral gene therapy</td>
<td>(Qian et al., 2012; Song et al., 2012)</td>
</tr>
<tr>
<td>Induction of pre-existing CM proliferation</td>
<td>Manipulation of signaling pathways via miRNAs</td>
<td>Intramyocardial, AAV9 viral delivery of miRNAs</td>
<td>Relatively cell specific (AAV9); efficient</td>
<td>Risk of uncontrolled proliferation; risks associated with viral gene therapy; in vivo delivery; cell-specific targeting (non AAV9)</td>
<td>(Eulalio et al., 2012; Lin et al., 2014)</td>
</tr>
<tr>
<td>Activation and directed differentiation of adult cardiac progenitors</td>
<td>Enhanced contribution of EPDCs to CMs, paracrine factors (VEGF)</td>
<td>Intramyocardial paracrine factor delivery via modified RNA</td>
<td>Non-integrating; non-immunogenic; high efficiency in vivo expression; transient pulse-like expression</td>
<td>No long-term expression; cell-specific targeting; cationic lipid-based carrier; improvement of chemical modifications; in vivo delivery</td>
<td>(Zangi et al., 2012)</td>
</tr>
</tbody>
</table>

CM, cardiomyocyte; BM, bone marrow; EPDCs, epicardium-derived cells; ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; IP, intraperitoneal.

**Phosphoinositide 3-kinase (PI3K) and to improve cardiac function after myocardial infarction (Fig. 3)** (Bersell et al., 2009; Engel et al., 2006; Kühn et al., 2007). Postnatal metabolic changes, from a glycolytic to an oxidative metabolism, and a coinciding DNA damage response have also been uncovered as a major mechanism to mediate permanent cell cycle exit in the majority of cardiomyocytes. Interference with these processes has been shown to prolong the postnatal proliferative window (Puente et al., 2014).
secretion of paracrine factors by the c-Kit+ cells was required for the generation of these new cells (Loffredo et al., 2011). There is an ongoing debate fuelled by contradictory studies as to whether c-Kit+ cardiac stem cells (CSC) exist and whether they can give rise to cardiomyocytes (Beltrami et al., 2003; Ellison et al., 2013; Jesty et al., 2012; van Berlo et al., 2014; Zaruba et al., 2010). The observed discrepancies could be due to different experimental methodologies (for further discussion see Molkentin and Houser, 2013; Molkentin and Houser, 2014; Nadal-Ginard et al., 2014; Torella et al., 2014). As c-Kit is a relatively ubiquitous marker (including endothelial, hematopoietic, tissue monocytes), its expression alone is insufficient to define a specific c-Kit+ cell population (Smith et al., 2014). Therefore, cell surface marker signatures will be needed to define such c-Kit+ subtypes. c-Kit+ cells further progressed towards their first therapeutic use by the initiation of a first clinical trial [stem cell infusion in patients with ischemic cardiomyopathy (SCIPIO)] in 2009 (Bolli et al., 2011). Apart from c-Kit, other markers have also been suggested to label endogenous cardiac progenitor cells, which may or may not reside in the heart (Bu et al., 2009; Chong et al., 2011; Laugwitz et al., 2005; Leobon et al., 2003; Leobon et al., 2009; Makino et al., 1999; Makkar et al., 2012; Martin et al., 2004; Messina et al., 2004; Oh et al., 2003; Sampaioles et al., 2005; Smith et al., 2007; Tselsou et al., 2014; Yamada et al., 2006). To this date, putative adult endogenous cardiac progenitor cells have generally been shown to contribute to the cardiomyogenic cell lineage either in vitro or in vivo when delivered after injury; however, so far, there is no convincing evidence that they can be reactivated in situ to induce cardiac regeneration following cardiac injury.

Although not traditionally considered cardiac progenitor cells, cells of the epicardium constitute another population of interest with regard to cardiac regeneration (Table 1). EPDCs are activated during injury by the transcription factor C/EBP, which functions together with HOX, MEIS and Grainyhead (GRH) transcription factors to reactivate an embryonic gene program in the epicardium (Huang et al., 2012). Such injury-mediated activation results in an intrinsic cell fate plasticity of EPDCs that can be influenced via extrinsic factors. There is controversial data as to whether priming of Wt1+ EPDCs by the peptide Thymosin-beta 4 (TMSB4) results in their contribution to new cardiomyocytes in the adult heart in response to injury, but if so, then it is likely to be a very minimal contribution (Smart et al., 2011; Zhou et al., 2012). The observed positive effects on cardiac function and scar size (Smart et al., 2011) appear more likely to be due to enhanced neovascularization, given that EPDCs activated by injury have also been identified as a source of pro-angiogenic paracrine factors, which may enhance cardiomyocyte survival (Smart et al., 2007; Zhou et al., 2011).

The injury-induced plasticity of EPDCs can also be exploited to modify the cardiac response to injury, as shown by a recent study. Here, delivery of vascular endothelial growth factor A (VEGFA) through injection of synthetic chemically modified mRNA (modRNA) to the site of injury after myocardial infarction in mice reactivated, expanded, and directed Wt1+ epicardial cells away...
from a fibroblast cell fate towards an endothelial and myocardial cell fate. This approach improved cardiac function, reduced scar size and prolonged survival (Zangi et al., 2013). The identification of further paracrine factors that may activate repair mechanisms, or activate and recruit endogenous progenitors towards the cardiomyogenic lineage, is now experimentally and clinically feasible with this new technological platform (Lui et al., 2013, 2014).

**De novo generation of cardiomyocytes in vitro**

In addition to *in vivo* approaches, another method to overcome the massive loss of cardiomyocytes from a myocardial infarction or other cardiac insult *in vivo* is to generate cardiomyocytes or cardiac progenitor cells *in vitro* and then deliver these cells back to the injured heart. This approach relies on recent advances in stem cell and reprogramming technologies, whereby both pluripotent stem cells (PSCs) and somatic cells can be forced to adopt a cardiomyocyte fate *in vitro* (Ieda et al., 2010; Kehat et al., 2001; Maltsev et al., 1994; Metzger et al., 1995; Song et al., 2012). Such an approach has a distinct advantage over endogenous approaches in that a vast number of starting cells is available for manipulation and eventual transplantation to the injured myocardium. This is made possible by the theoretically limitless self-renewal capacity of PSCs, as well as the expansion potential of somatic cells such as fibroblasts, which can also be converted to induced pluripotent stem cells (iPSCs) via direct reprogramming (Takahashi and Yamanaka, 2006) (Fig. 3, Table 1). The prospect of generating large numbers of cardiomyocytes has implications not only for cellular therapies but also for drug screening. Patient-specific iPSCs can be generated and converted into cardiomyocytes, in order to model particular diseases and to screen for candidate drug treatments.

In order to induce a cardiomyocyte fate from PSCs or somatic cells, a detailed understanding of cardiac muscle development *in vivo* is required. Directed differentiation approaches of PSCs into cardiomyocytes mimic the steps of embryonic development in the dish, starting from cardiac mesoderm formation all the way to cardiomyocyte specification and maturation. During *in vitro* directed differentiation, PSCs are exquisitely sensitive to their immediate environment, and much emphasis is placed on the particular signaling pathways and growth factors that drive commitment to the cardiomyogenic lineage. By contrast, direct lineage conversion of other somatic cells into cardiomyocytes typically involves the delivery of transcription factors as opposed to growth factors, and the focus is more directed at understanding the distinct gene regulatory network that instructs the cardiomyocyte phenotype. Despite these conceptual differences, both approaches rely on a detailed understanding of the fundamental principles that govern cardiac development *in vivo* in order to recapitulate these events *in vitro*.

**Directed differentiation of PSCs to cardiomyocytes**

Key signaling pathways, including the Wnt, Activin/Nodal/TGFβ, BMP and FGF signaling pathways, that coordinate cardiogenesis *in vivo* also play a pivotal role in controlling the differentiation of PSCs to cardiovascular progenitor cells and cardiomyocytes *in vitro*. They are used reiteratively during cardiac differentiation in the dish and must be activated and inhibited as the differentiating culture of cells passes through specific developmental stages (Figs 2 and 3) (Kattman et al., 2011; Laflamme et al., 2007; Lian et al., 2012; Noseda et al., 2011; Paige et al., 2010; Yang et al., 2008).

Initial efforts to differentiate human embryonic stem cells (ESCs) into cardiomyocytes employed the embryoid body (EB) technique in medium containing fetal calf serum, where spheres of pluripotent cells spontaneously recapitulate early steps of embryogenesis in an uncontrolled manner (Kehat et al., 2001). This method proved to be inefficient, only yielding a low percentage (~1-5%) of cardiomyocytes. Further studies revealed that the timed addition of defined extracellular molecules, either inhibiting or activating specific intracellular signaling pathways, improved the differentiation efficiency into cardiomyocytes (Fig. 3) (Noseda et al., 2011). The temporary and dose-controlled addition of activin A and BMP4 efficiently induced cardiac mesoderm formation in mouse and human EBs characterized by co-expression of Flk1 and platelet-derived growth factor receptor alpha (Pdgfra) or by the generation of a cardiovascular progenitor characterized by co-expression of KDR and c-KIT (Kattman et al., 2011; Yang et al., 2008). Addition of defined factors, such as FGF2, VEGFA and DKK1, and inhibition of Nodal or TGFβ receptor 2 signaling, enhanced the efficiency of cardiomyocyte differentiation to greater than 50% (Cai et al., 2013; Kattman et al., 2011; Laflamme et al., 2007; Willems et al., 2012; Yang et al., 2008). However, optimization is required for individual cell lines and the differentiation process needs to be monitored for the timely addition of factors to achieve optimal results. In order to generate a relatively pure cardiomyocyte culture (up to 98%), further enrichment steps were included, such as cell sorting with an antibody against signal-regulatory protein α (SIRPA) (Dubois et al., 2011), cell purification based on biochemical differences in glucose and lactate metabolism between cardiomyocytes and non-cardiomyocytes (Tohyama et al., 2013), or cell purification based on the relatively high content of mitochondria in cardiomyocytes using mitochondria-specific fluorescent dyes (Hattori et al., 2010).

As an alternative to EB differentiation, monolayer-based directed differentiation techniques using sequential exposure to growth factors in a defined RPMI/B-27 medium have been established, generating ~30% cardiomyocytes, although significant variability was observed between cell lines and experiments (Laflamme et al., 2007; Melkoumian et al., 2010; Paige et al., 2010). This variability could be due to differences in the sensitivity to growth factors, passage number and, in the case of iPSCs, also the organ of origin, which appears to affect the response to extracellular signaling molecules (Kim et al., 2010; Ohi et al., 2011; Paige et al., 2010; Xu et al., 2012). Finally, endogenous canonical Wnt signaling was shown to be required for human ESCs to differentiate into cardiomyocytes (Paige et al., 2010). Temporal modulation of canonical Wnt signaling in an otherwise fully defined culture setting, in which canonical Wnt signaling was first enhanced and then inhibited, was sufficient to produce a high yield of 80-98% cardiomyocytes from multiple human ESC lines (Lian et al., 2012, 2013). Such a biphasic Wnt regulation mechanism is also required during cardiogenesis *in vivo* (Naito et al., 2006; Ueno et al., 2007) (Figs 2 and 3), indicating the importance of following developmental progression in the *in vitro* setting.

For the development of cell replacement therapy, it will be important to not only obtain pure cardiomyocyte cultures in a large-scale format, but also to further select for the appropriate subtype of cardiomyocytes. Different subtypes of cardiomyocytes can be distinguished by their gene expression profile and electrophysiological properties (Box 2). Myocardial infarction and heart failure predominantly affect the ventricles of the heart, and it is therefore important to be able to selectively purify the ventricular subtype of cardiomyocytes to prevent arrhythmias after transplantation to the cardiac ventricles (Table 1). Recent studies have reported some success in biasing cardiomyogenic differentiation towards an atrial versus...
ventricular cardiomyocyte subtype by manipulation of retinoic acid signaling (Jiang et al., 2012; Weng et al., 2014; Zhang et al., 2011), or towards a conduction system-like phenotype via activation of the Notch signaling pathway (Rentschler et al., 2012). Despite this progress, differentiation protocols need to be optimized further to obtain pure cultures of cardiomyocyte subtypes for transplantation.

**Direct lineage conversion of somatic cells into cardiomyocytes**

The successful reprogramming of fully differentiated fibroblasts to pluripotent cells by forced expression of the pluripotency transcription factors octamer-binding transcription factor 4 [Oct4; also known as POU domain, class 5, transcription factor 1 (Pou5f1)], SRY (sex determining region Y)-box 2 (Sox2), Kruppel-like factor 4 (Klf4) and myelocytomatosis oncogene (Myc) (collectively OSKM factors) has fueled subsequent attempts to directly convert fully differentiated cells to those of another cell lineage, without going through the pluripotent state. By complementing the original iPSC reprogramming protocol, using only OSK factors, with a chemical cocktail and modifications of cell culture conditions, it has been shown that partial dedifferentiation of fibroblasts followed by cardiomyocyte differentiation can be achieved without reaching the pluripotent stage (Efe et al., 2011). Transdifferentiation through a cardiac progenitor stage instead of a pluripotent stage was achieved by the induced expression of the transcription factors Mesp1 and Ets2 in fibroblasts (Islam et al., 2012) or of Oct4 in combination with a mix of small molecules (Wang et al., 2014). Recent studies have demonstrated the feasibility of direct lineage conversion from fibroblasts to cardiomyocyte-like cells without passing through a stem cell-like stage (Fu et al., 2013; Ieda et al., 2010; Nam et al., 2013; Qian et al., 2012; Song et al., 2012). This was achieved by the forced expression of key cardiac lineage transcription factors (see below for details) leading to partial dedifferentiation of the existing cellular phenotype and instructing a cardiomyocyte-like phenotype (Fig. 3). These elegant studies were inspired by the original observation by Weintraub and colleagues that overexpression of the myogenic master gene MyoD can convert fibroblasts to muscle cells (Davis et al., 1987), followed by successful cell lineage conversion studies in the hematopoietic system (Laiosa et al., 2006; Xie et al., 2004). An important advantage of this direct lineage conversion approach is that, by bypassing pluripotency, the risk of tumor formation is reduced, which is a concern when using PSC-derived cells for therapy purposes.

The first direct lineage conversion of postnatal cardiac and dermal fibroblasts to cardiomyocyte-like cells *in vitro* was achieved by retroviral delivery of the transcription factors Gata4, Mef2c and Tbx5 (collectively known as GMT), and later also by GMT plus Hand2 (collectively GMTH) to cardiac and tail tip murine fibroblasts. These transcription factors are all well known for their role in controlling normal cardiac development during embryogenesis (Fig. 2) (Ieda et al., 2010; Song et al., 2012). The Srivastava and Olson laboratories then independently used the GMT and GMTH cocktails, respectively, to directly reprogram resident cardiac fibroblasts in 2-month-old mice by intramyocardial injection of retroviruses encoding these transcription factors. Interestingly, this resulted in improved cardiac function and reduced scar size, pointing to a potential therapeutic approach for heart repair (Ieda et al., 2010; Qian et al., 2012; Song et al., 2012). However, the direct reprogramming efficiency was rather limited (5-7% with GMT and ~20% with GMTH) and transdifferentiated cells did not adapt a mature cardiomyocyte phenotype but one that resembled that of neonatal ventricular cardiomyocytes. Given the rather low number of cardiomyocytes generated, the functional improvement is greater than expected and it will be important to clarify whether the observed improvements were due to other effects, such as the secretion of paracrine factors, new blood vessel formation, inhibition of fibrosis, or improvement of cardiomyocyte survival (Fig. 3, Table 1). The forced expression of the miRNAs miR-1, -133, -208 and -499 was also sufficient to directly convert mouse fibroblasts into cardiomyocytes *in vitro* and *in vivo*, which was enhanced by inhibition of Janus kinase 1 (JAK1) (Jayawardena et al., 2012) (Fig. 3). Importantly, the ability to generate functional cardiac-like myocytes via direct lineage conversion is not limited to the mouse, but has also been accomplished using human adult cardiac and dermal fibroblasts, and neonatal foreskin fibroblasts, where reprogramming was achieved within 4-11 weeks by a different combination of factors, including GATA4, HAND2, Tbx5, MYOCD, as well as two microRNAs, miR-1 and miR-133 (Nam et al., 2013). In another study, expression of GMT plus estrogen-related receptor gamma (ESRRG), MESP1, MYOCD and zinc finger protein, FOG family member 2 (ZFPM2) induced a cardiomyocyte-like phenotype in human fibroblasts derived from ESCs, fetal heart, and neonatal skin (Fu et al., 2013).

Other examples of induced conversions of cell fate within the cardiac lineage include demonstrations that cardiomyocytes can be forced to adopt the identity of other cardiomycogenic subtypes, such as cells of the CCS. A sinoatrial-like phenotype was induced by forced expression of Tbx18 or Tbx3, as verified by pacemaker characteristics *in vitro* and *in vivo*, whereas activation of Notch signaling in cardiomyocytes resulted in Purkinje-like cells (Fig. 3) (Bakker et al., 2012; Kapoor et al., 2013; Rentschler et al., 2012).

Much work remains to optimize direct reprogramming approaches and to fully understand the mechanistic aspects of directed reprogramming to cardiac lineages. A major problem remains the incomplete reprogramming into fully mature cardiomyocytes (Chen et al., 2012) and the rather heterologous population of predominantly atrial- and ventricular-like cardiomyocytes generated through reprogramming, which might increase the risk of arrhythmic events following transplantation. To further translate this method toward clinical application, many other issues need to be addressed, particularly with regard to efficacy and safety. Examples include the open chest surgical procedure currently used for cardiac delivery of molecules and/or cells that perhaps can be replaced by catheter-based delivery during a coronary artery intervention after myocardial infarction, and the need to move to non-integrative methods for delivery of reprogramming factors instead of viruses that integrate into the genome.

**From bench to bedside: functional maturation and delivery of *in vitro* derived cardiomyocytes**

In order to move *in vitro* derived cardiomyocytes into the clinical setting, it is important to directly compare the maturity, function and durability of these cells with the resident cardiomyocytes found in the adult heart. Characterization of PSC-derived cardiomyocytes has revealed that they share molecular, electrical, mechanical and ultrastructural features with primary cardiomyocytes, but also exhibit diverse characteristics of fetal rather than adult cardiomyocytes. A recent study has shown that long-term cultures of differentiated PSCs can result in structural and functional maturation to adult-like cardiomyocytes (Lundy et al., 2013), but achieving full maturation to an adult phenotype *in vitro* appears to remain elusive. However, it is important to recognize that this might not be a problem for transplantation purposes, as it has been shown that immature cardiomyocytes exhibit a markedly higher engraftment rate than adult
cardiomyocytes when transplanted to the injured heart, and immature cardiomyocytes are likely to mature as they integrate into the recipient myocardium (Reinecke et al., 1999). Two elegant studies have documented the feasibility of transplanting PSC-derived cardiomyocytes to injured hearts in model organisms. In the first study, ESC-derived cardiomyocytes were transplanted into rodent hearts and the results indicate that, at least to some extent, such transplants can integrate functionally with the recipient myocardium, couple electrically with cardiomyocytes from the host, suppress arrhythmias, and improve cardiac output by contributing to the pumping capacity of the heart (Shiba et al., 2012). The second study highlighted the feasibility of producing clinical-scale human ESC-derived cardiomyocytes in vast numbers, as well as their successful intra-myocardial delivery to a non-human primate model of myocardial ischemia followed by reperfusion, moving this approach a substantial step closer towards clinical application (Chong et al., 2014). Here, the human ESC-derived cardiomyocytes formed new muscle grafts that were perfused by host vasculature and electromechanically coupled to the host myocytes; however, non-ventricular arrhythmias were observed and these need to be overcome before further translation to the clinical setting is possible.

Recent advances also allow for the large-scale generation of human PSC-derived endothelial progenitors, which constitutes an attractive cell population for therapeutic regenerative vascularization (Sahara et al., 2014). The capacity to produce scalable cultures of purified human PSC-derived cardiomyocytes and endothelial cells will also be of importance for tissue engineering attempts to produce more organized cardiac tissue. Early experiments have shown that co-culture of PSC-derived cardiac patches with fibroblasts and/or endothelial cells lead to vascularization and better survival of the graft (Sekine et al., 2008; Stevens et al., 2009). With the enormous progress made in large-scale derivation of cardiomyocytes from human PSCs, additional progress in tissue engineering is required to improve the function, engrafting, survival, electromechanical integration and delivery of engineered cardiac tissue for use in transplantation therapies. At the same time, more advanced engineered cardiac tissue would provide for better drug screening and patient-specific disease modeling in vitro (further reviewed by Cinemta et al., 2013; Hirt et al., 2014; Vunjak-Novakovic et al., 2011).

Future directions

Over the past decade, a host of novel therapeutic strategies have been developed that are aimed at capitalizing on recent advances in our understanding of the pathways and principles that guide cardiogenesis itself. These include both cell-based and paracrine factor-based therapeutic approaches, each employing different technological platforms (peptide, recombinant protein, plasmid, adenoviral, adeno-associated viruses, retroviruses, miRNA, RNAi) in attempting to drive the generation of new cardiomyocytes, additional vascular components (both endothelial and vascular smooth muscle) and/or to inhibit fibrosis and accompanying scar formation (reviewed by Pttszke et al., 2012). More recent studies have suggested that direct reprogramming of non-myocytes in the intact heart to cardiomyocyte-like cells could be an alternative approach (Qian et al., 2012; Song et al., 2012). All these strategies have inherent advantages and limitations (regarding such as safety, scalability, efficiency and duration of expression, in vivo delivery, target specificity, off-target effects, other organ side effects), necessitating additional studies to determine which, if any of these approaches, could eventually be promoted to clinical practice.

We believe that the new technological platform of modRNAs provides a promising route for the short-term, localized expression of paracrine factors [such as VEGFA (Zangi et al., 2013)] in diseased hearts, but future studies will need to determine their safety and efficacy compared with alternative therapeutic approaches. In any case, the key to understanding how to remake a human heart might lie in unraveling the pathways that make the heart during cardiogenesis itself. A more refined map of human cardiogenesis and the development of novel human and non-human primate model systems are on the horizon, which should lay the foundation for the next generation of strategies for regenerative cardiology.

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Competing interests

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