Review

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Focus: Induced Pluripotency & Cellular Reprogramming

Programming and reprogramming a human heart cell

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Abstract

The latest discoveries and advanced knowledge in the fields of stem cell biology and developmental cardiology hold great promise for cardiac regenerative medicine, enabling researchers to design novel therapeutic tools and approaches to regenerate cardiac muscle for diseased hearts. However, progress in this arena has been hampered by a lack of reproducible and convincing evidence, which at best has yielded modest outcomes and is still far from clinical practice. To address current controversies and move cardiac regenerative therapeutics forward, it is crucial to gain a deeper understanding of the key cellular and molecular programs involved in human cardiogenesis and cardiac regeneration. In this review, we consider the fundamental principles that govern the "programming" and "reprogramming" of a human heart cell and discuss updated therapeutic strategies to regenerate a damaged heart.

Keywords cardiac progenitor cell; cardiac regeneration; cardiomyocyte proliferation; embryonic heart field; reprogramming

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Introduction

Heart disease is the leading cause of mortality in the industrialized world, with insufficient therapeutic options and poor prognosis (Lopez *et al*, 2006). The adult mammalian heart cannot sufficiently regenerate or replace damaged cardiac tissue with new functional muscle after injury. Given the drastic shortage of donor hearts for transplantation, this calls for an urgent need to develop novel regenerative therapies to repair severely diseased hearts (Hansson *et al*, 2009). In this regard, cell transplantation approaches are attractive, due to the potential of various stem cell populations to promote cardiac regeneration and repair in experimental models of heart disease and to their feasibility of use in the clinics (Sanganalmath & Bolli, 2013). There have been a number of attempts to transplant cells to diseased hearts using a wide range of cell types, such as autologous/allogenic non-cardiac somatic stem cells and putative endogenous cardiac progenitor cells (CPCs). However, they have at best yielded mixed results and are still far from clinical practice (Ptaszek *et al*, 2012).

Meanwhile, recent revolutionary work in the fields of stem cell biology and cardiac regenerative medicine has progressively moved our understanding of human cardiac development and homeostasis forward, opening novel paths toward cardiac regeneration. For example, since the breakthrough discovery that fully differentiated mouse and human fibroblasts can be reprogrammed into pluripotent stem cells by retroviral transduction of four defined factors (*Oct3/4*, *Sox2*, *c-Myc*, and *Klf4*) (Takahashi & Yamanaka, 2006; Takahashi *et al*, 2007), modifications to this original protocol have been developed to directly reprogram somatic cells into cardiac lineage cells, bypassing the pluripotent state. Pioneering this field, the Srivastava group showed successful direct conversion of murine fibroblasts to cardiomyocyte-like cells *in vitro* and *in vivo* by a specific combination of cardiac transcriptional factors (*Gata4*, *Mef2c*, and *Tbx5*) (Ieda *et al*, 2010; Qian *et al*, 2012). Despite these encouraging results, much more work will be needed to optimize the technology before it is transferred to clinical testing. Some of the critical issues that need to be resolved include the low reprogramming efficiency and the possible risk of viral transduction-mediated tumorigenesis, which remains a subject of debate.

Post-natal cardiomyocyte renewal/turnover in mammals is another recent discovery in this field (Garbern & Lee, 2013). Over the last decade, the classical 20th-century paradigm that the human heart is a post-mitotic and terminally developed organ with no cell renewal/replication capability has been overturned. Recent studies from several laboratories have demonstrated that cardiomyocyte turnover occurs throughout life in mammals, including humans (Bergmann *et al*, 2009; Kajstura *et al*, 2010; Mollova *et al*, 2013; Senyo *et al*, 2013). Although the estimated rate of mammalian cardiomyocyte renewal varies from study to study depending on the method used to measure it, most reports find a remarkably low annual turnover rate of approximately 1%, which increases modestly after injury but declines with age. This demonstrates afresh that the inherent capability in humans to regenerate myocardium with aging or after injury in adulthood is entirely insufficient, encouraging researchers to investigate strategies to increase human cardiomyocyte renewal.

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So where are we now on the translational road map from stem cell biology to true regenerative therapeutics for heart disease? Importantly, even though much has been uncovered over the decades, the key programs governing human heart development and regeneration/replication remain undetermined. To address current controversies and achieve authentic cardiac regeneration in the clinical setting, it is mandatory for us to understand cardiac development ("programming" a heart) and regeneration ("reprogramming" a diseased heart) on a much deeper level, by employing rigorous research to elucidate the core mechanisms underlying these processes. In this review, we discuss the fundamental principles that govern the "programming" of a developing heart at the cellular/molecular level. We then provide an overview of current and novel therapeutic strategies for heart regeneration in humans, including stem cell transplantation and cellular "reprogramming" approaches, some of which are being tested clinically. Finally, we consider current controversies and issues to be addressed, and show where the field of cardiac regenerative biology and medicine is headed.

Fundamental principles of cardiac development and regeneration

Embryonic heart fields and multipotent CPCs

First and second heart field CPCs The human heart is a complex organ system and is composed of highly diverse cell types, including cardiomyocytes, conductive cells of the cardiac conduction system (CS), vascular smooth muscle cells (SMCs), and endothelial cells (ECs). All of these cells must be assembled into discrete anatomic and functional structures at the earliest embryonic stages (Hansson *et al*, 2009; Vincent & Buckingham, 2010). This assembly is a complicated and sequential morphogenetic process that depends on the spatiotemporally regulated contribution of multipotent CPCs (Buckingham *et al*, 2005; Moretti *et al*, 2006; Wu *et al*, 2006; Musunuru *et al*, 2010). The first differentiated myocardial cells are detected in the cardiac crescent in the splanchnic mesoderm at murine embryonic day (E) 7.5 (Fig 1). The crescent region is referred to as the first heart field (FHF) and is marked by expression of a broad heart field marker gene, *Nkx2-5* (Wu *et al*, 2006; Brade *et al*, 2013), and also by expression of the ion channel *HCN4* (hyperpolarization-activated cyclic nucleotide-gated channel 4) (Liang *et al*, 2013; Spater *et al*, 2013). The crescent/FHF then fuses at the midline to form the primitive heart tube that begins to pump blood. The second heart field (SHF) is instead specifically marked by *Isl1* expression (Cai *et al*, 2003) and lies medially and posteriorly to the crescent/FHF (Fig 1). The SHF progenitors then migrate behind the heart tube and extend anteriorly and posteriorly into the pharyngeal mesoderm to form the looping heart tube at E9.0 in concert with the FHF progenitors (Laugwitz *et al*, 2005; Moretti *et al*, 2006; Nakano *et al*, 2008; Bu *et al*, 2009). As the embryo grows, FHF derivatives give rise to left ventricular myocardium, with partial contribution to the atria, whereas SHF derivatives contribute to myocardium of the right ventricle, parts of the atria, and the outflow tract, with some minor mutual contribution of FHF cells to the right ventricle and SHF cells to the left ventricle (Buckingham *et al*, 2005; Vincent & Buckingham, 2010). Lineage tracing experiments *in vivo* and clonal analyses *in vitro* demonstrated that the $Isl1^+$ SHF progenitors can give rise to various cardiac lineages, including cardiomyocytes, conductive cells, vascular SMCs, and ECs (Moretti *et al*, 2006; Sun *et al*, 2007; Bu *et al*, 2009). In contrast, the $HCN4^+$ FHF progenitors appear to be committed toward cardiomyocytes of the left ventricle and parts of the atria, and conductive cells of the atrio-ventricular (AV) node and ventricular CS (Liang *et al*, 2013; Spater *et al*, 2013). There is still controversy around the embryonic origin of pacemaker cells in the sino-atrial (SA) node. Interestingly, a recent study in chick embryo found that chick pacemaker cells arise from a discrete region outside the FHF/SHF, a so-called tertiary heart field (Fig 1A) (Bressan *et al*, 2013).

The molecular cues that spatiotemporally regulate embryonic CPC populations and promote their differentiation into diverse cell types through putative intermediates are still under investigation (Fig 1A) (Soh *et al*, 2014). Given that the embryonic FHF/SHF CPCs are multipotent, these CPCs are attractive therapeutic targets for cardiac regeneration (Domian *et al*, 2009). However, $Isl1^+$ SHF progenitors are no longer present in the adult heart (Laugwitz *et al*, 2005) and in addition, there is no convincing evidence that they can be reactivated post-natally *in situ* to produce sufficient quantities of cardiomyocytes to repair the injured heart (Weinberger *et al*, 2012). In light of this, understanding the mechanisms that regulate CPC behavior during embryogenesis and identifying the specific signals that govern the transition between multipotent CPCs and fully differentiated cardiac cells is essential to establish novel therapeutic avenues for heart regeneration.

EPDCs and cNCCs In addition to FHF and SHF CPCs, other cell populations also contribute to the formation of the heart. The proepicardial organ (PEO) is a transitory mesenchymal structure that forms near the posterior end of the heart tube at around E9.0 (Fig 1B) and then develops into the epicardium, the outer layer of the heart (Manner *et al*, 2001; Schlueter & Brand, 2012). Some epicardial cells undergo epithelial-to-mesenchymal transition (EMT) and enter the heart as epicardium-derived progenitor cells (EPDCs), which contribute to SMCs, cardiac fibroblasts, and possibly to ECs of the coronary vasculature (Fig 1A) (Christoffels *et al*, 2009; Katz *et al*, 2012). Whether the EPDCs can also contribute to myocardium is controversial. The PEO and epicardium are marked by expression of *Wt1* and *Tbx18* (Cai *et al*, 2008; Zhou *et al*, 2008a). Whereas previous fate-mapping studies in chick or mouse have shown no EPDC contribution to the myocardium (Winter & Gittenberger-de Groot, 2007), more recent reports using a *Wt1-Cre* or *Tbx18-Cre* conditional reporter mouse line suggest that EPDCs might contribute to a small population of cardiomyocytes (Cai *et al*, 2008; Zhou *et al*, 2008a). It should, however, be noted that *Wt1* and *Tbx18* expression may not be specific to the epicardium alone, thus making it difficult to unequivocally interpret the results of these fate-mapping experiments (Christoffels *et al*, 2009; Ruiz-Villalba *et al*, 2013). Nevertheless, the suggestion that embryonic EPDCs may contribute to a certain extent not only to the coronary SMCs/ECs but also to the myocardium is important. Unlike $Isl1^+$ SHF progenitors, EPDCs are maintained throughout life in the adult heart and may

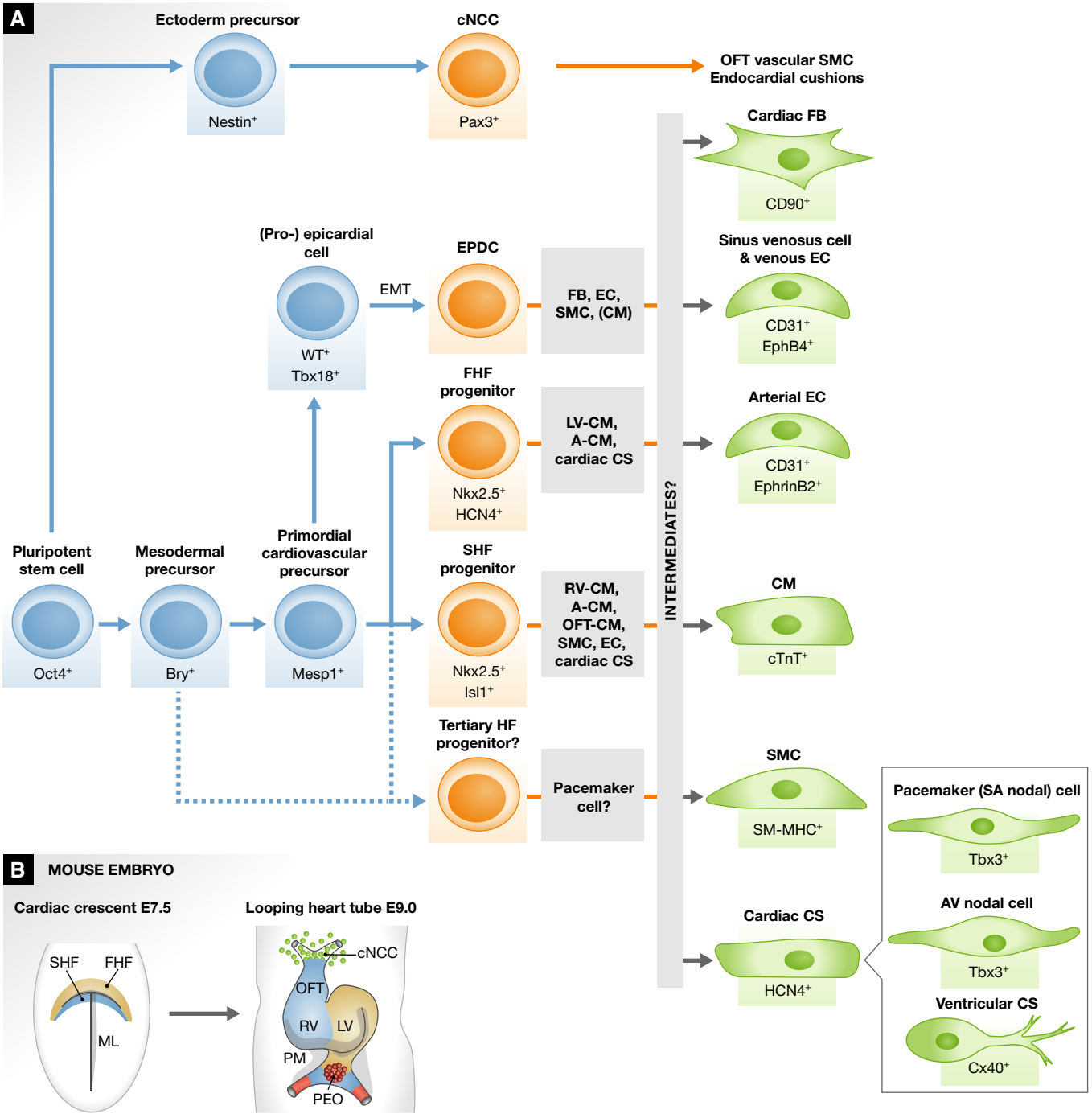

Figure 1. Fate map of cardiac cell lineages during development.
(A) A cellular flow chart shows the stepwise commitment of pluripotent stem cells via various cardiac progenitor cells, including the first and second heart field (FHF and SHF) progenitors, epicardium-derived progenitor cells (EPDCs) and so-called tertiary heart field (HF) progenitors, and putative intermediates toward mature cardiac cell types during heart development. Mature cardiac cells include cardiomyocytes (CMs), vascular smooth muscle cells (SMCs), arterial and venous endothelial cells (ECs), fibroblasts (FBs), and conductive cells of the cardiac conduction system (CS), which include pacemaker (sino-atrial [SA] nodal) cells, atrio-ventricular (AV) nodal cells, and the ventricular CS cells (ex. Purkinje fibers). The gray boxes in the middle indicate the major mature cell types that each cardiac progenitor differentiates into. Cardiac neural crest cells (cNCCs) originating from the ectoderm also contribute to vascular SMCs of the outflow tract (OFT) and thereby to OFT separation and patterning. (B) Cardiac development at the early stage of the mouse embryo. Developing hearts at murine embryonic day (E) 7.5 and 9.0 are shown. A, atria; EMT, epithelial-to-mesenchymal transition; LV, left ventricle; ML, midline; PEO, proepicardial organ; PM, pharyngeal mesoderm; and RV, right ventricle.

potentially represent an endogenous source of newly formed cardiomyocytes following injury.

Cardiac neural crest cells (cNCCs) originate from the dorsal neural tube and migrate through the posterior pharyngeal arches to the arterial pole of the heart tube at around E9.5 (Fig 1). cNCCs and their derivatives give rise to SMCs of the pharyngeal arch arteries and the outflow tract of the heart, contributing to septum and valve formation and thereby resulting in outflow tract separation and patterning into the pulmonary trunk and aorta (Hutson & Kirby, 2007; Hildreth *et al*, 2008).

Adult endogenous CPCs Cardiac progenitor cells are usually defined as self-renewing, clonogenic, and multipotent cells that can differentiate into cardiomyocytes, SMCs, and ECs both *in vitro* and *in vivo* (Beltrami *et al*, 2003; Garbern & Lee, 2013; Sanganalmath & Bolli, 2013). To date, various kinds of putative endogenous CPCs have been isolated from adult rodent and human hearts, although the magnitude of their contribution to heart homeostasis and repair remains controversial (Chong *et al*, 2014a). The presence of the tyrosine kinase receptor c-kit is often used to identify CPCs (Beltrami *et al*, 2003; Bearzi *et al*, 2007). c-kit$^+$ cardiac cells isolated from adult human heart and injected into infarcted rodent myocardium were shown to promote functional and structural cardiac improvement (Bearzi *et al*, 2007). However, whether endogenous c-kit$^+$ cardiac cells can contribute to differentiated cardiomyocytes during aging or after injury in adulthood is highly debated (Bolli *et al*, 2011; Jesty *et al*, 2012; Ellison *et al*, 2013; Molkentin & Houser, 2013, 2014; Torella *et al*, 2014). In mice, the ability of these cells to give rise to cardiomyocytes is elevated shortly after birth, but decreases significantly over time and is virtually negligible in adult animals (Zaruba *et al*, 2010). A recent study using genetic lineage tracing experiments with a *Kit-Cre* conditional reporter mouse line showed that the generation of new cardiomyocytes from endogenous c-kit$^+$ cells is a rare event (0.027%), even after cardiac injury, whereas c-kit$^+$ cells amply contribute to cardiac ECs (van Berlo *et al*, 2014). This is consistent with previous reports showing that c-kit$^+$ cardiac cells transplanted into injured rodent hearts are not likely to be the predominant source of newly formed cardiomyocytes, but instead promote cardiac proliferation/regeneration by secreting paracrine cytokines and growth factors (Tang *et al*, 2010; Loffredo *et al*, 2011). The cardiac-resident c-kit$^+$ CPCs originally described by Beltrami *et al* could originate from extra-cardiac sources, as shown by the fact that 74% of c-kit$^+$ cells found in the heart after myocardial infarction (MI) appear to be bone marrow derived (Fazel *et al*, 2006). As c-kit is broadly expressed in various cell types, including hematopoietic lineage cells (Smith *et al*, 2014), the use of this single marker to isolate CPCs from adult mammalian hearts is challenging and susceptible to contamination from non-CPC populations.

Aside from c-kit$^+$ CPCs, other types of CPCs, such as Sca1$^+$ cardiac cells (Oh *et al*, 2003; Matsuura *et al*, 2004), cardiospheres and cardiosphere-derived cells (Messina *et al*, 2004), and cardiac side population cells (Martin *et al*, 2004), have also been reported. Similar to c-kit$^+$ CPCs, they are heterogeneous, and whether they can be a reproducible source of newly generated cardiomyocytes after cardiac injury remains controversial. Furthermore, clear evidence that they can have clinically beneficial effects on global heart function and cardiac repair is still lacking (Garbern & Lee, 2013; Sanganalmath & Bolli, 2013).

Essential signaling pathways and molecular drivers of cardiogenesis

Extracellular signaling pathways and their interaction with transcriptional regulators are tightly regulated during embryonic cardiogenesis and control patterning of embryonic CPCs, including the FHF/SHF progenitors. The major signaling cascades involved in cardiac muscle creation include canonical Wnts, the transforming growth factor (TGF)β superfamily such as bone morphogenetic proteins (BMPs) and Activin/Nodal, fibroblast growth factors (FGFs), non-canonical Wnts, and Hedgehog and Notch pathways, all of which function sequentially and cooperatively (Fig 2). A complex network of these signaling pathways and transcriptional regulators controls cardiac progenitor specification, proliferation, and differentiation into diverse cardiac cell lineages, ultimately giving rise to the entire heart, as further reviewed elsewhere (Vincent & Buckingham, 2010; Noseda *et al*, 2011).

Mesodermal formation and cardiac specification Bone morphogenetic protein signaling is necessary for gastrulation and primitive mesoderm formation in mammals. Germline deletion of *Bmp4* or the BMP type I receptor (*Bmpr1a*) causes embryonic death before E9.5 (Mishina *et al*, 1995; Winnier *et al*, 1995). Abnormal cardiac morphogenesis occurs in mice upon conditional deletion of *Bmp4* using *Tnnt2-Cre* or *Nkx2-5-Cre* lines (Jiao *et al*, 2003; Liu *et al*, 2004; Jayawardena *et al*, 2012). When conditional deletion of *Bmpr1a* was introduced with a *Mesp1-Cre* allele, the cardiac crescent (FHF) was not formed, and the FHF markers *Hand1* and *Tbx5* were also absent (Klaus *et al*, 2007). This indicated an indispensable role of BMP signaling for cardiac specification in the mammalian heart.

Activin and Nodal belong to the TGFβ superfamily (Kitisin *et al*, 2007). This signaling pathway is essential in early mouse embryos for positional patterning, gastrulation, primitive streak, and mesoderm/endoderm formation, and later for cardiac myogenesis (Conlon *et al*, 1994; Schier, 2003). Activin A, in concert with BMPs, has been shown to successfully induce cardiac myogenesis in mouse and human embryonic stem cells (ESCs) and in human inducible pluripotent stem cells (iPSCs) (Burridge *et al*, 2007; Takahashi *et al*, 2007; Flaim *et al*, 2008).

The "canonical" Wingless-Int (Wnt) ligands include Wnt1, -2a, -3a, and -8, which require β-catenin for signaling translation into nuclei (Clevers, 2006; Gordon & Nusse, 2006). Before gastrulation, canonical Wnt/β-catenin signals are involved in primitive streak formation and the induction of primitive mesoderm and endoderm (Rivera-Perez & Magnuson, 2005; Barrow *et al*, 2007). However, after gastrulation, these signals are inhibited by a secreted Frizzled-related protein (sFRP) and Dickkopf1 (Dkk1), which are produced in the adjacent endoderm and are essential for further cardiac specification in the mesoderm (Foley & Mercola, 2005; Mii & Taira, 2009). This biphasic effect of canonical Wnt/β-catenin signals has been recapitulated in cultured mouse ESCs and human ESCs/iPSCs, collectively referred to as pluripotent stem cells (PSCs). The Wnt/β-catenin pathway is necessary for mesoderm and endoderm formation from mouse/human PSCs, cultured with Wnt3A-conditioned medium or the inhibitor of glycogen synthase kinase (GSK) 3β that phosphorylates and degrades β-catenin, yet the same signaling pathway inhibits cardiac myogenesis once mesoderm has been created (Lindsley *et al*, 2006; Naito *et al*, 2006; Ueno *et al*, 2007; Yoshida & Yamanaka, 2011).

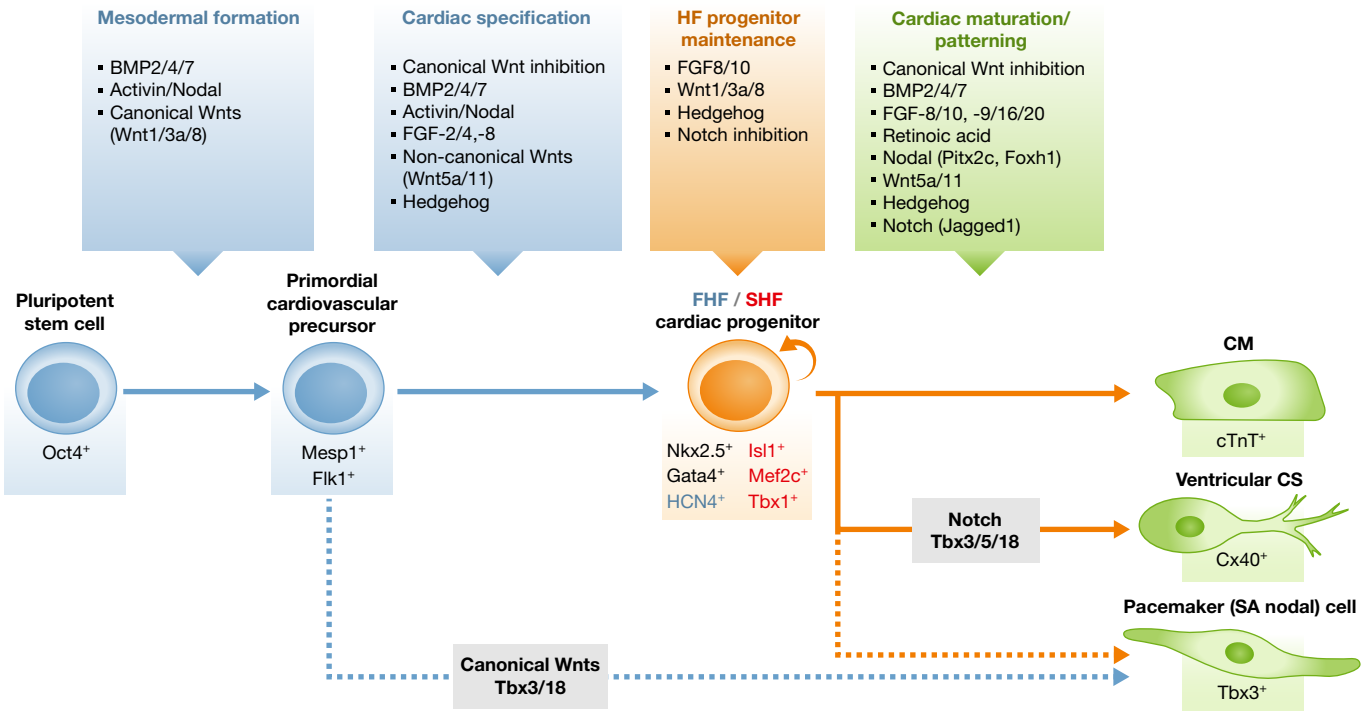

Figure 2. Signaling pathways programming a heart cell during embryogenesis.
The flow chart shows the representative signaling pathways and/or modulators working in a stepwise manner at various stages of cardiac development/cardiac cell differentiation, including mesodermal formation, cardiac specification, maintenance of heart field (HF) progenitors, and cardiac maturation and patterning (see text for details). A circular arrow denotes self-renewal. First heart field (FHF) marker gene is in blue, and second heart field (SHF) marker genes in red. CM, cardiomyocyte; CS, conduction system; and SA, sino-atrial.

The FGF pathway involves approximately 20 ligands and 4 transmembrane receptor tyrosine kinases (FGFRs) (Itoh & Ornitz, 2004; Turner & Grose, 2010). Studies using hypomorphic alleles or conditional deletion of *Fgf8* with a *Tbx1-Cre* allele demonstrated impaired outflow tract aligning and septation, indicating that mesodermal *Fgf8* expression is crucial for SHF development (Frank *et al*, 2002; Ilagan *et al*, 2006). At the cellular level, *Fgf8* regulates expression of the SHF marker genes *Isl1* and its target *Mef2c* (Park *et al*, 2006), leading to proliferation of the SHF progenitor population. *Fgf10* is also expressed in the SHF (Marguerie *et al*, 2006). In human ESCs, FGF2, in combination with Activin and BMP4, is known to specifically promote mesoderm-committed precursor formation (Evseenko *et al*, 2010).

HF progenitor maintenance and cardiac cell maturation/patterning Canonical Wnt/β-catenin signaling also plays important roles at later stages of embryonic cardiogenesis, in proliferation/maintenance of SHF progenitors and prevention of their differentiation (Cohen *et al*, 2008). Wnt/β-catenin signaling is activated in the SHF, and β-catenin can directly enhance expression of the SHF transcription factors *Isl1* and *Fgf10* (Cohen *et al*, 2007; Lin *et al*, 2007). Conditional deletion in the SHF of the β-catenin gene by using an *Isl1-Cre* or *Mef2c-Cre* driver mouse line causes right ventricular and outflow tract hypoplasia, probably due to impaired SHF proliferation. Conversely, stable expression of β-catenin in the $Isl1^+$ or $Mef2c^+$ SHF progenitor population leads to right ventricular enlargement and hyperplasia (Ai *et al*, 2007; Kwon *et al*, 2007; Lin *et al*, 2007; Qyang *et al*, 2007). Of interest, canonical Wnt signaling blocks differentiation of SHF progenitors. $Isl1^+$ cells in which β-catenin is specifically stabilized down-regulate the gene encoding *Myocardin*, which promotes myocardial and smooth muscle differentiation in concert with serum response factor (*SRF*) (Evans *et al*, 2010). Consequently, maintenance of $Isl1^+$ cells in the outflow tract causes a delay in cardiac differentiation (Kwon *et al*, 2009).

Bone morphogenetic protein signaling promotes cardiac specification and myocardial differentiation (Tirosh-Finkel *et al*, 2006). Conditional deletion of *Bmpr1a* in $Isl1^+$ SHF progenitors at late embryonic stages causes right ventricle and outflow tract hypoplasia with increased numbers of $Isl1^+$ cells, indicating failure of the SHF progenitors to differentiate (Yang *et al*, 2006; Klaus *et al*, 2007). BMP signaling, mainly through BMP2, BMP4, and BMP7, is hence likely to affect myocardium maturation. FGF signaling also affects the myocardium maturation step. FGF9 and its relatives FGF16 and FGF20 are expressed in both endocardium and epicardium at mid-gestation and contribute to myocardial proliferation (Lavine *et al*, 2005). Conditional deletion of the *Fgfr1* and *Fgfr2* genes with the ventricle-specific driver *Mlc2v-Cre* causes severe ventricular defects (Lavine *et al*, 2005).

Patterning of the SHF along the anterior/posterior axis is regulated by retinoic acid (RA) signaling (Sirbu *et al*, 2008). RA, a biologically active derivative of vitamin A, is produced by retinaldehyde dehydrogenase (Raldh) 2, and in the *Raldh2*-mutant mouse embryos, the anterior SHF marker genes such as *Tbx1* and *Fgf8/10* show abnormal expression patterns, which expand posteriorly

(Ryckebusch *et al*, 2008). In mice, RA and its receptors are essential for normal cardiac morphogenesis, with atrial development being more affected by loss of *Raldh2* than ventricular development (Niederreither *et al*, 2001).

Hedgehog ligands bind to patched 12-span transporter-like receptors that inhibit the function of Smoothened (Smo) serpentine receptors in the absence of ligands (Wilson & Chuang, 2010). In zebrafish, Hedgehog signaling has been shown to promote cardiomyocyte formation (Thomas *et al*, 2008), whereas in mice, it is involved in the establishment of left/right asymmetry, coronary vasculature, atrial septation, and outflow tract morphogenesis (Kolesova *et al*, 2008; Lavine *et al*, 2008; Hoffmann *et al*, 2009). Hedgehog signals have been shown to be crucial for normal induction of *Nkx2-5* or its equivalent in both zebrafish and mice (Zhang *et al*, 2001).

Non-canonical Wnt signaling (Wnt5a and Wnt11) is associated with cardiac specification and differentiation (Pandur *et al*, 2002; Palpant *et al*, 2007). *Wnt5a*- or *Wnt11*-null mice have pharyngeal artery patterning and outflow tract defects. However, expression of *Isl1* and other SHF markers, such as *Mef2c*, is normal, suggesting that Wnt5a and Wnt11 control outflow tract maturation by affecting the cNCCs, but not the SHF (Schleiffarth *et al*, 2007; Zhou *et al*, 2007).

Notch signaling is associated with a wide range of developmental processes, including cell fate decisions in various cell types (Andersson *et al*, 2011). During embryonic cardiogenesis, Notch signaling affects both the SHF and the cNCCs, hence controlling right ventricle and outflow tract formation, vascular smooth muscle development, chamber specification, and trabeculation (McCright *et al*, 2001; High *et al*, 2007, 2008; Xin *et al*, 2007; Varadkar *et al*, 2008). SHF-specific deletion of *Notch1* with an *Isl1-Cre* line promoted proliferation of $Isl1^+$ progenitors and caused overexpression of β-catenin in the SHF, resulting in defects of the arterial pole including the right ventricle (Cohen *et al*, 2007). Thus, Notch signaling interferes with canonical Wnt/β-catenin signaling in the SHF, thereby inhibiting proliferation of SHF progenitors and promoting their differentiation.

The Epstein group recently showed that forced expression of Notch signaling *in vitro* can reprogram neonatal murine cardiomyocytes to display a conduction-like phenotype, including action potential characteristics (Rentschler *et al*, 2012). This suggests that Notch signaling, similar to T-box transcription factor Tbx3, Tbx5, and Tbx18 (Hoogaars *et al*, 2007; Bakker *et al*, 2008; Wiese *et al*, 2009), plays an important role in the specification of cardiac conductive cells (Fig 2). Recent reports also showed that forced expression of *Tbx18* or *Tbx3* could reprogram mature ventricular cardiomyocytes to a pacemaker-like phenotype *in vitro* and *in vivo* (Bakker *et al*, 2012; Kapoor *et al*, 2013). The Mikawa group reported that the fate of pacemaker cells, derived from the "tertiary" heart field in the chick embryo, is controlled by canonical Wnt signaling at early stages (Fig 2) (Bressan *et al*, 2013).

Finally, chromatin remodeling has also been shown to be a key event in establishing the cardiomyogenic program. The Bruneau group demonstrated that a cardiac-specific subunit of the chromatin remodeling complex Baf60c (BRG1-associated factor 60c), in combination with the cardiac transcription factors *Gata4* and *Tbx5*, can ectopically trans-differentiate mouse mesoderm into beating cardiomyocytes (Takeuchi & Bruneau, 2009).

Heart regeneration/replication capabilities

The long-standing concept that human heart cells exit the cell cycle after birth and are unable to renew themselves with aging or after injury has been drastically overturned by a growing body of contradictory evidence recently reported (Garbern & Lee, 2013), although the estimated rate of mammalian cardiomyocyte renewal is remarkably low, even in the injured heart (Bergmann *et al*, 2009; Kajstura *et al*, 2010; Mollova *et al*, 2013; Senyo *et al*, 2013). This indicates that the inherent capacity of the mammalian heart to replenish damaged myocardium is far from being sufficient to exploit in a clinical setting. In this section, we discuss recent knowledge and advances in cardiac regeneration/replication in various model organisms, including lower vertebrates (zebrafish and amphibians), and consider their relevance for cardiac regenerative medicine (Fig 3).

Cardiac regeneration in zebrafish and amphibians Urodele amphibians, such as salamanders and newts, have a remarkable capacity to regenerate injured tissues, including the heart (Brockes & Kumar, 2005; Roy & Gatien, 2008). Early studies showed that newts could survive after resection of a significant portion (up to 50%) of apical myocardium and that cardiomyocyte regeneration was evident 30 days after injury (Becker *et al*, 1974; Oberpriller & Oberpriller, 1974; Oberpriller *et al*, 1988). Furthermore, recent studies showed that newts are able to fully regenerate cardiac tissue within 60 days after amputation of 10–25% of apical myocardium (Witman *et al*, 2011; Mercer *et al*, 2013). Following the initial response to injury, which includes blood/fibrin clot formation, macrophage and lymphocyte infiltration, and deposition of extracellular matrix, DNA synthesis is detected within cardiomyocytes at the injury site at day 16 after injury. Although subject of debate, recent evidence suggests that urodele heart regeneration likely occurs via partial dedifferentiation of mature cardiomyocytes into progenitor-like cells (Laube *et al*, 2006). How urodele cardiomyocytes reenter the cell cycle and regenerate cardiac tissue following injury remains unclear. In this regard, FGFs, platelet-derived growth factors (PDGFs), thrombin, BMP signaling, and miRNAs such as miR-128 have all been proposed to be involved (Singh *et al*, 2010; Witman *et al*, 2013). Unlike mammalian cardiomyocytes, which are mostly multinucleated and/or polyploid (4n), 98% of uninjured urodele cardiomyocytes are mononucleated and diploid, and this may contribute to their inherent regenerative capacity (Neff *et al*, 1996).

Decades after the early urodele amphibian studies, similar observations were made in zebrafish. The adult zebrafish heart can regenerate completely within 60 days after resection of up to 20% of apical myocardium (Poss *et al*, 2002; Raya *et al*, 2003). Other types of injury were also employed, such as genetic ablation (Wang *et al*, 2011) and cardiac cryoinjury (Chablais *et al*, 2011; Gonzalez-Rosa *et al*, 2011), to evaluate whether the same regenerative responses appeared (Fig 3). Genetic ablation is based on conditional expression of the cytotoxic diphtheria toxin A gene under the control of tamoxifen-inducible Cre recombinase driven by the promotor for the contractile gene cardiac myosin light chain-2 (*cmlc2*), which allows to specifically ablate up to 60% of cardiomyocytes (Wang *et al*, 2011). For cardiac cryoinjury, a nitrogen-cooled probe is used to damage 20–30% of the ventricular myocardium, together with endocardium and epicardium. As a first response, all of these injury models induce the reactivation of genes expressed during embryonic

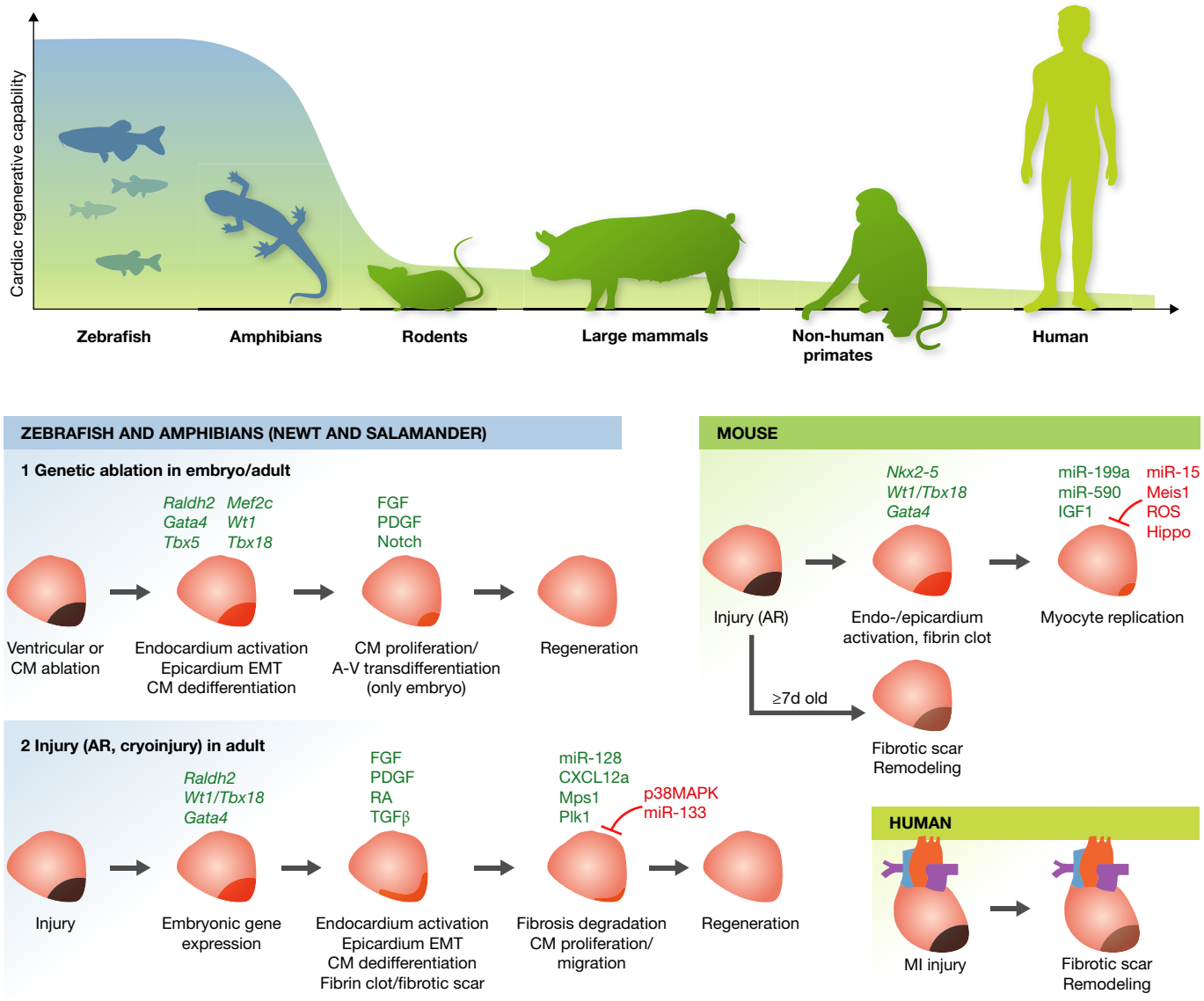

Figure 3. Heart regeneration in various model organisms.
(Top) Schematic representation indicates cardiac regenerative capabilities of various model organisms from lower vertebrates (zebrafish and amphibians) to human. (Bottom) Heart regenerative/replicative processes following injury in zebrafish and amphibians, neonatal/adult mice, and humans are shown. In zebrafish, various injury models (genetic ablation, apical resection (AR), and cryoinjury) are reported and exhibit different recovery processes, especially in terms of transient fibrotic scar formation. All ultimately lead to full regeneration of the ablated myocardium. Similar post-injury regenerative processes are seen in hearts of 1-day-old mice, but not after 7 days nor in humans, where fibrotic scar formation and pathological remodeling occur. Regeneration enhancers are indicated in green, inhibitors in red (see text for details). A-V, atrial-to-ventricular; CM, cardiomyocyte; EMT, epithelial-to-mesenchymal transition; MI, myocardial infarction.

heart development, such as *Gata4*, *Nkx2-5*, *Raldh2*, *Wt1*, and *Tbx18*, followed by activation of endocardium and epicardium, including EMT of epicardial cells (Kikuchi & Poss, 2012). While genetic ablation of cardiomyocytes does not induce deposition of collagen matrix but only formation of a blood/fibrin clot after amputation, the cryoinjury model produces massive, but transient, scar-like fibrosis around the injured area. Scar formation is mediated by the TGF-β/Activin signaling pathway and might be necessary for heart regeneration in this model, as, unlike the fibrotic scar that forms in injured mammalian hearts, it is later degraded (Fig 3) (Chablais & Jazwinska, 2012). Following scar formation and/or EMT of epicardial cells, which causes FGF- and PDGF-driven revascularization into the myocardium (Lepilina *et al*, 2006; Kim *et al*, 2010), proliferating cardiomyocytes appear around the injured sites. Several paracrine signals, including RA, synthesized by the epicardium and endocardium, and C-X-C motif chemokine 12a (CXCL12a, also referred to as stromal cell-derived factor 1 [SDF-1]), expressed in epicardial cells, have been suggested to promote cardiomyocyte proliferation (Kikuchi *et al*, 2011; Gonzalez-Rosa & Mercader, 2012; Itou *et al*, 2012b). In summary, although the injury models differ in the recovery process in regard to transient fibrotic tissue accumulation and time span required to obtain complete

heart regeneration, ranging from 30 days in the genetic ablation model to 60 days in the apical resection model to 130 days in the cryoinjury model (Poss *et al*, 2002; Schnabel *et al*, 2011; Wang *et al*, 2011), all of them ultimately lead to full regeneration of the ablated myocardium (Fig 3).

Importantly, zebrafish regenerative potential does not decrease with age (Itou *et al*, 2012a). The source of post-injury regenerated cardiomyocytes is a subject of debate. Genetic fate-mapping approaches, using a zebrafish strain in which tamoxifen-inducible Cre is driven by *cmlc2*, revealed that regenerated cardiomyocytes derive from preexisting mature cardiomyocytes that undergo partial dedifferentiation, as shown by disassembly of their sarcomeric structure and expression of *Gata4*, a regulator of embryonic heart development, and thereafter re-enter the cell cycle (Jopling *et al*, 2010; Kikuchi *et al*, 2010). Although the mechanisms through which cardiomyocytes dedifferentiate and then proliferate following injury have not been fully determined, recent studies suggest that mitotic checkpoint kinase *Mps1* and polo-like kinase 1 (*Plk1*) positively regulate heart regeneration in zebrafish, whereas cardiomyocyte proliferation is inhibited by miR-133 and p38 mitogen-activated protein kinase (MAPK) (Jopling *et al*, 2010, 2012; Yin *et al*, 2012). Of note, a recent report showed that an *in vivo* cardiac reprogramming event, the atrial-to-ventricular cardiomyocyte trans-differentiation, contributes to heart regeneration in zebrafish embryos, but not in adults (Zhang *et al*, 2013). The authors used a ventricle-specific genetic ablation system, in which metronidazole was administered to ablate transgenic ventricular cardiomyocytes expressing nitroreductase driven by the ventricular myosin heavy chain (*vmhc*) promoter. Ventricle-specific ablation of cardiomyocytes was performed 3–4 days post-fertilization, when the zebrafish heart has completed cardiac looping and cardiac chamber cardiomyocytes have fully differentiated (de Pater *et al*, 2009). Re-expression of key cardiogenic transcription factors, such as *Gata4*, *Nkx2-5*, *Hand2*, *Tbx5/20*, and *Mef2c*, occurs in injured hearts, and atrial cardiomyocytes adjacent to the atrio-ventricular canal dedifferentiate into intermediate reprogramming stages and then trans-differentiate into ventricular cardiomyocytes, thereby contributing to ventricular regeneration. This trans-differentiation capacity was shown to be age dependent and partly mediated by Notch signaling activation in the atrial endocardium following ventricular ablation (Fig 3) (Zhang *et al*, 2013).

Mammalian cardiomyocyte turnover There is now agreement that cardiomyocyte turnover does occur throughout life in mammals, including humans, although this turnover capacity is considerably limited (Bergmann *et al*, 2009; Kajstura *et al*, 2010; Mollova *et al*, 2013; Senyo *et al*, 2013). In a groundbreaking study, the Frisén group determined the birth date of cardiomyocytes in humans by measuring nuclear carbon-14 (^{14}C) content with accelerator mass spectrometry (Bergmann *et al*, 2009). They showed that new cardiomyocytes form in the human heart at a rate of around 1.5% per year at 25 years of age and that this turnover rate declines with age, and concluded that approximately 50% of human cardiomyocytes are replaced during an entire life span. Their data are consistent with those of newer studies in both mouse (Malliaras *et al*, 2013; Senyo *et al*, 2013) and human (Mollova *et al*, 2013). Using cardiomyocyte-specific fluorescent reporter mouse lines and multi-isotope imaging mass spectrometry, which monitors DNA synthesis at high resolution using the rare stable isotope of nitrogen (^{15}N), the Lee group showed that in a healthy mouse during normal aging (≥10 weeks old), the annual birth rate of cardiomyocytes is 0.76%, whereas 8 weeks after MI, roughly 3.2% of the cardiomyocytes adjacent to the infarct undergo cell division (Senyo *et al*, 2013). Although there is some variation among different reports (Laflamme & Murry, 2011), the estimated rate of mammalian cardiomyocyte turnover is approximately 1% per year, which increases modestly in response to injury, but declines with aging.

Multiple sources have been proposed to explain the origin of newly generated cardiomyocytes during both normal homeostasis and repair, including preexisting mature cardiomyocytes and quiescent CPCs (Garbern & Lee, 2013). Recent studies suggest that at least during normal homeostasis, preexisting cardiomyocytes that undergo dedifferentiation followed by proliferation might be the predominant source of newly formed cardiomyocytes (Mollova *et al*, 2013). However, CPCs may also participate in cardiomyocyte generation following injury (Porrello *et al*, 2011; Senyo *et al*, 2013). Importantly though, these two scenarios are not mutually exclusive, and both constitute possible avenues for increasing *de novo* cardiomyocyte generation for cardiac regenerative medicine.

Mammalian heart regenerative/proliferative response to injury In mammals, unlike zebrafish and amphibians, cardiac injury such as MI induces permanent cardiomyocyte cell death and the formation of an irreversible fibrotic scar. This leads to electrical uncoupling to the remaining myocardium, causing arrhythmias, unfavorable remodeling of ventricular walls, reduction of ventricular function, and finally heart failure (Fig 3) (Hasenfuss, 1998). Challenging this dogma, recent evidence suggests that similar to zebrafish and amphibian hearts, the 1-day-old neonatal mouse heart can regenerate completely 21 days after resection of approximately 15% of apical ventricular tissue (Porrello *et al*, 2011; Strungs *et al*, 2013; Naqvi *et al*, 2014). This regenerative capacity of the mouse heart is rapidly lost by 7 days after birth, when the injured heart develops fibrotic scars instead, as seen in adult mice and humans (Porrello *et al*, 2011; Mahmoud *et al*, 2013). A newer study, however, showed that in 1-day-old neonatal mice undergoing apical resection, the regeneration process is incomplete and accompanied by fibrotic scar formation, thereby questioning the cardiac regenerative capacity of the neonatal mouse (Andersen *et al*, 2014). Nevertheless, these experiments clearly show the highly activated regenerative capacity of the 1-day-old neonatal mouse heart, the extent of which diminishes rapidly after the first few days of life, suggesting that key programs/mechanisms regulating inherent regeneration must exist in the first week of life in mammals (Kotlikoff *et al*, 2014). Of interest, another recent study showed that during preadolescence (at post-natal day 15 in mice), a transient burst of cardiomyocyte proliferation occurs, with an increase in cardiomyocyte numbers by around 40%. Proliferation seems to be driven by a surge in the levels of thyroid hormones, which appear to activate the IGF-1/IGF-1-R/Akt pathway, although the causal relationship between thyroid hormones, IGF signaling, and this proliferative burst was not fully clarified (Naqvi *et al*, 2014). This indicates that, to a certain extent, mammalian cardiomyocyte proliferative capacities may persist beyond the perinatal period.

Why can the adult mammalian heart not regenerate? To understand the rapid regenerative loss upon birth and identify the mechanisms involved, multiple hypotheses are currently being

investigated (Fig 3). miRNAs, Hippo signaling, oxidative stress, and the transcription factor Meis1 have recently attracted attention in this regard and are described in detail below (see, "Cell-free therapies" section). In addition, cardiac regeneration following injury in the neonatal mouse is preceded by stronger activation of *Wt1* and *Tbx18* expression than observed in the adult, indicating that the enhanced epicardial response might play an important role in heart regeneration (Smart *et al*, 2011). A role of the epicardium as source of paracrine signals, including vascular endothelial growth factor-A (VEGF-A), SDF-1, and FGFs, is supported by experiments showing the efficacy of transplanted EPDCs or administered EPDC supernatant in promoting regeneration in injured mouse hearts (Winter *et al*, 2007; Zhou *et al*, 2011).

Therapeutic strategies for human cardiac regeneration

It has been estimated that after MI, a patient loses on average around one billion cardiomyocytes (Laflamme & Murry, 2005)—a massive amount that the human body cannot replace on its own, given the extremely low cardiomyocyte turnover rate (as discussed in the previous section). There are multiple different approaches to promote cardiomyocyte regeneration/proliferation in human injured hearts, including transplantation of autologous non-cardiac/cardiac somatic stem cells, injection of *in vitro*-derived cardiomyocytes, direct lineage conversion ("reprogramming") of cardiac fibroblasts into cardiomyocytes *in vivo*, stimulation of dedifferentiation/proliferation of preexisting cardiomyocytes, and activation of endogenous CPC populations (Fig 4). These therapeutic strategies, classified as either cell-based or cell-free, are currently being investigated for their cardiac regenerative potential and feasibility of clinical application (Vunjak-Novakovic *et al*, 2011). Here, we review recent advances in both groups of therapies.

Cell-based therapies

Cell-based therapies involve transplantation into the injured heart of cells that have the ability to repopulate the damaged myocardium and integrate functionally with preexisting tissue, ultimately restoring normal cardiac activity. Two types of cells can be employed: (i) *in vitro*-derived cardiomyocytes, obtained via PSC differentiation or via direct conversion of terminally differentiated somatic cells, or

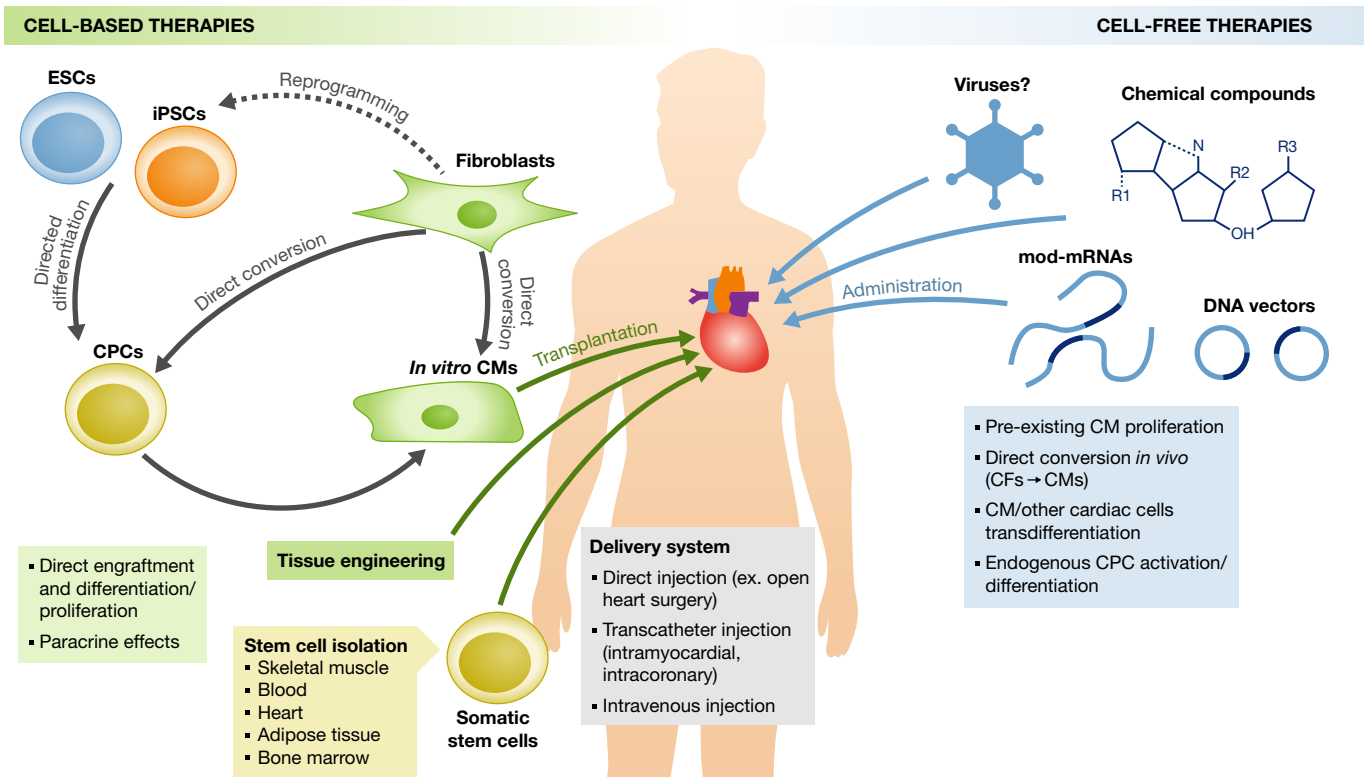

Figure 4. Therapeutic strategies for cardiac regeneration.
(Left) Cell-based therapies involve transplantation into the damaged heart of *in vitro*-derived cardiomyocytes (CMs) or somatic stem cells. CM differentiation can be induced from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) obtained by reprogramming differentiated somatic cells, such as fibroblasts. Alternatively, by forced expression of cardiac-specific factors, the pluripotent state can be bypassed and fibroblasts directly converted into CMs or cardiac progenitor cells (CPCs). Different types of cardiac and non-cardiac somatic stem cells can also be transplanted. Cell-based therapies can produce beneficial effects on heart function directly, by engrafting into the host tissue and differentiating/proliferating *in vivo*, or indirectly via paracrine effects that act on host cells. (Right) Cell-free therapies involve administration of chemical compounds or genes (via viral vectors, non-viral DNA vectors, or modified mRNAs) that act on host cells to stimulate cardiac regeneration. Mechanisms of action include the stimulation of proliferation of preexisting CMs, direct conversion of cardiac fibroblasts (CFs) into CMs, trans-differentiation of CMs or other cardiac cells from one cellular subtype to another, and activation and differentiation of endogenous CPCs. Cardiac delivery routes for both cell-based and cell-free therapeutic agents are also indicated.

(ii) adult stem/progenitor cells, which can differentiate into cardiomyocytes *in vivo* (Fig 4, *left*). In both cases, cells can be first expanded *in vitro* so that large amounts of starting material are readily available for manipulation and transplantation.

Directed cardiomyocyte differentiation from pluripotent stem cells The first way to derive cardiomyocytes for transplantation purposes is through directed differentiation from PSCs, such as ESCs. Alternatively, cardiomyocytes can be obtained from terminally differentiated non-cardiac somatic cells, provided that they are first converted into iPSCs via reprogramming (Takahashi & Yamanaka, 2006). Compared to ESCs, iPSCs have a critically important advantage: They can be derived from the somatic cells of any patient, thus circumventing graft rejection problems often associated with non-autologous cell transplants. A multitude of cardiomyocyte differentiation protocols have been developed over the years. Since their aim is to recapitulate embryonic development in a dish, protocol optimization requires a detailed understanding of the key signaling pathways that orchestrate heart development *in vivo* (Fig 2). Cardiomyogenic differentiation methods generally employ one of two alternative techniques, depending on whether the PSCs are cultured in three-dimensional aggregates, termed embryoid bodies (EBs), or in monolayer format.

In one of the first efforts to derive cardiomyocytes *in vitro*, spheres of human ESCs were generated in suspension cultures and allowed to spontaneously recapitulate early embryonic development (Kehat *et al*, 2001). The process, however, was extremely inefficient, with spontaneously contracting areas appearing in only 8% of EBs. It was clear that the timed addition of extracellular molecules acting on specific cardiogenic signaling pathways was going to be needed to improve the efficiency of differentiation. Key drivers of *in vivo* cardiogenesis have been described above and include Activin/Nodal-, BMP-, FGF-, and Wnt-mediated signaling cascades. The same pathways also play pivotal roles in promoting cardiomyogenic differentiation from PSCs. More than 30% cardiomyocytes can be obtained from human ESCs by exposure to Activin A and BMP4 (Laflamme *et al*, 2007). However, optimal levels of Activin, Nodal, and BMP signaling are required for cardiac lineage formation from different human ESC and iPSC lines (Kattman *et al*, 2011). Combinations of Activin A, BMP4, basic FGF (bFGF), VEGF-A, and Dkk1 have also been used to generate cardiovascular progenitors from human ESCs. This progenitor population, identified by low kinase insert domain receptor (KDR) expression and absence of c-kit, was able to give rise to more than 50% contracting cardiomyocytes (Yang *et al*, 2008). More recently, dual Nodal and BMP inhibition by antagonist ligand Cerberus-1 (Cer1) was shown to drive cardiomyocyte differentiation from both mouse and human ESCs (Cai *et al*, 2013).

Unlike EB differentiation, monolayer differentiation protocols involve culturing PSCs in standard two-dimensional format. Sequential treatment of high-density monolayer ESC cultures with Activin A and BMP4 was reported to yield more than 30% cardiomyocytes (Laflamme *et al*, 2007; Melkoumian *et al*, 2010), although not consistently. A different study in fact noted that the Activin A-/BMP4-directed differentiation protocol is not always successful and can sometimes yield less than 5–10% cardiomyocytes (Paige *et al*, 2010). The authors went on to show that efficient mesoderm induction and subsequent cardiac differentiation from human ESCs require fine-tuning of the cross talk between Activin A/BMP4 and Wnt/β-catenin signaling pathways (Paige *et al*, 2010). Recently, a robust cardiomyocyte differentiation protocol has been developed: By culturing pluripotent cells as monolayers and manipulating canonical Wnt signaling, around 80% cardiomyocytes can be reproducibly obtained from different human ESC lines (Lian *et al*, 2012, 2013).

Cardiomyocyte enrichment protocols have also been described that are able to yield, independently of the efficiency of differentiation, up to 99% pure PSC-derived cardiomyocytes. Different strategies have been used so far for such enrichment steps, including the use of mitochondria-specific fluorescent dyes that preferentially bind to cells with high mitochondrial content such as cardiomyocytes (Hattori *et al*, 2010), cell sorting with an antibody against the cardiomyocyte-specific marker SIRPA (signal-regulatory protein alpha) (Fujioka *et al*, 1996; Kharitonenkov *et al*, 1997; Dubois *et al*, 2011), and a biochemical purification method based on differences in sugar metabolism between cardiomyocytes and non-cardiomyocytes (Tohyama *et al*, 2013).

To date, there is no clinical test of human PSC-derived cardiomyocyte transplantation into human patients, but the first clinical-scale transplantation of *in vitro*-derived cardiomyocytes into a non-human primate (monkey) has been reported very recently (Chong *et al*, 2014b). One billion human ESC-derived cardiomyocytes were produced via the Activin A- and BMP4-mediated monolayer differentiation protocol (Laflamme *et al*, 2007) and delivered intramyocardially into the infarcted heart of a non-human primate model. After transplantation, the authors observed remuscularization of the damaged monkey heart, with formation of new muscle grafts that were electromechanically coupled to the host cardiomyocytes and rapidly perfused by the host vasculature (Chong *et al*, 2014b). However, the transplanted cells appeared quite diverse in terms of atrial–ventricular electrophysiological properties and were only partially mature, bearing more resemblance to fetal rather than adult cardiomyocytes. As a consequence, non-lethal ventricular arrhythmias were observed in the recipient monkeys. Other concerns, regarding the small number of animals studied, insufficient analyses of cardiac mechanics, and failure to provide evidence that transplanted cardiomyocytes improve cardiac function, have been raised and are discussed elsewhere (Anderson *et al*, 2014; Murry *et al*, 2014). While this study sheds hope on the possibility of remuscularizing a damaged human heart with a similar approach (Lian *et al*, 2014), it also highlights some of the crucial issues we need to address before *in vitro*-derived cardiomyocyte transplantation therapies can truly move into the clinical setting, as discussed below.

Direct conversion of differentiated somatic cells into cardiomyocytes In recent years, an alternative method to produce cardiomyocytes *in vitro* has been developed. It is often referred to as direct conversion, because it involves a cell fate switch from a fully differentiated cell type into another, without going through the pluripotent state (Sancho-Martinez *et al*, 2012). In analogy to conventional reprogramming, in which somatic cells are converted into pluripotent ones by overexpressing pluripotency-associated transcription factors (Takahashi & Yamanaka, 2006), direct conversion is achieved by forcing expression of key lineage-specific factors. The first experiment of this kind was performed almost 30 years ago, when overexpression of a single gene, the myogenic transcription

factor MyoD, was shown to be sufficient to convert fibroblasts into skeletal muscle cells (Davis *et al*, 1987). Similarly, overexpression of the smooth muscle coactivator myocardin (Myocd) can force fibroblasts into adopting a smooth muscle cell fate (Wang *et al*, 2003). Successful direct lineage conversions have since been reported for a plethora of cell types, including the hematopoietic system (Xie *et al*, 2004; Laiosa *et al*, 2006; Szabo *et al*, 2010), pancreatic exocrine cells (Zhou *et al*, 2008b), the hepatic system (Huang *et al*, 2011; Sekiya & Suzuki, 2011), and neuronal lineages (Vierbuchen *et al*, 2010; Caiazzo *et al*, 2011). Unfortunately, no single "master regulator" of cardiomyocyte development has been identified to date, but lessons from both iPSC generation and direct conversions in other systems suggest that combinations of specific factors can alter the gene expression profile of the donor cell and induce its conversion into cardiac cell types.

The therapeutic implications of being able to produce cardiomyocytes via direct conversion, rather than via PSC differentiation, are multiple. Firstly, the ability to bypass the pluripotent state may reduce the potential risk of tumorigenesis after transplantation. Secondly, similar to iPSC differentiation, immunologically matched tissue can be produced from a patient's own cells to circumvent graft rejection. Finally, the ability to directly convert non-cardiomyocytes into cardiomyocytes *in vitro* offers the enticing possibility to do the same *in vivo* and reprogram resident cardiac fibroblasts by introducing defined factors directly into the patient's heart.

A variety of recipes designed to steer fibroblast cells into a cardiomyogenic fate have been published so far, each employing a unique combination of cardiac-specific transcription factors, miRNAs, and/or chemical molecules (Table 1). The first study started out by testing fourteen different factors for their ability to induce a cardiomyocyte-like phenotype from mouse post-natal fibroblasts. Three were deemed sufficient for reprogramming: *Gata4*, *Mef2c,* and *Tbx5*, hereafter referred to as GMT factors (Ieda *et al*, 2010). The trans-differentiation process was found to be direct, with no transition through a multipotent cardiac progenitor-like state. Successful conversion into the cardiomyocyte lineage was judged by up-regulation of a cardiac-specific reporter gene, which occurred in up to 25% of transfected cells. Differentiation into cardiomyocyte-like cells was also observed when transduced fibroblasts were transplanted into immunocompromised mouse hearts 1 day after introduction of GMT factors. However, only 1% of induced cardiomyocytes (iCMs) displayed spontaneous contractions *in vitro*, suggesting overall inefficient conversion into fully mature, functional cardiomyocytes. Two additional studies later examined the utility of GMT factors for cardiac reprogramming (Chen *et al*, 2012; Inagawa *et al*, 2012). In one of them, expression of the three transcription factors via a single polycistronic vector, rather than three separate constructs, was found to enhance differentiation of iCMs obtained from mouse cardiac fibroblasts (Inagawa *et al*, 2012). When the GMT factors were delivered as separate viral vectors, most reprogrammed cells expressing cardiac markers remained smaller than endogenous ventricular cardiomyocytes and never displayed clear cross striations, even after 1 month from transduction. However, cardiomyocyte maturation was greatly enhanced upon GMT factor delivery as a single polycistronic vector – with cross striations appearing in 30% of transduced cells – highlighting the importance of choosing the appropriate delivery method and optimizing transcription factor dosage for optimal reprogramming. Interestingly, the second study that attempted to recapitulate findings by Ieda *et al* obtained markedly different results (Chen *et al*, 2012). GMT-mediated reprogramming efficiency was tested in fibroblasts derived from multiple transgenic mouse lines. In all of them, cardiac-specific gene expression was only marginally elevated as a result of GMT transduction. Importantly, the efficiency of reprogramming was extremely variable according to which reporter gene was chosen as read-out: 35% of GMT-transfected fibroblasts expressed *cTnT*, but none of these cells showed activation of two other cardiac-specific markers, *αMHC* and *Nkx2.5*. Unlike what reported by Ieda *et al*, GMT-overexpressing cells exhibited no spontaneous action potential *in vitro* and, when transplanted into injured mouse hearts, displayed poor survival and minimal activation of cardiac gene expression (Chen *et al*, 2012). While the reasons for such discrepancies may lie in the different experimental protocols and reagents used to achieve GMT overexpression, findings by Chen *et al* point out that the choice of reporters, cell types, and methods used to evaluate cardiac phenotypes have profound influences on the assessment of reprogramming efficiency and should therefore be standardized through further investigation.

Lipid-based transient transfection of four cardiac-specific miRNAs (miR-1, -133, -208, -499) was reported to convert mouse fibroblasts into cardiomyocytes *in vitro*, and the conversion efficiency enhanced up to almost 30% by inhibiting the JAK1 kinase (Jayawardena *et al*, 2012). However, no cardiac marker gene was activated when the same miRNAs were virally transduced into mouse fibroblasts by another group (Nam *et al*, 2013). Another example of experimental variables leading to markedly different reprogramming efficiencies is a study by the Olson laboratory (Song *et al*, 2012). In an effort to identify a better combination of cardiac reprogramming factors, they also transduced mouse fibroblasts with virally encoded GMT factors, followed by evaluation of the number of cells expressing both *αMHC* and *cTnT* reporter genes. Unlike Ieda *et al*, who reported a reprogramming efficiency of more than 20%, they found that GMT factors could only induce cardiac reprogramming, as assessed by expression of their two reporter genes, in around 6% of transfected cells. Adding one more cardiac transcription factor – *Hand2* – to the reprogramming cocktail, then referred to as GMTH, did however induce up to 20% of cells to become positive for both *αMHC* and *cTnT* expressions (Song *et al*, 2012). The importance of verifying the expression of multiple, lineage-specific genes when assessing reprogramming efficiency was also pointed out by another study, in which all triplet combinations of ten candidate factors were screened for their ability to induce a variety of cardiac-specific genes, whose expression reflects multiple heart functions (Protze *et al*, 2012). Interestingly, the broadest spectrum of cardiac genes was up-regulated not upon transfection of the GMT factors, but when *Gata4* was substituted with *Myocd*, suggesting that the GMT factors do not achieve full reprogramming of fibroblasts into cardiomyocytes. In conclusion, the ability to quantify reprogramming events necessarily relies on a reporter gene expressed in the desired final cell type, but such a reporter must accurately reflect the fully reprogrammed state. Overexpressing certain transcription factors might cause induction of the reporter (such as the αMHC-GFP reporter analyzed by Ieda *et al*), but not of the fully differentiated cell program. To avoid selecting for factor combinations that only achieve partial reprogramming, it is therefore essential to screen for other hallmarks of a differentiated

Table 1. Cardiac reprogramming studies.

(a) Fibroblasts → Cardiomyocytes

References	Species	Recipient cell type/Organism	Genes/Molecules	Gene delivery method	Reprogramming read-out	Reprogramming efficiency	*In vivo*
Ieda *et al* (2010)	M	Post-natal CFs and TTFs	Gata4, Mef2c, Tbx5	Retrovirus	αMHC reporter/cTnT	5–25%	–
Chen *et al* (2012)	M	CFs and TTFs	Gata4, Mef2c, Tbx5	Lentivirus	αMHC/Nkx2.5/cTnT reporters	0–35%	–
Inagawa *et al* (2012)	M	Adult CFs	Gata4, Mef2c, Tbx5	Retrovirus	αMHC reporter	3–7%	–
		MI model			αMHC reporter and α-actinin	1%	+
Qian *et al* (2012)	M	MI model	Gata4, Mef2c, Tbx5	Retrovirus	Postn/Fsp1 lineage tracing, α-actinin & sarcomeric structure	10–15%	+
Song *et al* (2012)	M	Adult CFs and TTFs	Gata4, Mef2c, Tbx5, Hand2	Retrovirus	αMHC reporter and cTnT	5–20%	–
		MI model			Fsp1/Tcf2l lineage tracing and cTnT	1–8%	+
Protze *et al* (2012)	M	Embryonic fibroblasts and neonatal CFs	Mef2c, Tbx5, Myocd	Lentivirus	αMHC reporter and cTnT	1–2%	–
Jayawardena *et al* (2012)	M	Neonatal CFs, adult CFs, and TTFs	miR-1, -133, -208, -499, +JI1	Lipid carrier	αMHC reporter	13–28%	–
		MI model	miR-1, -133, -208, -499	Lentivirus	Fsp1 lineage tracing and αMHC reporter	1%	+
Efe *et al* (2011)	M	Embryonic fibroblasts	Oct4, Sox2, Klf4, +JI1, +BMP4	Retrovirus	cTnT	40%	–
Wang *et al* (2014)	M	Embryonic fibroblasts and TTFs	Oct4, small-molecule cocktail	Retrovirus	Beating cell clusters	50–116 beating clusters/10,000 plated fibroblasts	–
Islas *et al* (2012)	H	DFs	ETS2, MESP1, +ActivinA, +BMP2	Lentivirus/ Recombinant proteins	NKX2.5 reporter/αMHC reporter and Ca^{2+} transients	2–9%	–
Nam *et al* (2013)	H	Neonatal FFs, adult CFs, and DFs	GATA4, HAND2, TBX5, MYOCD, miR-1, miR-133	Retrovirus	cTnT/tropomyosin	9–45%	–
Fu *et al* (2013)	H	ESC-derived fibroblasts, fetal CFs, and neonatal DFs	GATA4, MEF2C, TBX5, ESSRG, MESP1, MYOCD, ZFPM2 (+SIS3)	Retrovirus	αMHC reporter and cTnT	1–22%	–
Wada *et al* (2013)	H	CFs and DFs	GATA4, MEF2C, TBX5, MESP1, MYOCD	Retrovirus	α-actinin/cTnT	5%	–

(b) Cardiomyocytes → Conduction system cells

References	Species	Genes	Gene delivery/Expression method	Cell type obtained	Reprogramming readout	Reprogramming efficiency	*In vivo*
Bakker *et al* (2012)	M	Tbx3	Lentivirus	Pacemaker (SAN-like?) cells	Gene expression, electrophysiological parameters	N.D.	–
			Inducible transgene				+
Rentschler *et al* (2012)	M	NICD	Retrovirus	Purkinje-like conduction cells	Electrophysiological parameters	N.D.	–
		Notch activation	Inducible system				+
Kapoor *et al* (2013)	M	Tbx18	Adenovirus	SAN cells	Electrophysiological parameters	N.D.	–
	GP						+

GP, guinea pig; H, human; M, mouse; CFs, cardiac fibroblasts; DFs, dermal fibroblasts; MI, myocardial infarction; TTFs, tail tip fibroblasts; SAN, sino-atrial node; N.D., not determined.

cardiomyocyte, such as global cardiac gene expression, sarcomeric structure, and action potentials (Protze *et al*, 2012).

Cardiac reprogramming of fibroblast cells can be achieved by direct conversion, with no appearance of an intermediate cardiac precursor-like state, as reported by some of the studies above (Ieda *et al*, 2010; Song *et al*, 2012), or by an indirect switch in cell fate, which transitions through a cardiac progenitor state. By adapting the conventional iPSC reprogramming protocol, Efe *et al* achieved partial dedifferentiation of mouse embryonic fibroblasts, followed by differentiation into cardiomyocytes through a mitotically active intermediate expressing early (*Mesp1*, *Flk1*) and then mid-stage (*Nkx2.5*, *Gata4*, *Isl1*) cardiac progenitor markers (Efe *et al*, 2011). After initial overexpression via inducible viral vectors of the pluripotency factors *Oct4*, *Sox2,* and *Klf4*, cells were exposed to the small-molecule JAK inhibitor JI1, followed by culturing in a chemically defined medium containing the cardiomyogenic growth factor BMP4. With this protocol, the conventional reprogramming route toward pluripotency was shortcut and directed toward cardiac cytogenesis instead, with a conversion efficiency of 40% (Table 1) (Efe *et al*, 2011). In a similar recent study, mouse fibroblasts were initially transduced with *Oct4* alone and then exposed to a cocktail of lineage-specific soluble signals, including an anaplastic lymphoma kinase (ALK) inhibitor and a GSK3β inhibitor, to achieve transdifferentiation into the cardiac lineage (Wang *et al*, 2014). Despite initial *Oct4* overexpression from an inducible viral construct, converted cells never enter the pluripotent state but transition through a cardiac progenitor-like stage (as determined by expression of *Flk1*, *Mesp1*, *Isl1,* and *Gata4*) before turning into differentiated cardiomyocytes. Finally, viral-mediated co-expression of *Mesp1* and *Ets2* transcription factors or cell treatment with MESP1 and ETS2 recombinant proteins was reported to reprogram human dermal fibroblasts into cardiac progenitors, marked by expression of core cardiac transcription factors (*Nkx2-5, Isl1, Tbx5, Mef2c, Gata4*) (Islas *et al*, 2012).

Given the increased complexity of gene regulatory pathways in human cells, cardiomyocyte generation from human fibroblasts requires more factors than those needed to reprogram mouse fibroblasts. This observation is in line with what is reported for the generation of human iPSCs or neuronal cells, which also require different culture conditions and/or additional transcription factors compared to mouse cells. Species-specific requirements likely reflect differences in gene expression and regulation between mouse and human fibroblasts and different susceptibility of lineage-specific genes to activation in different cell types. For example, both GMT and GMTH factor combinations are insufficient for cardiac reprogramming of human fibroblasts (Islas *et al*, 2012; Nam *et al*, 2013; Wada *et al*, 2013). By employing a combination of four transcription factors (*Gata4, Hand2, Tbx5, Myocd*) and two miRNAs (miR-1, miR-133), human neonatal and adult fibroblasts were successfully converted into cardiomyocyte-like cells characterized by cardiac gene activation and sarcomeric-like structures (Nam *et al*, 2013). Despite initial high reprogramming efficiency (up to 45% depending on readout and fibroblast origin), human iCMs required longer maturation time compared to their murine counterparts and displayed low-amplitude calcium transients in response to electrical stimulation and extremely rare spontaneous contractions. Moreover, human iCMs appeared to be heterogeneous, with each cell expressing different levels of cardiac and non-cardiac genes (Nam *et al*, 2013). This heterogeneity may partly be ascribed to the mixed age and genetic background of the human fibroblasts tested, which will influence cell-to-cell variability in epigenetic landscapes and therefore in susceptibility to the reprogramming process. Importantly, utilizing different combinations of reprogramming factors does not seem to improve the outcome considerably. Induced cardiomyocytes generated by overexpressing GMT factors plus *Mesp1* and *Myocd* in human cardiac or dermal fibroblasts are functionally immature, as indicated by cell morphology, expression of embryonic cardiomyocyte marker genes, and slow calcium oscillations (Wada *et al*, 2013). Similar findings were reported when fibroblasts were transduced with GMT plus *Esrrg* (estrogen-related receptor gamma), *Mesp1*, *Myocd,* and *Zfpm2* (zinc finger protein, FOG family member 2) (Fu *et al*, 2013). Despite the appearance of a cardiomyocyte-like phenotype, with cardiac-specific gene up-regulation and sarcomere assembly, only a few of the reprogrammed cells fired action potentials upon electrical stimulation and none of them displayed any spontaneous contractions, even after a long time in culture.

Immediately after the first successful attempts at *in vitro* cardiomyocyte reprogramming of fibroblast cells were reported, several groups began to try the same *in vivo*, by injecting cardiac reprogramming factors directly into mouse hearts (Table 1). Intramyocardial delivery of retroviral vectors expressing the GMT factors in a mouse model of MI was shown to reprogram resident fibroblasts into cardiomyocyte-like cells (Qian *et al*, 2012). Interestingly, the initial reprogramming efficiency achieved *in vivo* (10–15%) was similar to that observed by the same group during *in vitro* conversion experiments, but *in vivo*-derived iCMs appeared more fully reprogrammed and more similar to endogenous cardiomyocytes than *in vitro*-derived ones (Ieda *et al*, 2010; Qian *et al*, 2012) (Table 1). This would suggest that factors within the native milieu that are absent in a cell culture dish – such as extracellular matrix and secreted proteins – enhance the cell fate switch. Importantly, *in vivo* cardiac reprogramming was accompanied by an improvement in cardiac function and a reduction in scar size following injury, but the functional improvement of GMT-injected hearts seemed greater than what one might expect from the relatively inefficient reprogramming of adult cardiac fibroblasts *in vitro*. This could mean that the observed benefits may arise from a combination of new muscle formation and other non-cell-autonomous effects, such as growth factor secretion. A similar conclusion was made when the GMTH factors were employed, as they only reprogrammed 1–8% of transduced cardiac fibroblasts but caused a much more pronounced improvement in heart function (Song *et al*, 2012). In another study, injection of GMT-expressing retroviral constructs *in vivo* only caused 1% of transduced fibroblasts to convert into cardiomyocyte-like cells, a strikingly lower reprogramming efficiency than the 10–15% claimed by Qian *et al* (Inagawa *et al*, 2012). As pointed out above for the *in vitro* studies, these differences may be due to a number of experimental variables that affect the efficiency of conversion *in vivo*, such as the mouse strain employed or the transgene expression levels achieved. Moreover, the study by Inagawa *et al* used immunosuppressed nude mice, because the number of retrovirus-infected cells was dramatically reduced 2 weeks after MI in immunocompetent mice (Inagawa *et al*, 2012), whereas no loss of reprogrammed cells was seen in the immunocompetent mice used by Qian *et al*, even four weeks post-infection (Qian *et al*,

2012). It is clear that additional experiments are needed to redeem these controversies before any conclusion concerning the utility of *in vivo* reprogramming therapies can be made.

Somatic stem cell transplantation therapies Since the realization that adult somatic stem cells can be isolated from many different tissue sources and can spontaneously differentiate *in vivo* in response to endogenous cues, the idea to use these cells to repair cardiac injury has been all the rage and translational efforts have proceeded at the speed of light (Hansson & Lendahl, 2013; Matar & Chong, 2014). Here, we briefly discuss some of the somatic stem cell transplantation therapies for heart diseases that have undergone clinical testing. A detailed review is obtained elsewhere (Rosen *et al*, 2014).

The first somatic stem cells to be tested were skeletal myoblasts (Taylor *et al*, 1998; Menasche *et al*, 2001). Upon muscle injury, skeletal muscle progenitor cells proliferate and promote regeneration by differentiating into new muscle fibers, making them attractive candidates for cardiac regeneration tools. The MAGIC (myoblast autologous grafting in ischemic cardiomyopathy) clinical trial examined the effects of intra-myocardial injection of skeletal myoblasts in 97 patients with severe ischemic heart failure (HF) (Menasche *et al*, 2008) (Table 2). Smaller clinical trials had previously yielded encouraging results, but MAGIC did not corroborate these findings. Although the occurrence of malignant arrhythmias between the myoblast-treated patients and controls was the same, at the end of the 6-month-long observation period, myoblast treatment did not cause any significant improvement in cardiac function or clinical status. Due to the overall discouraging results of the MAGIC trial, the risk of arrhythmias associated with skeletal myoblast transplantation as shown by the MARVEL (myoblast implantation into myocardium post myocardial infarction) trial (Povsic *et al*, 2011) (Table 2), and the availability of more attractive cell sources, interest in skeletal myoblasts has waned in recent years.

The majority of preclinical and clinical studies of somatic stem cell therapy for HF patients employed bone marrow-derived stem cells, including hematopoietic and non-hematopoietic stem cell populations, which can differentiate into diverse cellular phenotypes. Cells used in clinical trials include unfractionated bone marrow mononuclear cells (BMMNCs), $CD34^+$ and/or $CD133^+$ hematopoietic and endothelial stem/progenitor cells, and mesenchymal stem cells (MSCs). BMMNCs have been investigated multiple times in animal models of acute MI with encouraging results (Balsam *et al*, 2004; Murry *et al*, 2004). In the clinical setting, however, conflicting results have been obtained in patients with ischemic/non-ischemic HF treated with BMMNCs. The REPAIR-AMI (intracoronary progenitor cells in acute myocardial infarction) trial aimed to investigate the effects of intracoronary injection of autologous bone marrow cells in patients within 7 days after the onset of acute MI and successful reperfusion therapy. Published results reported improved ventricular function and event-free survival in cell-treated patients compared to the placebo group, up to 5 years post-transplantation (Schachinger *et al*, 2009; Assmus *et al*, 2014). However, other trials have failed to confirm the beneficial effects of intracoronary delivery of BMMNCs in ischemic HF (Ang *et al*, 2008; Yao *et al*, 2008). To definitively examine whether BMMNCs can reduce mortality after MI, a large multicenter European clinical trial called BAMI (the effect of intracoronary reinfusion of BMMNCs on all cause mortality in acute myocardial infarction) has been initiated (Table 2).

BMMNCs contain a low percentage (0.5–2.5%) of hematopoietic stem cells and endothelial progenitor cells, which are marked by cell surface markers CD34 and/or CD133 (Mackie & Losordo, 2011). Autologous $CD34^+$ cells have been transplanted into patients affected by either ischemic (Patel *et al*, 2005) or non-ischemic (Vrtovec *et al*, 2013) cardiomyopathy, and found to improve cardiac function. While transplanted $CD34^+$ cells are unlikely to transdifferentiate into cardiomyocytes (Murry *et al*, 2004), they might be inducing cardiac repair by promoting neovascularization via both direct engraftment and indirect paracrine effects (Mackie & Losordo, 2011). The RENEW study is planning to assess efficacy and safety of intramyocardial autologous $CD34^+$ cell transplantation in patients with refractory angina (Table 2) (Povsic *et al*, 2013). Effects of bone marrow-derived $CD133^+$ cells were examined in patients with ischemic HF (Stamm *et al*, 2007). Preliminary results indicate that intramyocardial $CD133^+$ cell transplantation improves perfusion and contractile function of the infarcted myocardium, presumably because of increased neovascularization. A larger study, termed PERFECT (intramyocardial transplantation of bone marrow stem cells in addition to coronary artery bypass graft surgery), is now planning to enroll 142 patients to determine the potential of bone marrow-derived $CD133^+$ cells to promote cardiac regeneration (Donndorf *et al*, 2012) (Table 2).

Mesenchymal stem cells (MSCs) are non-hematopoietic multipotent stem cells marked by expression of the cell surface markers CD105, CD73, CD90, and CD146 and by absence of the hematopoietic markers CD45, CD34, and CD14/CD11b (Barry & Murphy, 2004). MSCs can differentiate into adipocytes, osteoblasts, chondrocytes, and skeletal muscle cells and have also been shown to differentiate into endothelial cells and possibly cardiomyocytes (Pittenger *et al*, 1999; Reyes *et al*, 2002; Toma *et al*, 2002; Reinecke *et al*, 2008). Encouraged by animal studies reporting that transplanted MSCs could increase vascularization of the damaged heart and improve cardiac function (Silva *et al*, 2005), scientists initiated the PROMETHEUS (prospective randomized study of mesenchymal stem cell therapy in patients undergoing cardiac surgery) trial, in which autologous bone marrow-derived MSCs were administered to patients with chronic ischemic HF (Table 2). Recently published results show that intra-myocardial MSC injection resulted in scar size reduction, improvement in contractile function, and increased perfusion (Karantalis *et al*, 2014). However, it must be noted that the study was only conducted on a very limited sample population of six patients. The effects of bone marrow-derived MSCs on chronic ischemic HF were also examined by the POSEIDON trial (percutaneous stem cell injection delivery effects on neomyogenesis). Importantly, although initial results reported that MSCs exert favorable effects on ventricular remodeling, functional capacity, and patient quality of life (Hare *et al*, 2012), the POSEIDON trial failed to show an improvement in global ventricular function (Suncion *et al*, 2014).

Much controversy surrounds clinical trials employing putative CPCs, such as those marked by c-kit expression. Despite the heated debate around the origin and cardiomyogenic potential of c-kit^+ cells, SCIPIO (stem cell infusion in patients with ischemic cardiomyopathy)—the first CPC clinical trial—was initiated (Bolli *et al*, 2011) (Table 2). Autologous c-kit^+ CPCs were isolated from cardiac tissue obtained during surgery, expanded *ex vivo*, and delivered via intracoronary infusion in patients affected by ischemic HF. Although the planned two-year follow-up period still awaits completion, interim

Table 2. Selected clinical trials of stem cell therapy for cardiac regeneration.

Trial name/References	Classification	Cell type	Delivery method	Patient number	Follow-up period	Outcome	Side effects
MAGIC (Menasche *et al*, 2008)	Phase I/II	SKMs	Intramyocardial injection during CABG	97	6 m	Unchanged LVEF and regional wall motion/decreased LVEDV in patients receiving high dose	Ventricular arrhythmias in 9 of 63 patients, and 9 of 63 patients died
MARVEL (Povsic *et al*, 2011)	Phase I/II	SKMs	Transcatheter intramyocardial	20	3, 6 m	Improved 6-minute walk distance	Ventricular tachycardia in 7 of 14 patients
Repair-AMI (Assmus *et al*, 2014) (Schachinger *et al*, 2009)	Phase III	BMMNCs	Intracoronary	204	4, 12 m; 2,5 y	Reduced LV remodeling/Improved ventricular function	5 of 101 patients hospitalized for heart failure, and 7 of 101 patients died
BAMI (NCT01569178)	Phase III	BMMNCs	Intracoronary	3000	3 y	Currently recruiting	
RENEW (Povsic *et al*, 2013)	Phase III	BM-derived $CD34^+$ stem cells	Intramyocardial	444	12 m	Currently ongoing	
PERFECT (Donndorf *et al*, 2012)	Phase III	BM-derived $CD133^+$ stem cells	Intramyocardial injection during CABG	142	6 m	Currently recruiting	
POSEIDON (Hare *et al*, 2012) (Suncion *et al*, 2014)	Phase I/II	BM-derived MSCs	Intramyocardial (transendocardial)	30	13 m	Unchanged LVEF/decreased LVEDV and scar mass/improved physical performance	3 patients hospitalized for heart failure
PROMETHEUS (Karantalis *et al*, 2014)	Phase I/II	BM-derived MSCs	Intramyocardial injection during CABG	6	3, 6, 18 m	Increased LVEF/reduced scar mass	No major complications reported
TAC-HFT (Heldman *et al*, 2014)	Phase I/II	BMMNCs vs. BM-derived MSCs	Intramyocardial (transendocardial)	65	3, 6, 12 m	Unchanged LVEF and LV volumes/improved regional LV function and decreased infarct size (only with MSCs)	No major complications reported
ADVANCE (NCT01216995)	Phase II	Adipose-derived MSCs	Intracoronary	216	6, 12 m; 3 y	Currently ongoing	
SCIPIO (Chugh *et al*, 2012)	Phase I	$c\text{-}kit^+$ CPCs	Intracoronary	33	4, 12 m	Increased LVEF/reduced infarct size/increased viable mass	No major complications reported
ALCADIA (NCT00981006)	Phase I	CPCs	Intramyocardial injection during CABG	6	12 m	Currently ongoing	
CADUCEUS (Malliaras *et al*, 2014)	Phase I	Cardiosphere-derived CPCs	Intracoronary	25	6, 12 m	Unchanged LVEF and LV volumes/reduced scar mass/increased viable mass	Serious adverse events in 6 of 17 patients, and 1 of 17 patients died
ALLSTAR (NCT01458405)	Phase I/II	Cardiosphere-derived CPCs	Intracoronary	274	12 m	Currently recruiting	

For ongoing trials with no published results, the ClinicalTrials.gov Identifier (NCT...) has been indicated.
BM, bone marrow; BMMNCs, bone marrow mononuclear cells; CPCs, cardiac progenitor cells; MSCs, mesenchymal stem cells; SKMs, skeletal myoblasts; CABG, coronary artery bypass graft; LV, left ventricular; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; m, months; y, years.

observations indicate that infusion of c-kit$^+$ cells is associated with LVEF improvement and infarct size reduction that persist after one year (Bolli *et al*, 2011; Chugh *et al*, 2012). It must be noted though that concerns regarding patient randomization and the integrity of certain data generated during the SCIPIO trial have recently been raised (Nowbar *et al*, 2014; The Lancet Editors, 2014).

Another prominent clinical trial of autologous human CPC transplantation involved the use of cardiosphere-derived cardiac stem cells. Cardiospheres are multipotent cardiac-derived cells that can grow as self-adherent clusters and differentiate into all three major cardiac lineages *in vitro* (Messina *et al*, 2004). Cardiospheres isolated from human endomyocardial biopsies can be cultured *in vitro* to generate cardiosphere-derived cells (CDCs) (Smith *et al*, 2007). CDCs retain their cardiomyogenic differentiation potential *in vitro* and are able to promote cardiac regeneration and improve heart function *in vivo* in both murine and porcine MI models (Smith *et al*, 2007; Johnston *et al*, 2009). The regenerative potential of CDCs in human beings has been addressed by the CADUCEUS (cardiosphere-derived autologous stem cells to reverse ventricular dysfunction) trial (Table 2). Autologous CDCs derived from endomyocardial biopsies were expanded *in vitro*, followed by intracoronary infusion into patients with a recent episode of MI. CDC administration led to a reduction in scar size, an increase in the amount of viable myocardium but only regional improvement in left ventricular function (Makkar *et al*, 2012; Malliaras *et al*, 2014). Data from the CADUCEUS trial do not definitively prove CDC-mediated cardiac regeneration, and the clinical effects of CDCs thus remain unclear. It should also be noted that both cardiospheres and CDCs are heterogeneous mixtures containing different types of cells that express endothelial and mesenchymal antigens (Messina *et al*, 2004). Which of these cell types is responsible for the observed effects on heart remodeling and function is at this point unknown. A larger clinical trial, termed ALLSTAR (allogeneic heart stem cells to achieve myocardial regeneration), is now planning to investigate the effects of allogeneic CDC therapy in approximately 300 patients (Table 2).

In summary, although a multitude of clinical trials have been performed to date, their results remain ambiguous and no single-cell-based therapy for heart disease has been conclusively proven effective so far, particularly in improving life expectancy in patients. Trial outcomes are frequently affected by noise, poor trial design (especially the absence of blinding), and normal human tendencies toward optimism and denial, making it difficult to justify and conclude them clearly (Rosen *et al*, 2014). Rigorous long-term studies with adequate patient population size will have to be conducted in the future to solve the many controversies.

Cell-free therapies

Lessons from cell-based therapies indicate that, rather than transplanting cells that directly engraft and differentiate/proliferate in the host tissue, delivering paracrine factors alone to the damaged heart may be sufficient to activate repair mechanisms. This hypothesis has opened new avenues for cell-free therapies in cardiac regenerative medicine (Fig 4, right). Factors of choice can be delivered to the heart in one of multiple forms, including viral and non-viral DNA vectors, modified mRNAs, small-molecule chemical compounds, and recombinant proteins. Studies of mammalian and non-mammalian heart injury models have taught us that there are multiple mechanisms involved in cardiac regeneration/replication (Fig 3) and these pathways/modulators can be exploited to develop novel, non-cell-based therapies for heart disease. Current efforts are thus trying to identify clinically useful factors or molecules that can regenerate the damaged heart by (i) inducing proliferation of preexisting cardiomyocytes, (ii) reprogramming cardiac fibroblasts into cardiomyocytes by direct conversion *in vivo* (as described above) (iii) trans-differentiating one type of cardiomyocyte or other resident cardiac cell into another, or (iv) activating endogenous CPCs toward their differentiation. These mechanisms, together with other indirect ones acting on non-cardiomyocytes, such as the induction of angiogenesis and new vessel formation and the reduction of fibrotic scars, are likely to promote cardiac repair in a cooperative fashion (Fig 4).

Preexisting cardiomyocyte proliferation As detailed above (Fig 3), the neonatal mouse heart possesses a robust but transient regenerative capacity that rapidly disappears after the first few days of life. Manipulating the signaling pathways/drivers that control post-natal loss of cardiomyocyte proliferation represents an attractive strategy to reactivate proliferation mechanisms in the injured adult mammalian heart. One such target is the p38 MAPK signaling pathway, as cardiomyocytes seem to control cell cycle progression by modulating p38 MAPK activity. Overexpression of p38 was shown to block proliferation of rat fetal cardiomyocytes, whereas its inhibition in adult cardiomyocytes could promote cell division (Engel *et al*, 2005). However, p38 MAPK inhibition alone was not able to rescue heart function in adult rodent models of myocardial injury (Engel *et al*, 2006). An improvement in cardiac function was only observed when animals were also treated with FGF1, which may promote survival of newly generated cardiomyocytes by improving angiogenesis. A p38 MAPK inhibitor and FGF1 could therefore be employed together as therapeutic agents for cardiac regeneration.

High-throughput functional screening for human miRNAs has identified a subset of miRNAs, represented by miR-590 and miR-199a, as being able to induce both DNA synthesis and cytokinesis in neonatal mouse and rat cardiomyocytes (Eulalio *et al*, 2012). Following MI in mice, these miRNAs stimulated marked cardiac regeneration and significantly improved cardiac function. In contrast, miR-15 was shown to be up-regulated after the first post-natal week in mice, coinciding with the rapid loss of regenerative capacity (Porrello *et al*, 2013). miR-15 inhibition in mice improves cardiac function after injury, suggesting its potential as a therapeutic target.

The homeodomain transcription factor Meis1 has been identified as a critical transcriptional regulator of cardiomyocyte cell cycle and proliferation through activation of the cyclin-dependent kinase (CDK) inhibitors p15, p16, and p21 (Mahmoud *et al*, 2013). Meis1 represses neonatal cardiomyocyte proliferation and notably, *Meis1* deletion was sufficient to extend the post-natal proliferative window of cardiomyocytes in mice (Mahmoud *et al*, 2013), indicating that inhibition of this pathway may have therapeutic potential for cardiac regeneration/proliferation.

The oxygen-rich post-natal environment has been shown to induce cardiomyocyte cell cycle arrest through DNA damage response and to be associated with the post-natal loss of regenerative capacity (Puente *et al*, 2014). After birth, mammalian cardiomyocytes switch from a glycolytic to an oxidative metabolism. These post-natal metabolic changes are associated with marked increases of reactive oxygen species (ROS), oxidative DNA damage,

and DNA damage response markers in the heart. Of interest, interfering with these processes by reducing oxidative stress was shown to successfully increase cardiomyocyte proliferation beyond its normally permissive time frame, suggesting that the reduction of oxidative stress may be a novel mechanism to stimulate mammalian cardiomyocyte proliferation (Puente *et al*, 2014).

Hippo signaling, an ancient organ size control pathway, inhibits developing cardiomyocyte proliferation (von Gise *et al*, 2012) and a recent study showed that inactivation of this pathway prompts adult mouse cardiomyocytes to re-enter the cell cycle and undergo cytokinesis, promoting cardiac regeneration following injury in both adult and neonatal mice (at post-natal day 8) (Heallen *et al*, 2013). In concert with this finding, Yes-associated protein (YAP), the terminal effector of the Hippo signaling pathway, was shown to be crucial for regulating mouse cardiomyocyte proliferation (Lin *et al*, 2014) and thus might also become a novel therapeutic target for cardiac repair.

Positive regulators of cardiomyocyte proliferation include members of the neuregulin-1 (NRG1)/ERRB4 and periostin (POSTN) signaling cascades. Increasing signaling through the NRG1/ERBB4 pathway may represent a novel therapeutic strategy to promote cardiac regeneration, as shown by the beneficial effects of NRG1 injection in adult mice, which induced cardiomyocyte proliferation and improved heart function after MI (Bersell *et al*, 2009). Similarly, POSTN ligand can stimulate differentiated cardiomyocytes to re-enter the cell cycle by binding the cell surface integrin receptors αV, β1, β3, and β5 and inducing downstream PI3K activation, which is sufficient to promote cardiomyocyte renewal in the absence of POSTN (Kuhn *et al*, 2007). POSTN/PI3K-mediated cardiomyocyte proliferation was shown to have beneficial effects on heart morphology and function after MI (Kuhn *et al*, 2007).

Trans-differentiation of cardiomyocytes or other cardiac cells Direct cardiac reprogramming efforts have mainly focused on converting resident cardiac fibroblasts into cardiomyocytes, but there is no reason why the technology should be limited to these cell types. Successful trans-differentiation of cardiomyocytes into conduction system cells has been reported both *in vitro* and *in vivo* (Table 1) and may revolutionize current clinical treatment of cardiac conduction system disorders, which generally involves the implantation of costly electronic pacemakers. Ectopic activation of the pacemaker cell-related transcription factor Tbx3 causes cardiomyocytes to change their gene expression profile from that of working myocardium to that of pacemaker myocardium (Bakker *et al*, 2012). Unfortunately, although induced sinoatrial-like cells display pacemaker characteristics *in vitro*, no biological pacemaker activity was created *in vivo*. Tbx18 transduction also converts cardiomyocytes into sinoatrial-like cells and appears to be superior to Tbx3 in generating both automaticity *in vitro* and biological pacemaker activity *in vivo* (Kapoor *et al*, 2013). Finally, a third study recently reported *in vitro* and *in vivo* cardiomyocyte reprogramming into Purkinje-like cells by activating the Notch signaling cascade (Rentschler *et al*, 2012).

As described above (Fig 3), a recent study in zebrafish embryos showed that differentiated atrial cardiomyocytes can trans-differentiate into ventricular cardiomyocytes and contribute to ventricular regeneration upon injury, and that this process depends on Notch signaling activation (Zhang *et al*, 2013). Although it remains to be elucidated whether mammalian atrial cardiomyocytes have similar lineage plasticity, recent studies have shown that a cardiomyocyte progenitor population in mammalian fetal hearts is indeed enriched in the atrial chambers (Laugwitz *et al*, 2005; Bu *et al*, 2009; Genead *et al*, 2010). Together, these observations suggest that the trans-differentiating atrial cardiomyocytes found in zebrafish may be analogous to the atrial-resident multipotent CPCs found in mammals.

Finally, it has been recently shown that cardiac fibroblasts can trans-differentiate into endothelial-like cells after cardiac injury (Ubil *et al*, 2014). After acute ischemic heart injury, some cardiac fibroblasts rapidly adopt an endothelial-cell-like phenotype in response to p53 activation and contribute to neovascularization, which overall improves cardiac function. This native fibroblast-to-endothelial cell conversion could thus represent a novel therapeutic target to enhance cardiac repair.

Endogenous cardiac progenitor cell activation and differentiation Recent studies suggest that the predominant source of post-injury formed cardiomyocytes is preexisting cardiomyocytes (Garbern & Lee, 2013; Mollova *et al*, 2013). However, there is still the possibility for an alternative cardiac progenitor source to contribute to new generation of cardiomyocytes after injury (Senyo *et al*, 2013). Thus, activation of endogenous CPCs toward differentiation into cardiac cells is another important therapeutic strategy for cardiac repair.

The mammalian heart develops from multipotent cardiovascular progenitors, including the FHF and SHF CPCs, and EPDCs (Fig 1). The clinical utility of FHF- and SHF-derived progenitors is limited by their absence in the adult heart, making it unlikely that they could serve as a source of new cardiac cells after myocardial damage (Weinberger *et al*, 2012). Conversely, a number of resident cardiac progenitor populations have been identified in the adult epicardium, including EPDCs marked by expression of *Wt1* (Zhou *et al*, 2008a; Smart *et al*, 2011; Zangi *et al*, 2013), *Tbx18* (Cai *et al*, 2008; Zhou *et al*, 2011), or *Tcf21* (Braitsch *et al*, 2013). All of these EPDCs play a role in forming the fibrotic scar that arises after MI, and it remains unclear how to drive these cells away from the cardiac fibroblast fate and into a more useful cardiomyogenic cell fate. $Wt1^+$ EPDCs, for instance, are present at very low numbers in the normal adult heart and can readily differentiate into cardiac fibroblasts or smooth muscle cells in response to appropriate stimuli. However, they have limited potential to differentiate into endothelial cells and little, if any, capacity to form cardiomyocytes in both normal and pathological conditions (Smart *et al*, 2011; Zhou *et al*, 2011; Zangi *et al*, 2013). Priming, or pretreatment, of adult mouse hearts with the peptide thymosin β4 (Tβ4) was shown to drive endogenous EPDCs into a vascular cell fate that could promote neovascularization (Smart *et al*, 2007). In a follow-up study, the same group described Tβ4 priming before injury as a way to mobilize $Wt1^+$ progenitor cells and stimulate their differentiation into novel cardiomyocytes that can integrate structurally and functionally with the surrounding muscle after MI (Smart *et al*, 2011). Hence, Tβ4 has been heralded as a means to enhance response of the adult mammalian heart to injury, via initiation of a novel cardiomyogenic program. Unfortunately, the process is relatively inefficient and the Tβ4 peptide does not reprogram EPDCs into cardiomyocytes when administered after injury, making its use as a novel therapeutic agent extremely unlikely (Zhou *et al*, 2012).

Recently, direct injection after MI of a chemically modified mRNA (modRNA) encoding a single paracrine factor, VEGF-A, was shown to induce reactivation of the quiescent adult endogenous $Wt1^+$ EPDCs. The transient pulse of VEGF-A overexpression was sufficient to promote EPDC proliferation and to drive them away from the fibroblast, scar-forming fate, and toward an endothelial/smooth muscle cell fate that could ultimately improve cardiac function (Zangi *et al*, 2013). VEGF-A also appeared to stimulate adult EPDCs to differentiate toward the cardiomyocyte lineage, although the number of generated cardiomyocytes was extremely low (Zangi *et al*, 2013). It is likely that a number of other paracrine factors, aside from VEGF-A, will be able to activate endogenous progenitor cells and promote their cardiomyocyte differentiation once delivered into the infarcted mammalian heart via modRNA injection. It is therefore worth screening paracrine factor libraries to identify the best candidate for this novel, cell-free therapeutic approach (Chien *et al*, 2014; Lui *et al*, 2014).

Unresolved issues in human cardiac regenerative therapeutics

As described above, various cell-based and cell-free therapeutic strategies for cardiac regeneration achieved encouraging results in animal experiments, often leading to their rapid promotion to clinical testing. However, the benefits of such treatments, if any, remain controversial and these therapies are still far from widespread clinical application (Garbern & Lee, 2013; Hansson & Lendahl, 2013; Sanganalmath & Bolli, 2013; Matar & Chong, 2014; Nowbar *et al*, 2014). Before proceeding these strategies toward realistic clinical translation, many critical issues will have to be addressed.

Cell type and scalability Many different types of cells, including various CPCs, non-cardiac somatic stem cells, human PSC (hPSC)-derived cardiomyocytes, and more recently, directly reprogrammed cardiomyocyte-like cells have been considered for exogenous delivery. However, there is no consensus on the ideal cell type to adopt for cell transplantation in the setting of cardiac regenerative therapeutics for heart disease. Very few studies compared different cell types in regard to their therapeutic efficacy (Mathieu *et al*, 2009; Shintani *et al*, 2009; Mazo *et al*, 2010). In such studies, it is often hard to definitively conclude which cell type would be superior to others, given the difficulty to define the dose–response relationship for each cell type before the comparison is initiated. Conceptually, the ideal cell type should tolerate autologous transplantation, expand rapidly *in vitro*, differentiate specifically into cardiomyocytes, and couple electrically with the host cardiomyocytes. In addition, previous preclinical studies suggest that combinations of different cell types may be more efficient in promoting cardiac regeneration and function than a single-cell therapy, due to the complementary or even synergistic actions of different cell types (Bonaros *et al*, 2006; Williams *et al*, 2013), although further studies are needed.

The dose–response relationship and appropriate frequency of administration for each cell therapy are other undetermined issues that very few clinical studies tried to address so far (Menasche *et al*, 2008; Hare *et al*, 2012). Producing scalable cultures of hPSC-derived cells is challenging, but recent advances allow for large-scale production of hPSC-derived cardiomyocytes (Zhang *et al*, 2012; Lian *et al*, 2013) and/or endothelial progenitors (Sahara *et al*, 2014). Such technological advances encourage studies to determine the relationship between the number/frequency of cells administered and their effects on cardiac regeneration and function.

Mechanism by which cell therapy promotes cardiac regeneration The mechanisms of action by which cell therapy contributes to the generation of new cardiomyocytes and/or to an improvement in cardiac function remain unclear (Fig 4). It is possible that injected cells do so by engrafting directly into the damaged heart, proliferating, and differentiating into mature cardiac cell types, although previous studies suggest that these events are relatively rare (Tang *et al*, 2010; Loffredo *et al*, 2011). When $c\text{-}kit^+$ CPCs were transplanted into rats 1 month after coronary occlusion followed by reperfusion, the rats exhibited more viable myocardium in the injured region, less fibrosis, and improved ventricular function 5 weeks after transplantation. However, the number of transplanted $c\text{-}kit^+$ cell derivatives contributing to the newly formed myocardium was too small to account for the observed beneficial effects (transplanted $c\text{-}kit^+$ cell-derived cardiomyocytes only accounted for 2.6% of the total cardiomyocyte population in the injured region and 1.1% in the non-infarcted region) (Tang *et al*, 2010). Similar findings were also reported in a porcine ischemic model (Bolli *et al*, 2013). Collectively, these observations suggest that, rather than by direct engraftment and differentiation into cardiac lineages, transplanted cells may exert their salutary effects indirectly, by secreting paracrine signals that act on surrounding cells (Gnecchi *et al*, 2008). Paracrine signals such as cytokines and growth factors may promote cardiac repair through a variety of mechanisms, including preexisting cardiomyocyte dedifferentiation/proliferation, recruitment/activation of endogenous CPCs, induction of angiogenesis and new vessel formation, reduction of fibrotic scars, and inhibition of apoptosis, ultimately resulting in enhanced cardiac function and myocardial repair. To develop future cell-based and/or cell-free therapies for cardiac regeneration, it is important to define the extent to which direct cell engraftment followed by differentiation/proliferation or rather the paracrine effects of transplanted cells account for observed beneficial effects.

Subtype of generated cardiomyocytes Recent advanced differentiation protocols from hPSCs allow the generation of cardiomyocytes with unprecedentedly high efficiency (approximately 80%) (Zhang *et al*, 2012; Lian *et al*, 2013). However, the resultant cardiomyocyte populations are a heterogeneous and frequently uncharacterized mixture of different subtypes, such as atrial, ventricular, and conductive cells, which can be distinguished by their gene expression profile and electrophysiological properties (Xu *et al*, 2012; Weng *et al*, 2014). To obtain better effects on cardiac function and prevent arrhythmias after transplantation to the ventricle, it may be essential to selectively produce the ventricular subtype of cardiomyocytes through directed differentiation of hPSCs. In this regard, recent studies have reported that down-regulation of retinoic acid signaling (Zhang *et al*, 2011), or hypoxic conditions plus sequential addition of BMP4/Activin and a Wnt inhibitor (Weng *et al*, 2014) successfully generate ventricular cardiomyocytes, but not other subtypes, from hPSCs. Directed differentiation approaches need to be further optimized in this way to purify each subtype of cardiomyocytes for therapeutic transplantation purposes. hPSC-derived cardiomyocytes, as well as directly reprogrammed cardiomyocyte-like cells, exhibit structural and functional features

of neonatal, rather than adult, cardiomyocytes (Ieda *et al*, 2010; Song *et al*, 2012; Lundy *et al*, 2013), which could lead to increased risk of arrhythmias, lower long-term stability, and poor integration into the host myocardium after transplantation. Structural and functional maturation to adult-like cells can be achieved by longer *in vitro* culture times (Lundy *et al*, 2013), but the extent to which cells need to be matured *in vitro* before transplantation to achieve post-transplantation efficacy and safety remains an important unanswered question.

Electrical coupling One major caveat associated with cardiac cell therapy is the risk of arrhythmias, due to incomplete electrical coupling of transplanted cells with host cardiomyocytes. Ideally, transplanted cells have to align, engraft, and couple with host cardiomyocytes in an ordered fashion, although how this process is orchestrated is unclear. A recent study has shown that human ESC-derived cardiomyocytes can engraft into injured guinea pig hearts with 1:1 host–graft coupling, and this results in reduced risk of arrhythmias and improved cardiac function, indicating human ESCs as a potentially safe tool for cardiac regeneration (Shiba *et al*, 2012). As described above, human ESC-derived cardiomyocytes also seem to generate new muscle grafts when transplanted into the infarcted monkey heart (Chong *et al*, 2014b). However, non-fatal ventricular arrhythmias were observed in engrafted primates and further investigations are required to understand why electromechanical coupling appears to be incomplete (Anderson *et al*, 2014; Murry *et al*, 2014).

Teratoma formation Another concern upon transplantation of hPSC-derived cells is the risk of teratoma formation (Lensch *et al*, 2007), which is obviously harmful to cardiac function and electrical homeostasis. When unpurified human ESC-derived cardiomyocytes were transplanted into non-human primates following cardiac injury, microteratomas formed in the scar region (Blin *et al*, 2010). However, when cells were committed to the cardiac lineage, as identified by expression of stage-specific embryonic antigen-1 (SSEA-1), there was no evidence of teratoma formation at 2 months after transplantation (Bel *et al*, 2010; Blin *et al*, 2010), suggesting that adequate purification of hPSC-derived cardiac lineages before transplantation might be sufficient to prevent tumor formation.

Delivery systems In many preclinical and clinical tests, molecules and/or cells are often directly injected into the myocardium during open-heart surgery (Fig 4). A less invasive delivery system, such as transcatheter injection, is however desirable in the clinical setting. There are two kinds of transcatheter approaches, involving either intramyocardial (transendocardial) or intracoronary injection, both of which have advantages and disadvantages associated with cell engraftment efficacy and side effects (tissue disruption, microvascular embolism, etc.) (Beeres *et al*, 2007; Ang *et al*, 2008; Li *et al*, 2011). Further investigations are needed to determine which delivery system is the most appropriate for maximal efficacy and safety (Sanganalmath & Bolli, 2013).

Virus issues For direct fibroblast conversion into cardiomyocyte-like cells *in vitro* and *in vivo*, viral transduction of reprogramming factors is currently the main approach. Some viral vectors, however, integrate into the host genome, thereby carrying a potential risk of tumorigenicity. Before any clinical trial involving transplantation of induced cardiomyocytes can be designed, it is therefore imperative to develop non-integrative gene transfer approaches that can achieve efficient cell fate conversion without compromising on safety. Such non-integrative approaches include plasmid vectors, modRNAs, miRNAs, and chemical molecules (Fig 4) and are currently under investigation (Kim *et al*, 2009; Warren *et al*, 2010; Jayawardena *et al*, 2012).

Long-term engraftment and tissue engineering Another major issue in cell therapy is how to improve engraftment, because most cells transplanted into the heart do not survive long term. Independently of cell type and delivery system, more than 90% of injected cells disappear in the first few days and only 1–2% can still be detected 4 weeks after transplantation (Zeng *et al*, 2007; Hong *et al*, 2013). Massive loss of transplanted cells is likely to limit the efficacy of any type of cell therapy and may be one of the reasons behind the modest success and/or controversial results in the past trials of cardiac cell therapy (Sanganalmath & Bolli, 2013; Matar & Chong, 2014). To improve long-term cell engraftment in the ischemic heart, several strategies are under investigation, including pretreatment of transplanted cells by genetic modification or pharmacological preconditioning (Penn & Mangi, 2008), regression of fibrotic scar tissue (Chablais & Jazwinska, 2012), and cardiac tissue engineering (Hirt *et al*, 2014). Genetic modification strategies involve overexpression of anti-apoptotic genes, such as heme oxygenase-1 (HO-1) or proto-oncogene serine/threonine-protein kinase (Pim-1), which have been shown to improve the survival of mesenchymal or cardiac stem cells transplanted into ischemic hearts (Tang *et al*, 2005; Fischer *et al*, 2009). In addition, reestablishment of adequate vascularization might be essential for long-term engraftment of transplanted cardiac cells (Terrovitis *et al*, 2010).

Cardiac tissue engineering, which involves three-dimensional heart muscle constructs, has undergone remarkable progress in the past decades. Combining cells with natural or synthetic biomaterials, such as collagen, matrigel, fibrin, alginate, gelatin sponges, and polyglycolic acid, improves local cell retention and engraftment (Segers & Lee, 2011; Ye *et al*, 2011). More recently, biodegradable scaffolds/decellularized heart tissue (Schmidt *et al*, 2007; Ott *et al*, 2008) and cardiac patches/cell sheets containing cardiac cells (Wei *et al*, 2008; Kubo *et al*, 2013) have received much attention. Further details have been reviewed elsewhere (Cimetta *et al*, 2013; Hirt *et al*, 2014). Although cardiac tissue engineering is still in development, these approaches will enhance cell engraftment and likely result in better outcomes of cardiac cell therapy.

Future perspectives

Recent discoveries in the fields of stem cell and regenerative biology hold great promise for cardiac regenerative medicine. Some of the most recently developed therapeutic strategies, such as cardiac/somatic stem cell transplantation, have been or are currently being clinically tested. Despite encouraging results in experimental/preclinical settings, the clinical benefits of many of these therapies remain controversial. Moreover, there is no consensus on the strategy to use, including which cell type to transplant, which delivery system to adopt, and/or which biomaterials to co-administer, to improve efficacy and safety. Similarly, standardized protocols to produce hPSC-derived or directly reprogrammed cardiomyocytes exhibiting "appropriate" maturation levels and engraftment are

lacking. These controversies might be attributable, at least in part, to our incomplete understanding of how the human heart develops and can regenerate, and of which intrinsic factor(s) account for the ultimate differences in regenerative capacities between lower vertebrate or neonatal mammalian hearts and adult mammalian hearts. The key to successful human cardiac regeneration in the near future lies in our continuing efforts to explore these core issues and to unravel the essential pathways and factors that govern the programming and reprogramming of a human heart cell. Latest experimental and therapeutic tools, involving modRNA, cellular reprogramming, cardiac tissue engineering, and next-generation sequencing of genomes and transcriptomes at the single cardiac cell levels, as well as different model organisms such as lower vertebrates and non-human primates, will help advance our understanding of human cardiogenesis and heart repair, opening novel paths toward an ultimate goal of establishing cardiac regenerative therapeutics.

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Conflict of interest

The authors declare that they have no conflict of interest.

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