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Thymosin β4 and Cardiac Regeneration: Are We Missing a Beat?

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Abstract Epicardial resident stem cells are known to differentiate into cardiomyocytes during cardiac development, amongst other cell types. Whether epicardium-derived progenitor cells (EPDCs) retain this plasticity in the adult heart has been the topic of heated scientific debate. Priming with thymosin beta 4, a peptide which has been suggested to be critical for cardiac development and to have cardio-protective properties, was recently shown to induce differentiation of EPDCs into cardiomyocytes in a small animal model of myocardial infarction. This finding is in stark contrast to another recent study in which thymosin beta 4 treatment following myocardial infarction did not induce cardiomyocyte differentiation of EPDCs. While EPDCs seem to exhibit overall cardio-protective effects on the heart following myocardial infarction, they have not been shown to differentiate into cardiomyocytes in a clinically relevant setting. It will be important to understand why the ability of one therapeutic agent to induce cardiomyocyte differentiation of EPDCs seemingly depends on a single variable, i.e. the time of administration. Furthermore, in light of a recent report, it appears that thymosin beta 4 may be dispensable for cardiac development.

Keywords Stem cells · Progenitor cells · Cardiac development · Ischemic biology–basic studies · Myogenesis

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Introduction

Heart disease is the leading cause of death worldwide [1]. This is due to the fact that the human heart does not possess clinically significant ability to regenerate in response to injury, such as myocardial infarction [2]. Cardiomyocytes, the basic unit of the heart muscle, exit the cell cycle shortly after birth and remain in a quiescent state indefinitely [3]. Efforts in the field of cardiac regenerative medicine have focused on the use of stem cells, both non-resident and resident within the heart, in an attempt to generate new cardiomyocytes. So far, efforts to transplant stem cells have not resulted in adequate and sustained improvement in cardiac hemodynamics, nor evidence of actual cardiomyocyte differentiation arising from such cells.

This review aims to provide an overview of recent stem cell-based studies in cardiac regenerative medicine. Epicardium-derived progenitor cells, a cardiac progenitor cell type resident in the outermost layer of the heart [4] and thymosin beta 4, a peptide that has been suggested to be critical for cardiac development [5] and to have cardio-protective and–regenerative effects [6, 7], are discussed in detail as an excellent example of why it is crucial to avoid premature assumptions regarding the regenerative capacity of any given cell type or factor. Furthermore, a step-wise approach to testing candidate cell types and factors is outlined to ensure true regenerative potential.

Cardiac Regenerative Capacity

The mammalian heart has very limited regenerative capacity [3]. In mammals, cardiomyocytes exit the cell cycle shortly after birth and remain in a quiescent state indefinitely [2]. In

humans, the heart was traditionally believed to be a post-mitotic organ with a predetermined number of cardiomyocytes established at birth and preserved throughout life [8]. More recently, evidence of cardiomyocyte turnover in humans was found, and the numbers reported vary significantly. One recent study based on retrospective carbon 14 (^{14}C) birth dating of cells reported that the frequency of annual cardiomyocyte renewal ranges from 1 % in young adults to 0.45 % in the elderly [9]. These findings conflict with reports on the level of apoptosis in the adult human heart [10] and the progressive increase in cardiomyocyte turnover associated with aging [11, 12], ranging from 10 % to 40 % per year in female hearts 20 to 100 years of age and 7 % to 32 % per year in male hearts, respectively. These data indicate that the cardiomyocyte compartment is replaced 15 times in women and 11 times in men from age 20 to 100 [12]. Despite this evidence of cardiomyocyte turnover in the healthy adult heart, cardiac regeneration in response to injury, such as myocardial infarction, is very limited and its mechanisms are not well understood. A genetic pulse-chase experiment in mice using tamoxifen-induced Cre activation of a reporter marker traced its dilution following myocardial infarction and demonstrated that up to 18 % of cardiomyocytes present in the heart 3 months after infarction arose de novo from the infarct border zone, from a cell source intrinsic or extrinsic to the heart [13], and not from preexisting myocytes, as is the case in teleost fish [14].

Non-Resident Stem Cells

Investigators in cardiac regenerative medicine have tried to make use of a variety of stem cell populations, both intrinsic and extrinsic to the heart. For example, the question of whether bone marrow-derived cells (BMCs), in particular hematopoietic stem cells, can contribute to cardiac repair by transdifferentiation into cardiomyocytes [15, 16] has been the topic of heated scientific debates for years, as they were initially thought to differentiate into cardiomyocytes following transplantation [17–19] but later shown to rather adopt mature hematopoietic fates [20] and generate a small number of cardiomyocytes through cell fusion, rather than transdifferentiation [15]. The ability of autologous bone marrow-derived cells to improve cardiac function following myocardial infarction was tested in several large clinical trials in which BMCs were administered into the coronaries of patients suffering from acute myocardial infarction [21–23]. While a subset of patients in the REPAIR-AMI trial showed a 3 % increase in ejection fraction, the ASTAMI trial did not demonstrate any beneficial effect on left ventricular function. These contradictory results support the notion that BMCs may not be as promising a cell type as was originally thought.

Resident Cardiac Stem Cells

Endogenous cardiac stem cells are currently in the limelight as the study of this diverse group of cells that reside in the adult heart holds the promise of cardiac repair without the limitations and risks associated with BMCs. Several populations of cells resident in the adult heart have been identified. Although there is no definitive consensus yet on the markers that precisely define these cell populations, they are reported to share a set of cardiogenic markers including GATA-4, NKX2.5, TBX5 and MEF2c [24]. The markers used for enrichment are also used to group this diverse set of progenitor cells.

C-kit$^+$ cells were originally isolated from the adult rat heart and have been reported to adopt myogenic, endothelial and smooth muscle cell lineages in vitro, and to differentiate into cardiomyocytes, smooth muscle cells and vascular endothelium when engrafted into acutely ischemic myocardium [25, 26]. Transplantation of c-kit$^+$ cells in animal models of post-myocardial- infarction heart failure has proven effective in alleviating left ventricular remodeling and improving left ventricular function in acute and chronic myocardial infarctions [25–29]. Furthermore, initial results of the ongoing "Stem Cell Infusion in Patients with Ischemic cardiOmyopathy (SCIPIO)" trial are encouraging [30]. Intracoronary infusion of autologous c-kit$^+$ cells in patients with myocardial infarction and left ventricular ejection fraction <40 % resulted in significant long-term improvement of left ventricular ejection fraction and reduction in infarct size post coronary artery bypass grafting (CABG), whereas left ventricular ejection fraction and infarct size in control patients who had undergone CABG only did not change. ***Islet-1$^+$*** cardiac progenitor cells were first identified in postnatal rat, mouse and human myocardium [31, 32], and were recently shown to be able to differentiate into cardiac, smooth muscle and endothelial cells when isolated from neonatal mouse heart [33]. ***Sca-1$^+$ cells*** were first isolated from the adult mouse heart in 2003 and have been reported to express cardiac-specific markers in vitro in the presence of 5'-azacytidine. When administered intravenously following cardiac ischemia and subsequent reperfusion, Sca-1$^+$ cells were shown to home to the injured myocardium, however, transdifferentiation of Sca-1$^+$ cells was accompanied by fusion with native cardiomyocytes [34]. ***Side population (SP) cells*** are of interest to the field of cardiac regeneration because they have been shown to be able to serve as progenitors for hematopoietic cells [35], skeletal muscle [36] and endothelium [18]. SP cells have been identified in the bone marrow and non-hematopoietic organs, including the heart [37] and are named after their ability to efflux the fluorescent vital dye Hoechst 33342 which is readily taken up by live cells where it binds to DNA [38]. SP cells fall within a separate population to the side of the remainder of analyzed cells on a dot plot of emission data collected by

fluorescence-activated cell sorting. The ability of SP cells to efflux Hoechst 33342 is dependent on the expression of the Abcg2 protein, an ATP-binding cassette transporter [39]. $Abcg2^+$ SP cells have the ability to express sarcomeric α-actinin when co-cultured with adult cardiomyocytes [37] and have been shown to participate in cardiac repair in mice subjected to cardiac cryoinjury [40]. ***Cardiosphere-derived cells (CDCs)*** from explant cultures of endomyocardial biopsy material are being tested for efficacy in averting heart failure following myocardial infarction [41–43]. Previous reports support the notion that CDCs directly regenerate cardiomyocytes and blood vessels [42, 44, 45]. CDCs injected into the infarct borderzone of SCID beige mice have been reported to improve left ventricular function [42]. CDCs have also been shown to secrete prosurvival and proangiogenic growth factors in vitro and in a murine cardiac cell therapy model [43]. In the prospective, randomized phase 1 "CArdiosphere-Derived aUtologous stem CElls to reverse ventricular dySfunction (CADUCEUS)" trial [46], patients randomized to CDC instead of standard therapy were injected with autologous CDCs into the infarct-related coronary artery 1.5–3 months following myocardial infarction. While statistically significant reduction in scar mass, increase in viable heart mass, regional contractility and regional systolic wall thickening were noted compared with controls at 6 months, no statistically significant improvements in hemodynamic function were found.

Epicardial Stem Cells

Another group of cells currently being studied is found in the epicardium, the outermost layer of the heart [4], and has been explored as a potential source of generating new cardiomyocytes [47]. As most other internal organs, the heart is covered by a mesothelium which consists of epicardial cells that migrate from the proepicardium, an outgrowth of the septum transversum, and spread over the surface of the heart during cardiac development [48, 49]. Epicardial cells have been reported to contribute to formation of cardiac blood vessels by undergoing epicardial-mesenchymal transition (EMT) and subsequent differentiation into myocardial stroma as well as vascular smooth muscle and coronary endothelial cells [50–52]. However, Red-Horse et al. recently identified sprouting from the sinus venosus as the major source of coronary vascular endothelial cells in mice [53]. Furthermore, several independent lineage tracing experiments of epicardium-derived progenitor cells (EPDCs) during cardiac development using progenitor markers such as Tbx18 [51], Wt1 [54], Isl1 [33] and Nkx2.5 [55], reported that a subset of EPDCs also differentiates into cardiomyocytes. Specifically, Cai and colleagues suggested that Tbx18 –expressing epicardium provides a substantial contribution to cardiomyocytes in the ventricular septum as well as atrial and ventricular walls [51]. However, these findings were questioned by Christoffels and colleagues who demonstrated that Tbx18 itself is, in fact, expressed in the myocardium from embryonic day (E) 10.5 to at least E14.5 [56], thereby limiting the conclusion that $Tbx18^+$ epicardial cells contribute to the cardiomyocyte lineage in vivo. A genetic lineage study by Pu and colleagues indicated that $Wt1^{Cre}$ –expressing epicardial cells differentiate into cardiomyocytes during normal heart development and are derived from progenitors that express transcription factors Nkx2.5 and Isl1 [52], suggesting that they are of the same developmental origin as multipotent cardiogenic progenitors [57, 58]. Interestingly, Zeng and colleagues recently demonstrated Wt1 expression in the embryonic murine heart [59]. In contrast to the original study by Pu and colleagues [52], which found Wt1 expression to be confined to the proepicardium and epicardium from E9.5 to E15.5, here, Wt1 expression was confined to the epicardium only from E9.5 to E11.5 and began to be expressed in the myocardium at E12.5. However, Wt1 expression did not colocalize with Nkx2.5 [59], a well-characterized marker of early cardiomyocyte lineage [60]. Furthermore, Zeng and colleagues found robust Tbx18 expression in myocardium from E11.5, and its expression colocalized with Nkx2.5. These data on Tbx18 expression are in line with those previously reported by Christoffels et al. [56]. Taken together, the findings of Christoffels et al. and Zeng et al. raise doubts regarding the specificity of the Cre recombinase–based genetic lineage studies performed by Cai et al. [51] and Zhou et al. [52] and question the relevance of Tbx18 and Wt1 as epicardium-specific genetic markers.

A recent study by Katz et al. [61] demonstrated that the epicardium does not make significant contributions to the myocardium during development. Here, the authors employed fate mapping studies in mouse and chick, as well as in vitro analysis, to trace the fate of proepicardium-derived cells labeled with proepicardial markers Scleraxis (Scx) and Semaphorin3D (Sema3D) in an effort to answer the question whether the proepicardium contributes to the coronary endothelium. Previous data from chick and mouse lineage tracing studies conflict in that avian lineage tracing studies have established the proepicardium as a source of vascular smooth muscle and endothelial cells [62–66], whereas the aforementioned fating mapping studies in mice utilizing Tbx18 and Wt1 as proepicardial markers [51, 52] did not identify a significant proepicardial contribution to the endothelium. The authors showed here that Scx and Sema3D delineate proepicardial subcompartments which are largely nonoverlapping with Tbx18- and Wt1-expressing populations. It was demonstrated that both Scx^+ and $Sema3D^+$ epicardial cells give rise to endothelial cells, in addition to other cardiac fates including cardiomyocytes. However, only 6.6 % of Scx- and 0.36 % of Sema3D-lineage traced cells give rise to cardiomyocytes. On the one

hand, this study illustrates the complexity of the proepicardium, which consists of genetically distinct subcompartments that give rise to distinct yet partially overlapping cell fates, on the other hand, it demonstrates that the epicardium does not make significant contributions to the myocardium during development. Kikuchi et al. examined the developmental potential of epicardial tissue in a third model system, zebrafish, during embryonic development and injury-induced heart regeneration [67]. Their study provides convincing evidence that natural epicardial fates are limited to non-myocardial cell types in this key model system for embryonic heart development and function. It has previously been shown that zebrafish possess robust natural capacity for adult myocardial regeneration [68]. Surgical resection of the ventricular apex leads to significant proliferation of epicardial cells that subsequently incorporate into the regenerating tissue [69, 70]. While it is known that spared cardiomyocytes are activated and proliferate in response to myocardial injury and thus contribute significantly to cardiac regeneration in zebrafish [14, 69], Kikuchi et al. [67] aimed to examine whether epicardial cells differentiate into cardiomyocytes. Among several candidate genes screened for epicardial-specific expression, zebrafish tcf21 was selected for use in subsequent Cre recombinase based genetic lineage studies, as it displayed epicardial-specific expression during development and regeneration and was never detected within cardiomyocytes, in contrast to tbx18 and wt1b. Tcf21 was originally identified as a basic helix-loop-helix transcription factor which is expressed in the proepicardium, epicardium and other mesoderm-derived tissues during murine embryonic development [71–74]. A transgenic zebrafish line in which tcf21 regulatory sequences drive tamoxifen-inducible Cre recombinase was generated and crossed with the previously described gata5:RnG indicator line [71] to label cells with a nuclear localization signal-tagged EGFP after excision of loxP-flanked stop sequences. Tcf21:CreER; gata5:RnG double transgenic larvae were incubated with 4-hydroxytamoxifen (4-HT) at 3–5 days post fertilization (dpf), when proepicardium-derived epicardial cells have completely enveloped the developing zebrafish heart. An antibody against EGFP was used to increase sensitivity of detecting EGFP-labeled cells, and no cells that co-expressed the cardiomyocyte-specific markers myosin heavy chain or Mef2 were labeled, ensuring specificity. Labeled larvae were allowed to mature to adults and EGFP immunofluorescence was subsequently assessed. While the ventricular and atrial surfaces as well as the ventricular subepicardial areas were covered with $EGFP^{+}MHC^{-}Mef2^{-}$ cells, such cells were not found in the inner trabecular myocardium. Genetic labeling with a second indicator line, β-act2:RSG, which was previously shown to label cardiomyocytes with high efficiency in combination with the cmlc2:CreER line [71], was performed using tcf21:CreER; β-act2:RSG animals treated with 4-HT as larvae, and showed no EGFP labeled cells within the heart. Of note, it was found that larval tcf21+ epicardial cells contribute to perivascular cell types. These data indicate that the epicardium does not contribute to cardiac muscle during zebrafish development. The authors next assessed epicardial contributions during cardiac regeneration by growing 4-HT-labeled tcf21:CreER; gata5:RnG or tcf21:CreER; β-act2:RSG larvae and resecting ventricular apices at 4–5 months of age. Within 30 days post amputation (dpa), a substantial number of $EGFP^{+}$ cells was observed within tcf21:CreER; gata5:RnG animals, but without co-expression of MHC or Mef2. Instead, $EGFP^{+}$ cells resembled $DsRed2^{+}$ perivascular cells observed in regenerating tissue of the tcf21:DsRed2 reporter line. No EGFP + cells were detected in tcf21:CreER; β-act2:RSG regenerates. Incubation of 3- to 4-month-old tcf21:CreER; gata5:RnG animals with 4-HT and subsequent assessment for $EGFP^{+}$ cells in 30dpa regenerates revealed EGFP-labeling in the majority of adult epicardial cells as well as many EPDCs and perivascular cells, yet no $EGFP^{+}$ cells with co-expression of myocardial markers were observed. Also, no $EGFP^{+}$ cardiomyocytes were found in regenerating tissue of tcf21:CreER; β-act2:RSG animals treated with 4-HT at adult ages. These findings indicate that the zebrafish epicardium is a source of perivascular support cells, but not cardiomyocytes, during heart regeneration. In summary, this important study which used tcf21, an epicardial-specific marker, for Cre recombinase-based genetic lineage studies in zebrafish, found that the epicardium contributes perivascular support cells, but not cardiomyocytes, during development and cardiac regeneration. Since these findings conflict with previous reports in mice which employed Tbx18- and Wt1- based genetic fate mapping of epicardial cells [51, 52], use of the murine counterpart of tcf21 for genetic fate-mapping will help elucidate the natural potential of epicardial cells during vertebrate development. Furthermore, while the findings of Kikuchi et al. [67] indicate that epicardial cells are not a natural source of cardiomyocytes, it is possible that experimental manipulation can elicit a pro-myogenic potential in these cells.

Epicardial progenitor cells are currently an attractive target in cardiac regenerative medicine. However, the fact that EPDCs become dormant following embryonic development has been a great limitation regarding their use [75, 76]. Because EPDCs are of great clinical interest, recent efforts have been directed at reactivating or mobilizing EPDCs after ischemic heart injury such as myocardial infarction (M.I.).

Thymosin Beta 4 in Cardiac Repair

Reported Beneficial Effects

Recently, thymosin beta 4 (Tβ4) has drawn significant attention in cardiac regenerative medicine. Tβ4 is a polypeptide composed of 43 amino acid residues [77] and

member of the family of thymosin peptides that were initially isolated from the thymus, but have been shown to be present in other tissues as well [78]. While Tβ4 is the major regulator of the actin cytoskeleton in mammalian cells, it has also been reported to promote stimulation of wound healing and hair growth [79]. Furthermore, Tβ4 was detected in developing murine myocardium from E8.0 [80] and its temporal and spatial expression pattern during embryonic heart development from E9.5 to E12.5 was later characterized [6]. Based on these data, Tβ4 has been implicated as necessary for cardiac development and angiogenesis [5]. Data also suggests that Tβ4 has cardio-protective [6] and cardio-regenerative [7] effects in small and large animal models of myocardial infarction and ischemia/reperfusion [81]. In addition, ex vivo experiments have demonstrated that Tβ4 can promote neovascularization [82, 83]. In response to injury, Tβ4 is released by platelets, macrophages and other cell types to protect cells and tissues from further damage and reduce apoptosis, inflammation and microbial growth [84]. Tβ4 is thought to exhibit its beneficial effects on tissue regeneration by paracrine mechanisms [79]. In the field of cardiovascular regenerative medicine, research efforts have focused especially on confirming the suggested cardio-protective and -regenerative effects of Tβ4 in an effort to translate its use to clinical trials.

Were Hopes Premature?

Two recent reports have found contradictory results regarding the cardio-regenerative capacity of Tβ4 [47, 85] and a third report even calls into question its previously suggested essential role in cardiac development and function [86].

Recently, Smart et al. [47] reported that priming with Tβ4 before induction of myocardial infarction mobilizes EPDCs from the epicardium to the infarct zone where they begin to express markers specific for mature cardiomyocytes, such as cardiac Troponin T (cTnT), sarcomeric α-actinin (SαA) and Connexin-43 (Cx43). Employing Cre-lox and transgenic mice strain technologies, the authors were able to trace EPDCs in which GFP and YFP expression is driven by the Wt1 promoter and hence monitor the fate of stimulated epicardial progenitors as they migrated into the myocardium. In addition, Tβ4-induced GFP^+ progenitor cells were transplanted into wild-type mice following induction of M.I. and the GFP^+ cells appeared to differentiate into mature cardiomyocytes.

The mechanisms by which EPDCs are stimulated, mobilized and differentiated have yet to be defined. Furthermore, despite the elegant genetic fate-mapping approach employed in the study by Smart et al., demonstration of immunofluorescence data indicating expression of cardiac markers and calcium transients between YFP^- and YFP^+ cells as proof of functional coupling of existing and "de novo" cardiomyocytes is insufficient evidence of having generated functional cardiomyocytes. A hallmark of any cell type that can differentiate into a cardiomyocyte is the ability to beat. In order to circumvent any potential artifactual results, live imaging movies of beating cardiomyocytes are the only true test of cardiomyocyte differentiation. Thus, there is no conclusive data proving that Tβ4 can induce "de novo" cardiomyocyte formation. The findings of Smart et al., provocative as they may seem, leave some unanswered questions that must be resolved in order to fully explore a viable translational path for Tβ4.

An even more essential question than determining if Tβ4 can drive differentiation of EPDCs into cardiomyocytes is whether use of Tβ4 can be meaningfully translated into clinical practice. In stark contrast to the experimental design employed in the study by Smart et al. [47], administration of Tβ4 before M.I. will not be feasible in clinical trials. Therefore, it would seem more plausible to focus on the potential benefits of Tβ4 treatment after M.I. and evaluate its ability to induce differentiation of EPDCs into cardiomyocytes as well as its other reported cardioprotective properties in a more clinically relevant setting. Interestingly, Zhou et al. [85] addressed this very question in a recent report in which they asked whether Tβ4 treatment after M.I. could reprogram EPDCs into cardiomyocytes and augment the epicardium's response to injury. This study is of particular significance because the authors employed the same epicardium-specific inducible Cre-line for genetic fate mapping of EPDCs that was used in the study by Smart et al., i.e. WT1CreERT2/+, obtained clinical grade Tβ4 from the same source as Smart et al. and ensured similar systemic levels of Tβ4 by administering multiple injections. The principal difference was the timing of Tβ4 treatment, i.e. myocardial infarction was induced in the WT1CreERT2/+ mice and Tβ4 administered daily for 1 week as opposed to priming with Tβ4 before induction of M.I. The fate of GFP-expressing EPDCs was analyzed 2 weeks post M.I. and the data confirmed the previously described cardioprotective properties of Tβ4 [6, 83], namely significant reduction of infarct size, cardiac fibrosis, cardiomyocyte apoptosis and increased vessel density. Importantly, Tβ4 treatment also augmented thickening of the epicardial layer. Thickening of the epicardial layer, which is comprised of EPDCs and epicardial cells, in response to M.I. was previously reported by the same group and thought to mediate the secretion of paracrine factors that mitigate myocardial injury and adverse cardiac remodeling [87]. This finding also proves that the Tβ4 used in this study was biologically active. On the other hand, Tβ4 treatment post M.I. did not mobilize EPDCs so they would migrate into the myocardium, nor did it direct their differentiation into cardiomyocytes. The authors concluded that, while Tβ4 therapy post M.I. had an overall beneficial effect on the heart, it did not reprogram epicardial cells into cardiomyocytes.

These results raise the question why Tβ4 priming before M.I. induces EPDCs to differentiate into cardiomyocytes,

while Tβ4 treatment following M.I. does not. One possible explanation lies in the fact that myocardial injury alters epicardial cell properties and gene expression to a substantial degree. Following M.I., the angiogenic gene expression profile of epicardial cells remains largely unaltered when compared to the profile of EPDCs in the healthy heart, despite either up- or down-regulation of several angiogenic genes. However, gene expression of stromal cell-derived factor 1 (Sdf1) and monocyte chemotactic protein 1 (Mcp1), both chemotactic factors for circulating cells active in angiogenesis [88, 89], are highly up-regulated by M.I. This indicates that expression of additional epicardial genes relevant to the myocardial injury response is significantly altered following M.I., which is in line with other reported pro-angiogenic properties of EPDCs and stresses their significant contribution to increased angiogenesis in the infarcted heart [81]. These altered properties might "lock" EPDCs in their role as a mediator of increased angiogenesis and thus impair epicardial cell plasticity in response to Tβ4 treatment, preventing differentiation into cardiomyocytes. Myocardial infarction might also disrupt the architecture of an epicardial niche and thus deprive epicardial cells of factors required for reprogramming [85]. Another possible explanation why EPDCs do not differentiate into cardiomyocytes following M.I. may be because they also do not contribute significantly to cardiomyocyte formation during development [61, 67].

Taken together, Tβ4 therapy in a clinically relevant model of myocardial infarction has been shown to exhibit overall cardio-protective effects, mostly mediated by augmenting the pro-angiogenic response of the epicardium following M.I. through as yet unknown molecular mechanisms. However, in this model, Tβ4 has not been shown to be able to induce differentiation of EPDCs into cardiomyocytes, and, hence, is not able to generate "de novo" cardiomyocytes.

In light of a very recent report by Banerjee et al. [86], it appears that Tβ4 is dispensable for cardiac development and angiogenesis. Here, the authors generated two murine Tβ4 knockout models in which Tβ4 expression is ablated either globally or specifically in the heart and report that global ablation of Tβ4 does not interfere with cardiac development. While developmental studies in mice suggested that Tβ4 is required for embryonic heart development [5, 6, 80], Banerjee et al. [86] did not observe embryonic lethality, cardiac or other gross anatomic abnormalities in global Tβ4 knockout mice. Histological examination of Tβ4 knockout hearts at E14.5 did not reveal abnormal development of compact myocardium, detachment of epicardium or the presence of nodules on the epicardial surface, while these abnormalities were reported for E14.5 embryos in which Tβ4 gene expression was silenced using Tβ4-shRNA driven by Nkx2.5 ($T\beta4sh^{Nk}$) [5]. Furthermore, Banerjee et al. did not observe changes in the epicardial marker Wt1 in their Tβ4 knockout embryos at E14.5. Since Tβ4 was previously reported to induce mobilization of EPDCs and their differentiation into endothelial or smooth muscle cells (SMC) [5], the authors examined the microvasculature of embryonic Tβ4 knockout hearts by immunocytochemistry and found no difference in the total number, formation and distribution of CD31-positive endothelial cells in the myocardium compared to control littermate hearts. Also, there was no difference in the pattern and distribution of α-smooth muscle $actin^{+}$ SMC between Tβ4 knockout and control hearts. Large vessels revealed normal smooth muscle and endothelial cell layers. These results indicate that global ablation of Tβ4 does not alter the development of coronary vessels. Despite the suggested role of Tβ4 in coronary angiogenesis [5], Tβ4-knockout mice did not exhibit altered capillary bed density. As in embryonic hearts, adult hearts of Tβ4 global knockout mice did not reveal morphological abnormalities or changes in expression of epicardial marker Wt1 or Tbx18. Echocardiographic analysis up to the age of 12 months did not reveal any significant difference in cardiac function between Tβ4 global knockout mice and control littermates and no changes in survival were observed over a period of 12 months. As in Tβ4 global knockout mice, cardiac-specific ablation of Tβ4 did not result in embryonic lethality or cause abnormal cardiac function in adult animals.

Mouse embryos with heart-specific Tβ4 deficiency by transgenic conditional RNA interference have been reported to exhibit significant cardiac developmental defects [5]. Specifically, floxed Tβ4 short hairpin RNA (Tb4shRNA-flox) mice were crossed with one of two Cre-driver strains: Nkx2.5CreKI, which directs Cre expression throughout the majority of cardiomyocytes [90] or MLC2vCreKI, which directs Cre expression specifically to ventricular cardiomyocytes [91]. Banerjee et al. [86] did not observe any abnormalities in cardiac development in their murine global Tβ4 knockout model. A possible explanation for these different outcomes is that shRNA-mediated knockdown of Tβ4 may have had off-target effects [5, 86], i.e. unintended effects on gene expression mediated by RNA interference, a phenomenon associated with shRNA gene silencing [92, 93].

Previous reports suggested that Tβ4 is required for cardiac development and angiogenesis [5] and has cardioprotective [6] and cardio-regenerative [7] effects in animal models of myocardial infarction. However, according to the report by Banerjee et al. [86], Tβ4 appears dispensable for cardiac development and function and, while Tβ4 treatment following myocardial infarction exhibits overall cardioprotective effects in a small animal model of myocardial infarction [85], it does not reprogram EPDCs into cardiomyocytes [85], as opposed to priming with Tβ4 before induction of myocardial infarction [47].

Conclusions and Future Directions

Candidate Regenerative Cell Types and Factors

In light of the ever-growing number of reports that examine the regenerative potential of cardiac stem cells and trophic factors, and to avoid premature hopes of a certain candidate regenerative cell type or factor to be capable of mediating cardiac repair, it would be wise to ensure the respective cell or factor being tested meets minimum "quality standards" before drawing the conclusion that it actually aids in cardiac regeneration. This could be done by following a multi-step approach to test whether the candidate progenitor cell exhibits certain essential qualities, such as the ability to form spontaneously beating cardiomyocytes in vitro, as this is the ultimate test for any cell under study for its potential to generate real, functional cardiomyocytes. For example, our own laboratory recently demonstrated that fetal cells selectively home to the injured maternal heart and undergo differentiation into endothelial cells, smooth muscle cells and

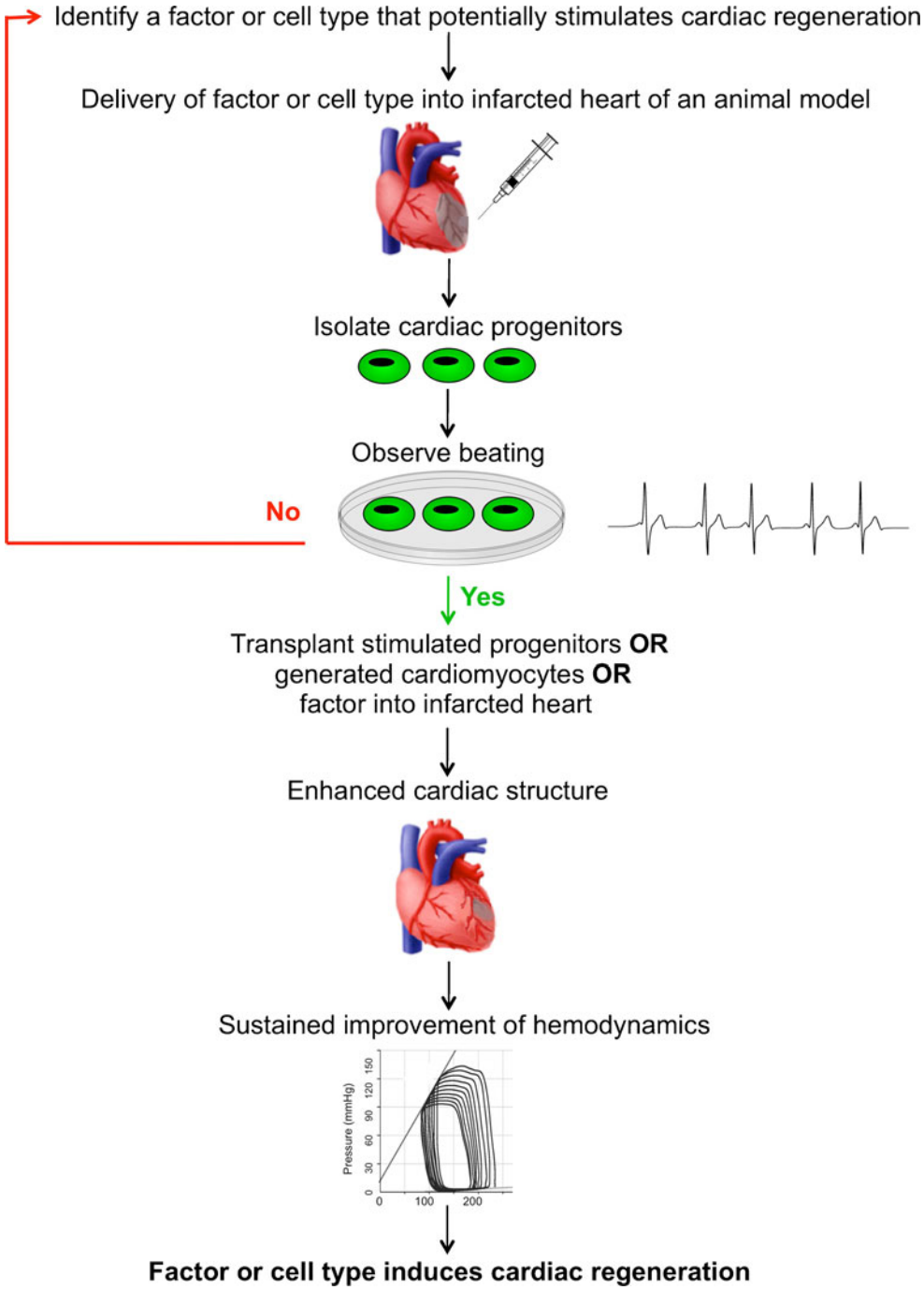

Fig. 1 Candidate regenerative factors and cell types. Isolation of putative cardiac progenitors that are either stimulated to adopt the cardiac fate or arise from transplanted cells should undergo ex vivo testing to document contractile ability using live imaging. Hemodynamic studies to support a true cardiac regenerative process should demonstrate sustained improvements over time

cardiomyocytes in a mouse model of mid-gestational myocardial injury. When isolated from the maternal heart, the fetal cells recapitulate these differentiation pathways and form beating cardiomyocytes in a fusion-independent manner [94]. If contractile ability is observed, the stimulated progenitors, newly generated cardiomyocytes or candidate factor(s) should be introduced into the infarcted heart to evaluate their ability to enhance cardiac structure, i.e. mitigate adverse remodeling following M.I. and to lead to sustained improvement of cardiovascular hemodynamics. Figure 1 depicts this suggested step-wise test approach. Clearly, monitoring cardiovascular hemodynamics for a time period of more than 28 days, which was the observational endpoint in the study by Smart et al. [47], is necessary to draw meaningful conclusions on whether the factor or cell type of interest induces true cardiac regeneration, as temporary improvements in left ventricular function may be attributable to paracrine effects [95, 96] that reduce cardiomyocyte hypoxia and necrosis rather than generate "de novo" cardiomyocytes, as is the case with Tβ4.

Although the excitement about Tβ4 as a modulator of cardiac repair has been lowered by recent reports, it is still worth investigating the molecular mechanisms by which Tβ4 exhibits its cardio-protective effects and why it fails to induce EPDC plasticity when given post M.I. With a variety of promising cardiac progenitor cell types at hand and intense efforts in the field of cardiac regenerative medicine, the hunt is on for the cell type or drug that can repair the most vital of all organs, the heart.

Conflicts of interest The authors declare no competing conflicts of interest.

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