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Review Article

Therapeutic potential of Pnmt+ primer cells for neuro/myocardial regeneration

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Abstract: Phenylethanolamine n-methyltransferase (Pnmt) catalyzes the conversion of norepinephrine into epinephrine, and thus serves as a marker of adrenergic cells. In adults, adrenergic cells are present in the adrenal medullae and the central and peripheral (sympathetic) nervous systems where they play key roles in stress responses and a variety of other functions. During early embryonic development, however, Pnmt first appears in the heart where it is associated with specialized myocytes in the pacemaking and conduction system. There is a transient surge in cardiac Pnmt expression beginning when the first myocardial contractions occur, before any nerve-like or neural crest cells appear in the heart. This early expression of Pnmt denotes a mesodermal origin of these "Instrinsic Cardiac Adrenergic" (ICA) cells. Interestingly, Pnmt+ cells are found in all four chambers of the developing heart, but by adult stages, are found primarily concentrated on the left side of the heart. This regionalized expression occurs in the left atrium and in specific regions of the left ventricle roughly corresponding to basal, mid, and apical sections. A second distinct population of Pnmt-expressing (Pnmt+) cells enters the embryonic heart from invading neural crest, and these "Neural Crest-Derived" (NCD) Pnmt+ cells appear to give rise to a subpopulation(s) of cardiac neurons. Pnmt expression thus serves as a marker not only for adrenergic cells, but also for precursor or "primer" cells destined to become specialized myocytes and neurons in the heart. This review discusses the distribution of Pnmt in the heart during development, including the types of cells where it is expressed, and their potential use for regenerative medicine therapies for cardiovascular disease.

Keywords: Phenylethanolamine n-methyltransferase (Pnmt), Pnmt+ primer cells, neuroregeneration, myocardial regeneration

Introduction

Cardiovascular disease is the leading cause of death in the U.S. Many forms of cardiovascular disease lead to congestive heart failure, for which there is poor prognosis and deteriorating health and quality of life. The recently published Heart Disease and Stroke Statistics – 2012 Update [1, 2] indicates that 5.7 million people suffered from congestive heart failure, with nearly 700,000 new cases reported each year. New strategies are clearly needed to provide more effective treatment options and improved outcomes.

Stem cells have the potential to regenerate cardiac muscle tissue, and several studies have shown that a variety of stem cells [3-11], including those recently identified in adult hearts [12], can be effectively transplanted into regions of the heart damaged by experimentally-induced myocardial ischemia or infarction. The ability of these transplanted cells to actually regenerate functional myocardium is an intense and sometimes controversial area of investigation [6, 7, 13-21]. Nevertheless, new cardiac stem and progenitor cell markers continue to be identified and characterized. Expression of these markers is being used to identify and isolate specific stem/progenitor cell populations from both embryonic and adult tissue sources [12, 22-27]. Further, the exciting developments demonstrating that somatic cells can be transformed into ES-like pluripotency upon transfection with four stem cell genes (Oct3/4, Sox2, c-Myc, and Klf4) [28, 29] or miRNA-based re-programming [30] creates for the first time the real possibility that autologous cell transplantation can be feasibly achieved.

For ethical and practical reasons, clinical studies have utilized adult stem cell populations

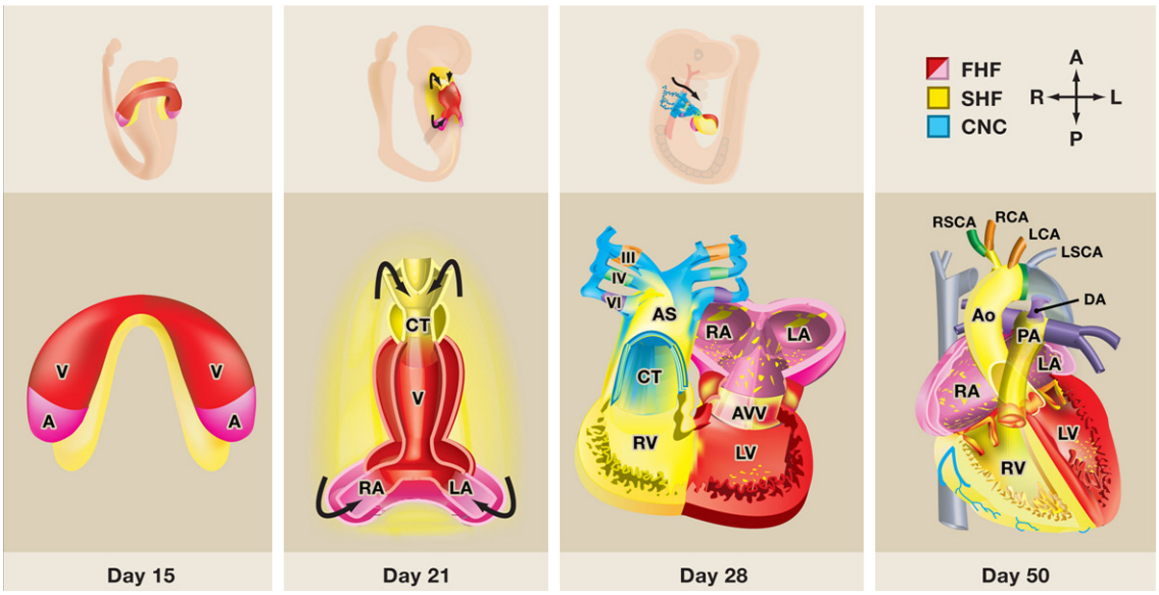

Figure 1. Mammalian Heart Development. Oblique views of whole embryos and frontal views of cardiac precursors during human cardiac development are shown. (First panel) First heart field (FHF) cells form a crescent shape in the anterior embryo with second heart field (SHF) cells medial and anterior to the FHF. (Second panel) SHF cells lie dorsal to the straight heart tube and begin to migrate (arrows) into the anterior and posterior ends of the tube to form the right ventricle (RV), conotruncus (CT), and part of the atria (A). (Third panel) Following rightward looping of the heart tube, cardiac neural crest (CNC) cells also migrate (arrow) into the outflow tract from the neural folds to septate the outflow tract and pattern the bilaterally symmetric aortic arch arteries (III, IV, and VI). (Fourth panel) Septation of the ventricles, atria, and atrioventricular valves (AVV) results in the four-chambered heart. V, ventricle; LV, left ventricle; LA, left atrium; RA, right atrium; AS, aortic sac; Ao, aorta; PA, pulmonary artery; RSCA, right subclavian artery; LSCA, left subclavian artery; RCA, right carotid artery; LCA, left carotid artery; DA, ductus arteriosus. *Reprinted with permission from Cell* [55].

from autologous sources such as bone marrow. This strategy also minimizes potential host immune rejection [31-36]. While many of these studies have indicated marginal improvement in cardiac hemodynamic function, there is little convincing evidence to suggest that there is substantial regeneration of new muscle tissue as a result of these interventions. A more recent phase I clinical study used autologous cardiosphere-derived stem cells (CDCs) and found reduced scar mass and increased viable heart mass; however, there were no significant changes in left ventricular function as measured by ejection fraction [37].

The specific type or types of stem cells needed for successful cardiac repair/regeneration strategies have not yet been established, and there are several different cell types currently under exploration in this regard. Recent studies have identified several key markers for cardiac stem cells that include, but are not limited to, Isl-1, flk-1, and c-kit [12, 24-26]. Cardiac stem cells can give rise not only to myocytes, but also to fibroblasts, endothelial, and smooth muscle cell types. Other studies have identified certain markers that represent specific derivatives of these stem cells with more limited lineage potentials within the heart. Some of these include Wnt11, Wnt7a, Id2, Tbx5, and Tbx18 [38-40], which appear to contribute to myocytes in the pacemaking and conduction system. The use of cardiac neuron progenitors is a relatively new and important area of research [41-44]. It is clear that significant neural damage occurs as a result of myocardial infarction and other insults that contribute to the development of heart failure [45-49]. Further, post-MI neural sprouting has been implicated as a potential pro-arrhythmic hazard [50-54]. Consequently, it is imperative to determine if cardiac neural progenitors are helpful or harmful in proper context.

A potentially useful reference context is that of "normal" cardiovascular development such as occurs during embryonic development. This highly orchestrated and dynamic process is

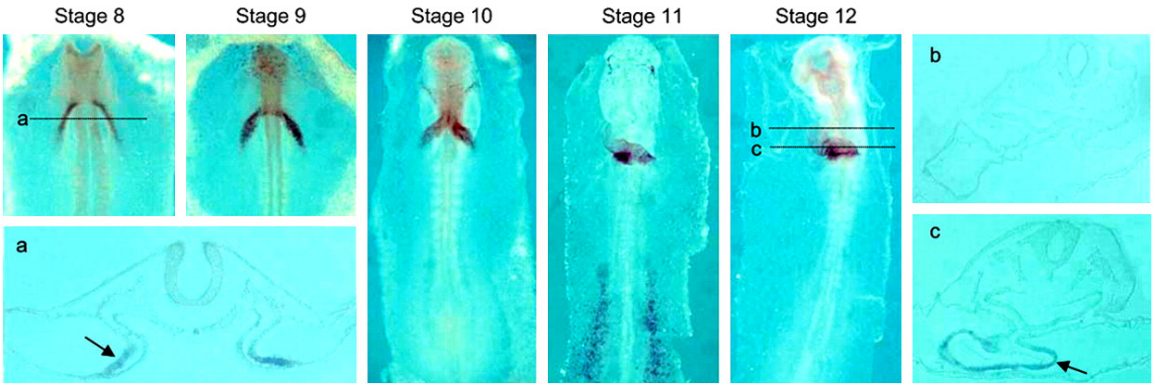

Figure 2. Tyrosine hydroxylase expression and activity in developing chick embryos. Whole-mount ISH for TH at st. 8-12. a, b, and c correspond to transverse paraffin sections at the levels indicated by the corresponding lines. Note that at st. 8 TH mRNA is restricted to the splanchnic mesoderm of the endocardial tubes, and later it is predominantly expressed in the myocardial layer of the atriogenic region (arrows in a and c). *Reprinted with permission from Cardiovascular Research* [57].

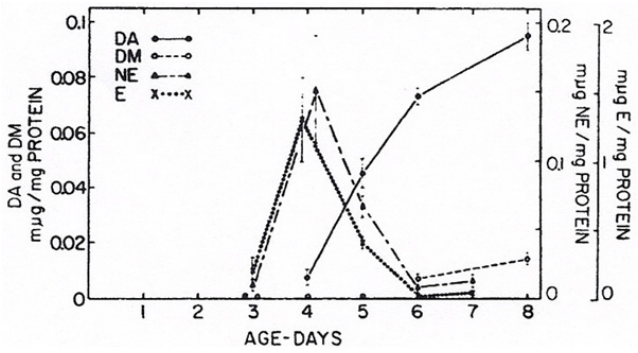

Figure 3. Appearance of dopa and catecholamines in the heart of the embryonic chick. Each point on the curves represents the mean of three to six determinations. Vertical lines represent ± S.E.M. DA, dopa; DM, dopamine; NE, norepinephrine; E, epinephrine. *Reprinted with permission from the Journal of Pharmcology and Experimental Therapeutics* [59].

schematically summarized in **Figure 1** [55] where color-coding aids our visualization of the three major contributing populations of cardiac progenitor cells. These include the primary or first heart field (FHF) shown in red/pink, anterior or secondary heart field (SHF) shown in yellow, and the cardiac neural crest (CNC) shown in blue. Cells from FHF give rise to the left ventricle and both atria, while the right ventricle and conotruncal regions are mainly derived from SHF. Both FHF and SHF are themselves derived from mesoderm origins, whereas the CNC cells are derived from neuroectodermal lineage. CNC cells invade the cardiac region several days after formation of the heart tube and these cells contribute to outflow tract, aortic arch formation, and cardiac neurons [56]. In theory, specific lineage-restricted progenitor or "primer" cells can be isolated and expanded from FHF, SHF, and/or CNC derivatives to provide customized repair/regeneration of specific cardiac regions. In some cases, more than one type of primer cell may be warranted depending on the nature of the damage/disease in need of repair. As we shall see in the next sections, progenitor cells transiently expressing the adrenergic biosynthetic enzyme, Phenylethanolamine n-methyltransferase (Pnmt), are supplied to the heart first through the FHF and SHF followed by a subsequent invasion from CNC.

Adrenergic cells in in the developing heart

The first and rate-limiting step in the production of adrenergic hormones is the enzymatic conversion of l-tyrosine to l-dopa via the action of tyrosine hydroxylase (TH). Although widely used as a marker for "neural" cells, its expression early in development is also associated with myocardial development, as recently shown by Lopez-Sanchez et al. [57]. They demonstrated that TH expression appeared coincident and in overlapping pattern with the formation of cells from the FHF in the developing chick embryo (**Figure 2**). Since there are no neurons in the chick heart at these early stages of development, this early TH expression constitutes a non-neuronal source of cells that have the potential to produce adrenergic hormones beginning at remarkably early stages of development.

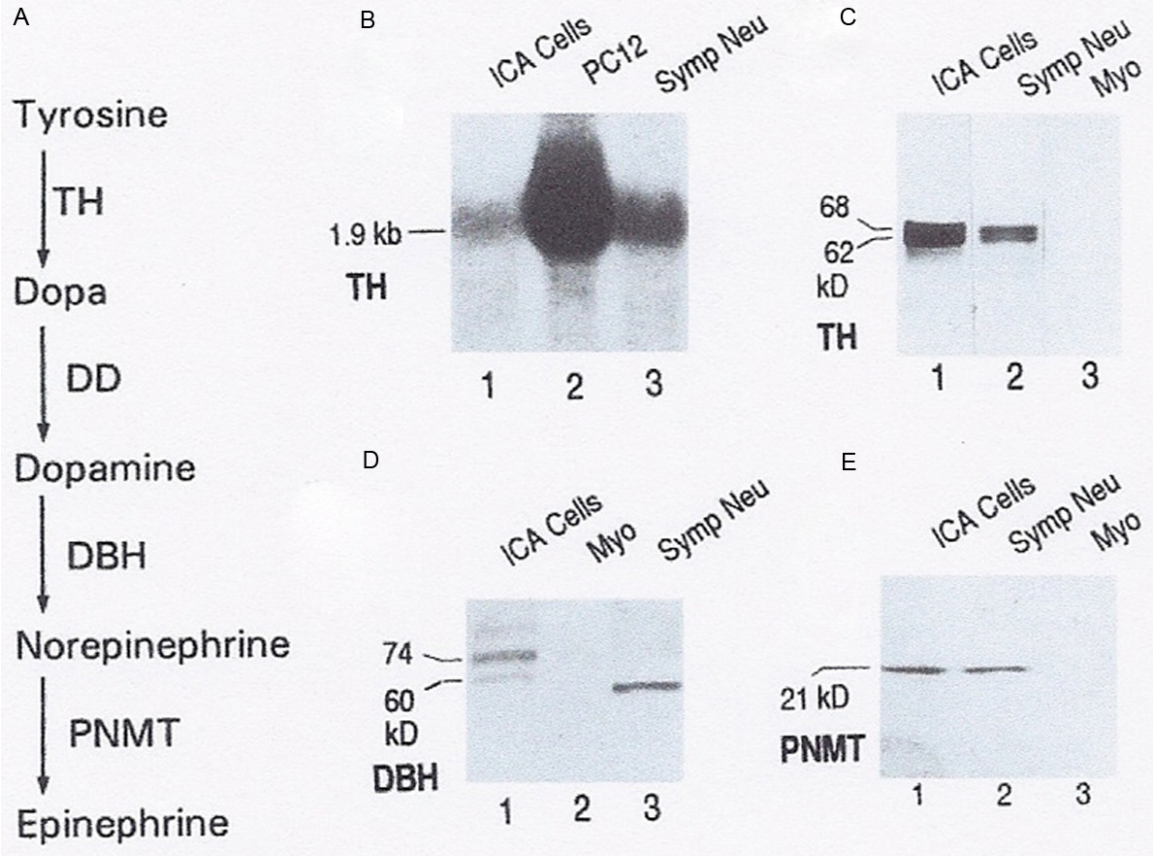

Figure 4. Catecholamine synthesizing enzymes in ICA cell lysates. A: Steps in the enzymatic synthesis of catecholamines are illustrated. TH, tyrosine hydroxylase; DD, dopa decarboxylase; DBH, dopamine beta-hydroxylase; PNMT, phenylethanolamine n-methyltransferase. B: Northern blot analysis of TH mRNA from total RNA. C: TH protein subunits were identified in ICA cells and cultured sympathetic neurons from rat stellate ganglia, but not in ventricular myocytes (Myo). D: Two major DBH isoforms were identified in ICA cells. No Dbh was detected in cardiomyocytes depleted of ICA cells (Myo). A single DBH isoform was detected in cultured rat sympathetic neurons. E: PNMT protein was detected in ICA cells and sympathetic neurons, but not in myocytes depleted of ICA cells. *Reprinted with permission from the Journal of Clinical Investigation* [62].

PNMT catalyzes the final step in the adrenergic biosynthetic pathway, the conversion of norepinephrine into epinephrine. The first description of PNMT activity in the heart was reported by Axelrod in 1962 [1]. Small but detectable levels were noted in both rabbit and monkey hearts from this study. Subsequent studies demonstrated that epinephrine, norepinephrine, and dopamine were all produced during early embryonic heart development in chicks [58-60], as shown in **Figure 3**. Similar results showing that adrenergic hormones are produced during embryonic heart development in mammals, including humans, have also been reported [61-65]. It has also been known for many years that non-neuronal sources of adrenergic cells are present in the hearts of numerous species both during early development and in adulthood [66-83]. Thus, there is a clearly an established presence of adrenergic cells in the heart throughout development, but what exactly is their role?

Adrenergic hormones play critical roles in heart development and disease [84-91]. Indeed, the absence of adrenergic hormones due to disruption of either the *tyrosine hydroxylase* (*Th*) or *dopamine β-hydroxylase* (*Dbh*) genes leads to heart failure in utero [92, 93]. Most cardiac adrenergic cells appear to be only transiently adrenergic during development, and then differentiate into other (non-adrenergic) phenotypes in all parts of the heart, including surprisingly large numbers of myocytes in distinctive regions of the left ventricle and atrium [64, 94]. These cells appear to be derived from *Intrinsic*

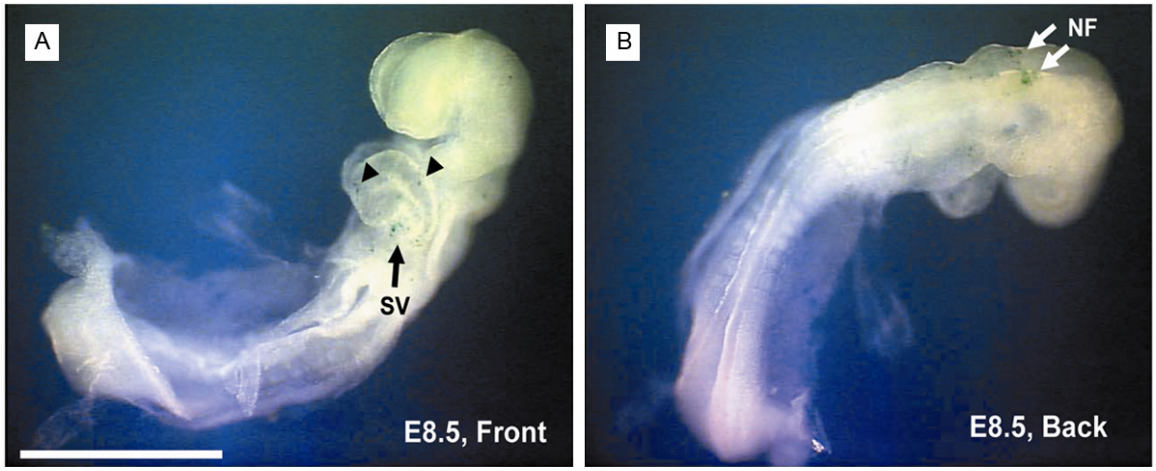

Figure 5. Whole-mount XGAL staining of an embryonic day (E) 8.5 Pnmt-Cre/R26R embryo. A: Left ventral view. Arrow points to LacZ^{+} blue-stained cells in the sinus venosus (SV) region. Arrowheads indicate positions of additional LacZ^{+} cells in this heart. B: Right dorsal view. Arrows point to LacZ^{+} blue-stained cells along the dorsal crest of the neural folds (NF). *Reprinted with permission from Developmental Dynamics* [64].

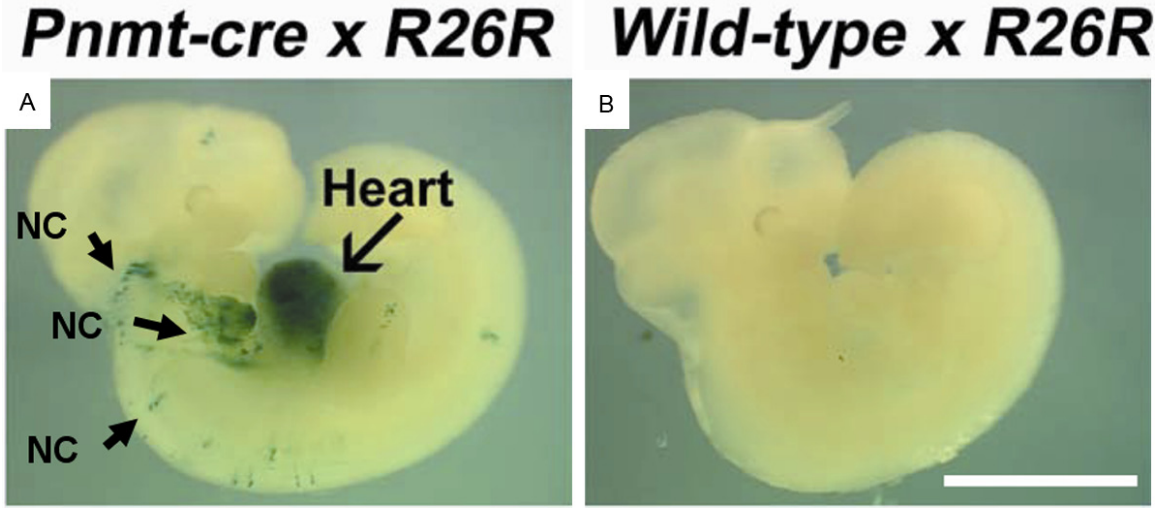

Figure 6. Whole-mount XGAL staining in embryonic day (E) 10.5 mouse embryos. (A, B) Pnmt^{Cre}/Pnmt+, R26R/+ (A) and Pnmt+/Pnmt+, R26R/+ (B) mouse embryos at E10.5. Right sagittal views are shown. The arrow depicts the heart. Staining can also be seen in the brainstem and 2nd branchial arch regions. Sparse patches of XGAL staining are also seen along the back (dorsal surface) and in the forelimb of the Pnmt-Cre x R26R embryo. Scale bar = 1.0 mm in B (applies to A, B). NCD, Neural crest-derived. *Reprinted with permission from Developmental Dynamics* [64].

Cardiac Adrenergic or *"ICA"* cells [62], which express all of the biosynthetic enzymes needed to produce norepinephrine (NE) and epinephrine (EPI), including the final enzyme in the pathway, phenylethanolamine n-methyltransferase (PNMT) (**Figure 4**). Further, ICA cells have been shown to have numerous clear vesicles [62], and thus appear capable of secretory activity.

Notably, however, ICA cells are not neurons nor are they derived from neural crest since their ontological appearance in the developing heart precedes neural crest invasion of the cardiac region, and their distribution within the heart is not consistent with the well-mapped anatomical locations of neural crest-derived cells in the heart [61-65]. In mice, for example, Pnmt+ cells appear independently in the heart and neural fold region of the embryo around embryonic day 8.5 (E8.5) (**Figure 5**) [64]. On the other hand, numerous Pnmt+ neural crest-derived cells appear to be invading the cardiac region by E10.5 (**Figure 6**) [64]. Consequently, there

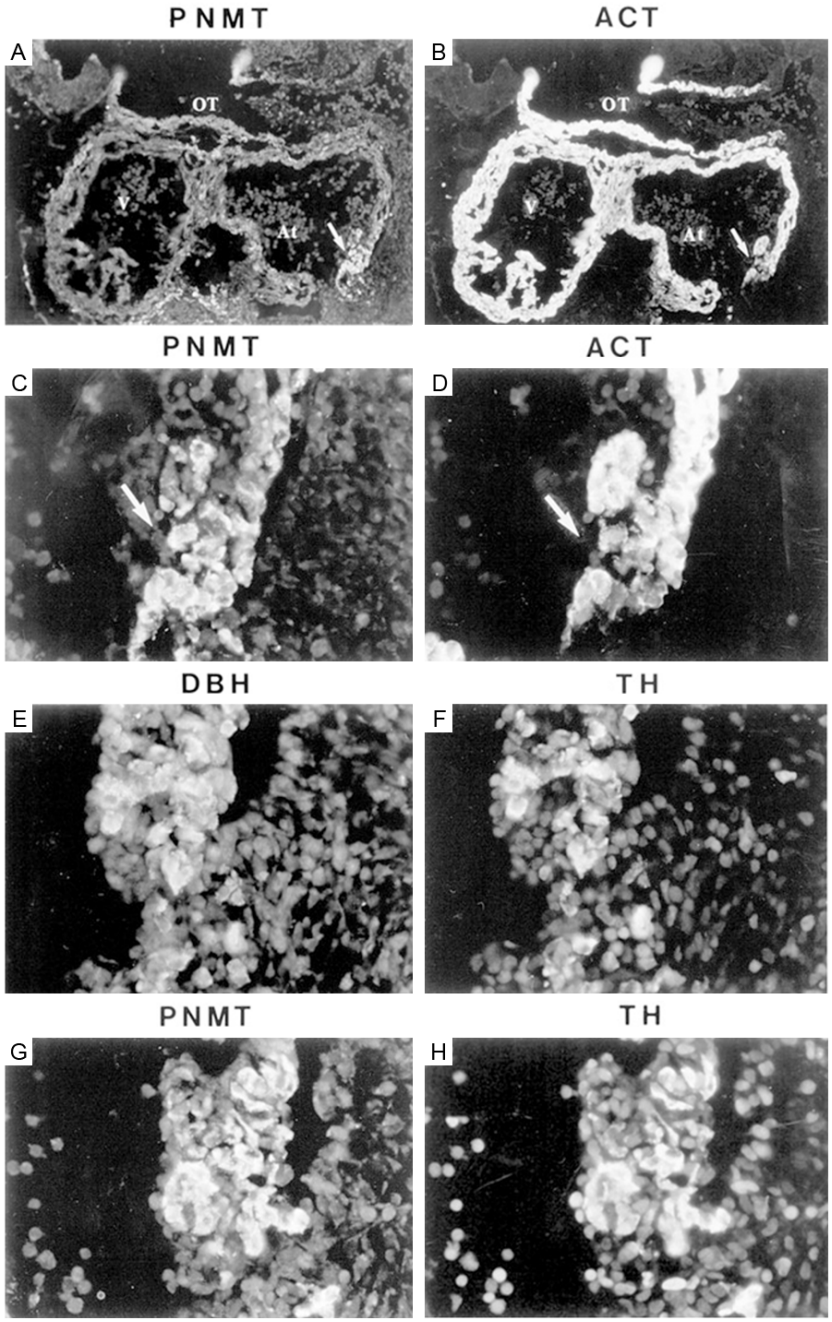

Figure 7. Coimmunofluorescent localization of catecholamine biosynthetic enzymes in the rat heart at E11.5. Sagittal sections (thickness = 10 μm) were costained for PNMT (A and C) and α-actinin (B and D) and visualized using fluorescence microscopy with FITC (A and C) and TRITC (B and D) filters. The arrows point to a region of the dorsal-caudal atrium that contains a clustering of PNMT-expressing cells (putative SA node). Note that some cells are clearly costained for PNMT and α-actinin when visualized at higher magnification (compare panels C and D, ×40 objective), whereas other cells express only one or the other of these markers. Sagittal sections adjacent to the one shown in A through D were double-stained for DBH and TH (E and F, respectively) or PNMT and TH (G and H, respectively). DBH and PNMT were visualized with the FITC filter, whereas TH was observed using the TRITC filter. Note the nearly identical patterns of staining for these enzymes in these atrial tissue sections. OT indicates outflow tract; At, atrium; V, ventricle. *Reprinted with permission Circulation Research* [63].

appear to be two separate populations of Pnmt+ cells that contribute to heart development. The first is derived from splanchnic mesoderm (FHF and SHF) while the second is derived from invading CNC cells.

Pnmt+ cells as cardiomyocyte progenitors

Initial examinations of Pnmt localization within the developing rat heart showed transient and progressive areas of cell concentrations in pacemaking and conduction system tissue. An example of such from the sinoatrial node region is shown in **Figure 6** where immunofluorescent histochemical staining revealed a cluster of cells that expressed PNMT, DBH, and TH at E11.5 in the rat heart [63]. Note, however, that cells expressing the myocyte-specific marker, sarcomeric α-actinin (ACT), generally displayed an inverse pattern of staining relative to cells expressing adrenergic enzymes (compare panels A and B, **Figure 7**). Nevertheless, a few cells appeared to be co-stained for PNMT and ACT when examined at higher magnification (compare panels C and D, **Figure 7**). Despite partial overlapping staining in a few cells, it is also clear that the vast majority of myocytes do not stain positive for PNMT, and conversely, most PNM-

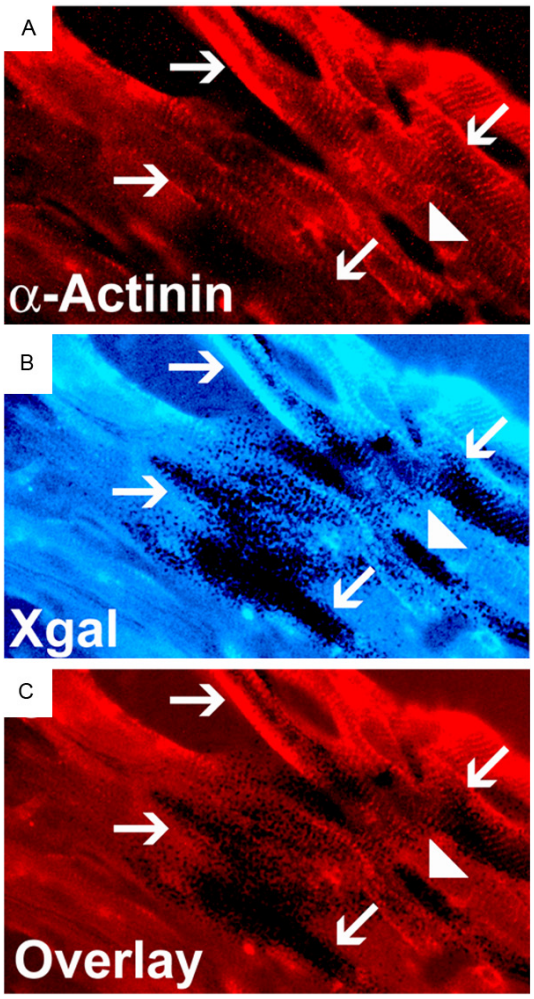

Figure 8. Co-localization of XGAL and sarcomeric α-actinin in LV cells of the adult mouse heart. Immunofluorescent staining for sarcomeric α-actinin in the adult mouse LV shows characteristic striations associated with cardiomyocytes (A, arrows). An adjacent serial section (10 μm) stained with XGAL shows dark (positive) staining, indicating a history of *Pnmt* gene expression (B, arrows). Overlay of the immunofluorescent and XGAL staining show a clear co-localization of cells that have a history of *Pnmt* expression and cells with a cardiomyocyte phenotype (C, arrows). *Reprinted with permission from PLoS One* [137].

T+ cells do not stain positively for ACT. Thus, adrenergic cells are generally not myocytes, and myocytes are generally not adrenergic.

Using a genetic marking strategy to identify historical descendants of Pnmt+ cells in the developing mouse heart, it was later found that many of the Pnmt-derived cells in the heart had become myocytes, as exemplified in **Figure 8** [94]. Consequently, the early PNMT expression seen in the previous figures appears to represent transient adrenergic cells, some of which later differentiate into cardiomyocytes. The distribution of these myocytes is widely spread through all four cardiac chambers through fetal and neonatal development periods, but remarkably becomes largely restricted to the left side of the heart by adult stages (**Figure 9**) [94]. It has certainly been known for many years that adult mammalian hearts have significant non-ganglionic sources of adrenergic cells [73, 95, 96], but only in the past few years has it become understood that adrenergic cells contribute directly to myocardial cell development through apparent trans-differentiation from an adrenergic phenotype into a myocyte phenotype. Further work still needs to be done to prove this hypothesis, but it is both plausible and consistent with the currently available data.

Pnmt+ cells as neuro-progenitor cells in the developing heart

The neural crest is a transient population of migratory cells that arise from the border of the neural plate and the epidermal ectoderm, located at the dorsal end of the developing neural tube [97, 98]. This population of migratory cells is often referred to as the fourth germ layer, because the cells are multipotent and crucial for proper organ development [99]. Ablation studies have confirmed the importance of these cells to normal development as their destruction in ovum leads to reproducible and often devastating organ defects [100-104]. Fate mapping studies have identified the heart, thyroid, parathyroid, thymus, sympathetic ganglia, parasympathetic innervations, and craniofacial bones as targets of migratory neural crest cells [105-107].

Of particular interest is the contribution of neural crest cells to the developing cardiovascular system. Neural crest cells have been shown to help direct formation of the aortic arches, the ventricular outflow tract, become the neurons of the autonomic nervous system within the heart, and they play a crucial role in development of the cardiac conduction system, as well as directing aorticopulmonary septation [108-111]. Furthermore, recent studies have identified dormant, neural crest-derived stem cells within the heart and these have been shown to migrate and differentiate in response to certain stimuli [111, 112].

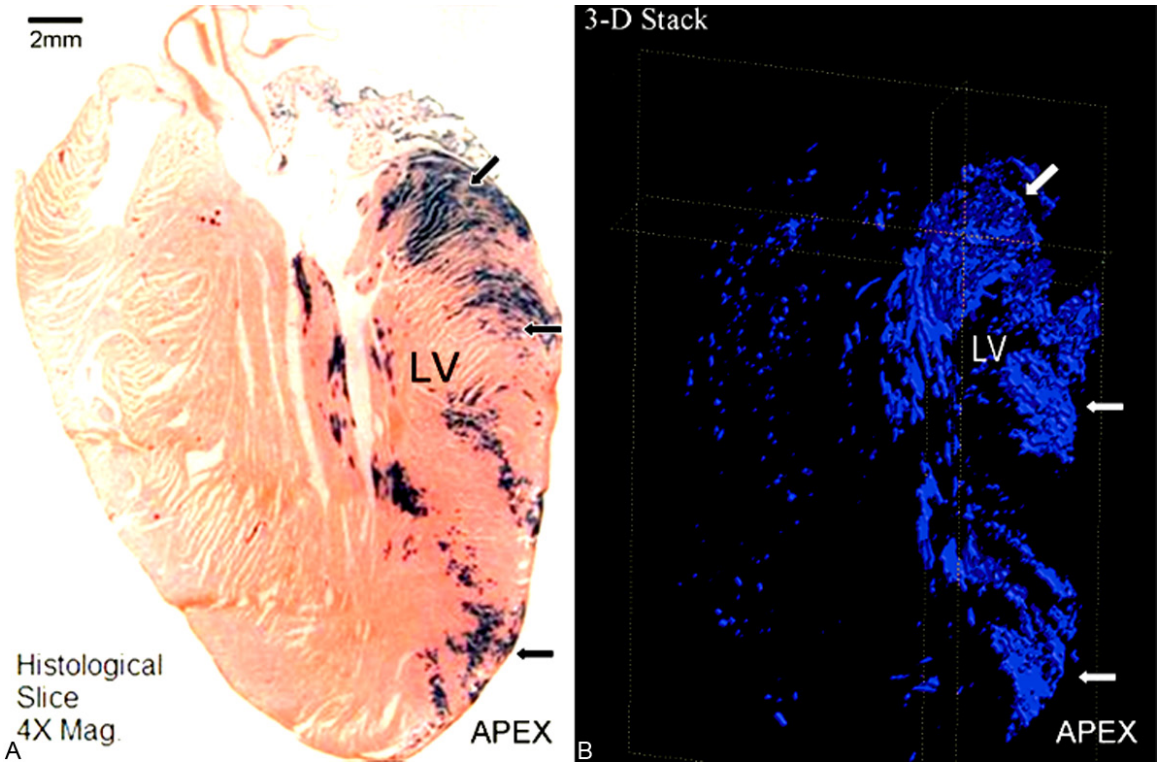

Figure 9. Three-dimensional (3D) reconstruction of the XGAL+ staining in the *Pnmt*$^{+/Cre}$, *ROSA26*$^{+/\beta gal}$ heart. A: Representative two-dimensional section of the adult mouse heart were cut at 20 microns. B: Still-shot of a 3D-rendered image generated from stacked 2D images as described in the methods section. The arrows point to equivalent regions in both panels that roughly correspond to basal, mid, and apical concentrations of XGAL+ cells in these hearts. *Reprinted with permission from PLoS One* [137].

There are three subpopulations of neural crest cells that contribute to heart development. The truncal neural crest cells become the sympathetic neurons innervating the heart, including the sympathetic ganglia and also the chromaffin cells of the adrenal gland [113]. Cranial neural crest cells become the parasympathetic neurons innervating the heart [114-116], and the cardiac neural crest directs development of the aortic arch arteries, the great arteries, and the outflow tract [108, 117]. While it is clear that a population(s) of Pnmt+ cells coming into the heart are derived from neural crest, it remains to be determined if these are exclusively cardiac neural crest cells or might also be contributed from cranial and/or truncal neural crest migrations as well.

Ablation studies

Neural crest-derived (NCD) cells are greatly outnumbered in the heart. While most of the myocardium is derived from mesoderm, the importance of the small population of cardiac neural crest cells that migrate during development is evidenced by the great array of cardiac defects seen upon destruction of pre-migratory neural crest cells. The various cardiac defects resulting from this sort of ablation include ventricular septal defects, variable regression of the great arteries, overriding aorta, and symptoms consistent with DiGeorge syndrome, including persisting truncus arteriosus, interrupted aortic arch, and Tetralogy of Fallot [100, 108, 118].

Furthermore, neural crest has been shown to contribute to the maturation of the cardiac conduction system. It has been shown that neural crest cells do not differentiate into the myocytes involved in conduction, but that NCD cells lie within the vicinity of ventricular (Purkinje) conduction fibers [119]. Ablation studies have confirmed the importance of neural crest cells to the proper development of these fibers and their destruction prevents the transition from immature base-to-apex mode of activation to the mature apex-to-base sequence of activation. Ablation also prevented insulation and

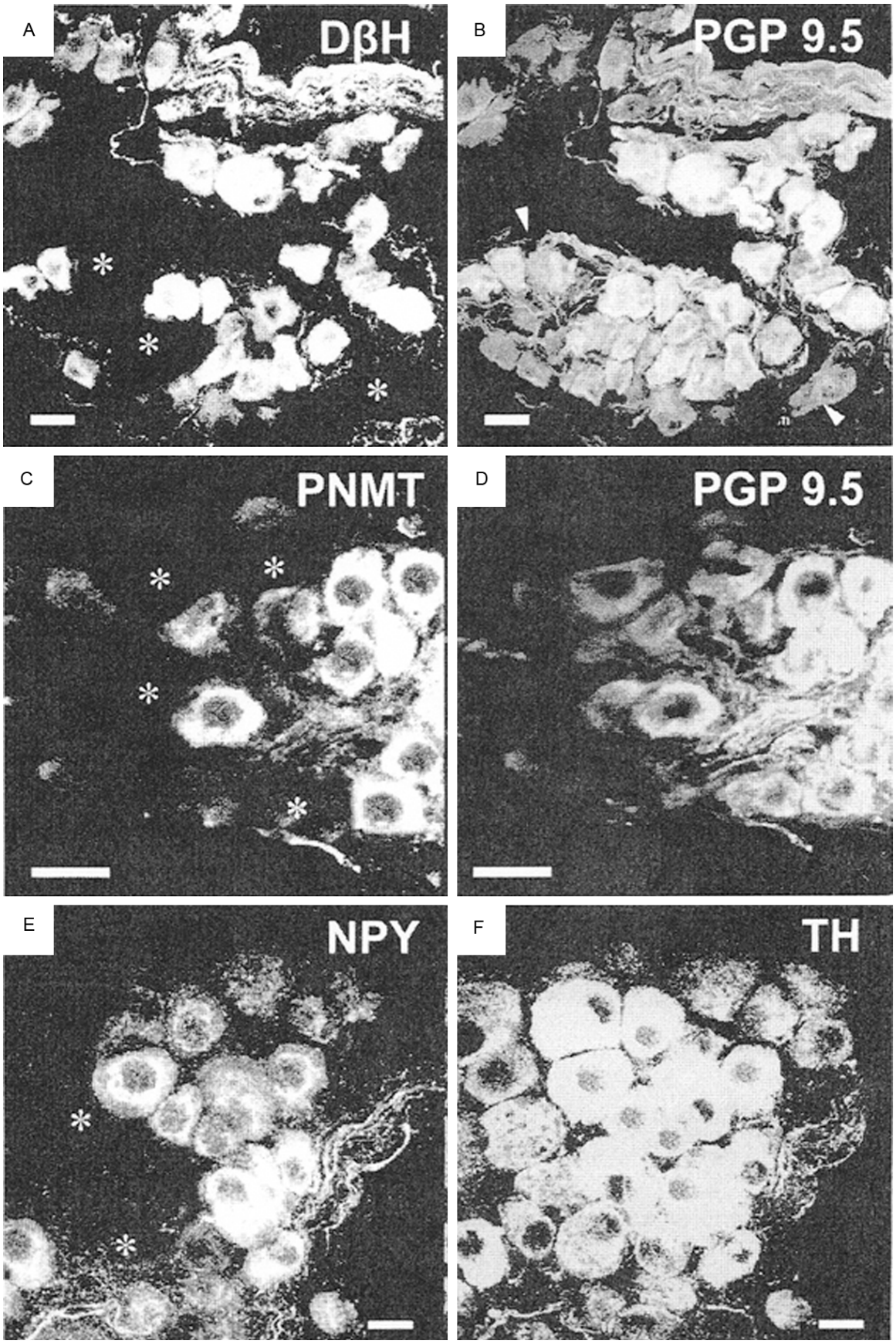
A
DβH
B
PGP 9.5
C
PNMT
D
PGP 9.5
E
NPY
F
TH

Figure 10. Expression of adrenergic biosynthetic enzymes in the rat intrinsic cardiac nervous system. (A and B) Immunoreactivities for DbH-FITC and PGP 9.5-TR in the same filed in section of the left (rat) atrium. (C and D) Immunoreactivities for PNMT-FITC and PGP9.5-TR in the same field in section of the left atrium. Note that not all of the large ganglion neurons, illustrated with PGP 9.5-IR (B, D), are also DbH-IR (asterisks in A) or PNMT-IR (asterisks in C). (E and F) Immunoreactivities for NPY-FITC and TH-TR in the same field in whole-mount preparation of the left atrium. A ganglion containing large-diameter neurons is seen. The somata express TH-IR (F), which co-localizes with NPY-IR in almost all neurons, with few exceptions (asterisk in E). Bar is 20 μm. *Reprinted with permission from Neurochemical Research* [125].

compaction of His fibers, and resulted in faster propagation of electrical currents along the His bundles [120].

The heart receives neural inputs from the sympathetic nervous system, which acts to increase overall cardiac output, and the parasympathetic system, which acts in contrast by ameliorating sympathetic effects. Sympathetic nerves innervate the conduction system [121], cardiomyocytes [122, 123], and the sinoatrial node [124].

It is now known that these ganglia do not act simply as bidirectional neural relay stations. Rather, they are complex, multidirectional stations containing interneurons whose activities are thought to be mediated through various neurotransmitters. A multitude of neurotransmitter-synthesizing enzymes have been identified within interneurons of these ganglia, including those that synthesize serotonin, histamine, norepinephrine, epinephrine, nitric oxide, and various neuropeptides [125-128].

Co-localization of TH, DBH, and PNMT has been demonstrated in some large diameter neurons, meaning they could act through the release of epinephrine to surrounding tissues (**Figure 10**) [125]. PNMT mRNA has been shown to be present in all heart compartments, but was found to be highest in the left atria [78, 125]. Kvetnansky [96] has found that the PNMT mRNA levels are about equal in ganglionic and nonganglionic tissue of the heart and also found a higher level in the left atria [79]. Interestingly, when rats were subjected to stress by immobilization, PNMT mRNA levels in nonganglionic areas of the heart rose, while those in ganglionic areas remained at basal levels [78, 129]. Taken together, these data suggest that there are at least two different populations of PNMT+ cells in the heart that appear to be under different regulatory influences. One hypothesis that could explain these results is that nonganglionic Pnmt+ cells arise from ICA cells while ganglionic Pnmt+ cells arise from NCD cells.

Therapeutic potential of Pnmt+ cells for cardiac repair/regeneration

The data outlined in this review suggest that Pnmt can serve as a useful marker for identifying cardiac myocyte and neuronal precursors. Substantial numbers of cardiac myocytes in the developing and mature mouse heart are derived from cells that expressed Pnmt [64, 94, 130, 131]. These include subsets of myocytes that are found in all four chambers of the heart and those comprising the pacemaking and conduction system [63, 64]. These myocardial precursors appear in the embryonic mouse heart between E7.5-E8.5 as part of the developing primary myocardium [64, 81]. In addition, a second set of Pnmt+ cells invade the developing heart several days later, beginning ~E10.5 [64]. These latter Pnmt+ cells appear to be of neural crest origin, and may give rise to cardiac neurons [94]. The developmental origins and functions of cardiac nerves is generally known to be derived from neural crest precursors, but the specific role and function of adrenergic neurons and their derivatives in the heart is not well described or understood at present. Further, most current stem cell strategies designed to treat cardiovascular disease tend to focus almost exclusively on regeneration of new muscle and/or blood vessel formation.

Here, we propose a new strategy to isolate and potentially amplify selected populations of cardiac neural progenitors to complement current angiomyogenesis approaches for cardiac regenerative medicine. It may be possible, for example, to use Pnmt (or other neurotransmitter-specific enzymes) as a marker to identify, isolate, and characterize specific subpopulations of cardiac stem/progenitor cells that have the potential to become either specialized myocytes or neurons during the course of normal heart development and when applied to damaged myocardium for repair/regeneration therapy. A schematic illustration of this idea is

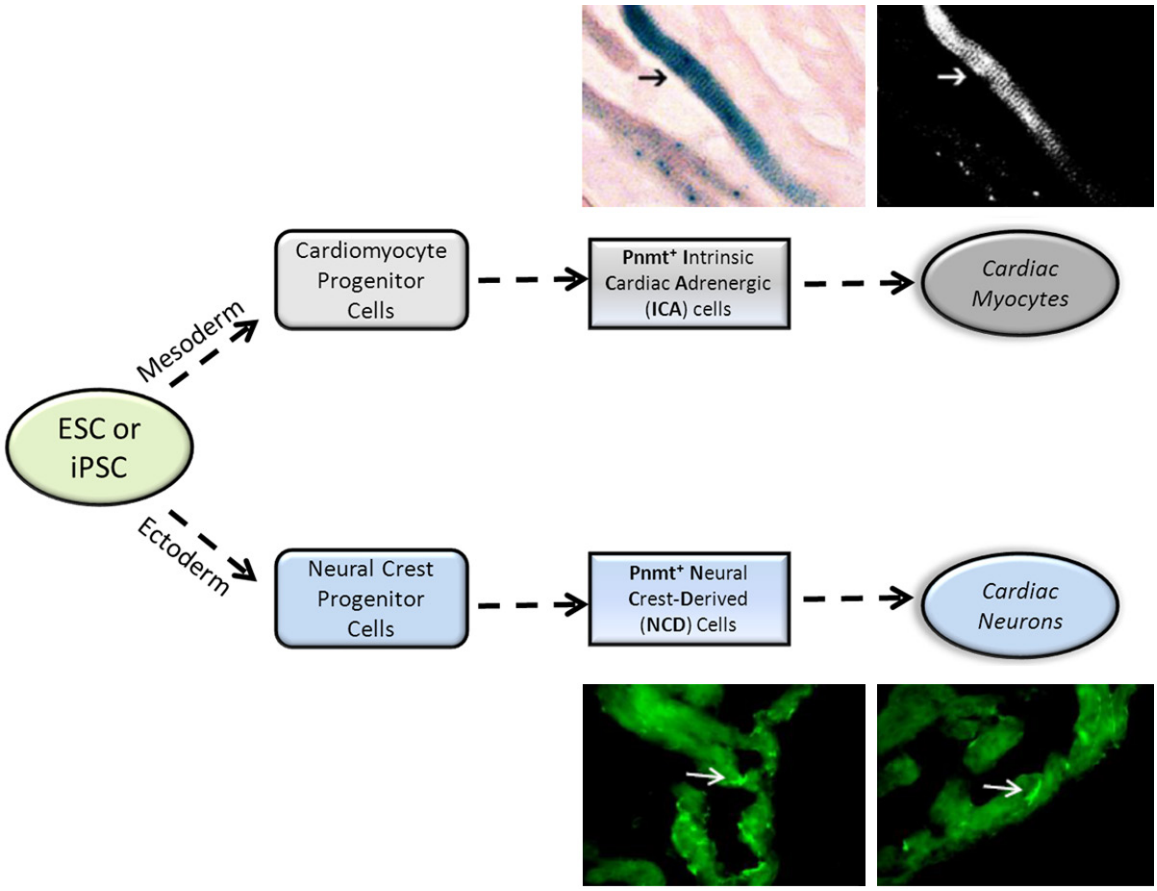

Figure 11. Schematic illustration of dual differentiation pathways for Pnmt+ cells in the heart. The pathway shown in the upper half of the diagram is of mesoderm origin that produces ICA cells, which in turn become specific myocytes within the heart. The pathway shown in the lower half of the diagram is of ectoderm origin that produces NCD cells, which in turn become specific types of cardiac neurons. *Upper panel pictures:* Pnmt-derived (XGAL+) myocyte (left panel, arrow) showing elongated rectangular shape with striations, characteristic of mature ventricular cardiomyocytes (right panel). *Lower panel pictures:* Pnmt+ neuron-like cells identified in adult mouse atrial tissue using anti-Pnmt immunofluorescent staining techniques (arrows). *Both picture sets are reprinted with permission from PLoS One* [137].

shown in **Figure 11**. Evidence supporting this hypothesis is provided in the pictures showing Pnmt-derived myocytes (top panels) and neuron-like cells (bottom panels) in **Figure 11** in the adult mouse heart [94].

By manipulating the differentiation pathway in culture, it is theoretically possible to generate both neuronal and myocardial primer cells that express Pnmt. Indeed, this has recently been shown using recombinant mouse embryonic stem cells (mESCs) that have the green fluorescent protein (GFP) reporter gene knocked into the endogenous Pnmt locus [132]. These results showed that GFP expression was induced in parallel with endogenous Pnmt mRNA following induction of cardiac differentiation in these cells using well-established protocols. With these and other new tools being developed to identify viable Pnmt+ cells, it will soon be feasible to isolate them for detailed characterization of their physiological properties and ability to differentiate and develop into functional myocardium.

In parallel, Pnmt+ cells could also be isolated following induction of neural crest differentiation to isolate neuronal precursors using a differentiation strategy to promote cardiac neural crest development and migration [133]. The two different Pnmt+ cell populations could be evaluated independently or combined to facilitate an integrated neuro/myocardial approach for cardiac repair and rehabilitation. Concei-

vably, other markers in this pathway such (e.g., TH or DBH) could have similar but somewhat different utilities in this context, and thus appear ripe for exploration.

It is important to keep in mind, however, that adrenergic primer cells may not necessarily provide beneficial results for cardiac regeneration. It is possible that they could instead show no significant change, or they might worsen an already deteriorating condition (e.g., heart failure). There are, for example, reports indicating that post-MI neural sprouting can lead to arrhythmogenic problems [50-52, 134-136]. An argument could be made that because adrenergic influence is one of the major problems associated with heart failure in the clinic, it might not make sense to add adrenergic-derived stem/progenitor cells into a failing heart. It will thus be important to know if these cells pose a benefit or risk for stem cell therapies used in the treatment of heart failure. It will be equally informative if they turn out to pose a risk because this may help future stem cell therapies selectively avoid using this subpopulation of Pnmt+ cells for therapeutic applications of this type. In either case, this work opens new possibilities for directed differentiation of semi-specialized "primer" cells that have great potential for cardiac regenerative medicine therapies, and thus promises to be an engaging area of research focus in the foreseeable future.

Disclosure of conflict of interest

None.

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