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CATECHOLAMINE SYNTHESIZING CELLS IN THE EMBRYONIC MOUSE HEART

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Abstract

The heart is a primary source of epinephrine and norepinephrine during embryonic development, yet little is known about the cardiac cells that produce these catecholamine hormones. To identify when and where catecholamine-synthesizing cells are found in the embryonic heart, we developed a novel mouse genetic model by “knocking-in” the cre-recombinase gene to the locus encoding for the epinephrine biosynthetic enzyme, phenylethanolamine n-methyltransferase (pnmt). When crossed with ROSA26 reporter (R26R) mice, the β -galactosidase gene is activated in adrenergic cells. A major advantage of this approach is that it allows detection of adrenergic cells and their progeny, regardless of whether the progeny cells retain an adrenergic phenotype or not. Our data show that adrenergic cells appear as early as embryonic day 8.5 (E8.5) and continue to accumulate in substantial numbers through birth in the mouse heart where they appear to share common ancestry with myocardial lineages. Large numbers of atrial and especially ventricular myocytes appear to be derived from embryonic adrenergic cells in the heart. In addition, many of the pacemaking cells in the sinoatrial and atrioventricular nodes also appear to be derived from an adrenergic lineage. Thus, our results suggest that catecholamine synthesizing cells serve as cardiomyocyte progenitors in the embryonic heart.

Keywords

PNMT; EPINEPHRINE; CRE-RECOMBINASE; PROGENITOR CELL; MOUSE; LAC-Z

INTRODUCTION

We and others have previously shown that the major catecholamine synthesizing enzymes, tyrosine hydroxylase (Th), dopamine β -hydroxylase (Dbh), and phenylethanolamine n-methyltransferase (Pnmt) are expressed in the embryonic heart during early stages of development^{1–7}. Detection of these enzymes first becomes apparent at about the time that the heart begins to beat, prior to neural crest invasion of developing myocardium and long before the appearance of nerves in and around the heart. Thus far, detection of adrenergic

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biosynthetic enzymes and their products (dopamine, DA; norepinephrine, NE; and epinephrine, EPI) has been demonstrated in chicks^{5,6}, rats¹⁻³, mice⁸, and humans².

Extra-neuronal sources of adrenergic cells have been found in adult hearts of numerous species⁹⁻¹⁴, but their specific origin and function in the developing heart are not well-understood. It is clear, however, that NE or EPI are essential for embryonic development because mice that have disrupted *Th* or *Dbh* genes die during mid-gestation of “apparent cardiac failure”^{15,16}. When we examined the distribution of adrenergic enzyme expression within the developing rat heart, we found that the catecholamine synthesizing cells were transiently and progressively associated with regions of the heart associated with pacemaking and conduction system development (e.g., sinoatrial node, SAN; atrioventricular node, AVN; His bundle, and Purkinje fibers)³. Notably, these cells were also found to be sporadically strewn within the myocardial layers of the developing cardiac chamber walls. Because of this early association with myocardial development, these catecholamine synthesizing cells have been designated as “intrinsic cardiac adrenergic” or “ICA” cells².

To determine the potential origin and possible fate of ICA cells in the developing heart, we created a novel mouse genetic model whereby the *Cre-recombinase* gene was “knocked-in” to the *Pnmt* gene locus, which effectively replaced *Pnmt* gene expression (due to disruption of *Pnmt* coding sequences in exon 1) with expression of the *Cre-recombinase* gene, now under control of *Pnmt* gene regulatory sequences (“Pnmt-Cre” mice)⁸. When crossed with the ROSA 26 Reporter (R26R) mouse strain¹⁷, the β -galactosidase reporter gene was activated by the action of Cre-recombinase, which removed a 5' transcriptional block sequence, thus permitting expression of β -galactosidase (Fig. 1). Because Cre-recombinase causes an irreversible genetic change, activation and expression of β -galactosidase does not require continuous expression of Cre-recombinase. Thus, even cells that only transiently express Cre-recombinase during development will thereafter express β -galactosidase. In our model, Cre-recombinase is expressed from the *Pnmt* gene locus such that we expect β -galactosidase to be expressed specifically in those cells that had a history of *Pnmt* gene expression (i.e., adrenergic cells). This general strategy is outlined in Fig. 1.

We have previously described the initial characterization of the Pnmt-Cre mouse model where we showed that β -galactosidase was indeed activated in adrenergic cells as expected, thereby demonstrating the specificity of this genetic model for marking adrenergic cell lineages within the heart and the adrenal gland⁸. In the present study, we expand upon these efforts to show that ICA cells appear to give rise to surprisingly large numbers of myocardial cells in all parts of the developing heart, thereby demonstrating that adrenergic cells likely serve as progenitors for substantial numbers of cardiomyocytes.

MATERIALS & METHODS

All materials and methods used for this study were performed as previously described^{3,8}. Animals were used in accordance with the guidelines provided by the Animal Care and Use Committees of the National Institutes of Health (Bethesda, MD) and the University of Central Florida (Orlando, FL).

RESULTS & DISCUSSION

In the process of creating the Pnmt-Cre knock-in mice, the Pnmt gene was disrupted such that in the homozygous condition (Pnmt-Cre/Pnmt-Cre), a “knock-out” (KO) of the Pnmt gene was also generated. To demonstrate the effectiveness of this strategy, we isolated, fixed, and stained adult mouse adrenal gland sections using immunofluorescent histochemical staining techniques. As shown in Fig. 2, the medulla of the adrenals from wild-type animals stained brightly when reacted with an anti-Pnmt antibody (panel A), indicating strong expression of endogenous Pnmt. In contrast, the medulla from Pnmt-Cre/Pnmt-Cre homozygous mice did not stain positively when reacted similarly with the anti-Pnmt antibody (panel C). The lack of Pnmt immunofluorescence was not due to an absence of medullary cells because these cells were readily identified in the same sections through co-staining with an anti-Th antibody as visualized in the red spectrum (Fig. 2, panels B and D). These results demonstrate that there is little or no Pnmt protein expressed in KO adrenals, consistent with our previous finding that EPI concentrations were not detectable in these animals⁸. Thus, disruption of the endogenous Pnmt alleles due to insertion of Cre-recombinase was effective in this model.

The absence of Pnmt and EPI in Pnmt-Cre/Pnmt-Cre KO mice did not produce any overt phenotype. As previously indicated, expected Mendelian ratios were observed and the KO mice survived to adulthood and were able to reproduce. Since these mice still produce NE, it likely compensates for the absence of EPI in most cases. One might expect, however, that a physiological phenotype may manifest if these mice are exposed to various stress paradigms, and this will be an important area for future investigations.

To test the ability of the Pnmt-Cre mice to “mark” adrenergic cells, we crossed Pnmt-Cre mice with R26R mice and performed XGAL staining on tissue sections to identify cells where β -galactosidase expression had been activated as outlined in the strategy depicted in Fig. 1. As shown in Fig. 2 (panel E), the adrenal medulla from these animals stained strongly positive with XGAL (blue stain). The surrounding adrenal cortex did not stain positive for XGAL, as expected since cortical cells do not normally express catecholamine biosynthetic enzymes. These results show that our adrenergic cell-marking strategy is specific and effective.

Perhaps the most notable finding from our initial characterization of Pnmt-Cre x R26R mice was the extensive XGAL staining in the developing heart⁸. An example of such staining is shown in panel F of Fig. 2 where heavy blue staining can be seen in the muscle layers of all four cardiac chambers. XGAL staining was most extensive in the ventricular septum, but both free walls of the ventricles and, to a lesser extent both atria, showed positive reactivity with XGAL. These results indicate that the transient Pnmt expression that we previously observed in the developing heart most likely reflects a temporary adrenergic phenotype in certain cardiac progenitor cells that eventually become myocytes. To investigate this possibility further, we have carefully evaluated XGAL staining patterns in the developing hearts from Pnmt-Cre x R26R mice as illustrated in Figs. 3–5.

The earliest developmental timepoint when positive XGAL staining appeared in Pnmt-Cre x R26R embryos was approximately E8.5, where it was found simultaneously in the heart and the neural folds along the nape of the neck⁸. To more specifically examine where and when ICA cells appear in the developing heart, we stained a series of sagittal sections through the cardiac region at E9.5 (Fig. 3) and at E11.5 (Fig. 4) with XGAL. Even from the earliest stages, the pattern of staining appears to be within the myocardial walls of the developing heart (indicated by arrows, Fig. 3), especially at junctional regions such as those between the atrium (AT) and ventricle (VE) as well as those bordering the outflow tract (OT). Positive XGAL staining was also observed in the trabeculated muscle of the ventricle at this early stage (Fig. 3, panel F). It is important to note that even though there is clearly some XGAL staining outside of the heart, the staining within the heart appears to arise from within the myocardial anlage itself rather than migrating into the heart from other outside sources such as invading neural crest cells. Whereas neural crest cells are just beginning to invade the cardiac region at E9.5 in the mouse, XGAL staining marking adrenergic cells is already fairly extensive by this time. Moreover, the pattern of XGAL staining in our mice is totally dissimilar to the well-established neural crest cell distribution in the heart¹⁸. These results, therefore, suggest that cardiac adrenergic cells share a common lineage with myocardial cells with little or no contribution from neural crest at these early stages of development.

The XGAL staining patterns observed at E9.5 continued at E11.5 (Fig. 4). Again, the heaviest labeling was observed along the border zones in the muscle layers of the developing cardiac chamber walls. By E11.5, XGAL staining is much stronger and there are many more cells labeled in these regions, thereby indicating that cells with a history of Pnmt expression accumulate over time in the heart. We cannot at present determine, however, if these cells are arising *de novo* or if they are simply the daughters of dividing myoblasts that had once expressed Pnmt. It may be that both mechanisms are operative at these early stages of development. There are, for example, some XGAL positive cells in the region just outside the heart, adjacent to the SAN area (see arrowheads, Fig. 4 panels E & F). These cells are located where myocardial progenitors have been found^{19–21}. Interestingly, the XGAL staining in these cells is much less intensive than it is in the myocardial cells at the junctional zones, perhaps reflecting *de novo* appearance of Pnmt expression in these putative myocardial progenitor cells. This idea is consistent with the previous staining patterns observed for endogenous Pnmt protein expression in cells along the myocardial progenitor ridge of tissue bordering the heart^{3,8}. Additional studies are needed to determine if this hypothesis is true.

It is clear from these results that adrenergic cells strongly contribute to myocardial development throughout the heart and especially at the atrioventricular junction. Compare, for example, the XGAL staining pattern at the atrioventricular junction at E9.5 (Fig. 3, panels E & F) with those at E11.5 (Fig. 4, panels G & H). Between E9.5 and E11.5, endocardial cushion tissue sprouts from the atrioventricular junction through a series of orchestrated inductive events²². Remarkably, there is no XGAL staining in the endocardial cushion tissue despite the strong positive staining observed in the muscle layer immediately underlying the cushions. These results further support the idea that adrenergic cells within the heart specifically contribute to the myocardial lineage.

To illustrate this point directly, we performed co-staining for XGAL and a myocardial-specific cell marker, sarcomeric α -actinin in E15.5 heart sections. As shown in Fig. 5, a substantial number of myocardial cells (stained red due to α -actinin immunofluorescence) were co-stained with XGAL. Note that the XGAL staining quenches the fluorescence, so we first captured the fluorescent images showing α -actinin staining (Fig. 5, panels A & D representing atrial wall and ventricular septum, respectively), and then performed XGAL staining (Fig. 5, panels B & E). When combined, it is clear that many of the cells that had stained positively for α -actinin were now also stained with XGAL (Fig. 5, panels C & F). These results suggest that Pnmt-expressing cells within the developing heart give rise to substantial numbers of myocardial cells both in the atria and ventricles.

In summary, we have shown that Pnmt-Cre mice provide an effective model for both the study of Pnmt/EPI function and as a means to identify and track descendent cells produced from cardiac adrenergic cells. Our results indicate that cardiac adrenergic cells give rise to cardiac muscle tissue in all four chambers of the heart, and especially along the crest of the ventricular septum. Although the specific function of transient adrenergic expression in the developing heart remains to be elucidated, the Pnmt-Cre x R26R mouse model provides a useful tool for examining the appearance, accumulation, and distribution of adrenergic cells in the heart and other organ systems.

Acknowledgments

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Fig. 1.
Schematic diagram of *Pnmt* cell tracking strategy.

Fig. 2. Characterization of Pnmt-Cre mice

(A–D) Immunofluorescent histochemical staining with anti-Pnmt and anti-Th antibodies reacted with secondary antibodies conjugated to fluorescein isothiocyanate (FITC) and Texas Red, respectively. (A&B) Wild-type (Pnmt+/+) adrenal sections. (C&D) KO (Pnmt-cre/Pnmt-cre) adrenal sections. (E) XGAL staining (blue) of adrenal medulla in Pnmt-Cre x R26R mouse. (F) XGAL staining in a frontal heart section from a postnatal day 2 (P2) Pnmt-cre x R26R mouse. LA, left atrium; RA, right atrium; LV, left ventricle; RV, right ventricle. Color figures available online only.

Fig. 3. XGAL staining in serial sagittal sections through an E9.5 *Pnmt-cre* (x *R26R*) mouse heart Arrows indicate regions of the myocardial walls where β -galactosidase activity was detected, as indicated by the blue XGAL stain. The tissue was counterstained with eosin (pink) to enhance contrast. Section thickness, 10 μ m. AT, atrium; BrA, branchial arch; OT, outflow tract; VE, ventricle.

Fig. 4. XGAL staining in serial sagittal sections through an E11.5 Pnmt-cre (x R26R) mouse heart

Arrows indicate regions of the myocardial walls where β -galactosidase activity was detected, as indicated by the blue XGAL stain. The tissue was counterstained with eosin (pink) to enhance contrast. Section thickness, 10 μ m. AT, atrium; IT, inflow tract (see accompanying arrow); OT, outflow tract; VE, ventricle. Arrowhead depicts XGAL-stained cells in the extracardiac region immediately adjacent to inflow tract (panel E). Color figures available online only.

Fig. 5. E15.5 frontal heart sections from a Pnmt-Cre (x R26R) mouse co-stained for the cardiac muscle-specific marker, sarcomeric α -actinin (red fluorescence) and ICA cell descendents (blue XGAL stain)

A region of the atrial wall (A–C) and the ventricular septum (D–F) were stained first for α -actinin alone (A&D), XGAL alone (B&E), and α -actinin together with XGAL (C&F). Color figures available online only.