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Nkx2-5- and Isl1-expressing cardiac progenitors contribute to proepicardium

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Abstract

Correct delineation of the hierarchy of cardiac progenitors is a key step to understanding heart development, and will pave the way for future use of cardiac progenitors in the treatment of heart disease. Multipotent Nkx2-5 and Isl1 cardiac progenitors contribute to cardiomyocyte, smooth muscle, and endothelial lineages, which constitute the major lineages of the heart. Recently, progenitors located within the proepicardium and epicardium were reported to differentiate into cardiomyocytes, as well as smooth muscle and endothelial cells. However, the relationship of these proepicardial progenitors to the previously described Nkx2-5 and Isl1 cardiac progenitors is incompletely understood. To address this question, we performed in vivo Cre-loxP-based lineage tracing. Both Nkx2-5- and Isl1-expressing progenitors contributed to the proepicardium and expressed Wt1 and Tbx18, markers of proepicardial progenitor cells. Interestingly, Nkx2-5 knockout resulted in abnormal proepicardial development and decreased expression of Wt1, suggesting a functional role for Nkx2-5 in proepicardium formation. Taken together, these results suggest that Nkx2-5 and/or Isl1 cardiac progenitors contribute to proepicardium during heart development.

Introduction

Regenerative approaches hold great promise to improving the treatment of congenital and acquired heart disease, the leading causes of morbidity and mortality in infancy and adulthood, respectively. Realizing this promise will require detailed understanding of the diverse cardiac lineages and their hierarchies during heart development.

Recent work indicates that a common multipotent precursor differentiates into cardiomyocyte, smooth muscle, and endothelial lineages, the major differentiated cell types of the heart [1–3]. This precursor is marked by expression of the transcription factors Isl1 and Nkx2-5. Lineage tracing studies indicate that these Isl1- and Nkx2-5-expressing precursors contribute to most of the cardiomyocytes and a subset of the smooth muscle and endothelial cells of the heart [3].

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Proper cardiac morphogenesis requires contribution of cells from the proepicardium (PE), an outpouching of the septum transversum that protrudes towards the heart. Proepicardial cells migrate onto the surface of the heart, forming an epithelial sheet termed the epicardium. Epicardial cells subsequently undergo epithelial to mesenchymal transition, migrate into the myocardium, and differentiate into smooth muscle and endothelial cells [4–6]. In the heart, expression of the transcription factor Wilm's Tumor 1 (*Wt1*) is confined to the proepicardium and epicardium [5]. We recently performed genetic lineage tracing studies based on *Wt1*-driven recombinase expression, and found that progenitors located within the PE and epicardium also differentiate into cardiomyocytes [7]. At the same time, Cai and colleagues reached the same conclusion using an independent lineage tracing system based on recombinase expression driven by *Tbx18* regulatory elements [8].

The lineage relationship between epicardial progenitors and the previously described multipotent *Nkx2-5*⁺/*Isl1*⁺ precursor is incompletely understood. While Cai et al. suggested that *Tbx18*-marked epicardial progenitors represent a novel lineage that is complementary to and distinct from *Isl1*-marked cells [8], we found that *Wt1*-marked epicardial progenitors are descended from precursors that express *Nkx2-5* and *Isl1* [7]. In this work, we provide further evidence indicating that *Wt1*⁺ progenitors within the PE are derived from *Nkx2-5*⁺/*Isl1*⁺ precursors. Furthermore, we show that *Nkx2-5* expression is required for normal development of the PE, suggesting that *Nkx2-5* functionally regulates formation of this structure.

Materials and Methods

Mice

Nkx2-5^{IRESCre/+}, *Nkx2-5*^{Cre/+}, or *Isl1*^{Cre/+} or mice were crossed with *Rosa26*^{fsLacZ} reporters [9–12] for fate map studies. We note that this *Rosa26*^{fsLacZ} reporter was more robustly recombined than a similar, independently generated reporter [13]. To generate *Nkx2-5* or *Isl1* knock out embryos, *Nkx2-5*^{Cre} mice or *Isl1*^{Cre} mice were intercrossed. Noon of the day of the plug was defined as E0.5. All procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of Children's Hospital Boston.

Histology and Immunohistochemistry

Whole-mount X-gal staining for β -galactosidase (β -gal) was performed as previously described [14]. Immunostaining was performed as described [15]. Briefly, the embryos were fixed with 4% paraformaldehyde and cryosectioned at 5–8 μ m. Cell membranes were permeabilized by washing with 0.1% Triton X-100 PBS. The tissues were subsequently pre-blocked in 5% donkey serum and PBS for 1.5 hour at room temperature. The primary antibodies *Wt1* (1:100, Santa Cruz), *Tbx18* (1:100, Santa Cruz), *Nkx2-5* (1:100, Santa Cruz), β -gal (1:5000, MP biomedical), *Isl1* (1:100, Iowa Developmental Studies Hybridoma Bank) were incubated for 1.5 hours at room temperature. Bound antibodies were visualized by secondary antibodies (Alex488 or 555, Invitrogen) or by the ABC method (ABC kit, Vector Laboratories). Nuclei were stained with 4',6-Di-amidino-2-phenylindole dihydrochloride (DAPI). The immunostained tissues were imaged by confocal microscopy (FV1000, Olympus).

Results and Discussion

To track the descendants of *Nkx2-5*-expressing progenitors, we used the Cre-loxP lineage tracing approach [12]. Prior to Cre recombination, a floxed stop cassette in *Rosa26*^{fsLz} prevented β -gal expression, as demonstrated by the absence of X-gal staining in *Nkx2-5*^{+/+};*Rosa26*^{fsLz} embryos (Fig. 1A). In *Nkx2-5*^{IRESCre/+};*Rosa26*^{fsLz/+} embryos, *Nkx2-5* regulatory elements drove expression of Cre recombinase, which removed the floxed stop cassette. This resulted in heritable expression of β -gal by *Nkx2-5*⁺ cells and their descendants

(Fig. 1A). At E9.5, *Nkx2-5^{IRESCre/+};Rosa26^{fsLz}* embryos exhibited strong X-gal staining in the developing heart. The proepicardium (PE), residing between atrium (A) and left ventricle (LV), was robustly stained. This finding was confirmed in tissue sections, which showed that most PE cells expressed the genetic lineage β -gal (white arrowheads), indicating that cells descended from *Nkx2-5⁺* precursors ("*Nkx2-5*-derived") contribute to PE.

Wt1 marks a subset of PE cells that differentiate into cardiomyocyte, smooth muscle, and endothelial lineages [7,8]. To test if *Wt1⁺* PE cells are derived from *Nkx2-5*-expressing precursors, we co-stained tissue sections of *Nkx2-5^{IRESCre/+};Rosa26^{fsLz}* embryos for Wt1 and the β -gal lineage tracer (Fig. 1B). A subset of *Nkx2-5*-derived PE cells (β -gal⁺; red) expressed Wt1 (green, Fig. 1B row 2), indicating that *Wt1⁺* PE cells originate from *Nkx2-5*-expressing precursors. The *Nkx2-5*-derived PE cells were not actively expressing *Nkx2-5* (green, Fig. 1B row 1), indicating that *Nkx2-5* expression occurs transiently in PE precursors and is not maintained in PE cells.

Knock-in of Cre into the same locus by different strategies can lead to distinct recombination patterns [9,10]. To reduce the likelihood of a false positive finding due to potentially idiosyncratic behavior of the *Nkx2-5^{IRESCre}* line, we used a different, independently generated *Nkx2-5* Cre knockin line, *Nkx2-5^{Cre}* [10], to confirm the above findings. In *Nkx2-5^{Cre/+};Rosa26^{fsLz/+}* embryos, β -gal⁺ cells were also present within the PE, although the extent of proepicardial labeling was reduced compared to *Nkx2-5^{IRESCre/+};Rosa26^{fsLz/+}*. These β -gal⁺ PE cells expressed Wt1 (Supplementary Fig. 1A) as well as *Tbx18* (Supplementary Fig. 1B), another marker of the PE [8].

Collectively, these data provide strong evidence that *Nkx2-5⁺* precursors contribute to the PE, suggesting that the PE shares a common developmental origin with the rest of the heart. The PE gives rise to much of the coronary vasculature [4,16,17]. Consistent with the finding that *Nkx2-5⁺* precursors contribute to PE, our recent work indicates that *Nkx2-5⁺* progenitors also contribute to a subset of coronary endothelial and smooth muscle cells in embryonic and adult hearts (Ma et al., submitted). While PE cells are originate from *Nkx2-5⁺* precursors, they no longer actively express *Nkx2-5*, suggesting that the *Nkx2-5⁺* progenitors are located higher in the progenitor hierarchy, before differentiation into and formation of the proepicardial/epicardial progenitors.

To test the hypothesis that *Isl1⁺* progenitors contribute to the PE, we again used the Cre-loxP lineage tracing approach, now with Cre recombinase driven by *Isl1* regulatory elements [11]. No β -gal⁺ activity was detected in negative control embryos (*Isl1^{+/+};Rosa26^{fsLz}*) (Fig. 2A). In *Isl1^{Cre/+};Rosa26^{fsLz}* embryos, the PE was robustly stained by X-gal (Fig. 2A). β -gal activity was also detected throughout the left ventricle (Fig. 2A). Extensive expression of β -gal within PE was confirmed in histological sections (Fig. 2A).

Next, we asked if the *Isl1*-derived PE cells expressed the proepicardial marker Wt1. We found that PE cells co-expressed Wt1 and the β -gal lineage tracer (Fig. 2B, arrows), indicating that *Wt1⁺* proepicardial cells originate from *Isl1*-expressing precursors. Consistent with this finding, both *Isl1⁺* cardiac progenitors and *Wt1⁺* proepicardial/epicardial progenitors differentiate into cardiomyocytes, smooth muscle and endothelial cells in vivo and in vitro [3,7,18]. By immunostaining, the PE did not actively express *Isl1* (data not shown), consistent with previous reports [19]. This positions the *Isl1⁺* progenitor high in the progenitor hierarchy, before differentiation into proepicardial cells.

Since both *Nkx2-5⁺* and *Isl1⁺* cardiac progenitors contribute to PE, we asked if *Nkx2-5* or *Isl1* are functionally required for proepicardium development. Therefore we studied proepicardial development *Nkx2-5* or *Isl1* knockout embryos (*Nkx2-5^{Cre/Cre}* or *Isl1^{Cre/Cre}*, respectively). *Nkx2-5* knockout embryos were developmentally delayed and showed severe

cardiac abnormalities (Fig. 3A), consistent with previously described *Nkx2-5* knockouts [20, 21]. *Nkx2-5* was not detectable by immunohistochemistry (Fig. 3B), indicating that the *Nkx2-5^{Cre}* allele is protein null. The PE was not clearly evident. Proepicardial *Wt1* expression was markedly downregulated (Fig. 3B, arrow), while *Wt1* expression in other regions of the embryo was intact (Fig. 3B, arrowheads). This suggests that *Nkx2-5* is functionally required for formation of PE and expression of *Wt1*.

Isl1 knockout also resulted in severe growth retardation and cardiac abnormalities (Fig. 3C). Although the PE was abnormally located towards the atrial pole of the heart, it was identifiable (black arrows, Fig. 3C) and *Wt1* expression was preserved (white arrows, Fig. 3D). This indicates that *Isl1* is not functionally required for PE development or *Wt1* expression. Furthermore, this suggests that abnormal PE development and *Wt1* expression in *Nkx2-5* null embryos is a specific consequence of *Nkx2-5* loss of function, rather than a non-specific consequence of developmental delay or abnormal cardiac development. The mechanisms by which *Nkx2-5* regulates PE development and *Wt1* expression merit further investigation.

In conclusion, we found *Nkx2-5*- and *Isl1*-expressing precursors contribute to the proepicardium, marked by *Wt1* and *Tbx18*. *Nkx2-5*, but not *Isl1*, is functionally required for PE development. These results place *Wt1*⁺ proepicardial/epicardial progenitors as an early branch of the multipotent *Nkx2-5*⁺/*Isl1*⁺ cardiac progenitor lineage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. *Nkx2-5*-derived cells contribute to proepicardium

(A) *Nkx2-5*^{IRES-Cre/+}; *Rosa26*^{fLz} and littermate control *Nkx2-5*^{+/+}; *Rosa26*^{fLz} embryos, stained in whole mount with X-gal and then sagittally sectioned. Arrowheads mark the X-gal positive cells in proepicardium. (B) Co-staining of *Nkx2-5* (green), *Wt1* (green), and β -gal (red) on the same slide. Nuclei were stained with DAPI (blue). Arrows indicate PE cells that are β -gal and *Wt1* positive, and *Nkx2-5* negative. Black bar = 500 μ m. White bar = 100 μ m. A, atrium; RV, right ventricle; LV, left ventricle; OFT, outflow tract; PE, proepicardium.

Fig. 2. *IsI1*-derived cells contribute to proepicardium

(A) Wholemount X-gal staining of *IsI1*^{Cre/+};*Rosa26*^{fsLz} and littermate control *IsI1*^{+/+};*Rosa26*^{fsLz} embryos. Sagittal sections of whole mount stained embryos are shown. Arrowheads mark the X-gal positive proepicardium. (B) Immunostaining of Wt1 and β -gal on sections from *IsI1*^{Cre/+};*Rosa26*^{fsLz} embryos. Asterisk indicates proepicardium. Arrows indicate cells positive for both β -gal and Wt1. Bar = 100 μ m. Other abbreviations as in Fig. 1.

Fig. 3. Loss of *Nkx2-5*, but not *Isl1*, leads to malformation of proepicardium

(A) Wholemount figure of *Nkx2-5^{Cre/Cre}* (*Nkx2-5 KO*) and littermate control *Nkx2-5^{+/+}*. Black arrow indicates PE in control embryo, and corresponding region of the mutant embryo. (B) Immunostaining of Wt1 (red) and *Nkx2-5* (green) shows decreased Wt1 expression and no *Nkx2-5* expression in mutants. Arrow indicates Wt1+ cells in PE of control embryo, and corresponding area deficient in Wt1 expression in mutant embryo. Arrowhead indicates preserved Wt1 expression outside of the heart-forming regions of the mutant embryo. (C) Wholemount figure of *Isl1^{Cre/Cre}* (*Isl1 KO*) and littermate control *Isl1^{+/+}*. Black arrow indicates PE in control and mutant embryos. (D) Immunostaining of Wt1 (red) and cardiac marker Desmin (green) shows existence of proepicardium. White arrow indicates Wt1-expressing proepicardium. A,C bar = 500 μ m; B,D bar = 50 μ m.