Pitx2c and Nkx2-5 Are Required for the Formation and Identity of the Pulmonary Myocardium

Mathilda T.M. Mommersteeg, Nigel A. Brown, Owen W.J. Prall, Corrie de Gier-de Vries, Richard P. Harvey, Antoon F.M. Moorman, Vincent M. Christoffels

Abstract—The pulmonary vein is sleeved by myocardium, which is a major source of atrial fibrillation and is involved in congenital sinus venosus defects. Little is known about the cellular origin and mechanism of formation of the pulmonary myocardium. We observed a biphasic process of pulmonary myocardium formation in mice. Firstly, a myocardial cell population forms de novo at the connection of the pulmonary vein and the atrium. Genetic labeling revealed that atrial cells do not contribute to this population, indicating it forms by differentiation of pulmonary mesenchymal cells. Secondly, these pulmonary myocardial cells initiate a phase of rapid proliferation and form the pulmonary myocardial sleeve. Pitx2c-deficient mice do not develop a pulmonary myocardial sleeve because they fail to form the initial pulmonary myocardial cells. Genetic-labeling analyses demonstrated that whereas the systemic venous return derives from Nkx2-5–expressing precursors, the pulmonary myocardium derives from Nkx2-5–expressing precursors, indicating a distinct origin of the 2 venous systems. Nkx2-5 and its target gap-junction gene Cx40 are expressed in the atria and in the pulmonary myocardium but not in the systemic venous return, which expresses the essential pacemaker channel Hcn4. When Nkx2-5 protein level was lowered in a hypomorphic model, the pulmonary myocardium switched to a Cx40-negative, Hcn4-positive phenotype resembling that of the systemic venous return. In conclusion, our data suggest a cellular mechanism for pulmonary myocardium formation and highlight the key roles played by Pitx2c and Nkx2-5 in its formation and identity. (Circ Res. 2007;101:902-909.)

Key Words: pulmonary myocardium | pulmonary vein | Nkx2-5 | Pitx2c | Hcn4 | Cx40 | differentiation | lineage | precursor

The pulmonary venous vessels are ensheathed by a myocardial layer known as the pulmonary myocardium. Besides its normal physiological functions,1 the pulmonary myocardium is a major source of atrial fibrillation.2 Several possible mechanisms for this pathology have been put forward, including intrinsic pacemaker activity and properties that facilitate reentry.3 A related issue, therefore, is whether the formed pulmonary myocardium shares both origin and phenotypic properties with nodal (pacemaker) tissues,4–7 such as the sinoatrial node and myocardium of the developing systemic venous return (sinus venosus/sinus horn), or with the atrial myocardium, which has a working myocardial phenotype much less prone to automaticity. The pulmonary myocardium is also involved in congenital cardiac malformations with anomalous connections to the atria or systemic venous return as major defects.8

Although the morphology and physiology of the pulmonary myocardium have been extensively studied, little is known about the developmental origin and the molecular mechanisms controlling its formation and phenotype. The pulmonary myocardium has been proposed to form from atrial myocardium, which migrates around the pulmonary vein after its connection with the atrium and differentiates into pulmonary myocardium.9,10 Alternatively, because α-smooth muscle actin expression in the developing pulmonary vein wall precedes that of myocardial markers, the lung vein mesenchyme has been proposed to differentiate into pulmonary myocardium.11 Furthermore, the systemic and pulmonary myocardial venous returns have been suggested to have either a similar12–14 or distinct15–18 origin. However, these morphogenetic and expression analyses did not allow assessment of a shared or distinct origin. In this study, we addressed these issues by means of genetic lineage and gene expression analyses. Using Pitx2c knockout and Nkx2-5 hypomorphic models, we identified critical roles of these genes in the differentiation and phenotypic identity of the pulmonary myocardium.

Materials and Methods

Mice
The Nppa-Cre,19 R26R,20 Nkx2-5IRESCre,21 Nkx2-5fl/fl22 Pitx2c−/−,23 and Pitx2c+/24 transgenic mouse lines have been described previously. Embryonic age was determined according to the vaginal plug.
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The base of the pulmonary vein but absent around its lumen. From E14.5 onward, the branches of the vein that penetrate deeply into the lungs were also covered by pulmonary myocardium (Figure 1G through 1L). This tree subsequently enlarges in the following period to reach far into the smallest lung veins.24 Taken together, between E10.5 and E12.5, a process of myocardium formation is observed around the pulmonary vein. Thereafter, this myocardial tree rapidly expands around the pulmonary vein branches.

Origin of the Pulmonary Vein Myocardium

We next asked whether the first pool of pulmonary myocardial cells at the base of the pulmonary vein at E11.5 to E12.5 was derived from atrial myocardial cells or from pulmonary mesenchymal cells that differentiate into myocardium. Markers or tools to assess a mesenchymal origin are lacking. Therefore, we used the genetic Cre-LoxP labeling system to label the atrial cells. By crossing *Nppa-Cre3* mice19 with the *R26R* reporter line,20 the atrial cells and their daughters irreversibly activate the lacZ gene between E10.5 and E12.5 (Figure 2A).25 At E17.5, the now well-established pulmonary myocardium did not express lacZ (Figure 2B), indicating that it is not derived from migrating labeled atrial myocardium. This experiment suggests that the first pulmonary myocardium forms de novo by differentiation of mesenchyme around the pulmonary vein.

The sinus horn myocardium and its mesenchymal progenitors do not express *Nkx2-5* (Figure 2C).18 To assess whether the sinus horn and the pulmonary myocardium have a shared or distinct origin, we analyzed *Nkx2-5<sup>RES-Cre</sup>*, *R26R* double transgenic embryos,21 in which the cells that express or once expressed *Nkx2-5*, and their daughters, are irreversibly labeled with an activated lacZ gene. All atrial and ventricular myocardium is labeled in these embryos, including the

Results

Development and Expression Analysis of the Pulmonary Vein Myocardium

We first analyzed the formation and differentiation of the pulmonary myocardium by in situ hybridization and 3D reconstruction. At E10.5, the pulmonary vein is surrounded by mesenchymal cells (Figure 1A). Using cardiac troponin I (*cTnI*) to mark all differentiated myocardium, the first myocardial cells became visible in this population at E11.5 to E12 (Figure 1A and 1B). Three-dimensional reconstruction of a heart of E12.5 (Figure 1B and 1C) shows that myocardium is present around the base of the pulmonary vein but absent around its lumen. These myocardial cells expressed connexin 40 (*Cx40*), an atrial marker initially absent from the systemic venous return myocardium.17 From E12.5 onward, the *Cx40*-positive myocardial sleeve around the pulmonary vein started to extend and cover the stem of the pulmonary vein but not the branches (Figure 1D through 1F). From E14.5 onward, the branches of the sinus horn myocardium and its mesenchymal progenitors do not express *Nkx2-5* (Figure 2C).18 To assess whether the sinus horn and the pulmonary myocardium have a shared or distinct origin, we analyzed *Nkx2-5<sup>RES-Cre</sup>*, *R26R* double transgenic embryos,21 in which the cells that express or once expressed *Nkx2-5*, and their daughters, are irreversibly labeled with an activated lacZ gene. All atrial and ventricular myocardium is labeled in these embryos, including the...
pulmonary myocardium (Figure 2C and 2D). Moreover, at E10.5 and E11.5, the mesenchymal cardiac precursors in the dorsal mesocardium were also found to be labeled (Figure 2C). Thus, the pulmonary myocardium and the sinus horn myocardium are derived from precursors that are distinct early in pulmonary myocardium development.

The transcription factor Is1 is required for the formation of the heart tube by recruitment of second heart field progenitor cells.26 Shortly after myocardial differentiation, Is1 expression is extinguished in the second heart field–derived cells,26 and this results in a transient coexpression of Is1 and myocardial markers.18 We investigated whether the pulmonary precursor population expresses Is1. At E9.5, the presumed precursors in the dorsal mesocardium around the pulmonary pit are highly positive for Is1 expression, indicating that the pulmonary vein precursors are a component of the second heart field (Figure 2E). However, the expression decreases sharply after E10 (Figure 2F and 2G), and by E11.5, the mesenchymal cells around the pulmonary vein are Is1 negative (data not shown). These data indicate that approximately 1 to 2 days before pulmonary myocardium formation, Is1 expression is turned off.

We next assessed the spatiotemporal pattern of proliferation of the pulmonary vein region. At E10.5 and E11.5, the mesenchymal cells at the base of the pulmonary vein revealed a moderate fraction of 5-bromodeoxyuridine (BrdUrd)-positive nuclei (19%, see below and Figure 5B) in comparison with the surrounding myocardial and mesenchymal tissue (Figure 3A). One day later, at E12.5, a very high fraction of the newly formed pulmonary myocardium showed BrdUrd labeling (37%, see below and Figure 5B), indicating that these cells, on their initial myogenic differentiation, underwent a phase of rapid proliferation (Figure 3B). At E13.5 and E14.5, the pulmonary myocardium still proliferated rapidly compared with the atrial myocardium (Figure 3C and 3D). These findings suggest that subsequent to its formation between E11.5 and E12.5, the pulmonary myocardium initiates rapid proliferation to envelope the pulmonary vein.

**A Pitx2c-Dependent Mechanism for the Formation of the Pulmonary Myocardium**

*Pitx2c*, a bicoid-related homeodomain protein, is required for left–right patterning of the visceral organs, including the atria and systemic venous return of the heart.27 *Pitx2c* is highly expressed in the pulmonary myocardium.28 Previously, *Pitx2c*-deficient fetuses were found to have a common medial sinus venosus, into which both the horns of the sinus venosus and the pulmonary vein drain.23 This raised the question of whether failure to specify the pulmonary and sinus horn myocardium underlies this defect in *Pitx2c*-deficient mice. Consistent with previous findings,23 we found that in *Pitx2c* mutants, the pulmonary vein connects in the midline of the right isomeric atria (Figure 4A and 4B). The sinus horns of the wild-type fetus and the right isomeric sinus horns of the *Pitx2c*-deficient fetus (aqua blue) did not express *Nkx2-5* (Figure 4A). However, in both wild types and mutants, the pulmonary vein enters at a separate position from the *Nkx2-5*-negative sinus horn myocardium and directly engages the *Nkx2-5*-positive atrial myocardial domain (gray) (Figure 4A). Thus, despite the apparent convergence of the systemic and pulmonary entrances in *Pitx2c* mutants, the sinus horn myocardium and the mediastinal myocardium around the pulmonary vein entrance retain their molecular identity.

We observed a striking absence of myocardium around the pulmonary vein in all (n=24) *Pitx2c* mutant fetuses studied (Figure 4A). Expression of early cardiac markers *Tbx5*, *Tbx20*, and *Nkx2-5* (Figure 4B and data not shown) and late markers for differentiated myocardium (cTnI and

**Figure 3.** Initiation of rapid proliferation in newly formed pulmonary myocardium. A through D, Sections of subsequent stages stained for cTnl, BrdUrd, and sytox green. Arrowheads depict high proliferation in the pulmonary myocardium. PV indicates pulmonary vein; RA, right atrium; WT, wild type. Scale bars=100 μm.

**Figure 4.** Formation and identity of *Pitx2c*-deficient fetuses. A, Three-dimensional reconstructions of an E14.5 wild-type embryo (left) and a *Pitx2c* knockout littermate (right) with a dorsal (top) and caudal view (bottom) on the atria, sinus horns, and pulmonary vein. Gray indicates *Nkx2-5*-positive myocardium; aqua, *Nkx2-5*-negative myocardium; red, lumen pulmonary vein; orange, lumen caval veins. B, In situ hybridization serial sections of an E14.5 wild-type heart (top images) and of a littermate *Pitx2c* knockout heart (bottom images) stained for cTnl, *Tbx5*, and *Nkx2-5*. PV indicates pulmonary vein; PM, pulmonary myocardium; (R/L)SH, (right/left) sinus horn; (R/L)A, (right/left) atrium. Scale bars=100 μm.
Serca2a; Figure 4B and data not shown) was absent around the pulmonary vein. However, the lumen of the pulmonary vein was normally formed, surrounded by an intact endothelial cell layer (data not shown). Detailed analysis showed the complete absence in mutants of the first pulmonary myocardial cell population that normally forms around E12 (Figure 5A). Consistently, in the absence of this population, the phase of rapid proliferation is not initiated (Figure 5A and 5B). In both mutants and controls, apoptosis, assessed by cleaved caspase 3-detection, did not occur in the region where the pulmonary myocardium develops at E11.5 and E12.5 (data not shown).

Closer examination of the Pitx2 pattern revealed its expression in the pulmonary mesenchyme at E10.5 to E12.5, before and during pulmonary myocardium formation (Figure 5C). In contrast, after E12.5, Pitx2 expression became confined to the pulmonary myocardium itself and was not seen in the pulmonary mesenchyme ahead of the expanding pulmonary myocardium (Figure 5D). A similar developmental expression profile was observed for Nkx2-5 (Figures 2C, 2D, and 5E), which is required for second heart field deployment and during pulmonary myocardium formation (Figure 5C). In Nkx2-5 mutants (Figure 5A and 5B), both proliferation and expansion (potentially migration) of the pulmonary myocardial cells. In the absence of Pitx2c, the formation and hence subsequent expansion of the pulmonary myocardium does not occur, resulting in a failure to form the pulmonary myocardial tree.

Nkx2-5 Is Required for Pulmonary Myocardial Gene Program

The sinus horn myocardium expresses neither Cx40 nor its essential activator Nkx2-5, whereas the pulmonary myocardium does express Cx40 and Nkx2-5 (Figures 1, 6A, and 6B).3,18 Furthermore, Hcn4, encoding an essential pacemaker channel,30–32 was found not to be expressed in the pulmonary myocardium, although it is abundantly expressed in the sinus horn myocardium (Figure 6B).3 Thus, the pulmonary myocardium and atrial myocardium share the expression profile of several key genes, whereas the sinus horn myocardium displays a distinct profile.

Nkx2-5 is among the earliest precardiac markers and is required for heart formation, chamber differentiation,33 and the expression of Cx40.34 This raised the question of whether Nkx2-5 is involved in the differentiation and phenotypic identity of the pulmonary myocardium. It is not possible to assess the function of Nkx2-5 in the pulmonary myocardium.

Figure 5. In the absence of Pitx2c, the formation and subsequent expansion of the pulmonary myocardium does not occur. A, E12.5 and E13.5 immunohistochemistry sections stained for cTnl, BrdUrd, and sytox green showing the difference in proliferation between the wild-type (WT) and Pitx2c−/− embryos (arrowheads). B, Assessment of the fraction of BrdUrd-positive nuclei in the area around the pulmonary vein (n=3). C, Subsequent sections stained with in situ hybridization for Pitx2 showing the expression of Pitx2 in the left lung mesenchyme at E9.5 and, later, the confinement to the pulmonary myocardium. D and E, In situ hybridization serial section stained for cTnl and Pitx2 (E13.5) (D) or cTnl and Nkx2-5 (E14.5) (E). Arrowheads show the confinement of Pitx2 and Nkx2-5 expression to the pulmonary myocardium. F, Pitx2+/+, Pitx2−/−; R26R and Pitx2+/−, Pitx2−/−; R26R lungs with the pulmonary myocardium. The absence of β-galactosidase activity in the Pitx2+/−; Pitx2−/− pulmonary vein indicates the absence of cells with Pitx2 gene activity. PV indicates pulmonary vein; RA, right atrium; LB, lung bud; (R/L)L, (right/left) lung. Scale bars 100 μm.
using Nkx2-5–null embryos, because their heart development arrests at E10, before the development of pulmonary myocardium. Therefore, we analyzed Nkx2-5IRES-Cre/GFP embryos, which have 1 null allele (Nkx2-5GFP)²² and 1 allele in which an IRES-Cre cassette has been inserted into the 3' untranslated region of the Nkx2-5 gene,²¹ resulting in an hypomorphic allele producing less than normal Nkx2-5 levels in the heart and pulmonary myocardium (Figure 6A).²⁹ Nkx2-5IRES-Cre/GFP fetuses have less severe defects than the null mutants and die at birth with atrial and ventricular septal defects.²⁹ cTnI-expressing myocardium around the pulmonary vein developed normally in Nkx2-5IRES-Cre/GFP fetuses, even though, because of the absence of an atrial septum primum, the orifice of the pulmonary vein appeared to enter more toward the middle of the common atrium (Figure 6B). However, a change in the gene program of the pulmonary myocardium was observed. The sinus horn marker Hcn4 was no longer restricted to the sinus horns but extended into the pulmonary myocardium (Figure 6B). Cx40, on the other hand, was almost completely absent from the pulmonary myocardium in these hypomorphic embryos, although atrial expression was still observed (Figure 6B). The expression of Pitx2 was unaltered (data not shown), suggesting that Nkx2-5 does not regulate Pitx2 expression in the pulmonary myocardium, as has been found to occur earlier in the cardiac lateral plate mesoderm via direct Nkx2-5 binding to a consensus DNA-binding site within the asymmetry enhancer element of Pitx2.³⁵ Thus, reduction of Nkx2-5 levels results in an expression pattern in the pulmonary myocardium partly resembling that of the sinus horn myocardium.

Discussion

Origin and Formation of the Pulmonary Myocardium

The origin of the pulmonary myocardial cells has remained controversial. Based on gene expression profiles, 2 possible pools of cells for the pulmonary myocardium have been hypothesized: atrial cells that migrate around the pulmonary vein⁹,¹⁰,³⁶ and mesenchymal cells that differentiate into myocardium.¹¹ Our analyses indicate a biphasic model for the development of the pulmonary myocardium that unites both hypotheses (Figure 7). Firstly, a myocardial population forms de novo at the connection of the pulmonary vein and the atrium. Secondly, the pulmonary myocardium expands by proliferation and expansion (potentially migration) to form the pulmonary myocardial sleeve.

During the first phase, the pulmonary myocardial cells seem to replace pulmonary mesenchymal cells around the proximal pulmonary vein. The absence of Nppa-Cre-derived atrial cells in the pulmonary myocardium indicates that atrial cells do not invade the pulmonary mesenchyme, which in turn suggests they form by differentiation of the mesenchyme into pulmonary myocardium. Furthermore, during the formation of the first pulmonary myocardial cells, we ob-
served expression of Nkx2-5 in the pulmonary mesenchyme. Because, during cardiac differentiation, Nkx2-5 expression normally precedes the expression of markers for differentiated myocytes such as cTnl, this further suggests that the first pulmonary myocardial cells are formed from the mesenchyme by direct differentiation. In the absence of Pitx2c, the formation of the first population of pulmonary myocardial cell does not occur. Because apoptosis was not observed in this region of mutants, this indicates that Pitx2c is not required for maintaining these cells. Therefore we propose that Pitx2c is required for the differentiation of the mesenchymal cells into pulmonary myocardium. Recently, Pitx2 has been implicated in branchial arch muscle formation, providing support for this possibility. Alternatively, Pitx2c may be required to bring about the correct juxtaposition of inducing tissue with pulmonary mesenchyme.

The second phase of pulmonary myocardium formation involves rapid expansion of pulmonary myocardium along the pulmonary vein (Figure 7) either by proliferation and potentially migration of the pool of pulmonary cardiac cells formed during the first phase or by progressive differentiation of mesenchymal cells adjacent to the pulmonary vein. Immediately after its formation, the pulmonary myocardial pool was seen to initiate very rapid proliferation, underlining the notion that outgrowth and possibly migration underlies expansion of the pulmonary myocardial sleeve. Furthermore, before pulmonary myocardium formation, the expression of Nkx2-5 and Pitx2 was observed in the mesenchyme, but during the second phase, their borders of expression coincided with that of cTnl. These findings argue against a wave of differentiation because, in this scenario, the expression of Nkx2-5 and Pitx2 would be expected to precede that of cTnl in the mesenchyme before its myocardial differentiation. Expression of the Pitx2 gene in the absence of Pitx2 protein (visualized by recombination by the Pitx2Cre allele in Pitx2Cre, Pitx2cR26R embryos) was strongly reduced or even absent around the pulmonary vein branches (Figure 5F). The failure to activate Pitx2Cre expression could indicate that Pitx2 is required to induce the Pitx2 gene in adjacent cells, i.e., through regulation of signaling molecules. Alternatively, Pitx2 could be required for its own activation. However, this is unlikely because Pitx2Cre expression is not affected elsewhere in the heart. Because Pitx2 mutants failed to initiate rapid proliferation of the cells around the pulmonary vein, we hypothesize that Pitx2-expressing daughter cells fail to proliferate or to migrate into the pulmonary vein in the absence of Pitx2 protein in Pitx2 mutants. Taken together, our results indicate that expansion of Pitx2/Nkx2-5/cTnl-positive cells by rapid proliferation underlies the formation of the pulmonary myocardium along the pulmonary vein branches.

Distinct Origin and Identity of Pulmonary and Sinus Horn Myocardium

Sinus venosus defects involve the formation of abnormal connections between the sinus venosus and the pulmonary vein. To understand the underlying mechanisms, insight into the developmental origin of the sinus horns and pulmonary vein is required. This issue has been controversial, with studies arguing in favor of a common or a distinct origin. We found that the pulmonary myocardium develops from Nkx2-5-positive precursors, whereas the sinus horn myocardium develops from Nkx2-5-negative precursors. Thus, the precursors of the pulmonary myocardium seem to derive from the second heart field, which is marked by Nkx2-5, the precursors of the sinus horn myocardium not. These 2 distinct precursor lineages are established already at E9.5, when the sinus horns form. Therefore, the mechanism underlying sinus venosus defects, which affects both sinus horns and pulmonary vein, may have to be sought in misspecification of mesenchymal precursors to a systemic or pulmonary myocardium fate before E9.5 or in a defect in a morphogenetic process such as proliferation or differentiation in otherwise correctly specified precursors, resulting in defects involving both venous returns.

The Phenotype of the Pulmonary Myocardium Depends on Nkx2-5

The pulmonary myocardium is an important source of ectopic electrical activity that initiates atrial fibrillation. Two potential arrhythmogenic mechanisms have been proposed. First, the pulmonary myocardium might have properties largely similar to that of the atrial working myocardium, but tissue architecture (fiber direction, fibrosis) could cause conduction delay favoring reentry, which may cause maintenance of atrial fibrillation. Secondly, the presence of automaticity and triggered activity in pulmonary myocardial cells has been found. This suggests that the pulmonary myocardium may have properties resembling those of nodal (automatic) cells such as conduction system cells or may have properties for triggered activity. Sinus node pacemaker cells have been found in pulmonary veins of rat hearts, as determined by light and electron microscopy. In the pulmonary veins of patients with atrial fibrillation, P cells, transitional cells, and Purkinje cells have been documented. Further indication of a relation between the conduction system and the pulmonary vein came from Leu and Hnk-1 expression analyses and analysis of the CCS-lacZ line, which marks both the conduction system and the pulmonary vein. However, because the relationship between the expression of these markers and the nodal conduction system phenotype has not been established, the significance of coexpression in conduction cells and pulmonary vein is not clear.

Our observations in normal embryologic development indicate that pulmonary myocardium from the outflow of its formation has an atrial myocardial-like phenotype. The expression of the conducting gap-junctional protein Cx40, essential for fast atrial conduction and not expressed in the sinoatrial and atrioventricular nodes, was initiated in the pulmonary myocardium with its formation. In addition, the pulmonary myocardium did not express Hcn4, a pacemaker channel essential for pacemaker activity in human and embryonic mice that is expressed specifically in nodal tissues. This is in contrast to the myocardium of the developing systemic venous return, including the developing sinoatrial node, which runs an Hcn4-positive, Cx40-negative sinoatrial node–like gene program, compatible with the ability to spontaneously generate impulse.
argue against the possibility of a nodal-like phenotype in the pulmonary myocardium in normal development.

Our data, however, present a possible scenario for how conversion to a nodal phenotype might occur during abnormal development. Whereas a reduced dose of Nkx2-5 allows formation of the pulmonary myocardium, we found an identity shift of the pulmonary myocardium to a sinus venosus–like (ie, more nodal) phenotype, with loss of Cx40 and ectopic expression of Hcn4. We observed previously that in Nkx2-5-deficient embryos, Hcn4 is activated in the entire embryonic heart tube, whereas Cx40 expression is lost. Thus, with the reduction in level of only a single transcription factor, a gene program potentially sufficient to provide automativeness will usually be suppressed by normal electric coupling, an increase in collagen deposition and architecture with major changes in fiber direction can cause disconnection between cells, thus facilitating the development of atrial fibrillation.

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Disclosures

None.

References


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Expanded Materials and Methods

Proliferation analysis by BrdU incorporation

BrdU (5-bromo-2'-deoxyuridine, Sigma, St. Louis, MO) was injected intraperitoneally into pregnant mice (50 mg/kg body weight in PBS). One hour after BrdU injection, the mice were sacrificed and the embryos harvested in ice cold PBS. The embryos were fixated with methanol:aceton:water (40%:40%:20%) for 4 hours to overnight, and processed further for immunohistochemistry.

β-galactosidase activity detection and immunohistochemical analyses

Detection of β-galactosidase activity on 20 µm cryostat sections was performed as described.1 For immunohistochemistry on 7 µm embryo sections, the following primary antibodies were used: polyclonal antibody against cTnI (1:1000; Hytest Ltd); polyclonal antibody against Nkx2-5 (1:250; Santa Cruz); monoclonal antibody against BrdU (1:100; BD-Biosciences); monoclonal antibody against cleaved caspase 3 (1:200; Cell Signaling). Nuclei were stained with Sytox green (1:40000; Molecular Probes). Sections were boiled for 10 minutes in antigen unmasking solution (H3300, Vector) and the signal was amplified with thyramide signal amplification (TSA NEL702, Perkin Elmer).

Non-radioactive in situ hybridization

Non-radioactive in situ hybridization of 12 µm embryo sections was performed as described.2 The probes for Isl13 and Hcn44 were kindly provided by S. Evans (Skaggs School of Pharmacy, University of California, San Diego) and B. Santoro (Center for Neurobiology and Behavior, Columbia University, New York). Other probes have been described previously.5
3D-reconstructions

Three-dimensional visualization and geometry reconstruction of patterns of gene expression determined by in situ hybridization were carried out as described previously. Files with reconstructions are available upon request.


