**Nkx2.5 cell-autonomous gene function is required for the postnatal formation of the peripheral ventricular conduction system**

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Abstract

The ventricular conduction system is responsible for rapid propagation of electrical activity to coordinate ventricular contraction. To investigate the role of the transcription factor Nkx2.5 in the morphogenesis of the ventricular conduction system, we crossed Nkx2.5+/− mice with Cx40eGFP/+ mice in which eGFP expression permits visualization of the His–Purkinje conduction system. Major anatomical and functional disturbances were detected in the His–Purkinje system of adult Nkx2.5+/−/Cx40eGFP/+ mice, including hypoplasia of eGFP-positive Purkinje fibers and the disorganization of the Purkinje fiber network in the ventricular apex. Although the action potential properties of the individual eGFP-positive cells were normal, the deficiency of Purkinje fibers in Nkx2.5 haploinsufficient mice was associated with abnormalities of ventricular electrical activation, including slowed and decremented conduction along the left bundle branch. During embryonic development, eGFP expression in the ventricular trabeculae of Nkx2.5+/− hearts was qualitatively normal, with a measurable deficiency in eGFP-positive cells being observed only after birth. Chimeric analyses showed that maximal Nkx2.5 levels are required cell-autonomously. Reduced Nkx2.5 levels are associated with a delay in cell cycle withdrawal in surrounding GFP-negative myocytes. Our results suggest that the formation of the peripheral conduction system is time- and dose-dependent on the transcription factor Nkx2.5 that is cell-autonomously required for the postnatal differentiation of Purkinje fibers. © 2006 Elsevier Inc. All rights reserved.

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Introduction

The cardiac conduction system (CS) is required for the generation and propagation of the electrical impulse responsible for the synchronization of atrial and ventricular contractions. The electrical impulse generated in the sino-atrial node is transmitted to the ventricles through the central CS including the atrio-ventricular node (AVN), the atrio-ventricular bundle (AVB, or His bundle) and the left and right bundle branches (Anderson and Ho, 2003; Gourdie et al., 2003). The rapid propagation of impulse in the ventricles is mediated by specialized cardiomyocytes, called Purkinje fibers (PF), which form a well-organized network under the endocardial surface of the ventricles, also referred to as the peripheral CS (Gourdie et al., 2003). In mice, the morphology of the His–Purkinje system is reproducible from one animal to another and is structurally well correlated with the His–Purkinje system of human hearts.
Mutations in genes encoding for proteins involved in cardiac conduction, including ion channels, sarcomeric proteins or gap junction channels are now recognized to be important contributing factors in arrhythmias and conduction disturbance in the heart (Roberts and Brugada, 2003). Interestingly, mutations in the gene encoding the cardiac transcription factor Nkx2.5 have been identified in patients with congenital heart diseases presenting atrial septal defects (ASD) and atrioventricular (AV) conduction blocks (Schott et al., 1998). Nkx2.5 is a homeodomain transcription factor which is necessary for early stages of cardiac morphogenesis (Tanaka et al., 1999). Despite this primordial role during early cardiac development, expression of Nkx2.5 continues throughout development and into adult life (Komuro and Izumo, 1993). Elevated Nkx2.5 transcript levels have also been detected in the developing CS, in particular in the AVB, bundle branches and PF (Thomas et al., 2001). Mice haplinsufficient for Nkx2.5 develop AV conduction blocks and susceptibility to arrhythmias (Jay et al., 2004; Tanaka et al., 2002). In Nkx2.5 mutant mice with a single allele deleted or with a ventricular-restricted Nkx2.5 conditional null deletion, AV conduction defects have been attributed to the smaller size of the AVN and AVB (Jay et al., 2004; Pashmforoush et al., 2004). Recently, morphological abnormalities associated with conduction defects have been described in Tbx5 haploinsufficient mice, where atrioventricular and right bundle branch (RBB) blocks are correlated with a foreshortened AVN and absence of the RBB (Moskowski et al., 2004). Hypocellularity of the central CS could therefore underlie conduction defects in different models and in man.

Recently, we have created a mouse model in which the entire His–Purkinje system can be visualized by eGFP (enhanced Green Fluorescent Protein) expression under the regulatory elements of the endogenous Cx40 gene (Miquerol et al., 2004). The presence of the vital marker allows a direct visualization of the PF network and also enables electrophysiological recording of isolated PF. While molecular cues responsible for the development of the ventricular conduction system are not well known, it has been recently established that Nkx2.5 may be involved in this process. Nkx2.5 haploinsufficiency results in hypocellularity of PF which can explain a significant prolonged QRS in these mice (Jay et al., 2004) and the absence of ventricular expression of Nkx2.5 disturbs trabeculation (Pashmforoush et al., 2004). To investigate the role of Nkx2.5 in the morphological development and formation of the peripheral CS, we crossed Nkx2.5 heterozygous null mice (Tanaka et al., 1999) with the transgenic line Cx40KleGFP. Our results demonstrate that ventricular conduction disorders in Nkx2.5+/CX40+/eGFP mice are associated with a severely hypoplastic PF network. Moreover, we show that the hypocellularity of the peripheral CS results from a cell-autonomous defect of postnatal cardiomyocytes to differentiate into PF and to form the peripheral PF network after birth.

Material and methods

Macroscopic and histological analyses

Macroscopic examination of the internal surface of the ventricles was previously described (Miquerol et al., 2004). For histological studies, E16.5, P0, P3 and adult hearts were dissected, fixed for one to four hours in freshly prepared 4% paraformaldehyde (wt/vol) in PBS, then embedded in OCT and cryosectioned. To quantify the number of eGFP-positive PF, transverse sections were counterstained with wheat germ agglutinin–TRITC (WGA–TRITC from Sigma-Aldrich) to label cell membranes as described previously (Jay et al., 2004). The number of eGFP-positive cells is the mean of eGFP-positive cells counted from three sections and for three independent hearts at each level (base, middle, and apex). Statistical significance for difference between groups was set at the P values less than 0.05 as determined by the Student’s t test.

Polycystic rabbit EH–myomesin antibody was generated in Perriard’s lab and used at a dilution of 1:1000 following techniques of immunofluorescence previously described (Agarkova et al., 2000; Agarkova et al., 2004). For scanning electron microscopy, P4 and adult hearts were opened longitudinally in order to visualize the left ventricular conduction system. The hearts were proceeded following standard protocol. Samples were finally coated with 30 nm of gold and observed with a 440 Leica microscope under 20 kV tension.

Optical mapping methods

The heart was excised and maintained in ice-cold, oxygenated Tyrode solution. The heart was quickly cannulated and perfused. The left ventricular free-wall was dissected from the heart so that the LBB and distal Purkinje fibers could be visualized with minimal pinning and retraction. In successful dissections a perfusion pressure of 130 mm Hg could be maintained with moderate flow rates. Hearts were mounted and perfused in a temperature-controlled bath (37±0.5 °C). Techniques for optical mapping of electrophysiological activation sequences has been recently described elsewhere (Hewett et al., 2005). Briefly, hearts stained with voltage-sensitive dye (di-4-ANEPPS, 15 μmol/l) via perfusion and superfusion. Motion artifact was suppressed by superfusion and perfusion with cytochalasin D (10 μmol/l). Briefly, vectors were estimated over a window of seven pixels after initial filtering, utilizing a two-point linear regression of activation times as a function of distance along perpendicular axes. Directly adjacent pixels and diagonally adjacent pixel were separately used for velocity determinations (rotating the latter by 45° into the former) were used for this calculation. The amplitude of the resulting vector was inverted to provide the conduction velocity.

Electrophysiological recordings of Purkinje fiber

Individual Purkinje cells were isolated from Cx40+/eGFP+/Nkx2.5+/+ and Cx40+/eGFP+/Nkx2.5−/− mice according to the procedure described previously (Miquerol et al., 2004). For electrophysiological recordings, cells were transferred in a glass-covered chamber and washed with normal Tyrode solution. Cx40+/eGFP+/Nkx2.5+/+ and Cx40+/eGFP−/Nkx2.5−/− cells were identified by epifluorescence. Action potentials and ionic currents were recorded at 35 °C by the β-escin perforated-patch technique (Fan and Palade, 1998). The liquid junction potential was estimated to 2.9 mV and used to correct voltage values. Data acquisition was performed by using the pClamp software (version 9,
Axon). 30 μM tetrodotoxin (TTX) was added to the Tyrode solution for recording of \( I_{h} \). \( I_{h} \) was elicited by applying 500-ms-depolarizing voltage steps from a HP of -70 mV. The cell was then superfused with 5 mM 4-aminopyridine (4-AP) and \( I_{h} \) current density was estimated as the net 4-AP-sensitive current component. Statistical significances were evaluated with Student’s t-test. Data are expressed as the mean ± the standard error of the mean (S.E.M.) accompanied by the P value. Except for the enzymes, all chemicals were from Sigma-Aldrich.

**TUNEL assay and BrdU incorporation**

After dissection, hearts at P0 and P4 were fixed and the terminal transferase end-labeling (TUNEL) assay was performed on 12 μm frozen sections using the Apop Tag Apoptosis detection kit (Intergen, NY) and using Rhodamine-conjugated anti-digoxigenin antibodies.

P0 and P3 mice were labeled for 6 hours with bromodioxouridine (BrdU) via intraperitoneal injection with 50 mg/kg of BrdU labeling solution (Sigma). Hearts were dissected, fixed, and embedded in OCT. Cryosections were then treated for 30 min at 37 °C with 2 N HCl in PBS containing 0.5% Triton X-100 to denature the DNA to single strand. Sections were rinsed in sodium tetraborate buffer (0.1 M, pH 8.5) to restore neutral pH and processed for immunohistochemistry using an anti-rat BrdU antibody (1:100, Immunologicals). The proliferative index of the apex of the ventricular myocardium was determined as the number of BrdU-positive nuclei divided by the total number of nuclei within a section. A proliferative index was determined for 3 mice per genotype and per stage.

**Production and analysis of mouse chimeras**

Morulae isolated from CD-1 females crossed with Cx40<sup>−/−</sup>eGFP or Cx40<sup>−/−</sup>eGFP/Nkx2.5<sup>−/−</sup> males were aggregated together following the procedure described by Nagy et al. (1993). Hearts were dissected, excised to expose the ventricular cavities for eGFP observation. Then, hearts were fixed, stained in X-Gal by standard procedures, and cryosectioned. To evaluate the cellular composition of chimeric heart, sections were counterstained with WGA–TRITC and Dapi. The number of PF was estimated by counting the number of eGFP-positive cells using the cell membrane marker WGA–TRITC. The numbers of PF from wild-type and mutant origins were estimated by counting the number of eGFP-positive cells (Figs. 2A-a, B-b), while the reduced number of eGFP-positive fibers was accompanied by a morphological disturbance of the trabecular network in mutant hearts (Figs. 2C-c, D-d). In adult mice, the PF network is physically distinct from the ventricular wall in the apex of the heart, perfectly matching the eGFP expression pattern (Figs. 2E-e, F-f). In Nkx2.5<sup>−/−</sup>/Cx40<sup>−/−</sup> hearts, PF were barely detectable at the subendocardial surface of the left ventricle corresponding to the fewer eGFP-positive fibers detected (arrowheads in Figs. 2G-g, H-h). However, we observed the presence of large and straight eGFP-negative trabecules that are detached in the apex of mutant hearts (arrows in panel G), while in control, trabecules are compacted with the ventricular wall (arrows in panel E). These observations support the hypothesis that the reduced number of eGFP-positive cells in Nkx2.5 heterozygous mutant hearts results from a physical deficiency in PF.

**Results**

**Nkx2.5 haploinsufficient mice display a hypoplastic His–Purkinje system**

The network of eGFP-positive PF in Cx40K1eGFP mice can be easily detected by analyzing the internal surface of both ventricles by epifluorescence (Miquerol et al., 2004). In adult Nkx2.5<sup>−/−</sup>/Cx40<sup>−/−</sup>eGFP mutant mice, the number of eGFP-positive fibers was strikingly reduced in both ventricles (Figs. 1B, D) in comparison with control hearts, Nkx2.5<sup>+/−</sup>/Cx40<sup>−/−</sup>eGFP (Figs. 1A, C). The RBB was thinner in Nkx2.5<sup>+/−</sup>/Cx40<sup>−/−</sup>eGFP than in Nkx2.5<sup>−/−</sup>/Cx40<sup>−/−</sup>eGFP hearts (inserts in Figs. 1A, B). In the left ventricle of mutant hearts, fewer eGFP-positive fibers were present in comparison with control hearts. Furthermore, a reticulon pattern comprised of elliptical arrangements of PF in the apical part of the left ventricle was absent in Nkx2.5<sup>−/−</sup> mice. In contrast, the morphology of the left bundle branch (LBB) in the upper part of the septum appeared less affected (Figs. 1C, D).

The reduced number of PF in Nkx2.5 heterozygous mutant was estimated by counting the number of eGFP-positive cells on transverse sections performed at three levels of the heart (apex, middle and base) (Fig. 1E). To delineate cells, the sections were counterstained with WGA–TRITC to label cell membranes (Fig. 1F). Quantitative analyses showed that the number of eGFP-positive cells begins to decrease in the apex of the heart relative to controls from birth, and that this reduction increases progressively with age (only 20% of eGFP-positive cells remain in the apex of Nkx2.5<sup>−/−</sup> adult heart) (Fig. 1G). In the medial part of the heart, the reduction of eGFP-positive cells followed approximately the same progression as in the apex. However, within the basal level, no significant reduction in the number of eGFP-positive cells was observed in young mice. A decrease of 30% of eGFP-positive cells occurred at the base of Nkx2.5<sup>−/−</sup> adult hearts relative to control hearts. These observations show that the peripheral conduction system, as seen in its medial and apical parts, is more severely affected than the proximal bundle branches at the base of the septum which are part of the central conduction system.

Scanning electron microscopy (SEM) investigations were performed to study the development and the structure of the PF network in both genotypes at postnatal day 4 and in adult heart. At the early stage, a network of trabecules forming ellipses is present in the apex of control heart and matches with eGFP-positive cells (Figs. 2A-a, B-b), while the reduced number of eGFP-positive fibers was accompanied by a morphological disturbance of the trabecular network in mutant hearts (Figs. 2C-c, D-d). In adult mice, the PF network is physically distinct from the ventricular wall in the apex of the heart, perfectly matching the eGFP expression pattern (Figs. 2E-e, F-f). In Nkx2.5<sup>−/−</sup>/Cx40<sup>−/−</sup> hearts, PF were barely detectable at the subendocardial surface of the left ventricle corresponding to the fewer eGFP-positive fibers detected (arrowheads in Figs. 2G-g, H-h). However, we observed the presence of large and straight eGFP-negative trabecules that are detached in the apex of mutant hearts (arrows in panel G), while in control, trabecules are compacted with the ventricular wall (arrows in panel E). These observations support the hypothesis that the reduced number of eGFP-positive cells in Nkx2.5 heterozygous mutant hearts results from a physical deficiency in PF.

**Hypoplasia of Purkinje fiber network is associated with abnormalities in ventricular activation but not with electrophysiological defects of the Purkinje fibers themselves**

Nkx2.5<sup>−/−</sup> mice display conduction defects characterized by AV blocks associated with a hypoplastic AVN (Tanaka et al., 2002). Moreover, QRS prolongation was observed in these mice that may be correlated with the hypocellularity of PF (Jay et al., 2004). To test whether the deficiency of eGFP-positive PF disturbed impulse conduction in the ventricle, the propagation of electrical activity was optically mapped on the left ventricular septal surface of Nkx2.5<sup>−/−</sup>/Cx40<sup>−/−</sup>eGFP and Nkx2.5<sup>−/−</sup>/Cx40<sup>−/−</sup>eGFP mice. These recordings showed markedly abnormal and decremental propagation on the mid-left septal surface of 3 out of 7 Nkx2.5<sup>−/−</sup> animals tested (Fig. 3A). Moreover, consistently, lower
global conduction velocity was recorded in all of the Nkx2.5+/− mice (32.8±22.1 cm/s for Nkx2.5+/− versus 53.6±34.2 cm/s for Nkx2.5+/+). Investigations of regional variation in activation spread indicated that conduction velocity in mutant mice was more reduced in the apical sector of the ventricular surface than in the basal part (Fig. 3B).

We then recorded automaticity and action potential properties of individual PF cells (PFCs) from Nkx2.5+/−/Cx40eGFP/+ mice. Inactivation of one Nkx2.5 allele had no effect on PFCs electrical capacitance indicating that the cell size is not affected by Nkx2.5 haploinsufficiency (Figs. 4A, E). We then compared the spontaneous activity of PFCs in both genotypes (Fig. 4B). Similar spontaneous firing rates were recorded in cells from Nkx2.5+/−/Cx40eGFP/+ and Nkx2.5+/−/Cx40eGFP/+ mice. Also, the action potential maximum upstroke velocity (dV/dt) and the action potential duration (APD) were not significantly changed. These observations indicate that Nkx2.5 haploinsufficiency had no effect on PFCs automaticity and action potential waveform (Fig. 4E). We then measured the density of the $I_{to}$ current, which constitutes an important regulator of the action potential repolarization in the mouse myocardium (Guo et al., 1999). $I_{to}$ was measured as the net 4-aminopyridine (4-AP)-sensitive component. No significant difference in the density and activation of $I_{to}$ was observed in $n=8$ PFCs from Nkx2.5+/−/...
Cx40eGFP/+ and Nkx2.5+/−/Cx40eGFP/+ mice (Figs. 4C, D). Similarly, the 4-AP-insensitive current component was not significantly affected. Taken together, these results indicate that Nkx2.5 haploinsufficiency does not affect cell size and electrophysiological properties of PFCs.

The reductions in number of Purkinje fibers in adult Nkx2.5+/− mice occurs postnatally and results from a progressive failure of eGFP+ cells to differentiate into Purkinje fibers.

To investigate the developmental stage at which hypoplasia was first appeared, eGFP expression was examined in Nkx2.5+/−/Cx40+/eGFP embryos. eGFP expression followed the same pattern of expression as the endogenous mouse Cx40 gene (Delorme et al., 1997; Delorme et al., 1995). The eGFP expression starts in the ventricles at day E9.5 and is mainly observed in the trabecular layer of both ventricles. By E15.5, eGFP expression is seen in the AVB and in the ventricular conduction system (Gros et al., 2005). eGFP expression in Nkx2.5+/−/Cx40+/eGFP embryos was indistinguishable from that of Nkx2.5+/−/Cx40+/eGFP embryos between E13.5 to E18.5 (Figs. 5A, C and data not shown). The left ventricular cavity of E16.5 embryonic hearts from both genotypes exhibited a similar eGFP-positive trabecular network (Figs. 5A, C). Sagittal sections showed similar expression of eGFP in atria and trabeculae of both ventricles and along both sides of the interventricular septum of E16.5 embryos from both genotypes (Figs. 5B, D). At birth (P0), macroscopic observation of left ventricles from both genotypes showed a similar network of eGFP-positive cells in the trabecular layer of both ventricles. By E15.5, eGFP-positive cells showed initial modifications in the organization of the network in Nkx2.5+/−/Cx40+/eGFP/+ mice, characterized by a
To validate the use of this molecular marker, we verified that EH–myomesin was not transcriptionally dependent on Nkx2.5 expression by analyzing EH–myomesin expression in E9.0 Nkx2.5+/− embryos. EH–myomesin is expressed in all cardiomyocytes of Nkx2.5+/− and Nkx2.5+/+ embryonic hearts, and is localized in the M-bands of the sarcomeres (Supplementary Fig. 2).

Nkx2.5 is cell-autonomously required for the development of postnatal PF

To determine whether the PF hypoplasia phenotype in Nkx2.5+/− mutants could be rescued by the presence of wild-type cells, we performed chimeric analyses by aggregation of Nkx2.5+/−/Cx40GFP/+ and Nkx2.5+/−/Cx40GFP/+ morulae. In this context, all myocytes bearing the Nkx2.5 mutant allele expressed LacZ and can be identified by X-Gal staining (blue), while PF of both genotypes are eGFP-positive. In adult chimeras, the PF network was examined for eGFP expression and subsequently for X-gal staining detecting areas composed of Nkx2.5 mutant myocytes. In the Nkx2.5 wild-type areas (X-Gal-negative), the PF network forms elliptical structures while in the Nkx2.5 mutant areas (X-Gal-positive) the PF network is disorganized (Figs. 7A–F and Supplementary Figs. 3A–D). The global number of these elliptical structures in the medial and apical part of the left ventricle was estimated for each chimera and for control mice. We group chimera relative to their degree of chimerism in three categories, low chimeras (up to 20% of Nkx2.5 mutant cells) and high chimeras (over 70% of Nkx2.5 mutant cells) and for control mice. We group chimera relative to their degree of chimerism in three categories, low chimeras (up to 20% of Nkx2.5 mutant cells) and high chimeras (over 70% of Nkx2.5 mutant cells) and for control mice.
mice (Fig. 8A). In contrast, if the percentage of chimerism exceeds 30%, the number of ellipses is close to the Nkx2.5+/− phenotype. This result suggests that the pattern of PF network is not rescued and depends on the percentage of chimerism.

We quantified the number of total eGFP-positive fibers at different levels of chimeric hearts on transverse sections using the WGA–TRITC as a cell membrane marker (Fig. 7G). The total number of eGFP-positive cells in the medial part of the heart decreases proportionally in relation to the degree of chimerism (Fig. 8B), while it remains constant at the base. In the apical part of the ventricle, the total number of eGFP-positive cells is similar for each category of chimera and is intermediate between control and mutant hearts (Fig. 1G), suggesting that PF number may be partially rescued in this part of the heart only.

We then studied the cellular composition of these fibers by counting the number of double X-Gal-positive/eGFP-positive cells (Nkx2.5 mutant PF) and the number of Dapi-positive/eGFP-positive cells (Nkx2.5 wild-type PF). X-Gal+ and Dapi+ nuclei are found in the same fibers, showing that eGFP-positive fibers can be a mixed population of PF from both origins (Fig. 7G). To estimate the proportion of PF from mutant origin, we compared the percentage of X-Gal-positive nuclei and the...
percentage of global chimerism for each section and at three levels of the heart (see Supplementary Table 1). The proportion of Nkx2.5+/− myocytes within PF is always significantly lower than the global percentage of Nkx2.5 chimerism (Fig. 8C), except at the base for chimeras displaying high levels of chimerism. These data suggest that the participation of Nkx2.5+/− myocytes in the formation of PF is impaired by comparison to myocytes of wild-type origin. Maximal Nkx2.5 levels are therefore required cell-autonomously in order to get a normal number of PF cells.

We also estimated the proportion of Nkx2.5 mutant myocytes in the eGFP-positive cell population in young chimeras (P0 and P3). For that, we have compared the average of eGFP-positive cells from mutant origin in the apical and middle parts of the left ventricle at P0, P3 and adult stages from chimeras with a medium percentage of chimerism (n=3 per stage). At early postnatal stages, the participation of Nkx2.5 mutant myocytes to PF is proportionally equivalent to the global chimerism combining counts from medial and apical regions of the hearts (Fig. 8D). These data are summarized in Fig. 8E. The difference in the percentage of Nkx2.5 mutant cardiomyocytes to the eGFP-positive PF between P0 and adult timepoints strongly suggest that Nkx2.5 is only required at postnatal stages for the differentiation of PF.

Discussion

In this report, we have investigated the impact of the loss of a single Nkx2.5 allele on the development and the function of the peripheral Purkinje fibers network by crossing Nkx2.5
heterozygote null mice with Cx40KleGFP mice. We show that Nkx2.5+/− mice display major anatomical defects in the formation of the His–Purkinje system. Our results are consistent with the hypoplasia of PF network being the main cause of abnormalities in ventricular structure and function observed in Nkx2.5 haploinsufficient mice. We have demonstrated that the deficiency of PF occurs postnatally and that Nkx2.5 is required cell-autonomously for the differentiation of a normal network of Purkinje fibers.

Physiological disturbances in Nkx2.5 haploinsufficient mice result from independent defects in the development and maintenance of the central and peripheral conduction systems

Morphological analyses of the entire ventricular CS in haploinsufficient Nkx2.5+/− mice revealed differences in the degree and the timing of PF defects at the base and apex of the ventricles. For the BB, the reduced number of eGFP-positive PF occurred late, being detectable only in adults. By contrast, in the peripheral CS at the apex of the heart, the deficiency of eGFP-positive cells occurred mainly around birth, though reductions in PF became progressively more striking with age. Moreover, in double transgenic Nkx2.5+/−/Cx40eGFP/+ hearts, the number of eGFP-positive cells in the His bundle was similar at birth but was reduced in adult hearts in comparison with control mice (not shown). These data are in accordance with AVN and AVB morphological defects observed in Nkx2.5 null-heterozygotes and Nkx2.5 ventricular conditional knock-out mice (Jay et al., 2004; Pashmforoush et al., 2004), and suggest that the two components of the ventricular CS (central and peripheral) develop independently. Retroviral lineage studies in chick have also indicated that the central conduction system differentiates independently from the peripheral network of PF (Gourdie et al., 1995).
Optical mapping analyses of electrical activity propagation have shown a slowed and decremental conduction velocity in the apical part of the ventricles, an observation which is consistent with the prolonged QRS recorded in Nkx2.5+/- mice (Jay et al., 2003; Tanaka et al., 2002). Prolonged QRS appeared early in Nkx2.5 +/- mice in correlation with the perinatal hypoplasia of PF network while PR lengthening appeared in 7-week-old mutant mice associated with reduced AVN and AVB size (Jay et al., 2004). Interestingly, conduction defects in human patients also worsened progressively with age (Schott et al., 1998). The electrophysiological profile of isolated PF is not significantly affected by Nkx2.5 haploinsufficiency, indicating that conduction defects observed in Nkx2.5 +/- mice are due to anatomical disturbance of the His–Purkinje system and not from a remodeling of the phenotype of PF conductive cells. This idea is strengthened by the fact that the level and density of connexins are not modified in Nkx2.5 +/- hearts (Jay et al., 2004).

Nkx2.5 haploinsufficiency has no effect on PFCs cell size, automaticity and action potential configuration parameters. These results indicate that remaining PFCs of wild-type and Nkx2.5 +/- hearts have similar electrophysiological profile. We cannot exclude the possibility that other cellular components (e.g. intracellular organelles, kinases and receptors) can be affected by Nkx2.5 haploinsufficiency. However, it is unlikely that such alterations can explain dysfunction in ventricular conduction of Nkx2.5 +/-/Cx40eGFP+/- hearts, since the action potential configuration of individual PFCs is not altered.

APD prolongation and moderate downregulation of Ito current has been previously reported in ventricular myocytes...
of Nkx2.5−/− adult mice (Tanaka et al., 2002). The lack of significant reduction of \( I_{to} \) in PFCs can indicate the existence of distinct mechanisms of regulation of ionic channel subunits expression in working cardiomyocytes and PFCs. Furthermore, the lack of an effect of Nkx2.5 haploinsufficiency on \( I_{to} \) density is consistent with the similar action potential duration observed in PFCs of Nkx2.5+/+/Cx40eGFP/+ and Nkx2.5+/−/Cx40eGFP/+ mice. Our data are strongly supportive of the view that conduction defects of Nkx2.5 mutant mice primarily results from severe defects in the patterning of the CS, and that the morphogenesis of the PF network may play a role in the conduction of the electrical activity through the ventricles. Our results are also consistent with recent data showing that conduction velocity is dependent on the architecture of the BB (van Veen et al., 2005).

**Perinatal development of the Purkinje fiber network is influenced by Nkx2.5 dosage**

It has been proposed that PF network develops from a pre-existing population of cells included in the trabecular layer of the embryonic heart (Moorman et al., 1998). In our model and based on anatomical criteria, we have found that trabeculation occurred normally during embryonic development in Nkx2.5+/− embryos. However, abnormal patterning of the PF network and a deficiency of eGFP-positive cells are detectable from birth in
Nkx2.5+/− hearts. These observations indicate that the deficiency of PF in Nkx2.5 haploinsufficient mice does not result from a failure of trabeculation or a lack of progenitor cells. The postnatal deficiency of PF in Nkx2.5 heterozygous mice suggests that a critical step for the development of the PF network take place around birth. The PF deficit in Nkx2.5 heterozygous mice may arise by either cell loss or impairment of proliferation and/or differentiation. No increased apoptosis or proliferation defects in eGFP-positive cells were detectable, while proliferation defects and misexpression of the EH–myomesin marker in eGFP-negative cells were observed. Together these results suggest that the loss of PF in mutant hearts was accounted for a defect in the differentiation of conductive cells. The EH–myomesin marker is normally expressed in all cardiomyocytes of the murine embryonic heart until the first week after birth, and then its expression is downregulated in working myocytes, while persisting in conductive myocytes. This is consistent with a postnatal regulation of the expression of this gene in cardiomyocytes. Interestingly, Nkx2.5 has been shown to be upregulated in the conduction system (Thomas et al., 2001), and more recently, in the avian system, precise regulation of Nkx2.5 levels has been found to be important for normal PF maturation during perinatal development (Harris et al., 2006). Moreover, Pashmforoush et al. (2004) have recently shown that conditional loss of Nkx2.5 in the ventricles induces cardiac hypertrophy characterized by hypertrabeculation in association with proliferation disturbance. It is noteworthy that we observed by SEM and on histological sections detached trabecular structures in the apex of Nkx2.5+/− recalling this hypertrabeculation. All these data strongly suggest that the postnatal differentiation of PF is Nkx2.5 dose-dependent.

The defect in the postnatal differentiation of PF may be explained by two hypotheses, either a defect in the differentiation of newly formed conductive myocytes, i.e. recruitment, or a defect in the maintenance of the PF phenotype associated with the loss of eGFP expression. From our results, we could not discriminate between these two hypotheses, however the information tends to favor the recruitment hypothesis. The number of PF evaluated by the number of eGFP-positive cells per transverse section is low in the medial and apical part of Nkx2.5+/− hearts, but remains relatively stable from birth to adult stage while this number increases in control hearts. This suggests that in the normal situation either more PF are recruited after birth or that PF proliferate. We could not detect proliferation defects in eGFP-positive PF suggesting that a new recruitment of PF occurs at birth. Nonetheless, we cannot exclude that eGFP expression is lost in a subset of cells at birth, and in the absence of expression of eGFP, we were not able to detect these cells. However, this phenomenon should be limited to a small population of cells since only a very small number of eGFP-negative myocytes continue to express the EH–myomesin marker, a marker possibly indicative of a more embryonic differentiation status. In the future, clonal analysis experiments will be undertaken to help us discriminate between these two hypotheses. Thus, Nkx2.5 plays a critical role in the postnatal differentiation of PF either by recruitment of newly conductive myocytes or in the maintenance of the conductive phenotype.

Our anatomical analyses of the PF network of Nkx2.5+/− mice indicate that a major remodeling of this network occurs during a short window after birth. The mechanisms by which the PF network is patterned are not known but our data suggest that this phenomenon is dependent on normal Nkx2.5 expression level. Other important parameters, such as changes in hemodynamic forces, may also contribute to the morphological changes observed in the heart after birth. Recently, it has been proposed that biomechanical forces acting on the cardiovascular system during embryogenesis play a crucial role in PF induction and patternning in the avian system (Hall et al., 2004; Reckova et al., 2003). However, our results do not address whether the effect of Nkx2.5 on the maturation of the PF network is direct or indirect, and extrinsic factors such as biomechanical forces may intervene in this maturation. Future work will investigate this point.

**Development of the Purkinje fiber network is cell-autonomously dependent on Nkx2.5 dosage: a two-wave model**

In the rescue experiments, two major parameters have been evaluated to explore the role of Nkx2.5 in the development of the PF, the pattern of the PF network and the number of eGFP-positive PF itself. Disturbances in the patternning of the PF network as seen by the disorganization of the elliptical structures of the fibers persist in chimeric hearts. This argues in favor of a non-cell autonomous role of Nkx2.5 in development of the network. However, we cannot exclude that this mispattering could occur as consequence of deficit within the PF cell itself. Indeed, the number of PF was partially rescued in the apical part of the ventricle and was dependent on the percentage of chimerism, but this partial rescue was not true for the patterning of the PF network, which still presents a deficit in elliptical structures. The partial rescue in the number of PF in the apical part of the chimeric heart can be explained by the disproportionate high numbers of PF of wild-type origin in comparison to the small percentage of PF of Nkx2.5 origin. Regarding the cellular composition of PF, we found a very high percentage of wild-type PF (85%) in comparison to mutant PF (15%) while the percentage of chimerism in the total of the myocytes was about 50%. This disproportionate number of mutant PF argues in favor of a cell-autonomous role of Nkx2.5 in the differentiation of PF. Moreover, analysis of P0–P3 chimeras shows that the proportion of eGFP-positive cells from both origins is equal at early stages (Fig. 8E). This clearly demonstrates that Nkx2.5 is cell-autonomously required in a postnatal stage for the differentiatiation of PF and suggests a non-cell autonomous role of Nkx2.5 in patterning the PF network. Altogether, these results lead us to propose a model in which the development and the formation of the peripheral PF network proceed in two waves. Consistent with a normal development of trabeculae in Nkx2.5 mutant embryos, we postulate that the establishment of a primary network of conductive myocytes occurs concurrently with trabeculation. At birth, a severe hypoplasia of PF is observed in Nkx2.5 haploinsufficient mice in association with a defect in conductive differentiation. This suggests that a second wave of differentiation of conductive
myocytes occurs culminating in the formation of the mature PF network (Fig. 8F). While the first wave of differentiation of conductive myocytes appears to occur normally in Nkx2.5 heterozygotic mice, the second wave of differentiation occurring by recruitment or maintenance of PF is Nkx2.5 dose-dependent.

In summary, we have shown that in addition to the hypoplasia of the central CS (AVN and AVB) (Jay et al., 2004; Pashmforoush et al., 2004) and PF hypocellularity (Jay et al., 2004), Nkx2.5 haploinsufficiency disturbs the development and the formation of the peripheral PF network, demonstrating a general role of this transcription factor in the development of the entire conduction system. Nkx2.5 mutations are a common cause of congenital heart diseases, and our new data may be important in understanding the mechanisms underlying cardiac malformations in humans.

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Appendix A. Supplementary data


References


