Nkx2.5 negative myocardium of the posterior heart field and its correlation with podoplanin expression in cells from the developing cardiac pacemaking and conduction system.
ABSTRACT

Recent advances in the study of cardiac development have shown the relevance of addition of myocardium to the primary myocardial heart tube. In wildtype mouse embryos (9.5-15.5 days post conception) we have studied the myocardium at the venous pole of the heart using immunohistochemistry and 3D reconstructions of expression patterns of MLC-2a, Nkx2.5 and podoplanin, a novel coelomic and myocardial marker. Podoplanin positive coelomic epithelium was continuous with adjacent podoplanin and MLC-2a positive myocardium that formed a conspicuous band along the left cardinal vein extending through the base of the atrial septum to the posterior myocardium of the atrioventricular canal, the atrioventricular nodal region and the His-Purkinje system. Later on podoplanin expression was also found in the myocardium surrounding the pulmonary vein. On the right side podoplanin positive cells were seen along the right cardinal vein, which during development persisted in the sinoatrial node and part of the venous valves. In the MLC-2a and podoplanin positive myocardium Nkx2.5 expression was absent in the sinoatrial node and the wall of the cardinal veins. There was a mosaic positivity in the wall of the common pulmonary vein and the atrioventricular conduction system as opposed to the overall Nkx2.5 expression seen in the chamber myocardium. We conclude that we have found podoplanin as a marker that links a novel Nkx2.5 negative sinus venosus myocardial area, which we refer to as the posterior heart field, with the cardiac conduction system.

INTRODUCTION

In early cardiac development the myocardium of the heart tube develops from two bilateral cardiogenic plates (primary heart field) that fuse to a common primary heart tube. The earlier observations by cell marker research in chicken embryos of De La Cruz that myocardium is added to this primary heart field, is now supported by several studies that in most cases refer to addition of myocardium at the outflow tract of the heart, being from the anterior or secondary heart field. Newly recruited myocardium is not only added at the outflow tract but also at the inflow tract. This myocardium is derived from the splanchnic mesoderm running from the arterial pole (outflow tract) to the venous pole (inflow tract) which is also referred to as second heart field, or second lineage. Recently a number of genes / proteins, considered as early markers of the second lineage, have been reported, such as fibroblast growth factor 8 and 10, Is11, inhibitor of differentiation Id2, GATA factors targeting Mef2c and Tbx1 and Tbx18.
Terminology in this rapidly evolving area of recruitment of new myocardium is still somewhat confusing as most cell differentiation markers and sometimes there lineage tracing have different spatio-temporal boundaries.

From 9.5 days post conception (dpc) onwards we have become particularly interested in recruitment of myocardium at the venous pole, which we refer to by the new positional term: posterior heart field (PHF), as an addition to the anterior heart field at the outflow tract. We have discovered that a novel gene in heart development, called podoplanin, not only demarcates a specific area of myocardium at the sinus venosus of the heart, but is also expressed in major parts of the cardiac conduction system (CCS). In the differentiation of the CCS a number of markers have already been reported that are expressed in the sinoatrial and atrioventricular conduction system such as HNK1 and Leu7,14-16 PSA-NCAM,17 Msx218 and the reporter genes CCS-LacZ,19-21 MinK,22 Tbx323 and cardiomyocytes – antigens.24 Very recently a Mesp-1 non-expressing myocardial population was reported in the ventricular conduction system.25 All these studies, however, concentrate on the differentiation of the CCS myocardium as opposed to the chamber myocardium and do not, as is suggested by our present findings, provide a link with the recruitment of second lineage myocardium.

Podoplanin is a 43-kd mucin type transmembrane glycoprotein,26 which has not been described during heart development. It was first called E11 antigen by Wetterwald as a new marker for an osteoblastic cell line. The protein is also found in other cell types including the nervous system, the epithelia of the lung, eye, oesophagus and intestine,27 the mesothelium of the visceral peritoneum26 and podocytes in the kidney.28 Furthermore, it has recently been investigated as a marker for lymphatic endothelium.29

Our study of podoplanin expression in the developing myocardium of the PHF is combined with a novel finding regarding Nkx2.5, which is an early marker of cardiac progenitor cells30 and demarcates the cardiac field31 in concert with GATA-4/5/6.32 Nkx2.5 is also shown to be essential for normal differentiation and function of the CCS in both human33 and mouse studies.34

In this study we will describe development of novel sinus venosus myocardium, in close correlation with the mesothelial lining of the pericardio-peritoneal coelomic cavity that is demarcated by positive podoplanin expression and Nkx2.5 non-expression. The podoplanin expression in the CCS provides a possible link between this novel myocardium from the PHF with the development of the sinoatrial node and other parts of the CCS.
MATERIAL AND METHODS

We studied the lining of the thoracic cavity and heart in wildtype mouse embryos of 9.5 dpc (n=8), 10.5 dpc (n=8), 11.5 dpc (n=7), 12.5 dpc (n=8), 13.5 dpc (n=8), 14.5 dpc (n=9) and 15.5 dpc (n=2). The embryos were fixed in 4% paraformaldehyde (PFA) and routinely processed for paraffin immunohistochemical investigation. The 5 μm transverse sections were mounted onto egg-white / glycerin coated glass slides in a 1 to 5 order, so that 5 different stainings from subsequent sections could be compared.

Immunohistochemistry

After rehydration of the slides, inhibition of endogenous peroxidase was performed with a solution of 0.3% H₂O₂ in PBS for 20 min. The slides were incubated overnight with the following primary antibodies: 1/2000 anti-atrial myosin light chain 2 (MLC-2a, which was kindly provided by S.W. Kubalak, Charleston, SC, USA), 1/4000 anti-human Nkx2.5 (Santa Cruz Biotechnology, Inc.,CA, USA) and 1/1000 anti-podoplanin (clone 8.1.1. Hybridomabank, Iowa, USA). All primary antibodies were dissolved in PBS-Tween-20 with 1% Bovine Serum Albumin (BSA, Sigma Aldrich, USA). Between subsequent incubation steps all slides were rinsed as follows: PBS (2x) and PBS-Tween-20 (1x). The slides were incubated with secondary antibodies for 40 min: for MLC-2a 1/200 goat-anti-rabbit-biotin (Vector Laboratories, USA, BA-1000) and 1/66 goat serum (Vector Laboratories, USA, S1000) in PBS-Tween-20; for Nkx2.5 1/200 horse-anti-goat-biotin (Vector Laboratories, USA, BA-9500) and 1/66 horse serum (Brunschiweg Chemie, Switzerland, S-2000) in PBS-Tween-20; for podoplanin 1/200 goat-anti-Syrian hamster-biotin (Jackson Immuno research, USA, 107-065-142) with 1/66 goat serum (Vector Laboratories, USA, S1000) in PBS-Tween-20. Subsequently, all slides were incubated with ABC-reagent (Vector Laboratories,USA, PK 6100) for 40 min. For visualisation, the slides were incubated with 400 μg/ml 3-3’-diaminobenzidin tetrahydrochloride (DAB, Sigma-Aldrich Chemie, USA, D5637) dissolved in Tris-maleate buffer pH 7.6 to which 20 μl H₂O₂ was added: MLC-2a 5 min; Nkx2.5 and podoplanin 10 min. Counterstaining was performed with 0.1% haematoxylin (Merck, Darmstadt, Germany) for 10 sec, followed by rinsing with tap water for 10 min. Finally, all slides were dehydrated and mounted with Entellan (Merck, Darmstadt, Germany).

3D reconstructions

We made 3D reconstructions of the atrial and ventricular myocardium of MLC-2a stained sections of 11.5 dpc and 13.5 dpc embryos in which podoplanin positive and Nkx2.5 negative myocardium from adjacent sections were manually superimposed to show overlapping areas. The reconstructions were made as described earlier²⁰ using the AMIRA software package (Template Graphics Software, San Diego, USA).
RESULTS

Below we will describe the expression patterns of MLC-2a, podoplanin and Nkx2.5 in the PHF in several subsequent stages of heart development (9.5-15.5 dpc), while in Figures 1-3 typical examples and 3D reconstructions of the expression patterns of these proteins are provided.

Stage 9.5 dpc
At this stage the heart is still in the looping phase and the boundaries of the primary heart tube can easily be demarcated by immunohistochemistry. The MLC-2a and Nkx2.5 staining of the myocardium stops at the transition with the negatively stained coelomic epithelium at the dorsal mesocardium. This squamous coelomic epithelium is part of the lining of the pericardio-peritoneal cavities, which are laterally flanked by the cardinal veins. Podoplanin is slightly positive at the left side and negative at the right side on the medial border of the cardinal veins wall. There is no podoplanin staining discernable at other sides at this stage yet.

Stages 10.5 dpc and 11.5 dpc
Serial MLC-2a stained sections have been reconstructed to form a 3D image. Figures 1a and 1b show the dorsal face of the heart in which the various staining patterns are depicted. The line in Figure 1a shows the level of the sections depicted in c-k. Septation of the ventricular inlet and atrium has started. On the right side the venous valves are already recognizable (Figure 1c). Podoplanin expression is observed in the coelomic lining and in the mesenchyme adjoining the medial wall of the left superior cardinal vein (Figure 1b and k) with light staining alongside the right superior cardinal vein at the position of the developing right sinoatrial node (Figure 1b, i and j). The left sided expression envelops the sinus venosus confluence of the cardinal veins (Figure 1b) and extends in the myocardium to the posterior region of the atrioventricular canal (Figure 1i and k), which is the site of the future atrioventricular node. The podoplanin positive mesenchyme is differentiating into myocardium as indicated by the overlapping expression with MLC-2a (compare Figure 1a and c-e with 1b and i-k). These overlapping areas are Nkx2.5 negative in contrast to the marked Nkx2.5 staining in the MLC-2a positive myocardium of the atria and the ventricles (Figure 1a and f-h).

Stages 12.5 dpc and 13.5 dpc
The 3D reconstruction of MLC-2a stained sections from an 13.5 dpc embryonic heart (dorsal face shown) are depicted in Figures 2a and 2b. The cardiac chambers are now clearly discernable. As expected the MLC-2a is more markedly expressed in the atrial and sinus venosus myocardium than in the ventricular myocardium (Figure 2c and e). The coelomic cavity is separated in pleural and pericardial cavities.
At the venous pole we now discern marked podoplanin expression in the myocardium of the developing right sided sinoatrial node and the patchy staining in the venous valves, while the adjoining atrial myocardium is podoplanin negative (Figure 2k and l). The sinoatrial nodal myocardium is still in close contact with the adjacent markedly podoplanin positive coelomic lining (Figure 2k and l). Bordering the left cardinal vein a similar podoplanin positive cell cluster is seen, as well as podoplanin positive myocardial strands running along the posterior left atrial wall that merge with the myocardial cells of the common pulmonary vein (Figure 2m and n). The continuity of these strands is obvious with patches of cuboidal podoplanin positive cells, as opposed to squamous podoplanin positive epithelial cells, lining both the pleural (Figure 2k-n) and pericardial cavity (Figure 2k-n). Both left and right sided podoplanin positive cell clusters as well as the myocardium of the wall of both cardinal veins are positive for MLC-2a although the staining is somewhat less intense compared to the main body of the atrial wall (Figure 2c-f).

The expression of Nkx2.5 (Figure 2a and g-j) does not overlap completely with the MLC-2a or the podoplanin staining. Nkx2.5 is negative in the right sinoatrial node, the posterior cell cluster between the left cardinal vein and the pulmonary vein and in the wall of the right and left cardinal veins (Figure 2g-j). A podoplanin and MLC-2a positive myocardial cell strand extends from the left side of the sinus venosus and stretches by way of the dorsal mesocardium and the spina vestibulum deep into the crux of the heart (Figure 2e, i and m). The staining encircles the orifice of the left cardinal vein, which opens into the right atrial cavity (not shown). This myocardial strand extends through the basis of the atrial septum to the position of the atrioventricular node and can be followed to the common bundle (Figure 2e, i and m), bundle branches (Figure 3a-d), the moderator band and the Purkinje system (not shown). Up to the level of the bundle branches this strand shows a mosaic Nkx2.5 staining which is

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**Figure 1.** Dorsal view of a reconstruction (a, b) of an 11.5 dpc wildtype mouse heart of the myocardium stained with MLC-2a (atria brown and ventricles grey). In (a) the Nkx2.5 negative pattern is added (lime green) and (b) shows the podoplanin positive pattern (turquoise). The left (LCV) and right (RCV) cardinal veins and their sinus venosus (SV) confluence are transparent blue. (c-e): Sections stained with MLC-2a (c: overview and details d: line box and e: dotted box) show marked expression in the myocardium of the wall of the atria (RA and LA). Also the anlage of the sinoatrial node (SAN) and a left sided mesenchymal population (asterisk in e) as well as the wall of the LCV show MLC-2a expression. (f-h): Staining in consecutive sections with Nkx2.5 (lime green in reconstruction (a) and overview in (f), with higher magnification in (g) and (h), show a marked expression in the atrial wall (g) and negativity in the mesenchyme (asterisk in h) and the SAN (g). There is no staining in the wall of the LCV. Podoplanin staining is positive in some parts of the coelomic cavities (arrows in h and k). This is not shown in the reconstruction (b) where only the overlap of MLC-2a and podoplanin (turquoise) is shown. Podoplanin is more intense at the left side at this stage of development (k) specifically in the pre-myocardial mesenchyme running from the left pericardioperitoneal canal, caudal of the anlage of the common pulmonary vein (PV; pink in a and b) through the base of the atrial septum to the posterior part of the atrioventricular canal dorsal of the inferior atrioventricular cushion (AVC) (i and k). Scale bars: (c-k) = 100μm.
therefore less marked than the surrounding myocardium (Figure 2i). A mosaic Nkx2.5 staining is also observed in the venous valves (not shown).

At stage 13.5 dpc the common pulmonary vein for the first time is clearly discernable with a myocardial sheath in which podoplanin positive cells are extending (Figure 2m and n).
MLC-2a and Nkx2.5 are positive in the pulmonary wall although both are less marked as compared to the adjacent atrial wall (Figure 2f and j). Between the left cardinal vein and the myocardial pulmonary venous wall a small cluster of podoplanin and MLC-2a positive and Nkx2.5 negative myocardial cells is still present (Figure 2f, j and n).

**Stages 14.5 dpc and 15.5 dpc**
The left sided podoplanin expression in the myocardium is disappearing. The staining is only retained in the right sinoatrial node and it has become more marked in the common and right and left bundle branches (Figure 3e and f).

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**Figure 2.** Dorsal view of a reconstruction (a, b) of an 13.5 dpc wildtype mouse heart of the myocardium stained with MLC-2a (atria brown and ventricles grey). The Nkx2.5 negative region is superimposed in (a), whereas the podoplanin positive region is presented in (b). The left (LCV) and right (RCV) cardinal veins which have an independent entrance into the right atrium are **transparent blue**. The transection (1) for the sinoatrial node (SAN) and the left sided podoplanin expression and pulmonary vein (PV in **pink**) (2) are indicated. (c-f): Sections stained with MLC-2a antibody (c and e: overviews at transsections 1 and 2 and magnifications d and f: boxed areas) show marked expression in the myocardium of the wall the atria (RA and LA) and somewhat lesser in the right (RV) and left (LV) ventricle. The LCV in (f), RCV in (d) and the SAN in (d) are positive. A cluster of moderately MLC-2a positive cells (**arrow** in f) is positioned in the mesenchyme between the LCV and PV. Nkx2.5 staining is markedly positive in all major components of the heart. Absence of staining (**lime green** in a) is seen in the wall of the RCV (h), the SAN (g and h), the LCV and the mesenchymal cell cluster (**arrow** in j). The PV has a less marked Nkx2.5 (mosaic) staining (j). The same accounts for a circular structure situated at the site of the common bundle at the top of the ventricular septum (VS) (dotted circle in e, i and m). Podoplanin staining is observed on both right and left sided MLC-2a areas (**turquoise** in b). This encomprises the SAN (k and l) and the left sided cluster between LCV, partly merging with the PV wall (**arrow** in n) and extending into the base of the atrial septum (AS). It is also positive in the common bundle (dotted circle in m) extending over the top of the VS (k). Podoplanin is also positive in the lining of the coelomic cavity. In areas with underlying podoplanin positive myocardial cells the coelomic cells are cuboid (**open arrow** in k-n). In the remaining coelomic lining, like the epicardium (**arrowhead** in n and o) the epithelium is squamous. The coelomic lining is always MLC-2a and Nkx2.5 negative. Scale bars: (c-n) = 100μm.
The contribution of myocardium to the primary heart tube has been acknowledged for many years by tracing cells with marker constructs\textsuperscript{3,35} as well as molecularly based tracing techniques using reporter mice.\textsuperscript{7,10-12,36} From these studies the addition of myocardium to, in particular the outflow tract, is obvious. Moreover, Kelly\textsuperscript{7} described the recruitment of cardiomyocytes from the splanchnic mesoderm to the outflow and inflow tract of the heart as a second myocardial lineage adding to the first lineage. The regulation of continued cardiogenesis at the inflow tract of the heart, which already starts at 8.5 dpc, is far from unravelled and has to fit in the multiple transcriptional domains of i.e. atrial chambers.\textsuperscript{37} This process will be complicated if

**DISCUSSION**

Figure 3. Reconstruction of the podoplanin expression (turquoise) depicting the various parts of the myocardium of the conduction system in the same 13.5 dpc embryo depicted in Figure 2a. Left frontal view shows the position of the sinoatrial node (SAN) next to the right cardinal vein (RCV), the presence in the right (RVV) and left (LVV) venous valves (b) merges in the region of the atrioventricular node (AVN) visible in the left lateral view. The expression is also found in a left atrioventricular ring of myocardium (LAVR). The AVN myocardium continues as a common bundle (CB) in the right (RBB) and left (LBB) bundle branches. (c-f). Sections of the thorax and heart of wildtype mouse embryos of 13.5 dpc (c with box magnified in d) and 15.5 dpc (e with box magnified in f) stained for podoplanin which is clearly visible in the common bundle (CB) in (c, d, e and dotted circle in f), as well as in the RBB and LBB (e and f) on top of the ventricular septum (VS). PV indicates Pulmonary vein; SS, Septum spurium. Scale bars: (c-f) = 100μm.
it is comparable with the situation at the outflow tract in which many genes are involved such as *Isll*, GATA factors targeting *Mef2c*, *Tbx1*, *Tbx4*, *Id2*, and many others including members of the fibroblast growth factor and TGF beta family. Our study adds podoplanin to this list for the PHF, which is most probably a subpopulation of the second lineage.

**Podoplanin and MLC-2a in the posterior heart field**

Podoplanin is expressed in several tissues in the developing embryo but for this study the reported expression in the coelomic lining, the underlying mesenchyme and the myocardium of the CCS is important. Expression in other tissues did not pose problems in interpretation as patterns are well separated in time and space. The coelomic epithelium was clearly activated at specific sites, being irregular and cuboidal which might indicate an ongoing process of epithelial-mesenchymal transformation (EMT). Similar EMT events have been described for the endocardium of the atrioventricular cushions as well as epicardium derived cells (EPDCs) expressing *Wt1;* and cytokeratin. As a podoplanin reporter mouse has not been developed we cannot unequivocally prove EMT. It is remarkable that the podoplanin expression is retained in the mesenchyme underlying the coelomic epithelium and that we have shown that we are dealing with a myocardial progenitor cell by the overlapping expression with MLC-2a. Although MLC-2a is described to be specific for atrial myocardium, it also stains the myocardium of the sinus venosus and somewhat weaker the ventricular cardiomyocytes. The contribution of novel myocardium to the PHF at the sinus venosus seems to stop after 15.5 dpc as the podoplanin expression diminishes and the coelomic epithelium becomes quiescent resuming a squamous phenotype.

A functional role for podoplanin is still to be found. Data are emerging describing an EMT process of podoplanin dependent downregulation of E-cadherin in invasive and migratory cells of oral mucosal cancer cells. Also an EMT independent process in adult tissues has been described, where podoplanin induces the reorganisation of ezrin-radixin-moesin (ERM) proteins and the actin cytoskeleton via downregulation of RhoA signal, resulting in collective tumor cell migration and conelike invasion. For our study it would support a possible role for podoplanin in migration and invasion of the PHF myocardium into parts of the CCS.

**Nkx2.5 expression and the posterior heart field**

As a marker for pre-cardiac mesoderm and myocardial cells we also used an antibody against human Nkx2.5. We found that the U-shaped PHF myocardium was negative for Nkx2.5. During development this resulted in a Nkx2.5 negative right sided sinoatrial node. In the podoplanin positive venous valves, the base of the atrial septum and the atrioventricular conduction system, there seemed to be a mosaic Nkx2.5 expression as opposed to the overall expression in the atrial and ventricular wall comparable to the heterogeneous pattern of Nkx2.5
expression pattern described previously. The myocardial contribution to the sinus venosus from precursors that are Nkx2.5 negative was also recently described.

The function of \( Nkx2.5 \) in cardiogenesis seems very important but is still far from clear. Different noggin-sensitive \( Nkx2.5 \) enhancers are found in various segments of the heart during development, indicative for chamber-specific functions, whereas cofactors such as GATA-4 are equally important. Furthermore, the differentiation of cardiac Purkinje fibers requires precise spatiotemporal regulation of \( Nkx2.5 \) expression, probably in a dose-dependent way. The mechanism of \( Nkx2.5 \) regulation is probably dependent on repressor systems for which strong candidates include Tbox family members, such as \( Tbx2 \) and \( Tbx5 \). Most studies have concentrated on \( Nkx2.5 \) in intracardiac patterning and differentiation. The implications of a population of \( Nkx2.5 \) negative myocardial cells in the PHF have to be evaluated further, while at least \( Tbx18 \) plays a role.

The posterior heart field and development of the cardiac conduction system

Several marker studies have linked sinus venosus myocardium to the development of the cardiac conduction system. These include HNK1 and Leu7, PSA-NCAM and more recently the transgenic reporter mice for CCS-\( \text{LacZ} \) and \( \text{Mink} \). Our own studies on HNK1 and Leu7 provide in general the same pattern for the CCS as now found in our study for podoplanin. The CCS-\( \text{LacZ} \) mouse shows that the complete cardiac conduction system myocardium is positive. CCS-\( \text{LacZ} \) does not differentiate between \( Nkx2.5 \) expressing and non-expressing myocardial cells as the right sinoatrial node is CCS-\( \text{LacZ} \) positive. Also other reported markers as \( Tbx3 \) do not reflect the described podoplanin positive PHF myocardium. In this respect the recent elegant reporter gene study of Mesp-1 expressing and non-expressing myocardial cells in the heart is of great interest. These authors show that there is a myocardial heterogeneity in the atrioventricular conduction system. They also show that this does not refer to a neural crest derived population. The latter origin was shown by our group to align with the CCS, although we never found the neural crest cells to attain a myocardial phenotype. The Mesp-1 study does speculate on the origin of the non-expressing Mesp-1 cells but has not traced them to the PHF. There are no data on their contribution to the sinoatrial and atrioventricular node.

In literature there are two main concepts for development of the CCS. The first one provides evidence for an autonomous origin of the central conduction system from cardiomyocytes residing in the primary heart tube. This myocardium retains a primitive phenotype after ballooning of the atrial and ventricular cavities has started. \( Tbx2 \) and \( Tbx3 \), and \( ANF \) are important genes guiding this process. In this concept the atrioventricular node derives from the primitive myocardium of the atrioventricular canal. The origin of the cells of the conduction
system and specifically the atrioventricular node is still under debate. It seems evident that part of the posterior atrioventricular node originates from the myocardium of the primary heart tube. Our current findings, supported by the Mesp-1 study do not exclude a contribution of myocardium from the PHF to the CCS, which is further strengthened by the extensive clonal cell tracing study of the Buckingham group.

The second concept on conduction system differentiation works along local differentiation pathways of the myocardium of the heart tube by induction and signaling. This concept is more in line with our data on secondary differentiation of the conduction system in which both EPDCs and neural crest cells might play the inductive role. It does not exclude secondary sources of myocardium, which in part correlate with migration pathways of EPDCs and neural crest cells.

**The posterior heart field and functional clinical implications**

Our data show an early and transient left sided counterpart of the sinoatrial node. In the early embryonic heart using voltage-sensitive dye, the pacemaking activity has initially been located to originate at the left side, which would fit with our observations. It also supports the reports on the anlage of a left sinoatrial node, which is found as an anomaly in left atrial isomerism. A possible role for podoplanin in the electrophysiology of CCS still has to be investigated. It has been reported, however, to be essential for water transport, dependence cell adhesiveness and cationic, anionic and amino acid transport. These aspects might be linked to cellular communications important for cardiac conduction.

Mutations of the *Nkx2.5* gene in human patients lead to conduction system disturbances and atrial septal defects. Comparable to these mutations in human patients is the *Nkx2.5* haploinsufficiency in mice embryos. The effects of *Nkx2.5* haploinsufficiency, described above, are weaker in mice but convergent with those in human. Our study provides a new insight in that *Nkx2.5* negative PHF myocardium is continuously added to the already *Nkx2.5* positive myocardium of the primary heart tube. We show that PHF myocardium forms the sinoatrial node, which is *Nkx2.5* negative. PHF myocardium might also add cells through the base of the atrial septum to the region of the atrioventricular conduction system and to the venous valves, which play a role in development of the conduction system as well as in the formation of the atrial septum. In this way atrial septal defects found in *Nkx2.5* human mutation patients may relate to a deficient contribution from the PHF myocardium to the venous valves. Most studies are dealing with *Nkx2.5* mutations with ensuing underexpression. In an overexpression study, which would influence the *Nkx2.5* negative sinoatrial node, defects in pacemaker activity with bradycardia have been described. In conclusion the temporo-spatial information in this study on the late contribution of *Nkx2.5* negative as well as positive myocardium might explain the cardiac abnormalities found in the human population.
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