Foxp1 regulates cardiac outflow tract, endocardial cushion morphogenesis and myocyte proliferation and maturation

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Summary

We have recently described a new subfamily of Fox genes, Foxp1/2/4, which are transcriptional repressors and are thought to regulate important aspects of development in several tissues, including the lung, brain, thymus and heart. Here, we show that *Foxp1* is expressed in the myocardium as well as the endocardium of the developing heart. To further explore the role of Foxp1 in cardiac development, we inactivated *Foxp1* through gene targeting in embryonic stem cells. *Foxp1* mutant embryos have severe defects in cardiac morphogenesis, including outflow tract septation and cushion defects, a thin ventricular myocardial compact zone caused by defects in myocyte maturation and proliferation, and lack of proper ventricular septation. These defects lead to embryonic death at E14.5 and are similar to those observed in other mouse models of congenital heart disease, including *Sox4* and *Nfatc1* null embryos. Interestingly, expression of *Sox4* in the outflow tract and cushions of *Foxp1* null embryos is significantly reduced, while remodeling of the cushions is disrupted, as demonstrated by reduced apoptosis and persistent *Nfatc1* expression in the cushion mesenchyme. Our results reveal a crucial role for *Foxp1* in three aspects of cardiac development: (1) outflow tract development and septation, (2) tissue remodeling events required for cardiac cushion development, and (3) myocardial maturation and proliferation.

Key words: *Foxp1*, Endocardial cushion, Outflow tract, Myocyte proliferation, Mouse

Introduction

Correct morphogenesis of the heart requires precise interactions between diverse cell types of different functional and embryonic origins. In particular, interactions between endocardium, myocardium and neural-crest-derived cells are essential for proper morphogenesis of cardiac structures such as the endocardial cushions that develop into the valves of the mature heart. Neural crest cells contribute to the mesenchyme of both the outflow tract cushions/valves as well as the smooth muscle component of the outflow tract vessels (reviewed by Brand, 2003). Valve formation is influenced by signals from the overlying endocardium. For example, *Nfatc1*, which is expressed in endocardium but not cushion mesenchyme, is essential for proper valve development (de la Pompa et al., 1998; Ranger et al., 1998). Alternatively, genes expressed in the cushion mesenchyme are also required for proper valve development. An example of this is the transcription factor *Sox4*. Lack of *Sox4* expression results in several defects in outflow tract and cushion development, including lack of proper outflow tract septation and defective cushion development (Schilham et al., 1996; Ya et al., 1998).

Interactions between the endocardium and myocardium are also thought to play an important role in the differentiation and maturation of cardiac myocytes. The endocardium lines the trabecular myocardium and is essential for guiding the differentiation of this tissue. The myocardium exhibits an increasing gradient of differentiation from the outer compact zone to the inner trabecular zone. The neuregulin signaling pathway, consisting of the *ErbB* receptors and neuregulin ligand, plays an important role in cardiac myocyte differentiation. Neuregulin is expressed in the endocardium, while the *ErbB* receptors are expressed primarily in myocardium (Carraway, 1996; Gassmann et al., 1995; Kramer et al., 1996; Lee et al., 1995; Meyer and Birchmeier, 1995). Both neuregulin and *ErbB4*-deficient embryos lack mature trabecular myocardium, leading to mid-gestation embryonic lethality (Gassmann et al., 1995; Kramer et al., 1996).

In the myocardium, recent evidence suggests that there are several key families of factors that regulate cardiac myocyte specific gene transcription and differentiation, including GATA, MEF, SRF and Nkx factors (reviewed by Brand, 2003). However, there is growing evidence that other transcription factor families also regulate cardiac morphogenesis, including members of the Fox gene family of winged-helix DNA-binding

domain transcription factors (Kume et al., 2001; Yamagishi et al., 2003).

Fox genes comprise a large family of genes that contain a homologous DNA-binding domain called either the forkhead or winged-helix DNA-binding domain (Kaestner et al., 2000). This DNA-binding domain binds to the consensus sequence 5′-TRTTKRY-3′ found in the promoters and enhancers of many genes (Costa et al., 2001). Recent evidence has demonstrated a role for Fox proteins in the regulation of cardiac development. *Foxc1* and *Foxc2* are expressed in vascular smooth muscle, endothelial cells of large blood vessels and the heart, and in head mesenchyme (Swiderski et al., 1999; Winnier et al., 1999). Inactivation of either *Foxc1* or *Foxc2* results in perinatal lethality due to cardiovascular defects, including coarctation of the aortic arch and ventricular septation defects (Winnier et al., 1999). Interestingly, compound heterozygous *Foxc1*/*c2* mutants also display similar cardiac defects and die perinatally, suggesting that *Foxc1* and *Foxc2* may play similar dose-dependent roles during cardiac development (Kume et al., 2001; Winnier et al., 1999). This is supported by a more dramatic cardiac phenotype in compound homozygous *Foxc1*/*c2* embryos, leading to embryonic demise at E9.5 (Kume et al., 2001). Finally, *Foxc1*/*c2* and *Foxa2* are thought to regulate expression of *Tbx1*, a gene implicated in DiGeorge's syndrome, a human genetic disease that causes severe cardiac developmental defects, including outflow tract and ventricular septation defects (Yamagishi et al., 2003).

We have recently reported the identification of a new subfamily of Fox genes, Foxp1/2/4. These genes are expressed in overlapping patterns in lung, neural, lymphoid and cardiac tissues (Lu et al., 2002; Shu et al., 2001; Wang et al., 2003). They have been implicated in regulating both lung and neural development and gene expression (Ferland et al., 2003; Lu et al., 2002; Shu et al., 2001; Wang et al., 2003). However, a role in cardiac development has not been reported for any Foxp family member. Here, we show that inactivation of *Foxp1* results in severe cardiac defects leading to embryonic death at E14.5. Ventricular and outflow tract septation, as well as valve formation, are defective in *Foxp1* null embryos. Furthermore, cardiac myocyte proliferation and maturation are defective in these embryos. These defects indicate a role for *Foxp1* in the regulation of cardiac myocyte maturation and proliferation as well as outflow tract and endocardial cushion development.

Materials and methods

Generation of *Foxp1* null mice

The targeting construct was generated in the pPNT vector containing neomycin resistance (neo) and herpes simplex virus thymidine kinase cassettes for positive and negative selection (Tybulewicz et al., 1991). Portions of the *Foxp1* gene were cloned from a mouse genomic library (129SvJ, Stratagene) using mouse *Foxp1A* cDNA (Wang et al., 2003) as probe. For the right arm, a 2.4 kb *Xho*I-*Sma*I genomic fragment 3′ to the forkhead exons was inserted between the *Xho*I and *Not*I sites of pPNT. For the left arm, a 3.3 kb *Kpn*I-*Pst*I fragment immediately 5′ to the forkhead exons was subcloned into the *Xba*I-*Kpn*I sites of the pPNT vector. Gene targeting deletes the forkhead domain of *Foxp1* and replaces it with the neomycin resistance gene.

SM1-129SVJ mouse embryonic stem (ES) cells were electroporated with the targeting vector, and correctly targeted clones that survived double selection in G418 and FIAU were identified by Southern blot analysis of genomic DNA. To screen for homologous recombination of the short arm, DNA from each clone was digested with *Pst*I, fractionated by electrophoresis through 0.8% agarose gels, transferred to Nitran+ (Amersham), and hybridized with a 700 bp *Pst*I-*Pst*I genomic fragment residing 3′ of the 2.4 kb *Xho*I-*Sma*I fragment (3′ arm). In wild-type ES cells, the *Pst*I fragment is 3.3 kb; in *Foxp1*$^{+/-}$ ES cells, the *Pst*I fragments are 3.3 kb (wild type) and 4.9 kb (mutant). For homologous recombination of the long arm (5′ arm, DNA was digested with *Xba*I and probed with a 1.2 kb *Xho*I-*Xho*I fragment 5′ to the *Kpn*I-*Pst*I fragment. In wild-type ES cells, the hybridized *Xba*I fragment is 7.5 kb; in *Foxp1*$^{+/-}$ ES cells, the hybridized *Xba*I fragments are 7.5 kb (wild type) and 11.5 kb (mutant) (data not shown).

Correctly targeted clones were injected into E3.5 C57BL/6 blastocysts, and the resulting chimeric males were mated to wild-type C57BL/6 females for germline transmission of the altered allele. For these studies, *Foxp1*$^{+/-}$ mice were backcrossed to C57BL/6 for at least four generations. Routine genotyping of wild-type and altered *Foxp1* alleles was done by PCR. The wild-type allele was identified by the production of a 430 bp PCR product when the primer pair 1 (5′-CCTCTGGCGATGAACCTAGTGGTTC-3′) and 2 (5′-AGCC-ACACTTTCTCTCAGGATGTCC-3′) was used. The altered *Foxp1* allele was identified by the production of a 280 bp PCR product when primer 1 was used with a primer in the neo cassette (5′-AGCGCATGCTCCAGACTGCCTTG-3′).

Histological procedures

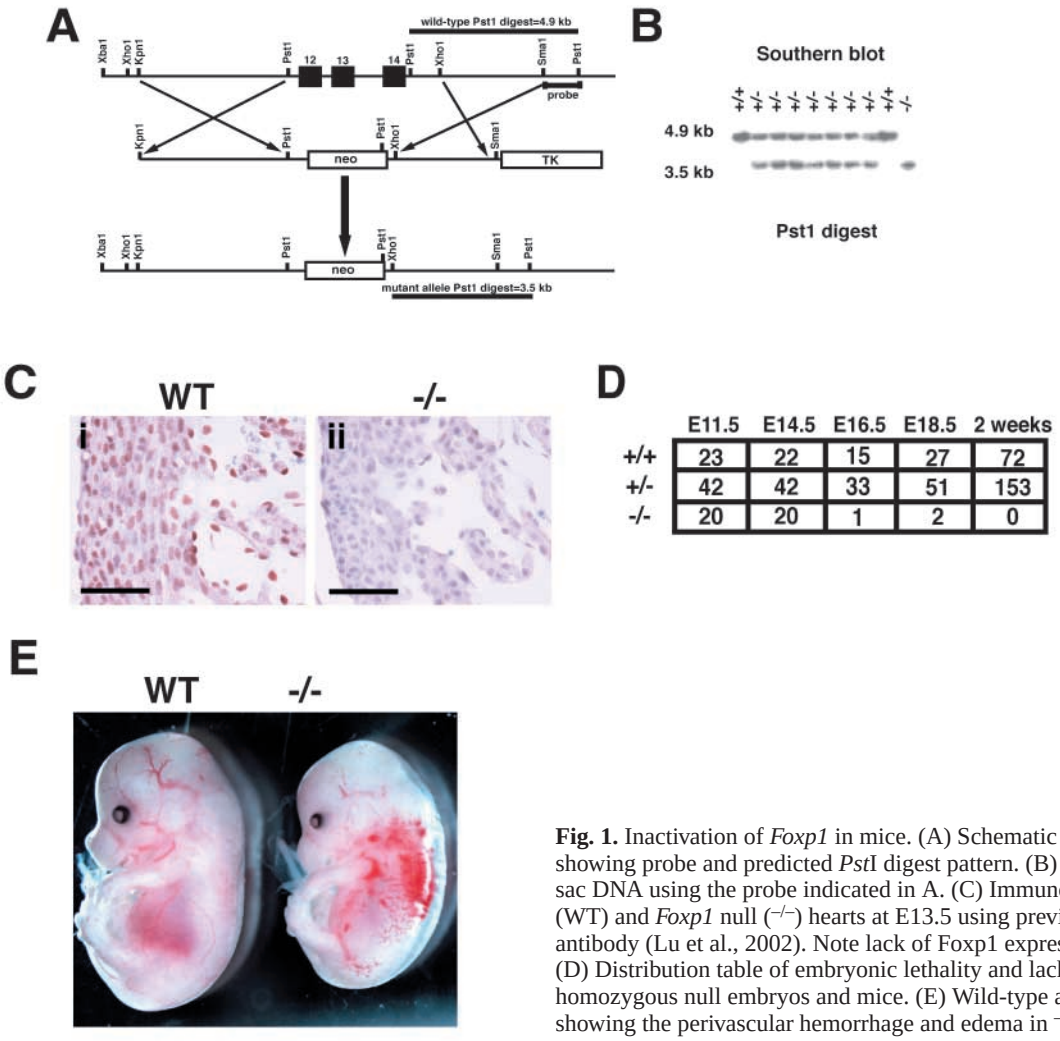
Embryos were collected at the days post conception as indicated and fixed in 4% paraformaldehyde for 24-48 hours. Embryos were then dehydrated through a series of ethanol solutions and were embedded in paraffin. In-situ hybridization, immunohistochemistry, and TUNEL staining were performed as previously described (Kuo et al., 1997; Shu et al., 2001). The *Anf* (*Nppa* – Mouse Genome Informatics) and *N-myc* in-situ probes have been previously described (Kuo et al., 1997; Sawai et al., 1993). The *Irx3* probe consisted of bp 196-711 of the published *Irx3* cDNA (Christoffels et al., 2000). The p21 (mouse monoclonal, 1:100), p27 (mouse monoclonal, 1:50), and p57 (mouse monoclonal, 1:100) antibodies are from Santa Cruz Biotechnologies and the phospho-histone H3 antibody (mouse monoclonal, 1:400) is from Cell Signaling Technologies. The fibronectin antibody (rabbit polyclonal, 1:100) is from Novus Biologicals. Electron microscopy studies were performed essentially as described (Kuo et al., 1997). Further details on histological procedures can be found at the University of Pennsylvania Molecular Cardiology Center website: http://www.uphs.upenn.edu/mcrc/.

To quantify the number of cells showing positive staining for TUNEL, PO4-histone H3, p21, and p27, three different fields of view for three embryos of each indicated genotype and age were viewed at 400X magnification. Positively stained cells were counted and the results are graphically shown ± standard error of the mean.

Results

Foxp1 null embryos die at E14.5 of cardiovascular failure

To determine the in-vivo role of *Foxp1* during development, we inactivated the mouse *Foxp1* gene using homologous recombination in ES cells. The targeting construct was designed to replace the three exons encoding the forkhead DNA-binding domain with the neomycin selection cassette (Fig. 1A). ES cells with homologous recombination of the targeting vector were identified using genomic Southern blotting with probes on the 5′ and 3′ side (Fig. 1A,B, and data not shown). Southern blotting and PCR confirmed germline transmission of the targeted allele (Fig. 1B and data not shown). RT-PCR demonstrated loss of *Foxp1* transcripts in null



	E11.5	E14.5	E16.5	E18.5	2 weeks
+/+	23	22	15	27	72
+/-	42	42	33	51	153
-/-	20	20	1	2	0

Fig. 1. Inactivation of *Foxp1* in mice. (A) Schematic of *Foxp1*-targeting construct showing probe and predicted *Pst*I digest pattern. (B) Southern blot analysis of yolk sac DNA using the probe indicated in A. (C) Immunohistochemistry of wild-type (WT) and *Foxp1* null ($^{-/-}$) hearts at E13.5 using previously characterized Foxp1 antibody (Lu et al., 2002). Note lack of Foxp1 expression in $^{-/-}$ heart tissue. (D) Distribution table of embryonic lethality and lack of postnatal survival in *Foxp1* homozygous null embryos and mice. (E) Wild-type and *Foxp1*$^{-/-}$ embryos at E14.5, showing the perivascular hemorrhage and edema in $^{-/-}$ embryos. Scale bars: 50 μm.

embryos (data not shown), and immunohistochemistry demonstrated lack of Foxp1 protein (Fig. 1C), indicating a functionally null allele.

Heterozygous *Foxp1* mice were born at the expected Mendelian ratio and appeared normal. However, no *Foxp1* homozygous null mice were recovered postnatally (Fig. 1D). This suggested that inactivation of *Foxp1* resulted in embryonic lethality. To determine the time of death, timed matings of *Foxp1* heterozygous mice were performed and embryos were collected at various stages of embryogenesis. These experiments determined that the vast majority of *Foxp1* null embryos died around E14.5 on the C57BL/6 background (Fig. 1D). Lethality at E14.5 was highly dependent on the mouse strain. Of the *Foxp1*$^{-/-}$ embryos on a C57BL/6 background, 92% died by E18.5, while 59% of mixed C57BL6/129SV background *Foxp1*$^{-/-}$ embryos died by E18.5 (Fig. 1D and data not shown). These data suggest that there are modifier genes that regulate the penetrance of the cardiovascular phenotype. The embryos used in these studies were from the fourth generation of outcrosses to C57BL/6 and are thus close to a pure C57BL/6 background.

Foxp1$^{-/-}$ embryos examined at E14.5 displayed several signs of cardiovascular failure, including edema and perivascular hemorrhage (Fig. 1E). The heart rate of E13.5 and E14.5 *Foxp1*$^{-/-}$ embryos was slower and more irregular than that of wild-type littermates (data not shown). To determine the cardiovascular defects responsible for the embryonic lethality of *Foxp1* null embryos, histological sections were generated from E11.5 and E14.5 wild-type and *Foxp1* null embryos (Fig. 2). H+E staining reveals several morphological abnormalities, including obvious ventricular septation defects (VSD) at E14.5 (Fig. 2F). Closer analysis revealed that the compact zone of the ventricular wall was thinner both at E11.5 and E14.5 (Fig. 2). Some areas of the compact zone of the myocardium were only two or three cells thick, suggesting severe defects in myocardial growth and/or differentiation.

Foxp1 is expressed in myocardium and endocardium

To determine precisely which cell types in the developing heart express *Foxp1*, we performed immunohistochemistry on embryonic heart sections using a previously characterized Foxp1-specific antibody (Lu et al., 2002). Staining of Foxp1 in the myocardium was observed from E9.5 through E14.5 (Fig. 3). Furthermore, Foxp1 myocardial expression was observed in a decreasing gradient from the compact zone to the

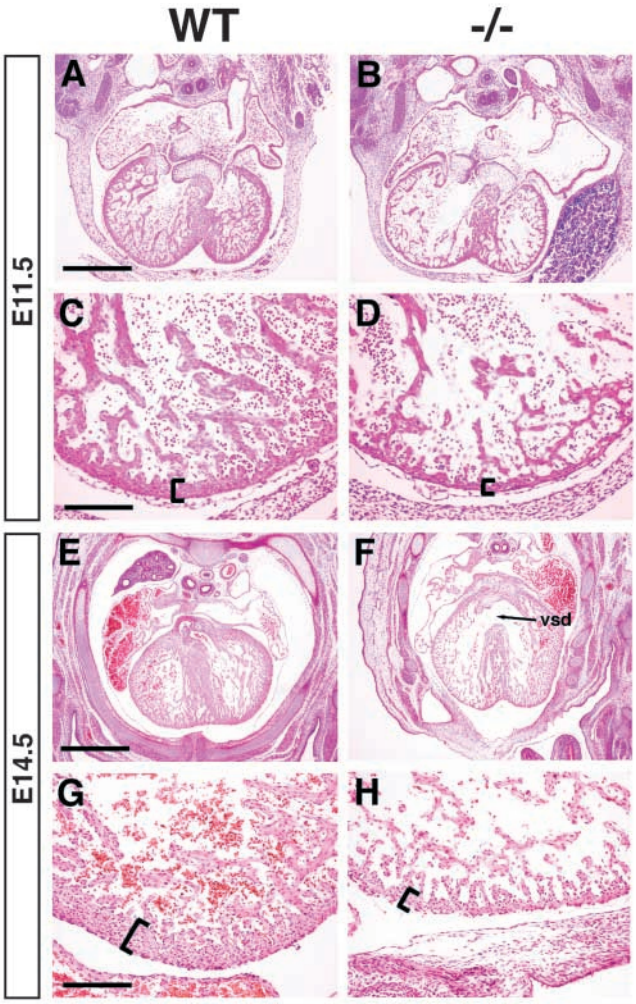


Fig. 2. Histology of *Foxp1*$^{-/-}$ hearts at E11.5 and E14.5. H+E staining was performed on wild-type (A,C,E,G) and *Foxp1*$^{-/-}$ (B,D,F,H) embryos to examine cardiac morphology at E11.5 (A-D) and E14.5 (E-H). *Foxp1*$^{-/-}$ hearts have a thin myocardial compact zone (compare C and D,G,H, brackets). *Foxp1*$^{-/-}$ hearts also show ventricular septation defects (vsd) (F). Scale bars: 400 μm in A,B; 200 μm in C,D,G,H; 800 μm in E,F.

trabecular zone (Fig. 3D-F,H). Expression of Foxp1 was observed in the endocardium and other endothelial cells throughout development (Fig. 3D-H) (Lu et al., 2002). In particular, *Foxp1* expression was prominent in the endocardium overlying the endocardial cushions and valves of the heart at E11.5 and later in development (Fig. 3C,G). Foxp1 expression was observed in endothelium and vascular smooth muscle of the outflow tract and abdominal aorta (Fig. 3I,J). Expression of Foxp1 was observed in cells underlying the cushion mesenchyme at E11.5 (Fig. 3C), but later in development, expression was extinguished in this region. Expression remained high, however, in the overlying endocardium (Fig. 3G).

Foxp1 null mice have defects in outflow tract septation

Previous studies have demonstrated a crucial role for certain endocardial-expressed genes such as *Nfatc1* and *Foxc1*/*c2* in regulating outflow tract development (de la Pompa et al., 1998; Kume et al., 2001; Winnier et al., 1999). This is thought to occur through endocardial–myocardial and endocardial–mesenchymal signaling mechanisms that are not well understood. Since *Foxp1* is expressed at high levels in the endothelium and vascular smooth muscle (VSMC) of the outflow tract (Fig. 3I,J), we sought to determine whether *Foxp1* null embryos exhibited defects in this region of the developing heart. During gestation, the pulmonary artery and aorta septate such that they become distinct vessels arising from the right and left ventricles, respectively (Fig. 4A-C,G-I). In 80% of *Foxp1*$^{-/-}$ embryos, the aorta and pulmonary trunk did not septate properly, leading to persistent truncus arteriosis (PTA), which forms a common trunk vessel originating from the right ventricle (Fig. 4D-F). In addition to PTA, 20% (4/20) of E14.5 *Foxp1*$^{-/-}$ embryos exhibited double outlet right ventricle (DORV), as marked by both the pulmonary trunk and aorta arising from the right ventricle (Fig. 4J-L). These data demonstrate a critical role for *Foxp1* in outflow tract septation and development.

Defects in valve formation in *Foxp1*$^{-/-}$ embryos

Since *Foxp1* is expressed at high levels in the cells underlying the endocardial cushion mesenchyme early in development and in the overlying endocardium throughout development, we sought to determine whether there were defects in valve formation in *Foxp1* null embryos. Histological analysis showed that there was significantly more cushion mesenchyme in the outflow and atrial-ventricular valves of *Foxp1*$^{-/-}$ embryos than in wild-type littermates at E14.5, indicating a lack of proper mesenchymal regression (Fig. 5A-D). The defects in the pulmonary and aortic valves were 100% penetrant, while defects in the mitral and tricuspid valves were observed in 45% (9/20) of *Foxp1*$^{-/-}$ embryos. Valve formation requires a remodeling of the endocardial cushions, which involves epithelial–mesenchymal transformation of endocardial cells and subsequent regression of the cushion mesenchyme by apoptosis to form the thin valve leaflets (Keyes and Sanders, 2002; Lakkis and Epstein, 1998). Thus, the increased size of the cushion mesenchyme could be due to either decreased apoptosis or increased cell proliferation. TUNEL assays reveal that there was an approximately 70% decrease in apoptosis in *Foxp1*$^{-/-}$ outflow tract cushions at E14.5 but not at E11.5 (Fig. 5E,F,M). Immunohistochemistry using a phospho-histone-H3 (PO4-H3) antibody detected no change in cell proliferation in outflow tract cushion mesenchyme at E14.5 or E11.5 (Fig. 5G,H,N and data not shown). These data suggest that decreased apoptosis contributes to the increased cellularity observed in the cushion mesenchyme of the outflow tracts of *Foxp1*$^{-/-}$ hearts at E14.5. In the atrial-ventricular cushions, however, little difference in cell proliferation or apoptosis was observed between *Foxp1*$^{-/-}$ and their wild-type littermates (data not shown). Thus, there are probably additional mechanisms responsible for defects in valve formation in *Foxp1*$^{-/-}$ hearts.

Nfatc1 is required for proper valve formation in mice and is expressed exclusively in the endocardium overlying the developing endocardial cushions (de la Pompa et al., 1998). Expression is extinguished as endothelial–mesenchymal transformation (EMT) occurs in these cells (de la Pompa et al., 1998). To determine whether *Nfatc1* was appropriately

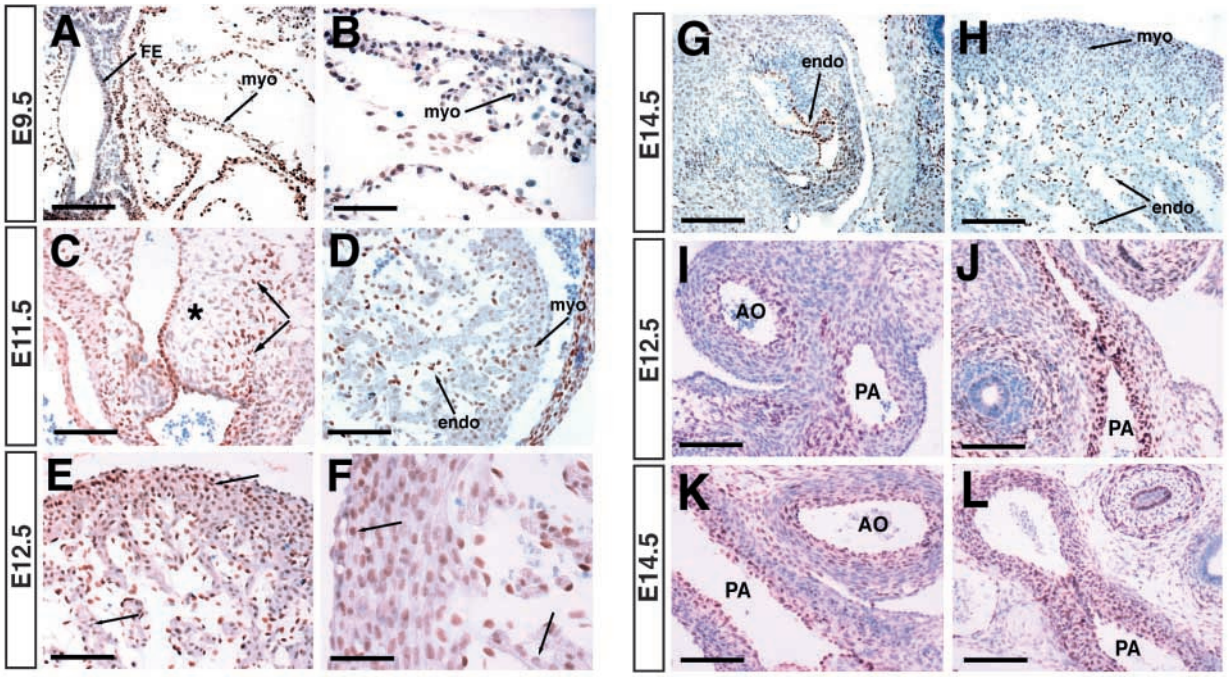


Fig. 3. Expression of Foxp1 protein during cardiac development. A Foxp1-specific antibody (Lu et al., 2002) was used to determine Foxp1 protein expression at E9.5 (A,B), E11.5 (C,D), E12.5 (E,F,I,J), and E14.5 (G,H,K,L) in the heart and outflow tract vessels. Foxp1 expression is observed in the ventral aspect of the foregut endoderm and the myocardium (A,B, arrows). Foxp1 expression is observed throughout the heart, including myocardium, endocardium and endocardial cushion tissue at E11.5 (C,D). Foxp1 is expressed in cells underlying the mesenchyme of the forming endocardial cushion (C, arrows). Foxp1 is expressed in a decreasing gradient from the compact zone to the trabecular zone as early as E11.5 through E14.5 (D-F,H; note arrows in E,F and H showing decreased expression in trabecular myocardium). By E14.5, expression in endocardial cushion mesenchyme is extinguished but remains high in the overlying endocardial cells (G, arrow). Foxp1 expression is observed in the endothelium of the aorta and pulmonary artery at E12.5 (I) and E14.5 (K). Foxp1 expression is also observed in the VSMCs of these vessels at E12.5 and E14.5 (J,L). The wall of the ductus arteriosus can be observed in J,L. FE, foregut endoderm; myo, myocardium; endo, endocardium; Ao, aorta; Pa, pulmonary artery. Scale bars: 200 μm in A,G,H; 100 μm in B,C-E,I-K; 50 μm in F, 150 μm in L.

expressed in *Foxp1*$^{-/-}$ embryos, immunohistochemistry was performed with an Nfatc1 antibody. Both wild-type and *Foxp1*$^{-/-}$ embryos expressed Nfatc1 in the endocardium overlying the endocardial cushions (Fig. 5I-L). Interestingly, at E14.5, persistent Nfatc1-expressing cells were consistently observed in the mesenchyme of the endocardial cushions in *Foxp1*$^{-/-}$ embryos, while these were never seen in wild-type cushions (Fig. 5J,L). This phenomenon was not observed at E11.5 (data not shown). Since *Foxp1* is not expressed in cushion mesenchyme at E14.5 (Fig. 3), the ectopic presence of Nfatc1-expressing cells in the cushion mesenchyme of *Foxp1*$^{-/-}$ embryos may suggest a defect in the EMT process required for endocardial cushion remodeling. However, since decreased levels of apoptosis and persistent *Nfatc1* expression were not observed at E11.5, these defects could be secondary to others in tissue remodeling and gene expression, which in turn, perturbs the EMT process.

Sox4 expression is significantly reduced in *Foxp1* null hearts

In addition to *Nfatc1*, several other genes expressed in either mesenchyme or endocardium are known regulators of endocardial cushion and outflow tract septation development. *Sox4* and fibronectin are expressed primarily in the cushion and valve mesenchyme with lower expression in the overlying endocardium, while *Foxc1* and *Foxc2* are expressed primarily in the overlying endocardium (Bouchey et al., 1996; Hiltgen et al., 1996; Kume et al., 2001; Winnier et al., 1999; Ya et al., 1998). To determine whether their expression was altered in *Foxp1*$^{-/-}$ hearts, we carried out either in-situ hybridization (for *Sox4*, *Foxc1* and *Foxc2*) or immunohistochemistry for fibronectin expression (Fig. 6). A significant reduction in *Sox4* expression was observed in the cushions and myocardium of the outflow tract at both E11.5 and E14.5 (Fig. 6A-E). Expression of fibronectin, *Foxc1* or *Foxc2* was not effected in either outflow or atrial-ventricular regions in *Foxp1*$^{-/-}$ hearts (Fig. 6F-K and data not shown). Together, these data suggest that *Foxp1* may reside upstream of *Sox4* in the same regulatory pathway in cardiac cushion development. This is supported by the similarities between the cardiac phenotype in *Sox4* and *Foxp1* mutant mice, including outflow septation defects, cushion development defects and DORV (Ya et al., 1998).

Increased proliferation in *Foxp1* null myocardium

In *Foxp1* null hearts, thinning of the compact zone suggested that cardiac myocyte proliferation and/or maturation was disrupted. In normal cardiac development after E12.5, the compact zone of the ventricular myocardium continues to exhibit significant cell proliferation while the trabecular zone, which is considered more mature, shows little to no cell

proliferation (Fig. 7A) (Moorman and Christoffels, 2003; Sedmera et al., 2000). In *Foxp1*$^{-/-}$ hearts, however, PO4-H3 antibody staining revealed a threefold increase in cell proliferation in the trabecular zone, while compact zone proliferation was unaffected (Fig. 7A,B,I).

Immunohistochemistry was performed to determine whether the cell cycle was perturbed at the level of cyclin kinase inhibition in *Foxp1*$^{-/-}$ hearts. Interestingly, p21 was upregulated by almost fourfold in the trabecular zone but was unchanged in the compact zone of *Foxp1*$^{-/-}$ myocardium (Fig. 7C,D,J). By contrast, p27 was downregulated in the compact zone by almost 50% but was unchanged in the trabecular zone in *Foxp1*$^{-/-}$ myocardium (Fig. 7E,F,K). p57, which is expressed primarily in the endocardium, remained unchanged (Fig. 7G,H). These data suggest that *Foxp1* regulates the cardiac myocyte cell cycle in a complex manner; increasing the expression of some cyclin dependent kinase inhibitors and decreasing expression of others.

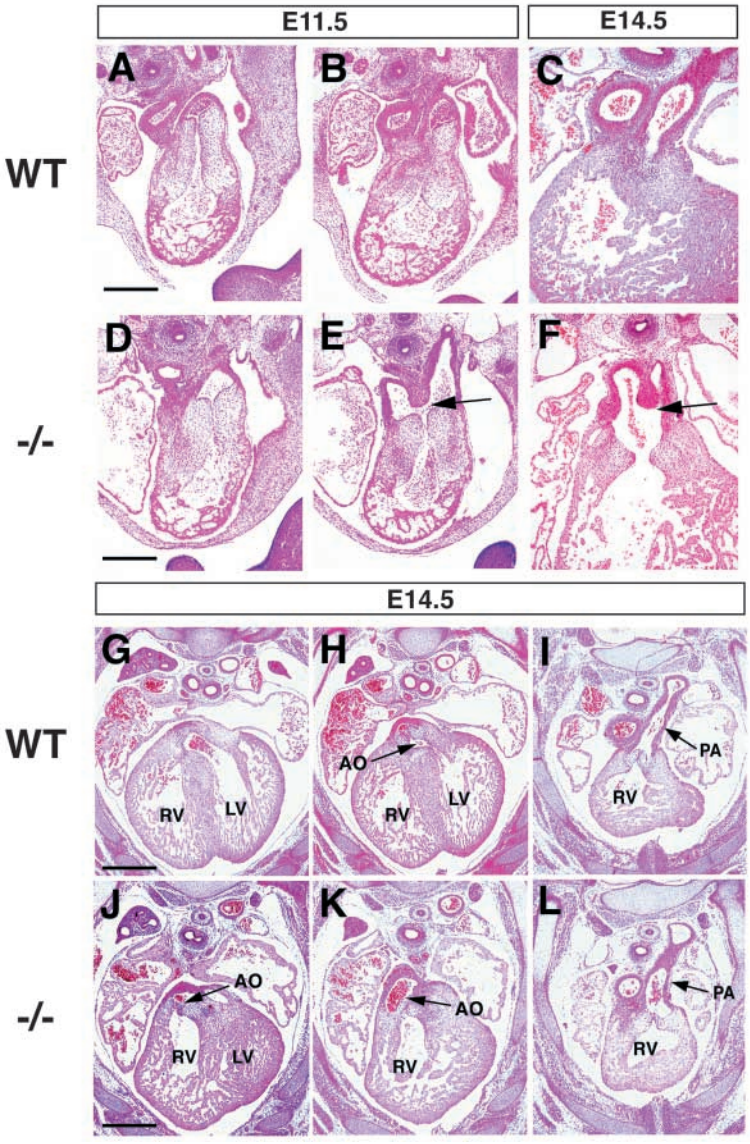


Fig. 4. *Foxp1*$^{-/-}$ embryos have severe defects in outflow tract development. H+E staining of embryonic sections of wild-type (A-C,G-I) and *Foxp1*$^{-/-}$ (D-F,J-L) were performed to characterize defects in outflow tract development. *Foxp1* null embryos show PTA at E11.5 (E, arrow) and at E14.5 (F, arrow). Of *Foxp1* null embryos, 20% have DORV, with both aorta and pulmonary artery arising from the right ventricle (J-L), whereas wild-type littermates exhibit the correct septation of these vessels, with the aorta arising from the left ventricle and the pulmonary artery arising from the right ventricle (G-I). RV, right ventricle; LV, left ventricle; AO, aorta; PA, pulmonary artery. Scale bars: 400 μm in A-F; 600 μm in G-L.

Defective myocardial maturation in *Foxp1* null hearts

During heart development, myocytes mature to form both compact and trabecular layers within the ventricular wall (reviewed by Mikawa and Fischman, 1996). This maturation process results in changes in cardiac-specific gene expression. In early cardiac development, *Irx3*, an iroquois homoeobox transcription factor, and atrial naturetic factor (*Anf*; *Nppa* – Mouse Genome Informatics) are expressed exclusively in trabecular zone myocytes, while *N-myc* is expressed exclusively in the compact zone of the developing heart (Charron et al., 1992; Christoffels et al., 2000; Sawai et al., 1993). To determine whether expression of these genes was altered in *Foxp1* null hearts, we performed in-situ hybridization analysis. Expression of *Irx3* was expanded in *Foxp1* null hearts at E14.5 to encompass both the trabecular and compact zone (Fig. 8A,B). By contrast, *Anf* expression remained unchanged (Fig. 8C,D). *N-myc* expression was significantly reduced in the ventricular walls of *Foxp1* null hearts at E11.5 (Fig. 8E,F) and E14.5 (Fig. 8G,H), which correlates with a thinner compact zone.

Transmisssion electron microscopy was performed to further analyze myocardial maturation in *Foxp1*$^{-/-}$ hearts. In normal hearts at E11.5, cardiac myocytes form a laminated array of similarly shaped cells in the compact zone with obvious aligned myofibers, while myocytes in the trabecular zone appear more irregular and epithelial in shape (Fig. 8I,K). In *Foxp1*$^{-/-}$ hearts, there was little or no organization of myocytes in the compact zone (Fig. 8J,L). Poor organization of myocytes in *Foxp1* null hearts is unlikely to be secondary to heart failure, as these experiments were performed 3 days before the time of embryonic death. These data support the histological and gene expression findings and suggest that cardiac myocyte maturation and proliferation in *Foxp1* null hearts are disrupted, leading to thinning of the ventricular wall, hemodynamic failure, and embryonic death.

Discussion

Regulation of outflow tract and endocardial cushion development by *Foxp1*

Our data demonstrate a critical role for *Foxp1* in regulating multiple aspects of cardiac morphogenesis. Inactivation of *Foxp1* leads to defects in outflow tract septation, endocardial cushion development, cardiac myocyte proliferation and maturation, and ventricular septation. Combined, these defects lead to mid-gestation embryonic death in *Foxp1*$^{-/-}$ embryos. The defects observed in *Foxp1* null embryos encompass a

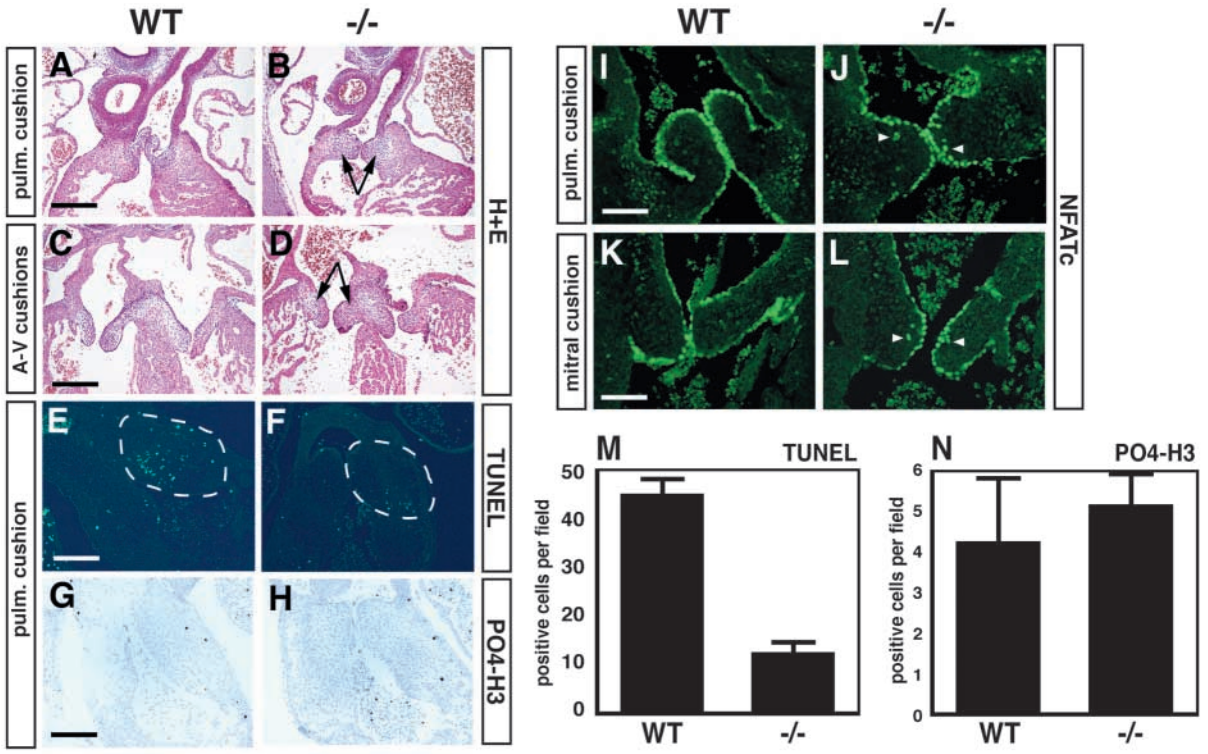


Fig. 5. Defects in endocardial cushion and valve formation in *Foxp1*$^{-/-}$ hearts. H+E staining of embryonic sections of wild-type (A,C) and *Foxp1*$^{-/-}$ (B,D) were performed to characterize defects in endocardial cushion and valve development at E14.5. By E14.5, endocardial cushion mesenchyme in wild-type heart valves has already begun to regress to form the mature pulmonary (A) and atrial-ventricular heart valves (C). In *Foxp1*$^{-/-}$ hearts, the cushion mesenchyme has not regressed as far and the cushions appear as large bulges (B,D, arrows). At E11.5, TUNEL assays reveal decreased levels of apoptosis in the outflow tract endocardial cushion mesenchyme in *Foxp1*$^{-/-}$ hearts (E) relative to wild type (F). At E11.5, cell proliferation as determined by phospho-histone H3 immunostaining is unchanged in *Foxp1* outflow tract cushions (G,H). Nfatc1 expression (J,L, arrowheads) in *Foxp1*$^{-/-}$ hearts persists in the endocardial cushion mesenchyme in both pulmonary (J) and mitral cushions (L) compared with wild-type pulmonary (I) and mitral (K) cushions at E14.5. Quantification of both TUNEL-positive cells (M) and phospho-histone H3-positive cells (N) for data shown in E-H. E,G and F,H are adjacent histological sections. Scale bars: 400 μm in A-D; 200 μm in E-L.

variety of outflow tract defects observed in human congenital heart disease, including PTA and DORV (Conway et al., 2003; Dees and Baldwin, 2002). Our data suggest that *Foxp1* is an important and previously unrecognized factor in the molecular pathway(s) disrupted in human congenital heart abnormalities.

Formation of the outflow tract of the developing heart is regulated by a complex series of morphogenetic events. These events are required for proper septation and alignment of the aorta and pulmonary artery such that the aorta emerges from the left ventricle and the pulmonary artery emerges from the right ventricle. Disruptions in this process can lead to a constellation of congenital defects in humans, such as PTA, DORV, and transposition of the great vessels (Conway et al., 2003; Dees and Baldwin, 2002). However, how this process is regulated at the molecular level is only recently being unraveled and these defects may represent a spectrum of phenotypes regulated by common mechanisms. Neural crest cells are thought to play an important role in the development of the outflow tract of the heart. Several transcription factors and signaling molecules expressed in neural crest and endocardium have been implicated in regulating endocardial cushion and outflow tract development. Endocardial cells migrate into and populate the cushion mesenchyme, losing expression of many endocardially specific genes, such as *Nfatc1* (Brand, 2003). Targeted inactivation of *Nfatc1* results in defective outflow tract cushion and vessel development, which leads to embryonic death (de la Pompa et al., 1998). However, *Nfatc1* expression persists in the mesenchyme of *Foxp1* null endocardial cushions at E14.5. This could be due to defects in EMT or subsequent tissue remodeling events involving apoptosis, which may eliminate these cells through programmed cell death. The lack of defects in apoptosis and Nfatc1 expression earlier in development (i.e. E11.5) in *Foxp1*$^{-/-}$ hearts when EMT is actively occurring is supportive of a tissue remodeling event rather than a direct effect on endocardial cushion EMT.

Targeted inactivation of *Foxc1*, *Foxc2* and *Sox4* also leads to embryonic lethality due to defects in cardiac cushion formation and outflow tract septation (Kume et al., 2001; Schilham et al., 1996; Winnier et al., 1999). *Sox4* is normally expressed in both cushion mesenchyme and the overlying endothelial cells in the outflow tract region (Maschhoff et al., 2003; Schilham et al., 1996; Ya et al., 1998). We observed that *Sox*4, but not these other factors, is downregulated in *Foxp1*$^{-/-}$ hearts. This result suggests that *Foxp1* may reside upstream of *Sox4* in a molecular pathway regulating cardiac cushion and outflow tract development. The phenotype in *Sox4* null embryos bears striking similarities to the cardiac defects in *Foxp1* null

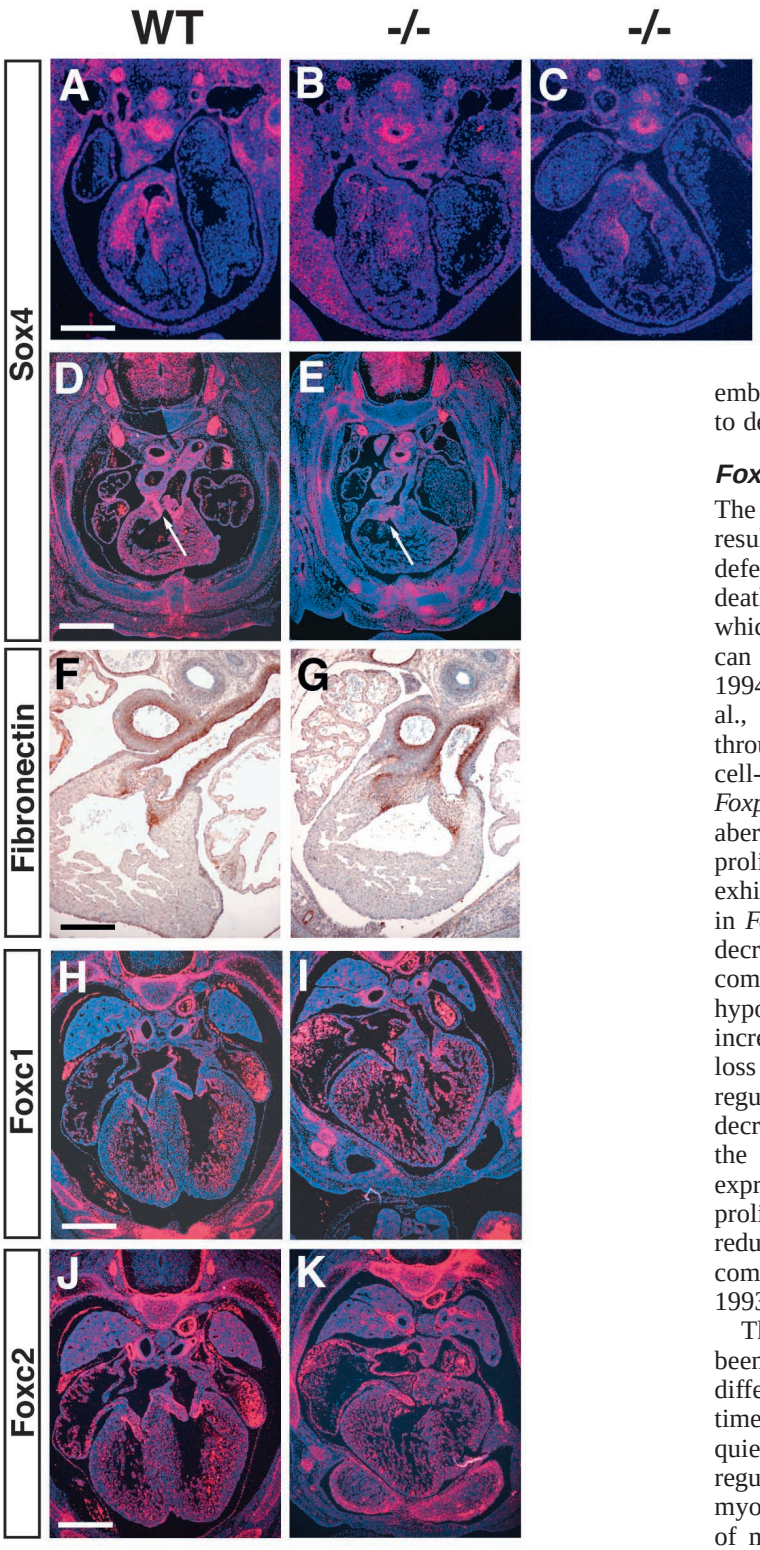


Fig. 6. *Sox4* is downregulated in *Foxp1* null hearts. In-situ hybridization for *Sox4* (A-E), *Foxc1* (H,I) and *Foxc2* (J,K) and immunohistochemistry for fibronectin (F,G) was performed on wild-type (A,D,F,H,J) and *Foxp1*$^{-/-}$ (B,C,E,G,I,K) E11.5 (A-C) and E14.5 (D-K) embryo sections. *Sox4* gene expression is significantly decreased in the outflow tract region of *Foxp1*$^{-/-}$ hearts at both E11.5 and E14.5 (A-C), while *Foxc1*, *Foxc2* and fibronectin expression is unchanged. Scale bars: 800 μm in D,E,H-K; 400 μm in A-C,F,G.

embryos, including a similar embryonic stage of lethality, PTA, presence of VSD, defective outflow tract endocardial cushions development marked by increased cellularity within the cushion mesenchyme, and double outlet-right ventricle in a subset of embryos (Ya et al., 1998). Since *Foxp1* is expressed in cells underlying the cushion mesenchyme only in early development, direct regulation of *Sox4* by *Foxp1* in this tissue would have to occur early. Alternatively, *Foxp1* may regulate *Sox4* gene expression in the overlying endocardium. The reduction in *Sox4* expression could also be a secondary result of defective EMT or other tissue remodeling processes in the endocardial cushions of *Foxp1*$^{-/-}$ embryos. Tissue-specific inactivation of *Foxp1* will be required to determine which cell type confers these cushion defects.

Foxp1 and cardiomyocyte proliferation

The increased ratio of trabecular to compact zone myocardium resulting in a thin ventricular wall probably contributes to defects in ventricular hemodynamics that lead to embryonic death in *Foxp1*$^{-/-}$ embryos. Thinning of the ventricular wall, which is seen in several mouse models with cardiac defects, can be attributed to non-cell autonomous effects (Chen et al., 1994; Schilham et al., 1996; Svensson et al., 2000; Tevosian et al., 2000). However, the high level of *Foxp1* expression throughout the myocardium early in development suggests a cell-autonomous cause for the thin ventricular compact zone in *Foxp1*$^{-/-}$ embryos. Cell proliferation in *Foxp1*$^{-/-}$ hearts is aberrantly regulated as demonstrated by the increase in cell proliferation in the trabecular zone, a region which normally exhibits little proliferation. However, trabecular myocardium in *Foxp1*$^{-/-}$ embryos also exhibited increased p21 levels and decreased p27 levels, suggesting that cell cycle regulation is compromised in a complex manner in *Foxp1* null hearts. One hypothesis is that p21 levels are upregulated in response to increased proliferation and thus its elevation is secondary to loss of *Foxp1* expression. Alternatively, *Foxp1* may positively regulate p21 expression, but this is insufficient to overcome decreased p27 levels, which, along with other disruptions in the cell cycle, lead to increased proliferation. Interestingly, expression of *N-myc*, which is known to positively regulate cell proliferation, was reduced in *Foxp1* null hearts, although this reduction could be secondary to an overall reduction in compact zone development (Charron et al., 1992; Moens et al., 1993).

The regulation of cell proliferation in cardiac myocytes has been the subject of much study. As with other terminally differentiated cells, cardiac myocytes proliferate for a short time in utero and only briefly postnatally before becoming quiescent. However, the exact cell cycle machinery involved in regulating embryonic proliferation and keeping mature cardiac myocytes quiescent is not well defined. Research into this area of myocyte biology is important for future development of therapies involving myocyte replacement through either activation of resident stem cells or transplantation of stem cells from an external source. Our data showing that loss of *Foxp1* results in increased cell proliferation suggests that *Foxp1* may regulate an important step in this process. In light of these findings, it is interesting to note that *Foxp1* has been implicated as a tumor suppressor gene (Banham et al., 2001). Decreased expression of *Foxp1* is observed in the majority of colon and

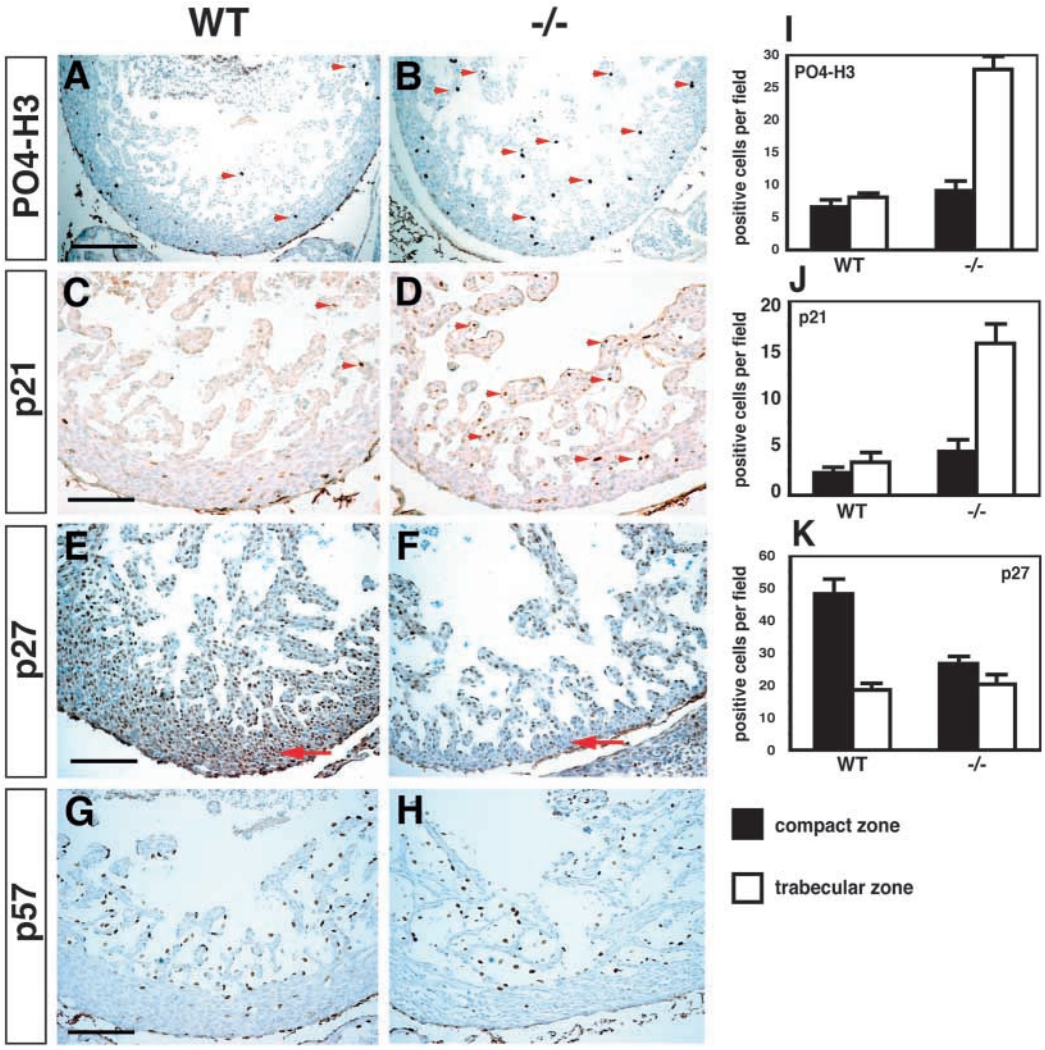


Fig. 7. Cell proliferation and expression of cyclin-dependent kinase inhibitors in *Foxp1*$^{-/-}$ hearts. Immunohistochemistry was performed on wild-type (A,C,E,G) and *Foxp1*$^{-/-}$ (B,D,F,H) hearts at E13.5 using antibodies for phospho-histone H3 (A,B), p21 (C,D), p27 (E,F), and p57 (G,H). Significant increases in phospho-histone H3 (B, red arrowheads) and p21 staining (D, red arrowheads) were observed, while a decrease in p27 expression was observed in the compact zone of *Foxp1*$^{-/-}$ hearts (F, red arrow). Quantifying the number of phospho-histone H3 positive cells in the trabecular zone for wild-type and *Foxp1*$^{-/-}$ hearts shows an approximately threefold increase in the number of mitotic cells in the trabecular zone of *Foxp1*$^{-/-}$ hearts but no change in the compact zone (I). Identical analysis of p21 expression levels reveals an approximate fourfold increase in trabecular myocardium but no significant difference in compact myocardium (J). p27 levels were decreased by almost 50% in *Foxp1*$^{-/-}$ compact myocardium, while levels in trabecular myocardium were unchanged (K). Scale bar: 200 μm.

stomach tumors tested (Banham et al., 2001). Thus, *Foxp1* may regulate specific aspects of cell proliferation required for normal organogenesis that, when disrupted, lead to defective development or tumorigenesis.

Foxp1 and cardiomyocyte maturation

In conjunction with increased myocardial proliferation, cardiomyocyte maturation in *Foxp1*$^{-/-}$ hearts is disrupted, as shown through expanded expression of *Irx3*, a transcription factor normally expressed only in trabecular myocardium (Christoffels et al., 2000) and through the disorganized appearance of compact zone myocardium, where myocytes lack their normal laminated organization. The disruption in *Irx3* expression was specific, since expression of *Anf*, another gene expressed exclusively in trabecular myocardium in early development (Brand, 2003), was unchanged. However, appropriate myofiber assembly was apparent in *Foxp1* null embryo hearts when viewed by transmission electron microscopy (data not shown). The reduced level of *N-myc* expression also supports the hypothesis that compact zone development is disrupted in *Foxp1* null hearts. Although these defects could be secondary to other defects in outflow tract or cushion development, *Foxp1* is expressed in the myocardium in a pattern that strongly suggests a direct effect on myocyte development. Taken together, our data suggest that inactivation of *Foxp1* locks myocytes in a proliferative state where they do not mature properly, leading to increased proliferation and defective maturation in mutant hearts.

Trabecular myocardium is considered to be more

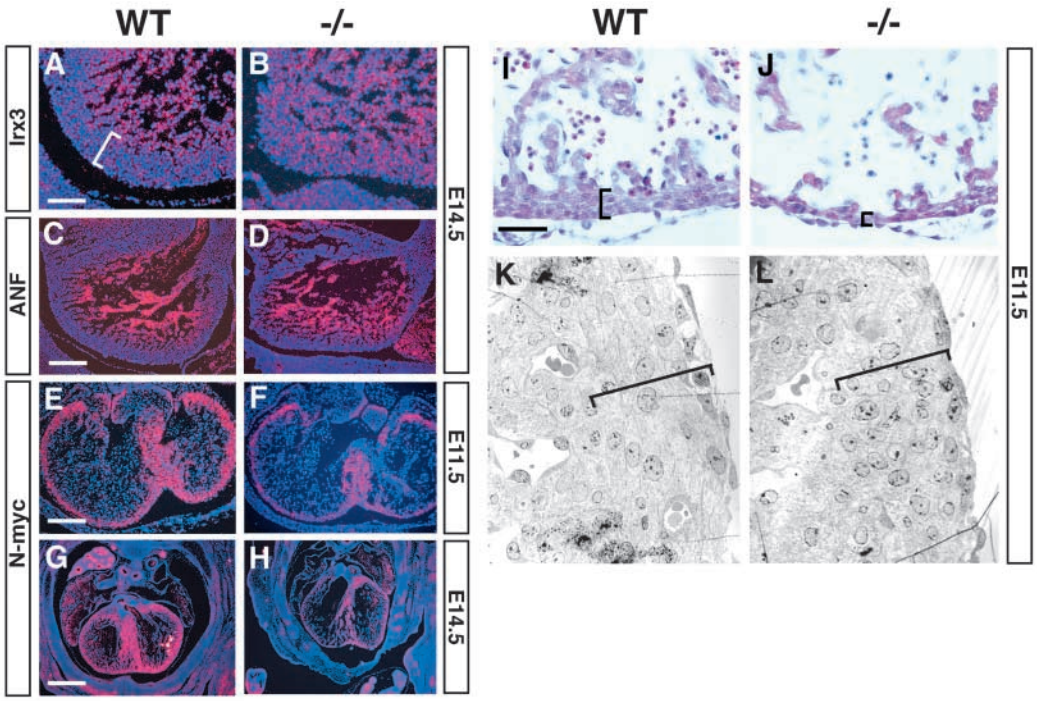


Fig. 8. Myocardial differentiation is disrupted in *Foxp1*$^{-/-}$ hearts. In-situ hybridization was performed using probes specific for *Irx3* (A,B), *Anf* (C,D) and *N-myc* (E-H) on wild-type (A,C,E,G) and *Foxp1*$^{-/-}$ (B,D,F,H) hearts at E11.5 (E,F) and E14.5 (A-D,G,H). *Irx3* expression is expanded to encompass almost all compact and trabecular myocardium (B). *N-myc* expression was significantly reduced in the ventricular wall of *Foxp1*$^{-/-}$ embryos (compare E,F,G,H). H+E staining of wild-type and *Foxp1*$^{-/-}$ hearts at E11.5 shows a thin compact zone in the myocardial wall of the ventricle wall of *Foxp1*$^{-/-}$ hearts (I,J, brackets). Transmission electron microscopy was performed on E11.5 wild-type (K) and *Foxp1*$^{-/-}$ (L) hearts. Wild-type compact zone myocardium shows a laminated appearance, while *Foxp1*$^{-/-}$ heart compact zone myocardium has a disorganized appearance (compare bracketed regions). Scale bars: 250 μm in A,B; 400 μm in C-F; 800 μm in G,H; 100 μm in I,J.

differentiated and mature than compact zone myocardium, and this maturation is essential for proper heart development (reviewed by Mikawa and Fischman, 1996; Sedmera et al., 2000). Current models using retroviral tagging of primitive cardiac myocytes suggest a single myocardial precusor that gives rise to both compact and trabecular myocytes (Mikawa and Fischman, 1996; Ong et al., 1998). However, the molecular pathways controlling this process are not well defined. Defects in the neuregulin signaling pathway result in lack of cardiac trabecular formation, leading to early embryonic death (Carraway, 1996; Gassmann et al., 1995; Kramer et al., 1996; Lee et al., 1995; Meyer and Birchmeier, 1995). Since neuregulin is expressed exclusively in the endocardium and the *ErbB* receptors are expressed in the myocardium, these data indicate an essential role for endocardial–myocardial signaling in trabecular myocyte differentiation. Expression of neuregulin is normal in *Foxp1*$^{-/-}$ hearts (data not shown), suggesting that *Foxp1* may play an important role in regulating endocardial–myocardial interactions in a pathway distinct from neuregulin signaling.

The cardiovascular abnormalities observed in *Foxp1* null embryos are similar to those seen in multiple forms of congenital heart disease in humans (Conway et al., 2003; Dees and Baldwin, 2002). Some of these are caused by mutations in transcription factors such as *Tbx1*, *Foxc1* and *GATA4*. However, the genes causing many congenital heart defects remain unknown. The data presented in this report suggest that *Foxp1* may be added to the growing list of candidate genes that cause congenital heart disease in humans.

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