Foxp1 regulates cardiac outflow tract and endocardial cushion morphogenesis and myocyte proliferation and differentiation

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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 ES 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 differentiation 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. Remarkably, expression of Sox4 in the outflow tract and cushions of Foxp1 null embryos is significantly reduced while NFATc1 expression persists as endocardial cells migrate and transform into cushion mesenchyme. Our results reveal a crucial role for Foxp1 in three aspects of cardiac development: 1) outflow tract development and septation, 2) the endothelial-mesenchymal transformation required for cardiac cushion development, and 3) myocardial differentiation and proliferation.

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 which 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 in (Brand, 2003). Valve formation is influenced by signals from the overlying endocardium. For example, NFATc, 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 differentiation (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 midgestation 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 in (Brand, 2003). These factors are expressed in the myocardium and DNA binding sites for these factors are found in almost all of the cardiac specific promoters analyzed to date, including those for MHC, ANF, MLCv and cTNC. 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 proteins 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 perinataly, 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 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 differentiation are defective in these embryos. These defects indicate a role for Foxp1 in the regulation of both cardiac myocyte differentiation and proliferation as well as outflow tract and endocardial cushion development.

MATERIELS 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 XhoI-SmaI genomic fragment 3' to the forkhead exons was inserted between the XhoI and NotI sites of pPNT. For the left arm, a 3.3 kb KpnI-PstI fragment immediately 5' to the forkhead exons was subcloned into the XbaI-KpnI 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 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 PstI, fractionated by electrophoresis through 0.8% agarose gels, transferred to Nitran+ (Amersham), and hybridized with a 700 bp PstI-PstI genomic fragment residing 3' of the 2.4 kb XhoI-SmaI fragment (3' arm). In wild-type ES cells, the PstI fragment is 3.3 kb; in Foxp1 +/- ES cells, the PstI 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 XbaI and probed with a 1.2 kb XhoI-XhoI fragment 5' to the KpnI-PstI fragment. In wild-type ES cells, the hybridized XbaI fragment is 7.5 kb; in Foxp1 +/- ES cells, the hybridized XbaI fragments are 7.5 kb (wild-type) and 11.5 kb (mutant) (data not shown).

Correctly targeted clones were injected into day 3.5 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 4 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'-AGCCACACTTTCTCTCAGGATGTCC-3') were 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 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. EM 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 web site: http://www.uphs.upenn.edu/mcrc/.

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 embryonic stem 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 and 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 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. 92% of Foxp1 -/- embryos on a C57BL/6 background 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 rates of E13.5 and E14.5 Foxp1 -/- embryos was slower and more irregular than 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 one or two cells thick, suggesting severe defects in myocardial growth and/or differentiation.

Foxp1 is expressed in myocardium and endocardium

To precisely determine 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 trabecular zone (Fig. 3F). High expression of Foxp1 in the endocardium was

observed throughout development (Fig. 3D-F and data not shown). In particular, Foxp1 expression was prominent in the endocardium overlying the endocardial cushions and valves of the heart at E12.5 and later in development (Fig. 3C, E, and G). Initially, expression of Foxp1 was observed in cushion mesenchyme at E11.5 (Fig. 3C), but later in development, expression was extinguished in mesenchyme and remained high in the overlying endocardium (Fig. 3E and G).

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 endocardium of the outflow tract, 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 Foxp1 -/- embryos, the aorta and pulmonary trunk did not septate properly leading to a double outlet right ventricle (DORV), which forms a common trunk vessel (Fig. 4D-F). In addition to DORV, 20% (4 out of 20) of E14.5 Foxp1 -/- embryos exhibited complete transposition of the great vessels as marked by the pulmonary trunk arising from the left ventricle and the aorta arising from the right ventricle (Fig. 4G-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 in 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 litter-mates 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 of 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 a significant decrease in apoptosis in Foxp1 -/- outflow tract cushions (Fig. 5E and F). Immunohistochemistry using a phospho-histone-H3 (PO4-H3) antibody detected no change in cell proliferation in outflow tract cushion mesenchyme (Fig. 5G and H and data not shown). These data suggest that decreased apoptosis is a major contributor to the increased cellularity observed in the cushion mesenchyme of the outflow tracts of Foxp1 -/- hearts. In the atrial-ventricular cushions, however, little difference in cellproliferation or apoptosis was observed between Foxp1 -

/- and their wild-type littermates (data not shown). Thus, there are additional mechanisms likely responsible for defects in these valves 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 occurs in these cells (de la Pompa et al., 1998). To determine whether NFATc1 was appropriately expressed in Foxp1 -/- embryos, immunohistochemistry was performed with a NFATc1 antibody. Both wild-type and Foxp1 -/- embryos expressed NFATc1 in the endocardium overlying the endocardial cushions (Fig. 5I-L). Interestingly, 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 and L). Since Foxp1 is not expressed in cushion mesenchyme at this time point (Fig. 3), the ectopic presence of NFATc1 expressing cells in the cushion mesenchyme of Foxp1 -/- embryos indicates a defect in the endothelial-mesenchymal transformation process required for endocardial cushion remodeling.

Sox4 expression is significantly reduced in Foxp1 null hearts

In addition to NFATc, 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). Expression of fibronectin, Foxc1, or Foxc2 was not effected in either outflow or atrial-ventricular regions (Fig. 6C, D, E, F, G, H and data not shown). In contrast, a significant reduction in Sox4 expression was observed in the cushions and myocardium of the outflow tract (Fig. 6A and B). Together, these data suggest that Foxp1 may reside upstream of Sox4 in the same regulatory pathway in cardiac cushion development.

Increased proliferation in Foxp1 null myocardium

In Foxp1 null hearts, thinning of the compact zone suggested that cardiac myocyte proliferation and/or differentiation was disrupted. In normal cardiac development after E12.5, the compact zone of the ventricular myocardium exhibits significant cell proliferation while the trabecular zone, which is considered more differentiated 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 3-fold increase in cell proliferation in the trabecular zone (Fig. 7B and 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 consistently upregulated while p27 was down-regulated in Foxp1 -/- myocardium (Fig. 7C-F). p57, which is expressed primarily in the endocardium, remained unchanged (Fig. 7G and H). These data suggest that Foxp1 regulates cardiac myocyte cell cycle in a

complex manner; increasing the expression of some cyclin dependent kinase inhibitors and decreasing expression of others.

Defective myocardial differentiation in Foxp1 null hearts

During normal heart development, myocytes differentiate and migrate from the compact zone to the trabecular zone with subsequent changes in cardiac specific gene expression. In early cardiac development, Irx3, an iroquois homoeobox transcription factor, and atrial naturetic factor (ANF) 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 and B). In contrast, ANF expression remained unchanged (Fig. 8C and D). N-myc expression was significantly reduced in the ventricular walls of Foxp1 null hearts at E11.5 (Fig. 8E and F) and E14.5 (Fig. 8G and H). Together, these data indicate defects in compact zone-trabecular zone myocyte differentiation in Foxp1 -/- hearts.

Transmisssion electron microscopy was performed to further analyze myocardial differentiation 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 in shape (Fig. 8I and K). In Foxp1 -/- hearts, there was little or no organization of myocytes in the compact zone (Fig. 8J and L). Poor organization of myocytes in Foxp1 null hearts is

unlikely to be secondary to heart failure as these experiments were performed three days before the time of embryonic death. These data support the histological and gene expression findings and suggest that cardiac myocyte differentiation 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 differentiation, and ventricular septation. Combined, these defects lead to midgestation embryonic death in Foxp1 -/- embryos. The defects observed in Foxp1 null embryos encompass a variety of outflow tract defects observed in human congenital heart disease including DORV and transposition of the great vessels (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 multiple congenital defects in humans such as truncus arteriosus, double outlet-right ventricle, 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. Neural crest cells are thought to pay an important role in the development of the outflow tract of the heart. These cells migrate in from the dorsal neural tube and populate the smooth muscle component of the outflow tract vessels as well as the cardiac cushions in the outflow tract, which are the precursors of the mature aortic and pulmonary outflow valves. 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, loosing 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 cushions and its persistence there mau perturbsuggesting perturbation in the normal endothelial-mesenchymal transformation process in cushion development. This hypothesis is supported by our finding of decreased apoptosis in the developing cushions, an event required for regression of cushion mesenchyme to form mature valve leaflets. Endothelial-specific loss of the neurofibromatosis type 1 gene ($NF1^{endo}$ -/-) results in a similar constellation of defects as observed in Foxp1 null hearts including outflow tract cushion defects and thin myocardial compact zone (Gitler et al., 2003). NFATc1 expression was also perturbed in $NF1^{endo}$ -/- embryos but instead of persistent expression in the cushion mesenchyme, increased levels of nuclear expression were observed prematurely in the endocardium overlying the cushions (Gitler et al., 2003). Both Foxp1 and $NF1^{endo}$ null mouse models could lead to increased NFATc1,

resulting in endothelial-mesenchymal transformation defects and valve malformation. Alternatively, persistent NFATc1 expression may be secondary to other as yet uncharacterized defects in cushion development in Foxp1 null hearts. Further experiments will be required to resolve this issue.

Targeted inactivation of Foxc1, Foxc2, and Sox4 also lead 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 Sox4, but not these other factors, is down-regulated in Foxp1 -/- hearts. This result indicate that Foxp1 resides 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 embryos including a similar embryonic stage of lethality, septation defects in the outflow tract, and lack of proper cushion regression to form the outflow tract valves. Since Foxp1 is expressed in cushion mesenchyme only in early development, direct regulation of Sox4 by Foxp1 in this tissue would have to occur early. Alternatively, the reduction in Sox4 expression may be a secondary result of defective endocardial-mesenchymal transformation in the endocardial cushions caused by loss of Foxp1 in the overlying endocardium. Tissue specific inactivation of Foxp1 will be required to determine which cell type confers these cushion defects. Together with persistent expression of NFATc in cushion mesenchyme, our data implicate Foxp1 in the regulation of endothelial-mesenchymal transformation involved in

cardiac cushion development and suggest that Foxp1 lies upstream of Sox4 in a pathway regulating this process.

Foxp1 and cardiomyocyte proliferation

The increased ratio of trabecular to compact zone myocardium resulting in a thin ventricular wall likely 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 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 up-regulated in response to increased proliferation and thus its elevation is secondary to loss of Foxp1 expression. Alternatively, Foxp1 may, indeed, positively regulate p21 expression but this is insufficient to overcome decreased p27 levels, which, along with other disruptions in the cell cycle machinery, lead to increased proliferation. Interestingly, expression of N-myc, which is known to positively regulate cell proliferation, was reduced in Foxp1 null hearts (Charron et al., 1992; Moens et al., 1993). Thus, the increased ratio of trabecular to compact zone in Foxp1 -/- hearts indicates a

defect in the coupled proliferation-differentiation process of cardiac myocytes during normal development.

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 postnataly 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. Futhermore, the complexity with which the cell cycle is disrupted in Foxp1 null cardiomyocytes illustrates the numerous and likely redundant mechanisms underlying cell cycle progression in these cells. 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 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 differentiation

In conjunction with increased myocardial proliferation, cardiomyocyte differentiation in Foxp1 -/- myocardium 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 the myocardium, where myocytes lack their normal laminated organization but instead appear irregular in shape. 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). Trabecular myocardium is considered to be more differentiated than compact zone myocardium and the differentiation of compact to trabecular myocardium is essential for proper heart development but the molecular pathways controlling this process are not well defined (Sedmera et al., 2000). 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, although further experimentation will be required to verify these findings.

The cardiovascular abnormalities observed in Foxp1 null embryos are similar to those seen in multiple forms 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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FIGURE LEGENDS

Figure 1. Inactivation of Foxp1 in mice. (A) Schematic of Foxp1 targeting construct showing probe and predicted Pst 1 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.

Figure 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 and H-brackets). Foxp1 -/- hearts also show ventricular septation defects (F). Scale bars: A and B = 400 μm; C, D, G, H = 200 μm; E and F = 800 μm.

Figure 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 and B), E11.5 (C and D), and E14.5 (E-H) in the heart and outflow tract vessels. Foxp1 expression is observed in the ventral aspect of the foregut endoderm and the myocardium (A and B, arrows). Foxp1 expression is observed throughout the heart including myocardium, endocardium and endocardial cushion tissue at E11.5 (C and D). By E14.5, expression in endocardial cushion mesenchyme is extinguished but remains high in the overlying endocardial cells (E-arrow). Foxp1 expression decreases in the trabecular zone of the heart by E14.5 but is still observed in the compact zone and the endocardium (F). Foxp1 expression is observed in vascular smooth muscle of the major

arteries such as the aorta and in the smooth muscle and epithelium of the esophagus (G and H). FE-foregut endoderm, myo-myocardium, endo-endocardium, Ao-aorta, Eso-esophagus. Scale bars: C and G = 400 μm; B and D = 100 μm; A, E, F, H = 200 μm.

Figure 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 double outlet-right ventricle (DORV) at E11.5 (E, arrow) and at E14.5 (F, arrow). Some Foxp1 null embryos have a complete transposition of the aorta and pulmonary arteries resulting in the aorta arising from the left ventricle instead of the right (L, arrow) while the pulmonary trunk arises from the right ventricle (J and K, arrows) instead of the left ventricle as in wild-type embryos (H, arrow). RV-right ventricle, LV-left ventricle. Scale bars: A-F = 400 μm; G-L = 800 μm.

Figure 5. Defects in endocardial cushion and valve formation in Foxp1 -/- hearts. H+E staining of embryonic sections of wild-type (A and C) and Foxp1 -/- (B and 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 and D). 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

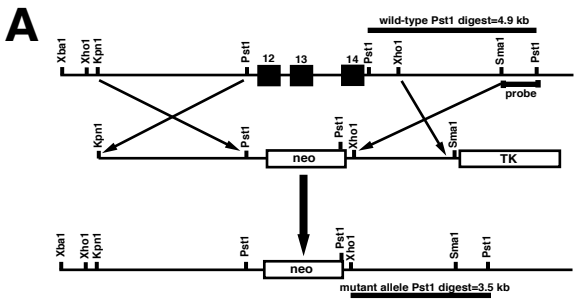
phospho-histone H3 immunostaining is unchanged in Foxp1 outflow tract cushions (G and H). NFATc expression (J and L, arrowheads) in Foxp1 -/- hearts persists in the endocardial cushion mesenchyme in both pulmonary (J) and A-V cushions (L) as compared to wild-type pulmonary (I) and mitral (K) cushions at E14.5. Scale bars: A-D = 400 μm; E-L = 200 μm.

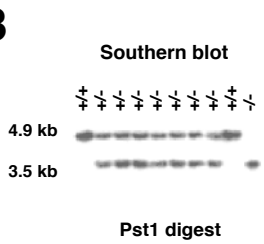
Figure 6. Sox4 is down-regulated in Foxp1 null hearts. In situ hybridization for Sox4 (A and B), Foxc1 (E and F), and Foxc2 (G and H) and immunohistochemistry for fibronectin (C and D) was performed on wild-type (A, C, E, G) and Foxp1 -/- (B, D, F, H) E14.5 embryo sections. Sox4 gene expression is significantly decreased in the outflow tract region of Foxp1 -/- hearts (A and B, arrows) while Foxc1, Foxc2, and fibronectin expression is unchanged. Scale bars: A, B, E-H = 800 μm; C and D = 400 μm.

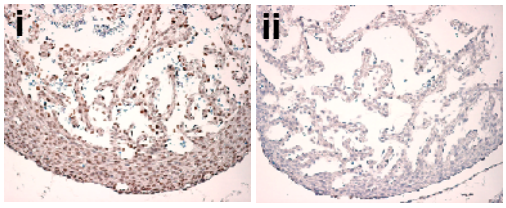
Figure 7. Cell proliferation and expression of cyclin dependent kinase inhibitors in Foxp1 -/- hearts. Immunohistochemistry was performed on wild-type (A, C, E, G) and Foxp1 -/- (B, D, F, H) hearts at E13.5 using antibodies for phospho-histone H3 (A and B), p21 (C and D), p27 (E and F), and p57 (G and H). Significant increases in phospho-histone H3 (B, red arrowheads) and p21 staining (D, red arrowheads) were observed while a decrease in p27 expression (F, red arrow) was observed in Foxp1 -/- hearts. Quantifying the number of phospho-histone H3 positive cells in the trabecular zone for wild-type and Foxp1 -/- hearts shows an approximately three-fold increase in the number of mitotic cells in Foxp1 -/- hearts (I). Scale bar = 200 μm.

Figure 8. Myocardial differentiation is disrupted in Foxp1 -/- hearts. In situ hybridization was performed using probes specific for Irx3 (A and B), ANF (C and D), and N-myc (E-F) on wild-type (A, C, E, G) and Foxp1 -/- (B, D, F, H) hearts at E11.5 (E and F) and E14.5 (A-D, G and H). Irx3 expression is expanded to encompass all ventricular myocardium (B). N-myc expression was significantly reduced in the ventricular wall of Foxp1 -/- embryos (compare E and F, G and 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 and 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: A-F = 400 μm; G and H = 800 μm; I and J = 100 μm.

Figure 1. Wang et. al.

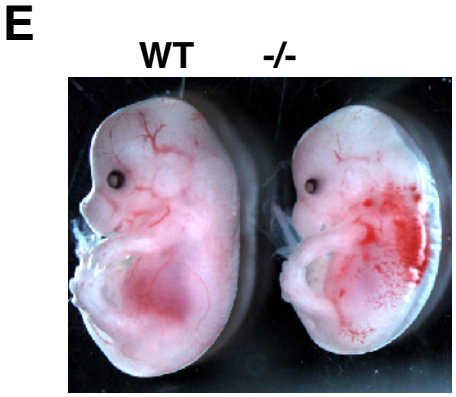






D

	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



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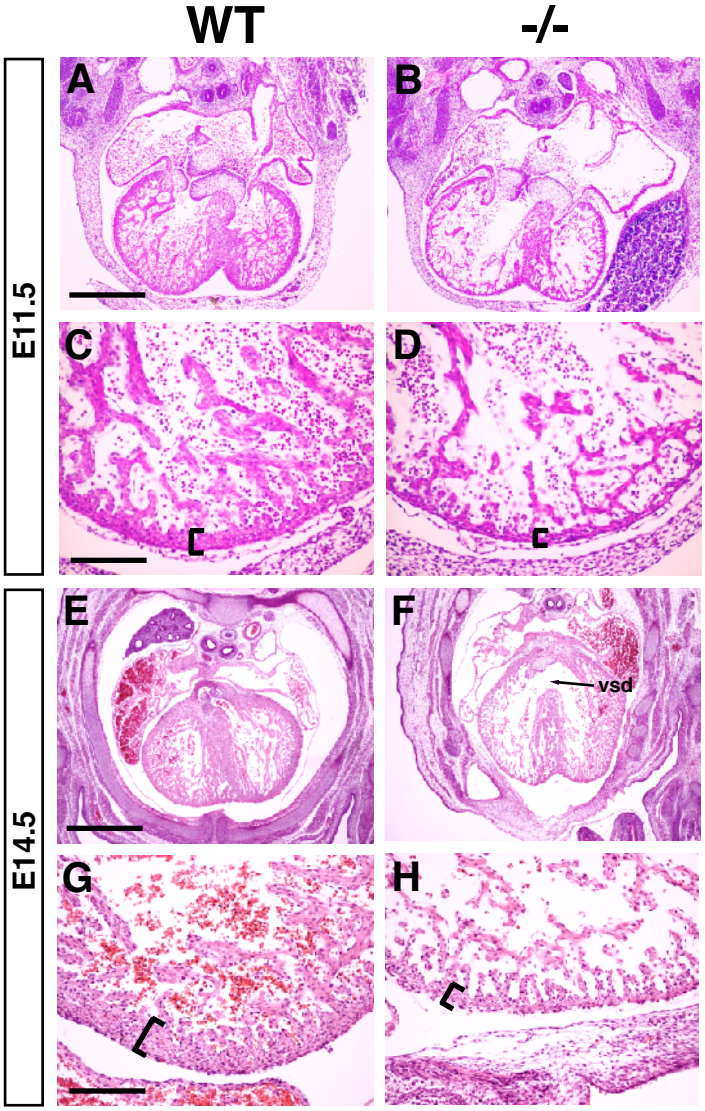

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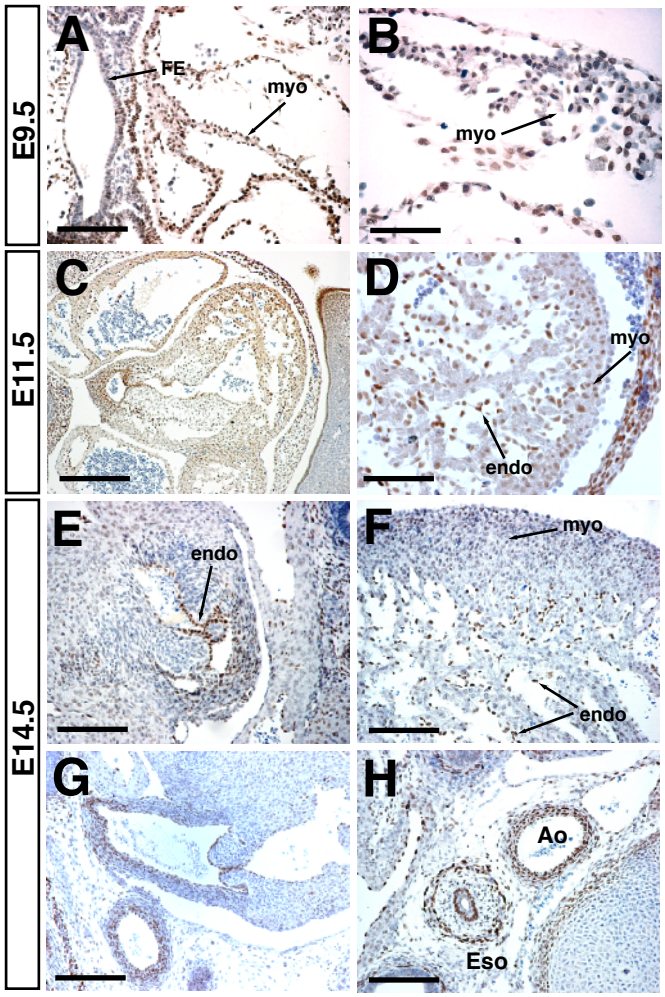

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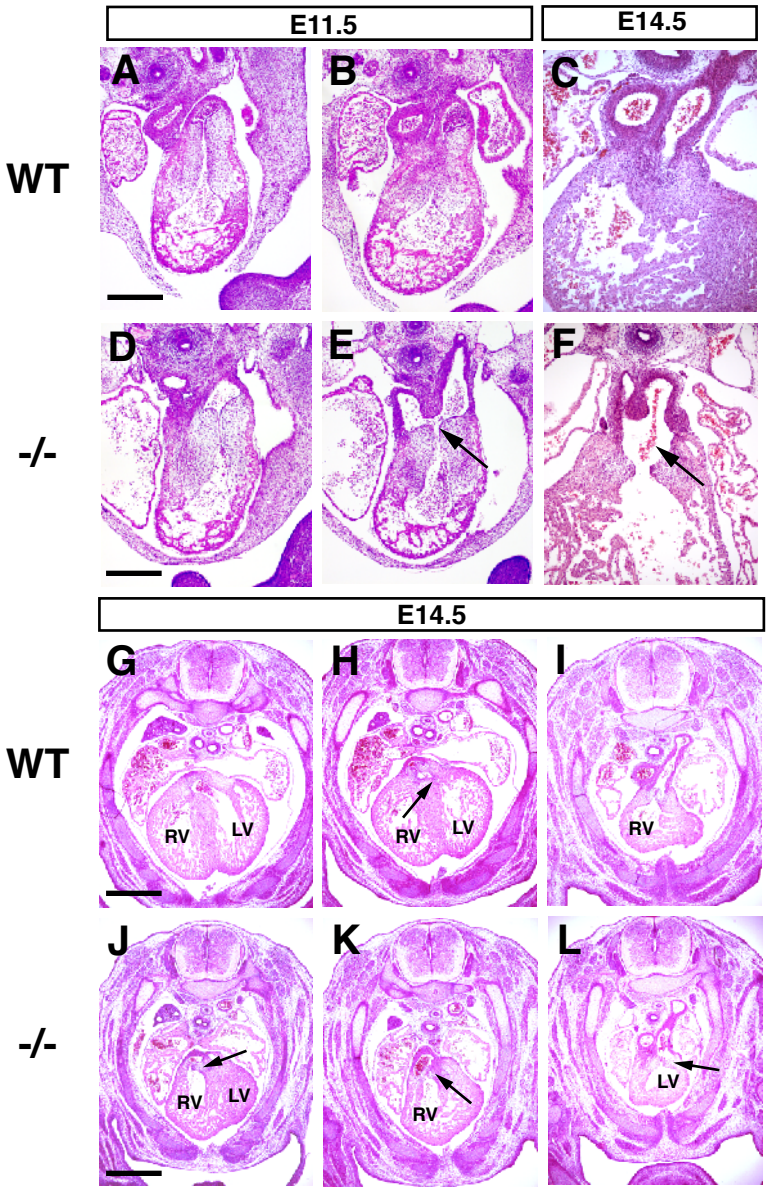

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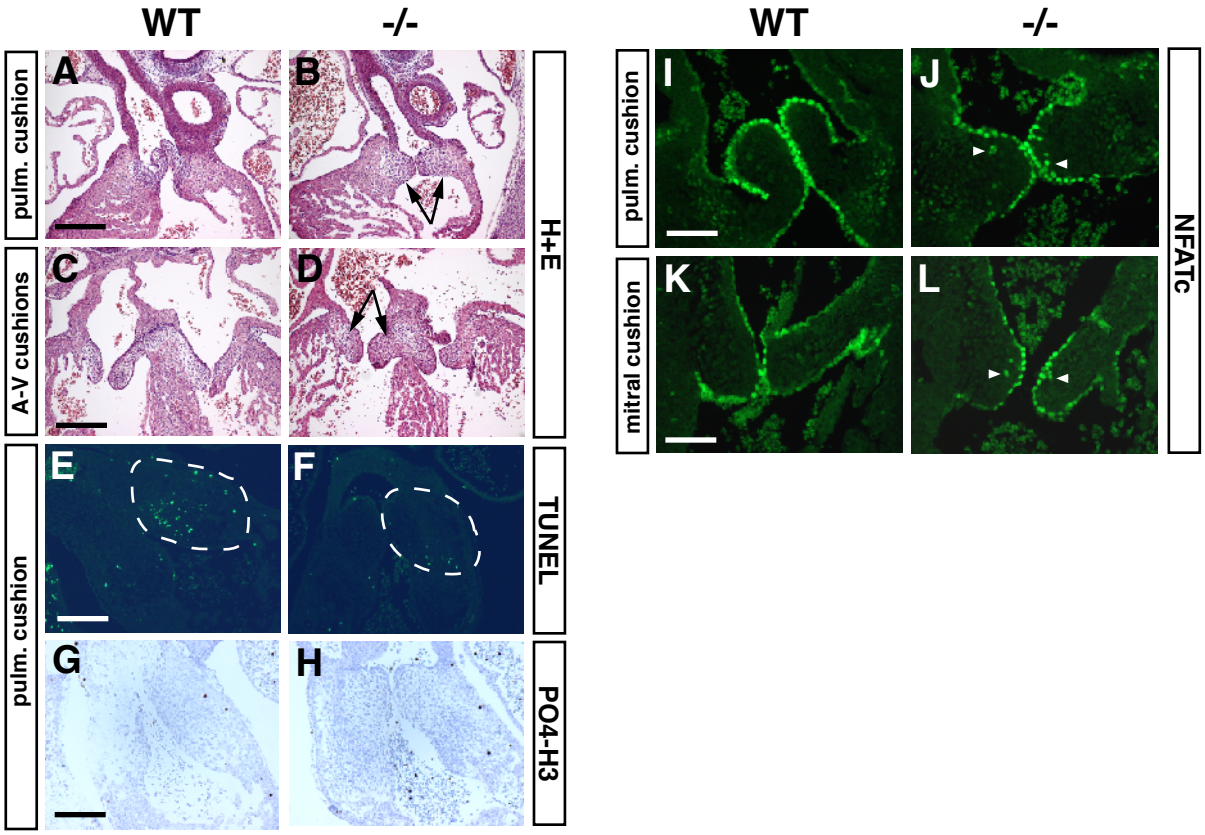

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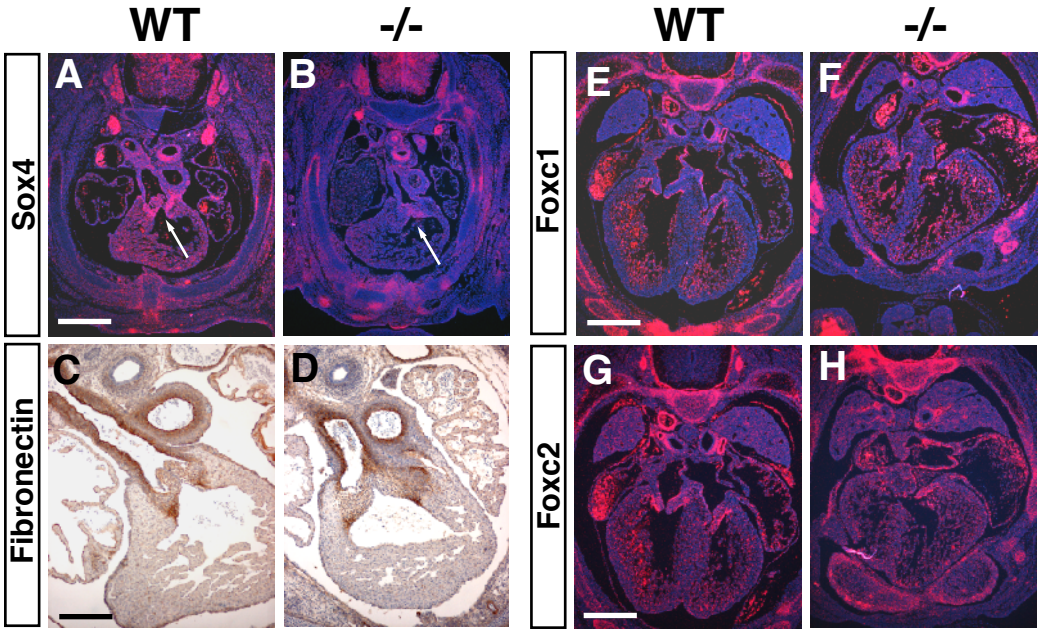

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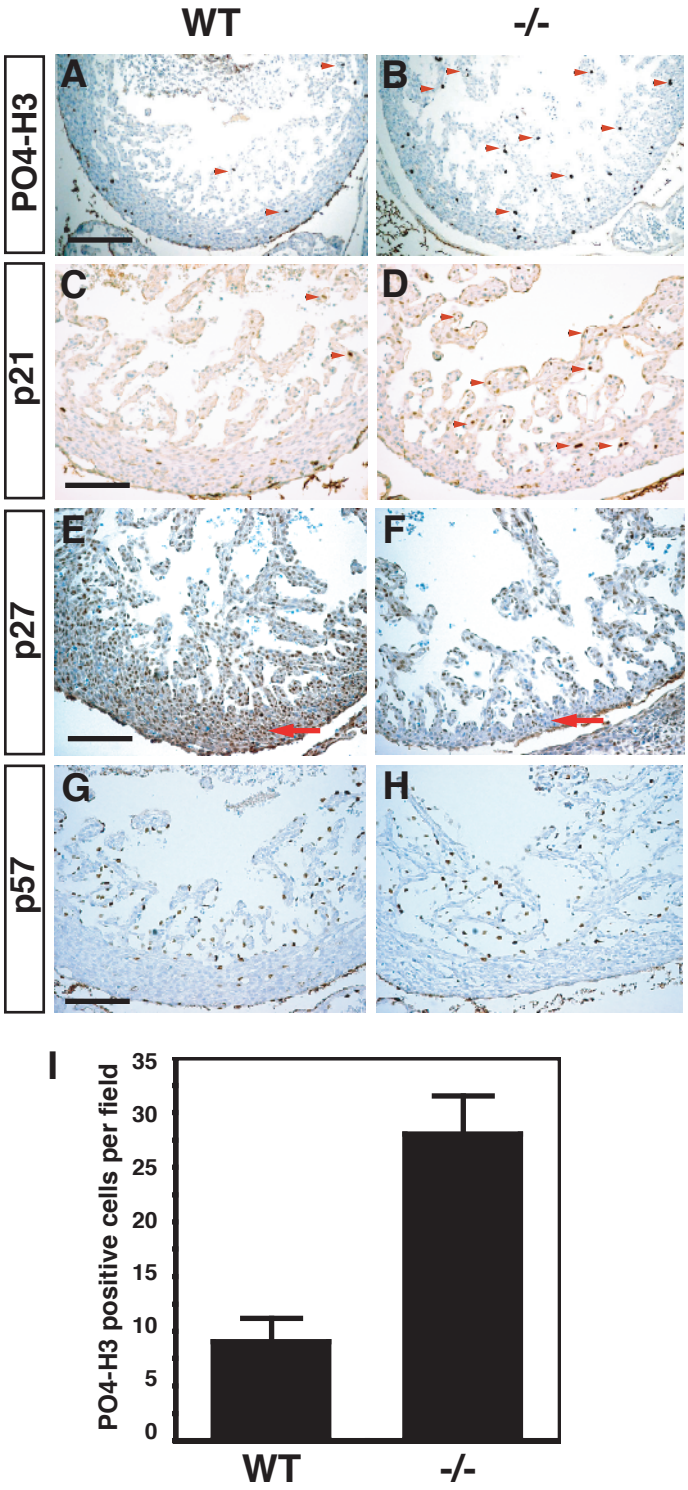

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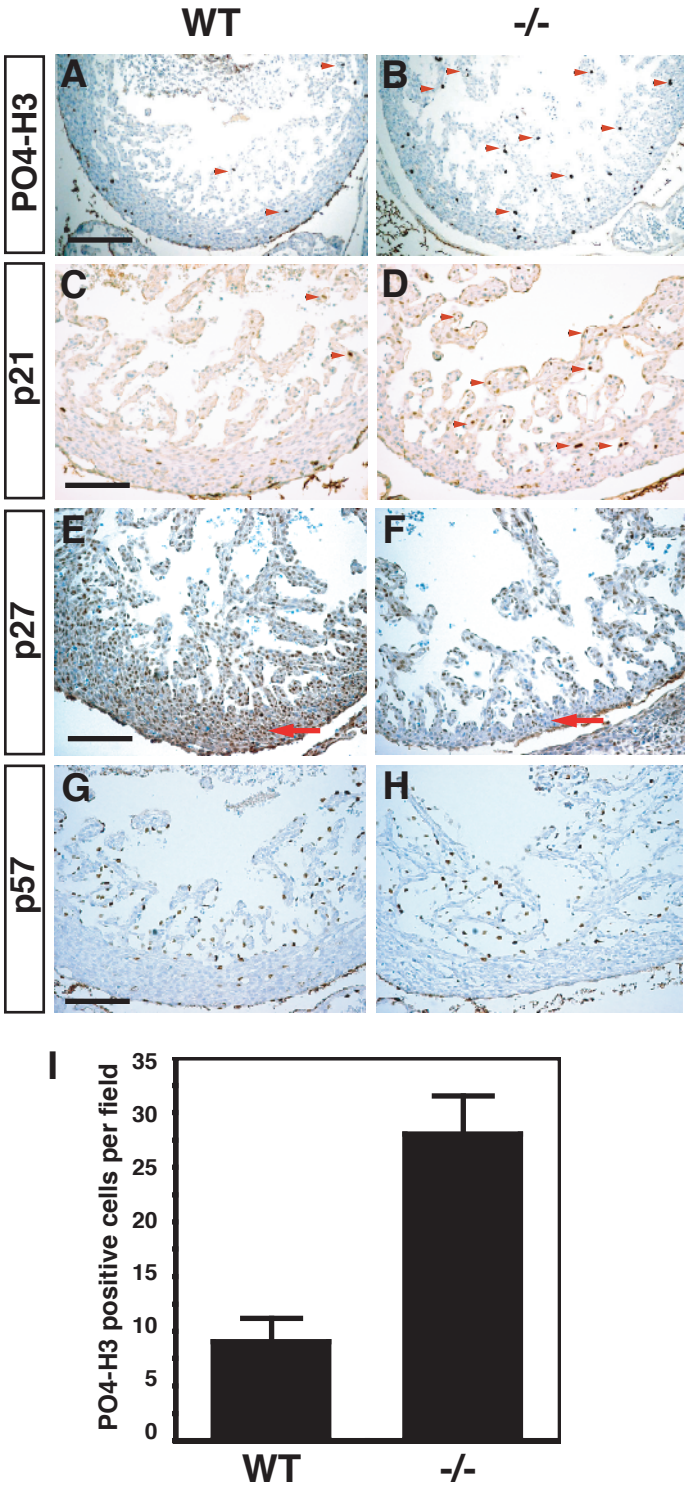

Figure 8. Wang et. al.