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Tgfβ signaling is required for atrioventricular cushion mesenchyme remodeling during in vivo cardiac development

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The transforming growth factorβ (Tgfβ) signaling pathway plays crucial roles in many biological processes. To understand the role(s) of Tgfβ signaling during cardiogenesis in vivo and to overcome the early lethality of *Tgfbr2*$^{-/-}$ embryos, we applied a Cre/loxp system to specifically inactivate *Tgfbr2* in either the myocardium or the endothelium of mouse embryos. Our results show that *Tgfbr2* in the myocardium is dispensable for cardiogenesis in most embryos. Contrary to the prediction from results of previous in vitro collagen gel assays, inactivation of *Tgfbr2* in the endocardium does not prevent atrioventricular cushion mesenchyme formation, arguing against its essential role in epithelium-mesenchyme transformation in vivo. We further demonstrate that Tgfβ signaling is required for the proper remodeling of the atrioventricular canal and for cardiac looping, and that perturbation in Tgfβ signaling causes the double-inlet left ventricle (DILV) defect. Thus, our study provides a unique mouse genetic model for DILV, further characterization of which suggests a potential cellular mechanism for the defect.

KEY WORDS: Tgfβ, Cardiogenesis, AV remodeling, DILV, Congenital heart disease, Mouse

INTRODUCTION

Congenital heart diseases (CHDs) occur in as many as 1% of newborns, and are the leading cause of infant morbidity and mortality (Hoffman, 1995; Hoffman and Kaplan, 2002). The most common disorders are caused by malformations in septation and valvulogenesis. Epithelium-mesenchyme transformation (EMT) in the atrioventricular canal (AVC) and outflow tract (OFT) regions of the heart is a crucial process regulating initial valve formation, in which a subpopulation of endocardial cells invade the extracellular matrix as the result of regional interaction between the myocardium and endocardium (Barnett and Desgrosellier, 2003). These mesenchymalized cushions serve as the primordia of valves and septa, and develop into mature structures through complicated remodeling processes. Proper cushion formation is required for simultaneous and subsequent events in cardiac development, including chamber formation and septation. Impairment of cushion development can cause various forms of CHD, such as misalignment between atria and ventricles, and yet the underlying molecular and cellular machineries are poorly understood.

The Tgfβ family of cytokines play crucial roles in many biological processes, including cardiovascular development (Azhar et al., 2003; Barnett and Desgrosellier, 2003). Tgfβ signaling is initiated when homodimers of ligands (Tgfβ1, Tgfβ2, Tgfβ3) bind to and bring together the type-I and type-II receptor kinases on cell membranes, and the ligand/receptor complex subsequently activates downstream target genes (de Caestecker, 2004; Shi and Massague, 2003). Three type I receptors (Alk1, Alk2, Alk5) and a single type II receptor (Tgfbr2) have been found to mediate Tgfβ signaling. Tgfbr2 can bind only Tgfβ ligands, and not other Tgfβ superfamily members (de Caestecker, 2004; Shi and Massague, 2003). In addition, Tgfbr3 (previously known as betaglycan, TβRIII) and endoglin function as co-receptors in concert with Tgfbr2 to regulate Tgfβ ligand/receptor interaction (Barnett and Desgrosellier, 2003). Thus, inactivation of *Tgfbr2* eliminates known Tgfβ signaling by inhibiting ligand/receptor interaction (Bhowmick et al., 2004) without directly affecting other signaling pathways.

Crucial functions of Tgfβ ligands during EMT have been suggested from previous studies using a 3D in vitro collagen gel system. In these AV explant cultures, Tgfβ ligands can substitute for the overlying myocardium to activate EMT (Nakajima et al., 2000; Potts and Runyan, 1989; Ramsdell and Markwald, 1997), and inhibition of Tgfβ signaling with an antisense oligonucleotide complementary to *Tgfb3* mRNA, or with neutralizing antiserums against Tgfβ ligands or receptors, blocks EMT (Boyer et al., 1999; Brown et al., 1996; Brown et al., 1999; Camenisch et al., 2002). However, the central role of Tgfβ signaling during EMT has not been supported by mouse genetic studies. No obvious valvular defect has been observed in *Tgfb1*$^{-/-}$ (Dickson et al., 1995), *Tgfb3*$^{-/-}$ (Kaartinen et al., 1995) or *Tgfbr3*$^{-/-}$ mice (Stenvers et al., 2003). *Tgfb2*$^{-/-}$ mice display a range of cardiac defects with partial penetrance, and the valvuloseptal defects of these mice were thought to be caused by abnormal cushion morphogenesis at later stages (Bartram et al., 2001; Sanford et al., 1997). The discrepancy between in vitro and in vivo data may result from the complementation by the remaining Tgfβ ligands. To circumvent the functional redundancy of Tgfβ ligands and the early lethality of *Tgfbr2*$^{-/-}$ mice (Oshima et al., 1996), and to identify the primary cardiac cell type that is responsive to Tgfβ signaling, we applied a Cre/loxp system to specifically inactivate *Tgfbr2* in the myocardium or endothelium of developing mouse hearts.

MATERIALS AND METHODS

Mouse and embryo manipulations

All procedures are approved by the Institutional Animal Care and Use Committee at Vanderbilt University. *Tie2cre* mice (the Jackson Laboratory) or *cTnTcre* mice (Jiao et al., 2003) were crossed with *Tgfbr2*$^{loxp/loxp}$ mice (Bhowmick et al., 2004; Chytil et al., 2002) to acquire *Tie2cre;Tgfbr2*$^{loxp/+}$

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and *cTnTcre;Tgfbr2*$^{loxp/+}$ mice, respectively. *Tgfbr2*$^{loxp/loxp}$ mice were crossed with *Rosa26R* (*R26R*) mice (Soriano, 1999) (the Jackson Laboratory) to acquire *Tgfbr2*$^{loxp/+}$*;R26R* mice, which were then intercrossed to generate *Tgfbr2*$^{loxp/loxp}$*;R26R/R26R* mice. In performing conditional gene inactivation experiments, we always used male *Tie2cre;Tgfbr2*$^{loxp/+}$ or male *cTnTcre;Tgfbr2*$^{loxp/+}$ mice to cross with female *Tgfbr2*$^{loxp/loxp}$*;R26R/R26R* mice. Embryo dissection, sectioning and X-gal staining were performed as described previously (Jiao et al., 2003; Jiao et al., 2002).

Laser microdissection and PCR analysis

Frozen sections of E10.5 embryos (15 μm) were dehydrated and AV endothelial/mesenchymal cells isolated using a PixCell II Laser Capture Microdissection Scope (Arcturus), following the manufacturer's instructions. Genomic DNA or RNA was isolated from the laser-captured cells using either the PicoPure DNA Extraction Kit (Arcturus) or the PicoPure RNA Isolation Kit (Arcturus). The RiboAmp HS RNA Amplification Kit (Arcturus) was used to perform two rounds of linear amplification of mRNA on the isolated total RNA samples. The primers used to amplify the recombined and unrecombined *Tgfbr2*^{loxp} allele, and for reverse transcriptase (RT)-PCR analysis of *Tgfbr2* mRNA, have been described previously (Bhowmick et al., 2004). Quantitative real-time PCR analysis was performed using the LightCycler-DNA Master SYBR Green I Kit on a LightCycler Instrument (Roche). Semi-quantitative RT-PCR analysis was performed using the OneStep RT-PCR Kit (Qiagen).

In vitro collagen gel assay

In vitro collagen gel assays were performed as described previously (Camenisch et al., 2002).

Measurement of sizes of ventricles and AV cushions

The procedure for quantification of ventricular and cushion volumes was essentially as previously described (Kubalak et al., 2002). Briefly, E12.5 embryonic hearts were sectioned in the frontal plane at 8.0 μm intervals and photomicrographs of all sections were taken with a digital camera (RTSlider 2.3.0, Diagnostic Instruments) under identical conditions. The sizes of the ventricular chambers and AV cushions of each section were measured in artificial units using the software MetaMorph 5.0 (Universal Imaging), following the manufacturer's instructions, and the sum of all sections for each structure was then calculated as its 'raw size'. The relative cushion size was determined by dividing the raw size of the inferior (or superior) cushion with the raw size of the left ventricle (LV) to minimize the effect of variations in developmental stages among embryos. The relative cushion size of control embryos was artificially set as 100%. The raw size of the right ventricle (RV) was divided by the raw size of the LV to acquire the ratio of RV/LV size. In total, sections from four control and four mutant embryos were measured.

Immunofluorescent studies and in situ hybridization analysis

Immunofluorescent studies were performed as described previously (Jiao et al., 2003). The primary antibodies used in this study included a cyclin D1 monoclonal antibody (BD Biosciences), an anti-Ki67 antibody (Novocastra), a Pecam antibody (BD Biosciences) and an α-smooth-muscle actin antibody (Sigma). Cy2 conjugated secondary antibodies were used for visualization. Samples were examined with a laser-scanning confocal image system (Zeiss LSM510). Quantification of the intensity of the fluorescence was performed using MetaMorph 5.0. Section in situ hybridization analysis was performed as described previously (Jiao et al., 2003), using a probe corresponding to the first four exons of *Tgfbr2*.

RESULTS

Tgfbr2 in the myocardium is dispensable for cardiogenesis

The multiple cardiac defects in *Tgfb2*$^{-/-}$ mice include double-outlet right ventricle (DORV), atrial and ventricular septal defect (ASD and VSD), overriding tricuspid valve and failure in myocardialization (Bartram et al., 2001; Sanford et al., 1997), some of which are likely to be caused by the disturbance of cardiac looping (Bartram et al., 2001), suggesting potential roles for Tgfβ signaling in the myocardium. To study myocardial-specific attenuation of Tgfβ signaling, we inactivated *Tgfbr2* by crossing *cTnTcre;Tgfbr2*$^{loxp/+}$ mice with *Tgfbr2*$^{loxp/loxp}$ mice (Chytil et al.,

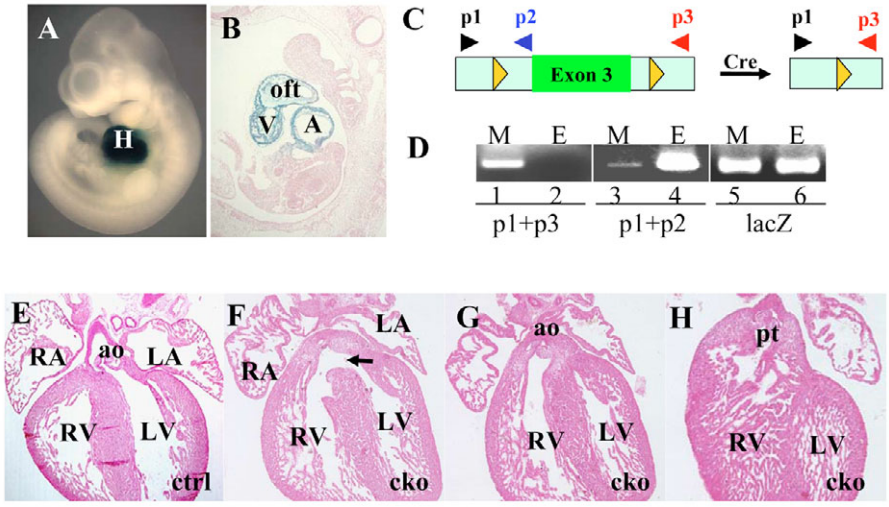

Fig. 1. Myocardium-specific inactivation of *Tgfbr2*. (**A**,**B**) Whole-mount (A) and sectional (B) examination of a *cTnTcre;Tgfbr2*$^{loxp/loxp}$*;R26R* embryo at E10.5 stained with X-gal. (**C**,**D**) PCR analysis of DNA isolated from either the myocardium (M) or endocardium (E) of the AVC region of an E10.0 *cTnTcre;Tgfbr2*$^{loxp/loxp}$*;R26R* embryonic heart. (C) Primers p1 and p2 detected the unrecombined *Tgfbr2*^{loxp} allele, whereas primers p1 and p3 detected the recombined allele (Bhowmick et al., 2004). (D) The recombined allele can be detected only from myocardial cells (lane 1) and not endocardial cells (lane 2), whereas the unrecombined allele is hardly detected from myocardial cells (lane 3 versus 4). The *lacZ* primers were used as a control to show that a similar amount of DNA template was used for the myocardium (lane 5) and endocardium (lane 6) in the PCR analysis. (**E-H**) Frontal sections of a wild-type (E) and a *cTnTcre;Tgfbr2*$^{loxp/loxp}$ (F-H) embryonic heart. The arrow in F indicates the VSD. Both the aorta and pulmonary trunk are connected to the right ventricle (G,H), resulting in a DORV defect. A, atrium; ao, aorta; cko, *cTnTcre;Tgfbr2*$^{loxp/loxp}$; ctrl, wild-type embryos; E, endocardial cells; H, heart; LA, left atrium; LV, left ventricle; M, myocardial cells; oft, outflow tract; pt, pulmonary trunk; RA, right atrium; RV, right ventricle; V, ventricle.

2002). *cTnTcre* induces recombination early in the cardiomyocyte lineage (E7.5), and efficiently inactivates target genes between E9.5 and E10.5 (Jiao et al., 2003) (Fig. 1A,B). The myocardium-specific deletion of *Tgfbr2* was confirmed by PCR analysis using genomic DNA isolated from myocardial cells or endocardial cells (Fig. 1C,D). The recombined allele could be detected only from myocardial cells and not from endocardial cells, whereas the unrecombined allele was hardly detected from myocardial cells.

The *cTnTcre*;*Tgfbr2*$^{loxp/loxp}$ animals were isolated with an expected Mendelian ratio (~25%) at all stages examined (Table 1), excluding embryonic lethality of these animals. Two out of 24 mutant animals displayed cardiac defects, with one embryo (E17.5) showing VSD (data not shown), and the other (E16.5) showing VSD and DORV (Fig. 1E-H). The low incidence (8.3%) of cardiac defects in *cTnTcre*;*Tgfbr2*$^{loxp/loxp}$ animals suggests that myocardial Tgfβ signaling is not essential for normal cardiogenesis. However, perturbation of myocardial Tgfβ signaling may cause embryonic hearts to be more susceptible to deleterious genetic and/or environmental factors, and may thus contribute to congenital abnormalities.

Inactivation of *Tgfbr2* in the endothelium does not prevent EMT in vivo

To directly address the roles of Tgfβ signaling on AV cushion morphogenesis in vivo, we inactivated *Tgfbr2* in endothelium by crossing *Tie2cre;Tgfbr2*$^{loxp/+}$ mice with *Tgfbr2*$^{loxp/loxp}$*; R26R/R26R* mice. *Tie2cre* efficiently inactivates target genes in endothelium and its derivatives, including in AV cushion mesenchymal cells (Koni et al., 2001; Liebner et al., 2004). The *R26R* (Soriano, 1999) allele was introduced into the cross for monitoring recombination efficiency and specificity, as we observed unexpected ectopic recombination induced by *Tie2-Cre* starting from E10.5. Approximately 30% of embryos at E10.5 displayed ectopic recombination throughout the whole embryo, as determined from expression of the *R26B* reporter, whereas the remaining 70% of embryos displayed the expected endothelium-specific recombination. No live *Tie2cre;Tgfbr2*$^{loxp/loxp}$*;R26R* embryos with ectopic recombination were recovered beyond E11.5; the embryonic lethality is presumably due to broad inactivation of *Tgfbr2* throughout these embryos. To overcome the ectopic recombination problem, embryos in this study were stained with X-gal when possible, and only those with proper endothelium specific recombination at the *R26* locus were included for further analysis.

Approximately 65% of the conditional knockout embryos (51/92; excluding embryos with ectopic recombination) isolated from E9.0 to E11.5 were arrested at the 20-25 somite stage (Fig. 2A-C). Their yolk sacs showed severe anemia and a deficiency in vasculogenesis that was indistinguishable from that of *Tgfbr2*$^{-/-}$ embryos, whose growth was arrested at the same stage (Oshima et al., 1996). No mutant embryo with apparent retardation was recovered beyond E11.5, and demise was most likely to be secondary to yolk sac vascular insufficiency, as previously described (Oshima et al., 1996). However, the remaining *Tie2cre;Tgfbr2*$^{loxp/loxp}$*;R26R* embryos did not display overall growth retardation or obvious abnormalities in their yolk sac vasculatures, and were able to survive to the E12.5 stage (Fig. 2D,E, Fig. 3A). As the mice were from a mixed genetic background, the mutant embryos with normal growth probably have inherent beneficial genetic factors that influence Tgfβ signaling in vivo, similar to those previously described (Tang et al., 2003). Alternatively, there may have been a delay in *Tgfbr2* excision in yolk sac endothelia of this group of embryos. The *Tie2cre*-mediated endothelial recombination at the *R26R* locus in these embryos was indistinguishable from the control embryos, as determined from both whole-mount and section studies (Fig. 3A,C-F), suggesting that the grossly normal growth of these mutant embryos is unlikely to be caused by insufficient recombination in their endothelium. No live mutant embryos between E13.5 and E15.5 (0/81) were recovered. Dead mutant embryos were often found at E13.5 with hemorrhage and edema, presumably caused by cardiovascular insufficiency (data not shown).

Section studies indicate that a high level of recombination occurs in the endothelia, including in the AVC region, of embryos at E9.0 (n=5), before the onset of EMT (Fig. 3B). In a subsequent analysis of AV cushion morphogenesis, we focused on the mutant embryos that did not show apparent growth retardation in order to avoid complicating the interpretation of data. This group represents a small but informative subset of embryos that have escaped the global vascular compromise evoked by endothelial *Tgfbr2* deletion. Histological examination of E9.5 and E10.5 embryos showed that AV cushion mesenchymal cells were normally formed in mutant embryos, and β-galactosidase-positive

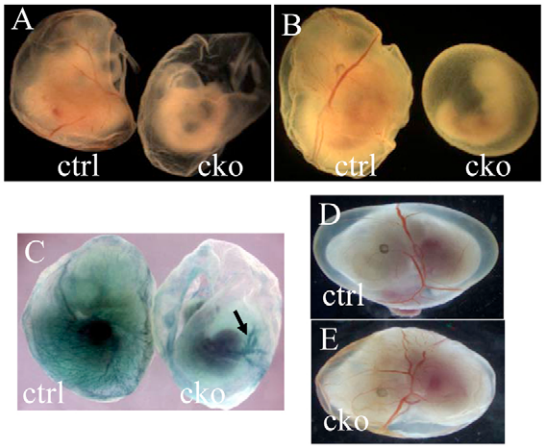

Fig. 2. Defective vasculogenesis in the yolk sacs of *Tie2cre;Tgfbr2*$^{loxp/loxp}$*;R26R* embryos. (**A**,**B**) Embryos with yolk sacs were isolated at E10.5 (A) and E11.5 (B). The *Tie2cre;Tgfbr2*$^{loxp/loxp}$*;R26R* embryos were arrested at around the 20-25 somite stage. No blood vessels were visible in their yolk sacs. (**C**) Embryos from A stained with X-gal to detect endothelial cells. In contrast to the well-formed blood vessel nets in the control embryo yolk sac, formation of blood vessels in the mutant was initiated only in the region close to the allantois (arrow). (**D**,**E**) Mutant embryo (E) that escaped global vascular compromise and survived to E12.5 without growth retardation or obvious yolk sac vascular abnormalities. cko, *Tie2cre;Tgfbr2*$^{loxp/loxp}$*;R26R* embryos; ctrl, *Tie2cre;Tgfbr2*$^{loxp/+}$*;R26R* embryos.

Table 1. Summary of myocardium-specific inactivation of *Tgfbr2*

Stage	Number of total animals	Number of *cTnTcre; Tgfbr2*$^{loxp/loxp}$ animals
E12.5	22	5
E15.5	21	6
E16.5	8	2 (1)
E17.5	26	6 (1)
P0	22	5
Total	99	24

The number in parentheses indicates the number of embryos with cardiac defects.

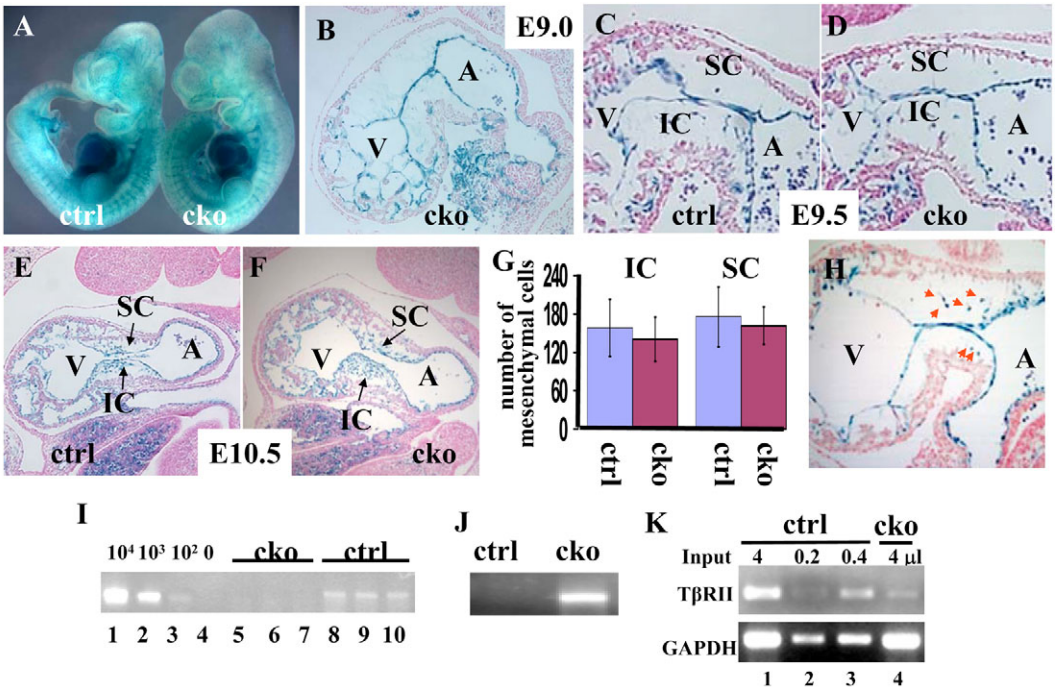

Fig. 3. *Tgfbr2* is not required for AV cushion mesenchyme formation. (**A**) The conditional knockout embryo (right) isolated at E10.5 was grossly normal compared with the control littermate (left). Embryos were stained with X-gal. The *Tie2cre*-mediated recombination in the mutant embryo occurred at the same level and with the same pattern as that in the control embryo (as judged from the *R26R* locus). (**B**) Mutant embryo isolated at E9.0, immediately before the onset of EMT in the AVC region, stained with X-gal and sectioned. (**C-F**) Control (C,E) and mutant (D,F) embryos isolated at E9.5 (C,D) and E10.5 (E,F), stained with X-gal and sagittally sectioned. The AV cushion mesenchymal cells formed normally in mutant embryonic hearts. (**G**) Count of the number of AV cushion mesenchymal cells in AVC regions of mutant and control embryonic hearts (E9.5). Data were averaged from four embryos of each genotype. Error bars indicate the s.d. (**H**) Sagitally sectioned mutant embryo isolated at E10.5, in which growth was arrested at around the 25 somite stage. Mesenchymal cells (arrowheads) can be observed in the AV cushions. (**I**) Quantitative analysis of the recombined *Tgfbr2*^{loxp} allele amplified by quantitative real-time PCR analysis from genomic DNA isolated from AV endocardial/mesenchymal cells of control or mutant embryonic hearts (Fig. 1C). Lanes 1-3 represent a positive control in which a plasmid containing the recombined *Tgfbr2*^{loxp} allele was used as a template, the copy number of which ranged from 10^4 to 10^2. Lane 4 is a no template control. Lanes 5-7 and lanes 8-10 represent the mutant and control samples, respectively, from three independent experiments. (**J**) The PCR product of the recombined *Tgfbr2*^{loxp} allele could only be detected in the mutant samples. (**K**) Semi-quantitative RT-PCR analysis was performed with RNA isolated from the AV endocardial/mesenchymal cells. Lanes 1-3 are control samples with 100, 5 and 10% of input RNA. Lane 4 is the mutant sample with 100% of input RNA. GAPDH was used as a loading control. A, atrium; cko, *Tie2cre;Tgfbr2*$^{loxp/loxp}$*;R26R*; ctrl, *Tie2cre;Tgfbr2*$^{loxp/+}$*;R26R*; IC, inferior cushion; SC, superior cushion; V, ventricle.

cells (indicative of recombination occurring at the *R26R* locus) were evident in their AV cushions (n=15; Fig. 3C-F). The lack of EMT defect was further confirmed with quantification analysis (Fig. 3G); the number of mesenchymal cells formed in both inferior and superior AV cushions was comparable between control and mutant embryos at E9.5, the stage when cushion mesenchymal cells are just formed, and thus cell proliferation and apoptosis are unlikely to play a major role in determining the total mesenchymal cell number. To determine if defects in EMT might be present in embryos that demonstrated early gross developmental delay and were thus excluded from analysis, we sectioned three mutant embryos at E10.5 with growth arrest at the 25 somite stage when compared to littermates at the 25-40 somite stage. As shown in Fig. 3H, mesenchymal cells were evident in AV cushions of these embryos, suggesting that EMT occurs in both groups of mutant embryos.

To directly evaluate the recombination efficiency at the *Tgfbr2* locus, we purified genomic DNA and total RNA from AV cushion endocardial/mesenchymal cells isolated with a laser capture microdissection system. PCR analysis with genomic DNA showed that the unrecombined *Tgfbr2*^{loxp} allele was undetectable in cells of *Tie2cre;Tgfbr2*$^{loxp/loxp}$*;R26R* embryos (Fig. 3I), by both conventional and real-time PCR analysis, indicating that recombination occurred in most, if not all, of the AV cushion endothelial/mesenchymal cells. As expected, the recombined allele was detected only from mutant, and not from control, embryos (Fig. 3J). Semi-quantitative PCR analysis using linearly amplified RNA showed that *Tgfbr2* in the mutant samples was expressed at less than 10% of the wild-type level (Fig. 3K). These results confirmed the efficient inactivation of *Tgfbr2* at both the DNA and mRNA levels. Thus, we concluded that EMT of AVC endothelial cells occurs normally in vivo, even when transcription of *Tgfbr2* is dramatically reduced.

Deletion of *Tgfbr2* in endocardial cells blocks EMT in in vitro cultures

Because our results were contrary to previous in vitro studies, we performed collagen gel assays, and found that very few mesenchymal cells were formed in mutant AV explants (Fig. 4). In contrast to the well-formed mesenchymal cells in the control explant (Fig. 4A), in the mutants, endothelial cells were associated with neighbor cells, and were expanded on the collagen

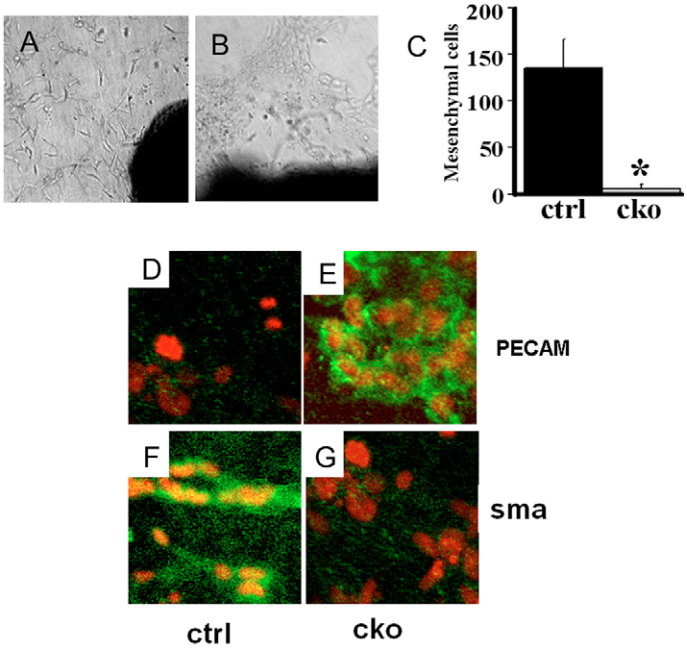

Fig. 4. Inactivation of *Tgfbr2* in endocardial cells prevents EMT in vitro. (**A**,**B**) In vitro collagen gel assays performed with AV explants isolated from control (A) or mutant (B) embryonic hearts at E9.25. (**C**) The number of mesenchymal cells formed in control (*Tgfbr2*$^{loxp/loxp}$*;R26R*) explants (n=18) is dramatically higher than that in mutant explants (n=14). *P<0.005. (**D-G**) Control (D,F) and mutant (E,G) explant cultures stained with an antibody (green) against the pan-endothelium marker Pecam (D,E), or against the mesenchyme marker α-smooth-muscle actin (F,G). Nuclei were visualized by staining with propidiumiodide (red). In the control, the mesenchymal cells expressed α-smooth-muscle actin (F) and lost the expression of Pecam (D). By contrast, the endocardial cells in mutant explant cultures expand on the gel surface with the expression of Pecam (E) but not of α-smooth-muscle actin (G). cko, *Tie2cre;Tgfbr2*$^{loxp/loxp}$*;R26R* embryos; ctrl, control embryos.

gel but did not undergo activation or transformation (Fig. 4B). This result was confirmed by a quantitative assay (Fig. 4C), and by immunofluorescent studies (Fig. 4D-G) using antibodies against Pecam (an endothelium-specific marker) and α-smooth-muscle actin (a mesenchymal marker). Significantly, this phenotype is identical to the result obtained in wild-type AV explant cultures treated with anti-Tgfβ2 antiserum (Camenisch et al., 2002), confirming that Tgfβ signaling is required for EMT in AV explant assays.

Endocardial inactivation of *Tgfbr2* causes a DILV defect

The E11.5 *Tie2cre;Tgfbr2*$^{loxp/loxp}$*;R26R* embryonic hearts (n=18) were largely normal, except for the reduced size of their inferior AV cushions (data not shown). Cardiac defects were observed in all E12.5 mutant embryos (n=6). Whole-mount examination showed that, in the mutant hearts, the interventricular sulcus at the apex was reduced and that the RV adapted a more ventral orientation (Fig. 5A,B), indicating a cardiac-looping defect. As we did not observe any morphological abnormality in the initial bending of the linear tubes in mutant mice, the looping defect appears to occur during chamber septation and remodeling of the AVC [the second phase of looping (Azhar et al., 2003)]. This result is consistent with a previous study, which showed that some *Tgfb2*$^{-/-}$ embryos displayed defects during the second phase of looping (Bartram et al., 2001).

Section studies showed that the inferior cushion of the mutant hearts had a dramatically reduced size and failed to fuse with the mesenchymal cap of the atrial septum primum (ASP), whereas the size of the superior cushion appeared normal (Fig. 5C-H). The size reduction in the inferior cushion but not in the superior cushion was further confirmed with quantification analysis (Fig. 5I). Significantly, in contrast to the control hearts in which the left and right atria were connected separately with the left and right ventricles (Fig. 5C,F), both atria of the mutant hearts were open only to the LV (Fig. 5D,G,H) in all sections that were examined, resulting in a DILV defect. All mutant embryonic hearts (Fig. 5L) showed a ventricular septal defect (VSD), which is a hemodynamic necessity for DILV. The DILV defect was not due to underdevelopment of the RV (Fig. 5J-M), nor to abnormal yolk sac vasculogenesis (Fig. 2D,E). In addition, we observed defects in septation of the OFT in all mutant hearts. Detailed characterization of OFT and general endothelial defects is currently underway.

To test whether the uneven development of AV cushions (inferior cushion versus superior cushion) in mutant embryonic hearts is due to differential expression of *Tgfbr2*, we performed radioactive in situ hybridization analysis on sections of wild-type embryos from E9.5 to E11.5. As shown in Fig. 5N-Q, no obvious asymmetric expression of *Tgfbr2* in AV cushions was observed, although we cannot rule out a potential subtle difference beyond our detection.

Inactivation of *Tgfbr2* in endocardial cells preferentially reduces cell proliferation in the inferior AV cushion

We did not observe any enhanced apoptosis in the mutant embryonic hearts at E11.5 and E12.5 (data not shown). To test whether proliferation of mesenchymal cells was affected in AV cushions, we stained embryo sections with an anti-Ki67 antibody (Fig. 6A-I). Our result shows that the growth rate of inferior cushion mesenchyme of mutant hearts was significantly reduced at E11.5, whereas that of the superior cushion was normal. Thus, Tgfβ signaling is required for the proper inferior cushion mesenchyme proliferation.

To gain insight into the mechanism of Tgfβ signaling in regulating the AV mesenchyme cell cycle, we performed immunofluorescent studies with a cyclin D1-specific antibody. Cyclin D1 promotes cell-cycle progression by regulating Rb phosphorylation in nuclei (Sherr and Roberts, 1999), and it was previously reported that elimination of Tgfβ signaling in palatal mesenchyme reduced cyclin D1 expression and inhibited cell proliferation (Ito et al., 2003). Using quantitative immunofluorescence analysis, we observed a significant reduction in cyclin D1 expression in the inferior cushion but not in the superior cushion of mutant hearts (Fig. 6J-Q). This is consistent with the observation that cell proliferation is only significantly reduced in the inferior AV cushion. In addition to the overall cyclin D1 expression reduction, we found that the percentage of mesenchyme with nuclear-localized cyclin D1 is also significantly reduced in the inferior cushion of mutant embryonic hearts (Fig. 6J-Q). Cytoplasmic cyclin D1 is nonfunctional and targeted for proteolysis (Alt et al., 2000; Sherr and Roberts, 1999), and cyclin D1 cytoplasmic sequestration serves as an important regulatory mechanism for controlling cell proliferation, survival and fate determination (see Sumrejkanchanakij et al., 2003; Tamamori-Adachi et al., 2003).

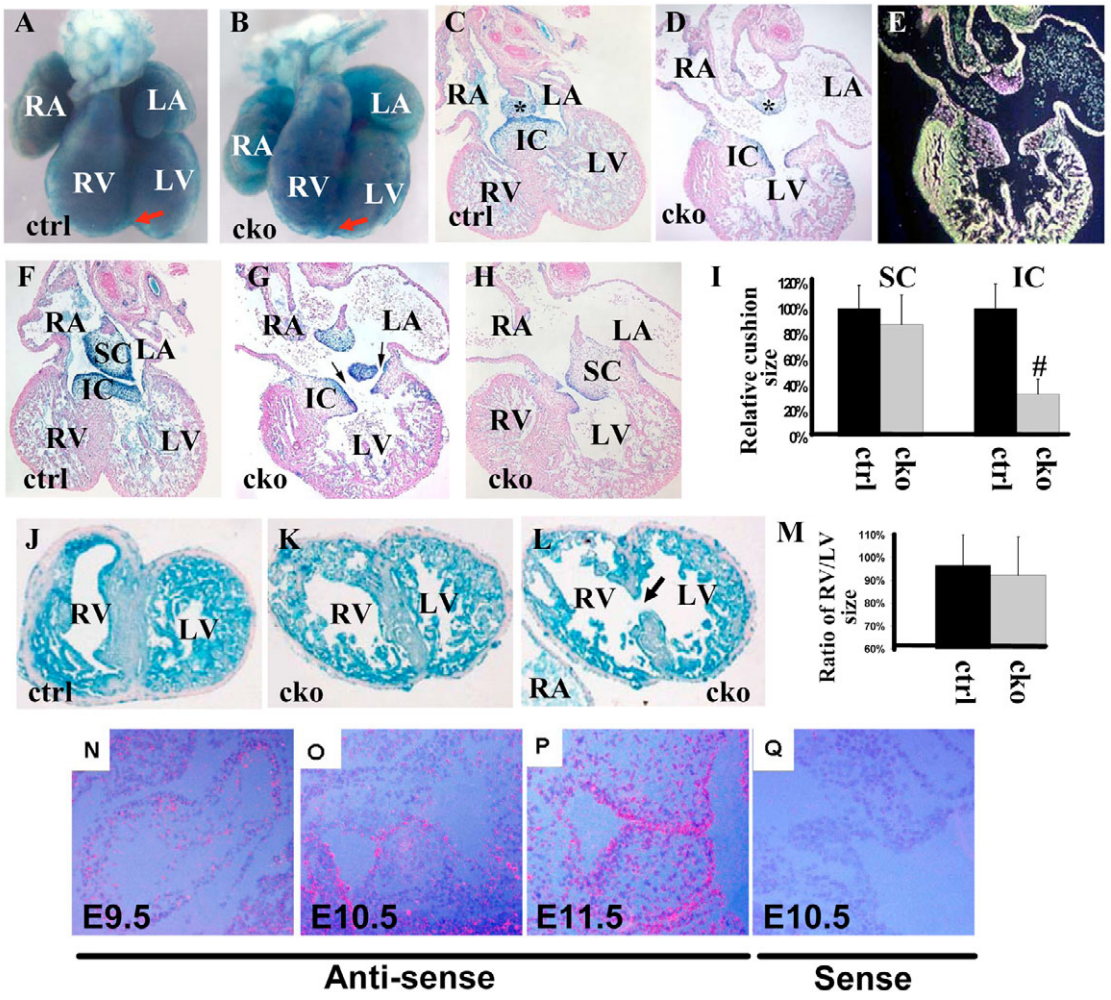

Fig. 5. Endocardial inactivation of Tgfβ signaling causes DILV. (**A**,**B**) Whole-mount examination of E12.5 embryonic hearts from a control (A) and a mutant (B) embryo stained with X-gal. Red arrows show the interventricular sulcus at the apex of the heart. (**C-H**) Frontal sections of E12.5 hearts from control (C,F) or mutant (D,E,G,H) embryos stained with X-gal. Asterisks in C and D indicate the mesenchymal cap of the ASP. E represents the dark field view of D. Owing to penetration problems, which are common in X-gal staining studies, results of X-gal staining of E12.5 embryonic hearts varied dramatically among samples. X-gal staining products appear pink in the dark field, and can be detected more easily than in the bright field. Arrows in G indicate that both the right atrium (RA) and left atrium (LA) empty through separated 'inlets' to the left ventricle, resulting in a DILV defect. (**I**) The relative cushion sizes of mutant embryonic hearts versus control hearts were measured as described in the Materials and methods. $^{\#}P<0.05$. (**J-L**) Cross sections of E12.5 hearts from control (J) and mutant (K,L) embryos show normal growth of the right ventricle. The section in K is close to the apex of the heart, and the section in L is at the level at which the VSD (black arrow) is obvious. (**M**) The size of the right ventricle is approximately the same as the left ventricle in both control and mutant embryos. (**N-Q**) Section in situ hybridization analysis performed on sagittally sectioned embryos (E9.5-E11.5) with a [^{35}S]-labeled antisense probe (N-P) corresponding to the first four exons of *Tgfbr2*, or a sense probe as a negative control (Q). ASP, atrial septum premium; cko, *Tie2cre;Tgfbr2$^{loxp/loxp}$;R26R*; ctrl, *Tie2cre;Tgfbr2$^{loxp/+}$;R26R*; IC, inferior endocardial cushion; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle; SC, superior endocardial cushion.

DISCUSSION

EMT by endocardial cells in the AVC and OFT is the initial step for valvulogenesis, and yet the molecular mechanisms governing this process have not been fully understood. Previous studies using the in vitro collagen gel culture system have suggested that Tgfβ signaling is required for EMT; however, thus far, this conclusion has not been supported by in vivo mouse genetic studies, including those using *Tgfb1$^{-/-}$*, *Tgfb2$^{-/-}$*, *Tgfb3$^{-/-}$* and *Tgfbr3$^{-/-}$* mouse models (Bartram et al., 2001; Dickson et al., 1995; Kaartinen et al., 1995; Sanford et al., 1997; Stenvers et al., 2003). In this study, we specifically inactivated *Tgfbr2*, which encodes the only known type II receptor for Tgfβ1-3 ligands, in the endothelium. Our studies using the collagen gel system essentially confirm previous in vitro studies in that no EMT occurs in AV explants of *Tie2cre;Tgfbr2$^{loxp/loxp}$* embryos (Fig. 4). However, the current study fails to provide evidence supporting an essential role of *Tgfbr2* during EMT in living embryos. We showed that transcription of *Tgfbr2* in mutant embryos is reduced to less than 10% of the wild-type level and, because of to technical difficulties, we failed to detect Tgfbr2 in AV cushions using the currently available antibody. Therefore, we cannot exclude that residual Tgfbr2 can sufficiently activate EMT; however, the failure in EMT observed from the collagen gel assay using mutant explants provided functional evidence showing that the expression of *Tgfbr2* was efficiently inactivated.

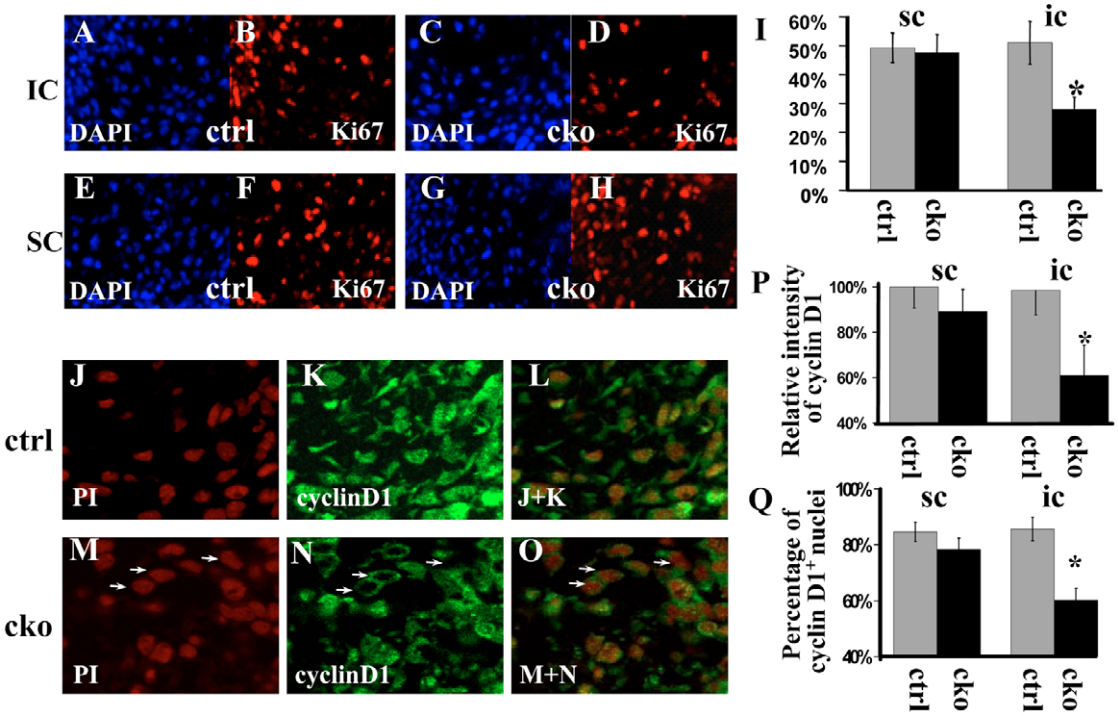

Fig. 6. Cell proliferation study of AV cushions. (**A-H**) Frontal sections of control (A,B,E,F) and mutant (C,D,G,H) embryonic hearts at E11.5 stained with DAPI to visualize all nuclei (A,C,E,G) and with anti-Ki67 antibody to identify proliferating cells (B,D,F,H). A-D and E-H show the inferior and superior AV cushions, respectively. (**I**) The number of Ki67-positive nuclei as a percentage of total nuclei. Data (mean±s.d.) were collected from four embryonic hearts of each strain, and at least 500 nuclei were counted for each heart. (**J-O**) Frontal sections of control (J-L) and mutant (M-O) embryonic hearts (E11.5) stained with propidiumiodide (J,M) and an anti-cyclin D1 antibody (K,N). Only results from inferior cushions are shown. Identical confocal microscopic conditions were used to acquire the images of control and mutant samples. (L) Merged images of control (J,K) samples. (O) Merged images of mutant (M,N) samples. Arrows in M-O indicate examples of nuclei without cyclin D1. (**P**) Relative intensity of cyclin D1 immunostaining in mutant versus control hearts. The total intensity of cyclin D1 antibody staining (green) was determined using MetaMorph 5.0, and was averaged with the number of nuclei to acquire the average intensity. The intensity of the control superior cushion was set as 100%. (**Q**) The number of cyclin D1-positive nuclei as a percentage of total nuclei. For P and Q, data (mean±s.d.) were collected from five embryonic hearts of each strain, and at least 500 nuclei were counted for each heart. **P*<0.05. cko, *Tie2cre;Tgfbr2*$^{loxp/loxp}$*;R26R*; ctrl, *Tie2cre;Tgfbr2*$^{loxp/+}$*;R26R*; IC, inferior cushion; SC, superior cushion; PI, propidiumiodide.

The absence of EMT defects in *Tie2cre;Tgfbr2*$^{loxp/loxp}$ embryos suggests that functions of Tgfβ signaling during EMT may be compensated for by other members of the Tgfβ superfamily of cytokines. A good candidate for complementing the loss of Tgfβ signaling is Bmp2 (Ma et al., 2005; Sugi et al., 2004). It has been reported that Bmp2 can induce EMT in in vitro culture assays (Sugi et al., 2004) and, furthermore, that myocardium-specific inactivation of *Bmp2* blocks EMT in mouse embryos (Ma et al., 2005). We cannot exclude the possibility that Tgfβ ligands may act through other type II receptors (such as ActRIIB; Acvr2b – Mouse Genome Informatics) when Tgfbr2 is absent in the AV cushions of live embryos, although Tgfbr2 still remains the only known type II receptor for Tgfβ signaling that has been substantiated by clear experimental evidence. The possibility that Tgfβ1-3 ligands act through other type II receptors is certainly an intriguing avenue of future investigation, but clearly beyond our current knowledge. An alternative explanation to reconcile the discrepancy between in vivo and in vitro studies is that although it is not absolutely required for EMT, *Tgfbr2* promotes survival and proliferation of mesenchymal cells, and hence very few mesenchymal cells were observed in the collagen gel system. Although we cannot fully exclude this explanation, we do not favor it for the following reason: given the dramatic difference in the number of mesenchymal cells observed in the collagen gel between control and mutant explants (>20-fold; Fig. 4), the *Tgfbr2*-mediated Tgfβ signaling would have a very potent effect on mesenchymal cell proliferation and/or survival. In our study, we did not observe increased apoptosis of AV mesenchymal cells in mutant embryos. The reduction of cell proliferation in the AV cushions of mutant embryos is relatively moderate (~50% Ki67-positive nuclei in the inferior cushions of controls versus ~30% in mutants; Fig. 6), and is limited to within the inferior cushion, whereas both inferior and superior cushions were included in a single explant in the collagen gel assay. Thus, the reduction in cell proliferation is unlikely to account for the dramatic decrease of mesenchymal cells in mutant explant cultures. Taken together, our results suggest that complementary mechanisms exist for the loss of *Tgfbr2* in living embryos to support EMT. The collagen gel culture system may be particularly useful in studying Tgfβ signaling during EMT, as compensatory pathways are not present to confound experimental results.

An unexpected result obtained from this study is that inactivation of *Tgfbr2* has different effects on proliferation of mesenchymal cells in the superior and inferior cushions, which are thought to be formed under the same genetic control. We did not observe obvious asymmetric expression of *Tgfbr2* in AV cushions (Fig. 5N-Q). It is well known that the superior cushion is adjacent to the OFT

(Mjaatvedt et al., 1999). We speculate that some morphogenic signaling molecules expressed in the OFT, such as Bmp4, Bmp6, Bmp7 and Fgf8 (Delot, 2003; Hu et al., 2004; Jiao et al., 2003; Kim et al., 2001b), can diffuse into the superior cushion to compensate for the loss of Tgfβ signaling, but are inaccessible to the inferior cushion.

This study describes a unique mouse genetic model with the DILV defect. This form of CHD accounts for the most common type of single ventricle physiology (Vanpraagh et al., 1965). The defect results from the failure to establish a direct communication between the right atrium and the RV, a crucial step for transforming the single-channeled cardiac primordia into a mature double-channeled organ. Formation of the right-chamber connection is achieved through the rightward expansion of the AVC in human embryos from 6.5 to 7 weeks (Kim et al., 2001a) – corresponding to ~E11.5-E12.5 in mice. The involvement of Tgfβ signaling in alignment between atria and ventricles was first demonstrated in a study of *Tgfb2* knockout mice; 25% of the *Tgfb2*$^{-/-}$ embryos demonstrated an overriding of the tricuspid valve (Bartram et al., 2001; Sanford et al., 1997), which may be considered as a mild phenotype in the spectrum of DILV. However, it is unclear from the above studies what is the primary defective cell population responsible for the malalignment defect. Although the development of AVC myocardium was thought to be the primary driving force for this rightward expansion (Kim et al., 2001a), the fact that endocardial but not myocardial depletion of *Tgfbr2* (Fig. 1) results in DILV demonstrates for the first time that misalignment between atria and ventricles can be primarily caused by impaired Tgfβ signaling in the valvular mesenchyme derived from endocardium. We propose that the unbalanced growth between the inferior and superior AV cushions in mutant embryos distorts the AVC, leading to aberrant cardiac looping during the second phase, and at least partially contributes to DILV. Alternatively, Tgfbr2 has distinct roles in promoting mesenchyme proliferation in the inferior AV cushion and in patterning embryonic hearts for proper looping.

In summary, in vivo tissue-specific inactivation of *Tgfbr2* identifies a novel role for *Tgfbr2* in stimulating mesenchymal cell proliferation in the inferior cushion through regulating the expression and subcellular localization of cyclin D1, which may be important for understanding the mechanisms related to the development of DILV and other pathologies involving unbalanced ventricular inflow. Our study does not support an essential role for *Tgfbr2*-mediated Tgfβ signaling either in myocardial development or during initial EMT.

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References

Alt, J. R., Cleveland, J. L., Hannink, M. and Diehl, J. A. (2000). Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. *Genes Dev.* **14**, 3102-3114.

Azhar, M., Schultz Jel, J., Grupp, I., Dorn, G. W., 2nd, Meneton, P., Molin, D. G., Gittenberger-de Groot, A. C. and Doetschman, T. (2003). Transforming growth factor beta in cardiovascular development and function. *Cytokine Growth Factor Rev.* **14**, 391-407.

Barnett, J. V. and Desgrosellier, J. S. (2003). Early events in valvulogenesis: a signaling perspective. *Birth Defects Res. Part C Embryo Today* **69**, 58-72.

Bartram, U., Molin, D. G., Wisse, L. J., Mohamad, A., Sanford, L. P., Doetschman, T., Speer, C. P., Poelmann, R. E. and Gittenberger-de Groot, A. C. (2001). Double-outlet right ventricle and overriding tricuspid valve reflect disturbances of looping, myocardialization, endocardial cushion differentiation, and apoptosis in TGF-beta(2)-knockout mice. *Circulation* **103**, 2745-2752.

Bhowmick, N. A., Chytil, A., Plieth, D., Gorska, A. E., Dumont, N., Shappell, S., Washington, M. K., Neilson, E. G. and Moses, H. L. (2004). TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* **303**, 848-851.

Boyer, A. S., Ayerinskas, I. I., Vincent, E. B., McKinney, L. A., Weeks, D. L. and Runyan, R. B. (1999). TGFbeta2 and TGFbeta3 have separate and sequential activities during epithelial-mesenchymal cell transformation in the embryonic heart. *Dev. Biol.* **208**, 530-545.

Brown, C. B., Boyer, A. S., Runyan, R. B. and Barnett, J. V. (1996). Antibodies to the Type II TGFbeta receptor block cell activation and migration during atrioventricular cushion transformation in the heart. *Dev. Biol.* **174**, 248-257.

Brown, C. B., Boyer, A. S., Runyan, R. B. and Barnett, J. V. (1999). Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart. *Science* **283**, 2080-2082.

Camenisch, T. D., Molin, D. G., Person, A., Runyan, R. B., Gittenberger-de Groot, A. C., McDonald, J. A. and Klewer, S. E. (2002). Temporal and distinct TGFbeta ligand requirements during mouse and avian endocardial cushion morphogenesis. *Dev. Biol.* **248**, 170-181.

Chytil, A., Magnuson, M. A., Wright, C. V. and Moses, H. L. (2002). Conditional inactivation of the TGF-beta type II receptor using Cre:Lox. *Genesis* **32**, 73-75.

de Caestecker, M. (2004). The transforming growth factor-beta superfamily of receptors. *Cytokine Growth Factor Rev.* **15**, 1-11.

Delot, E. C. (2003). Control of endocardial cushion and cardiac valve maturation by BMP signaling pathways. *Mol. Genet. Metab.* **80**, 27-35.

Dickson, M. C., Martin, J. S., Cousins, F. M., Kulkarni, A. B., Karlsson, S. and Akhurst, R. J. (1995). Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* **121**, 1845-1854.

Hoffman, J. I. (1995). Incidence of congenital heart disease: II. Prenatal incidence. *Pediatr. Cardiol* **16**, 155-165.

Hoffman, J. I. and Kaplan, S. (2002). The incidence of congenital heart disease. *J. Am. Coll. Cardiol.* **39**, 1890-1900.

Hu, T., Yamagishi, H., Maeda, J., McAnally, J., Yamagishi, C. and Srivastava, D. (2004). Tbx1 regulates fibroblast growth factors in the anterior heart field through a reinforcing autoregulatory loop involving forkhead transcription factors. *Development* **131**, 5491-5502.

Ito, Y., Yeo, J. Y., Chytil, A., Han, J., Bringas, P., Jr, Nakajima, A., Shuler, C. F., Moses, H. L. and Chai, Y. (2003). Conditional inactivation of Tgfbr2 in cranial neural crest causes cleft palate and calvaria defects. *Development* **130**, 5269-5280.

Jiao, K., Zhou, Y. and Hogan, B. L. (2002). Identification of mZnf8, a mouse Kruppel-like transcriptional repressor, as a novel nuclear interaction partner of Smad1. *Mol. Cell. Biol.* **22**, 7633-7644.

Jiao, K., Kulessa, H., Tompkins, K., Zhou, Y., Batts, L., Baldwin, H. S. and Hogan, B. L. (2003). An essential role of Bmp4 in the atrioventricular septation of the mouse heart. *Genes Dev.* **17**, 2362-2367.

Kaartinen, V., Voncken, J. W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N. and Groffen, J. (1995). Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat. Genet.* **11**, 415-421.

Kim, J. S., Viragh, S., Moorman, A. F., Anderson, R. H. and Lamers, W. H. (2001a). Development of the myocardium of the atrioventricular canal and the vestibular spine in the human heart. *Circ. Res.* **88**, 395-402.

Kim, R. Y., Robertson, E. J. and Solloway, M. J. (2001b). Bmp6 and Bmp7 are required for cushion formation and septation in the developing mouse heart. *Dev. Biol.* **235**, 449-466.

Koni, P. A., Joshi, S. K., Temann, U. A., Olson, D., Burkly, L. and Flavell, R. A. (2001). Conditional vascular cell adhesion molecule 1 deletion in mice: impaired lymphocyte migration to bone marrow. *J. Exp. Med.* **193**, 741-754.

Kubalak, S. W., Hutson, D. R., Scott, K. K. and Shannon, R. A. (2002). Elevated transforming growth factor beta2 enhances apoptosis and contributes to abnormal outflow tract and aortic sac development in retinoic X receptor alpha knockout embryos. *Development* **129**, 733-746.

Liebner, S., Cattelino, A., Gallini, R., Rudini, N., Iurlaro, M., Piccolo, S. and Dejana, E. (2004). Beta-catenin is required for endothelial-mesenchymal transformation during heart cushion development in the mouse. *J. Cell Biol.* **166**, 359-367.

Ma, L., Lu, M. F., Schwartz, R. J. and Martin, J. F. (2005). Bmp2 is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning. *Development* **132**, 5601-5611.

Mjaatvedt, C. H., Yamamura, H., Wessels, A., Ramsdell, A. F., Turner, D. and Markwald, R. R. (1999). Mechanisms of segmentation, septation, and remodeling of the tubular heart: endocardial cushion fate and cardiac looping. In *Heart Development* (ed. R. P. Harvey and N. Rosenthal), pp. 159-174. San Diego: Academic Press.

Nakajima, Y., Yamagishi, T., Hokari, S. and Nakamura, H. (2000). Mechanisms involved in valvuloseptal endocardial cushion formation in early cardiogenesis:

roles of transforming growth factor (TGF)-beta and bone morphogenetic protein (BMP). *Anat. Rec.* **258**, 119-127.
Oshima, M., Oshima, H. and Taketo, M. M. (1996). TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. *Dev. Biol.* **179**, 297-302.
Potts, J. D. and Runyan, R. B. (1989). Epithelial-mesenchymal cell transformation in the embryonic heart can be mediated, in part, by transforming growth factor beta. *Dev. Biol.* **134**, 392-401.
Ramsdell, A. F. and Markwald, R. R. (1997). Induction of endocardial cushion tissue in the avian heart is regulated, in part, by TGFbeta-3-mediated autocrine signaling. *Dev. Biol.* **188**, 64-74.
Sanford, L. P., Ormsby, I., Gittenberger-de Groot, A. C., Sariola, H., Friedman, R., Boivin, G. P., Cardell, E. L. and Doetschman, T. (1997). TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* **124**, 2659-2670.
Sherr, C. J. and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* **13**, 1501-1512.
Shi, Y. and Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700.
Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.
Stenvers, K. L., Tursky, M. L., Harder, K. W., Kountouri, N., Amatayakul-Chantler, S., Grail, D., Small, C., Weinberg, R. A., Sizeland, A. M. and Zhu, H. J. (2003). Heart and liver defects and reduced transforming growth factor beta2 sensitivity in transforming growth factor beta type III receptor-deficient embryos. *Mol. Cell. Biol.* **23**, 4371-4385.
Sugi, Y., Yamamura, H., Okagawa, H. and Markwald, R. R. (2004). Bone morphogenetic protein-2 can mediate myocardial regulation of atrioventricular cushion mesenchymal cell formation in mice. *Dev. Biol.* **269**, 505-518.
Sumrejkanchanakij, P., Tamamori-Adachi, M., Matsunaga, Y., Eto, K. and Ikeda, M. A. (2003). Role of cyclin D1 cytoplasmic sequestration in the survival of postmitotic neurons. *Oncogene* **22**, 8723-8730.
Tamamori-Adachi, M., Ito, H., Sumrejkanchanakij, P., Adachi, S., Hiroe, M., Shimizu, M., Kawauchi, J., Sunamori, M., Marumo, F., Kitajima, S. et al. (2003). Critical role of cyclin D1 nuclear import in cardiomyocyte proliferation. *Circ. Res.* **92**, e12-e19.
Tang, Y., McKinnon, M. L., Leong, L. M., Rusholme, S. A., Wang, S. and Akhurst, R. J. (2003). Genetic modifiers interact with maternal determinants in vascular development of Tgfb1(–/–) mice. *Hum. Mol. Genet.* **12**, 1579-1589.
Vanpraagh, R., Vanpraagh, S., Vlad, P. and Keith, J. D. (1965). Diagnosis of the anatomic types of single or common ventricle. *Am. J. Cardiol.* **15**, 345-366.