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Developmental Biology 286 (2005) 299 – 310

DEVELOPMENTAL
BIOLOGYwww.elsevier.com/locate/ydbio

Atrioventricular cushion transformation is mediated by ALK2 in the developing mouse heart

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Received for publication 29 March 2005, revised 29 July 2005, accepted 31 July 2005

Available online 2 September 2005

Abstract

Developmental abnormalities in endocardial cushions frequently contribute to congenital heart malformations including septal and valvular defects. While compelling evidence has been presented to demonstrate that members of the TGF- β superfamily are capable of inducing endothelial-to-mesenchymal transdifferentiation in the atrioventricular canal, and thus play a key role in formation of endocardial cushions, the detailed signaling mechanisms of this important developmental process, especially in vivo, are still poorly known. Several type I receptors (ALKs) for members of the TGF- β superfamily are expressed in the myocardium and endocardium of the developing heart, including the atrioventricular canal. However, analysis of their functional role during mammalian development has been significantly complicated by the fact that deletion of the type I receptors in mouse embryos often leads to early embryonal lethality. Here, we used the *Cre/loxP* system for endothelial-specific deletion of the type I receptor *Alk2* in mouse embryos. The endothelial-specific *Alk2* mutant mice display defects in atrioventricular septa and valves, which result from a failure of endocardial cells to appropriately transdifferentiate into the mesenchyme in the AV canal. Endocardial cells deficient in *Alk2* demonstrate decreased expression of *Msx1* and *Snail*, and reduced phosphorylation of BMP and TGF- β Smads. Moreover, we show that endocardial cells lacking *Alk2* fail to delaminate from AV canal explants. Collectively, these results indicate that the BMP type I receptor ALK2 in endothelial cells plays a critical non-redundant role in early phases of endocardial cushion formation during cardiac morphogenesis.

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Keywords: Atrioventricular cushion; ALK2; BMP; TGF- β ; Transformation; Cardiac development

Introduction

Cardiovascular malformations are the most common life-threatening birth defects in humans, affecting approximately one in one hundred live births (Srivastava, 2001; Hoffman and Kaplan, 2002). The etiology of these conditions is still very poorly known, but it often appears to be multi-factorial, involving both environmental and genetic causes. Among

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cardiac deformities, atrioventricular (AV) septal defects are very frequent (Schroeder et al., 2003). They arise from a failure of endocardial cushions to fuse, which leads to a range of secondary abnormalities, including incomplete formation of the septum between atria and ventricles and malformation of the AV valves. AV cushion formation is initiated by factors that induce endothelial-to-mesenchymal transdifferentiation (EMT) (Eisenberg and Markwald, 1995), and indeed, it has been shown that the vast majority of cushion mesenchymal cells are derived from the endothelium (Kisanuki et al., 2001; Anderson et al., 2003; de Lange et al., 2004). Subsequent morphogenetic events lead to elongation, outgrowth and remodeling of cushions to form mature septa and delicate thin valve leaflets. During EMT, endothelial cells delaminate from the endocardium into the underlying acellular hyaluronate-rich substance called cardiac jelly that separates endocardial and myocardial layers, and transform into cushion mesenchymal cells. Co-culture experiments have shown that signals from both the adjacent myocardium and from the extracellular matrix of the cardiac jelly play a critical role in the endocardial cell EMT (Eisenberg and Markwald, 1995).

Many soluble growth factors, transmembrane receptors, intracellular signaling molecules, nuclear factors and glycosaminoglycans are known to induce EMT. Initially, it was shown that members of the TGF- β superfamily are able to induce EMT in the chick during cardiac morphogenesis (Potts and Runyan, 1989; Eisenberg and Markwald, 1995). The Wnt/ β -catenin pathway was shown to be required for EMT, first in zebrafish (Hurlstone et al., 2003) and subsequently also in mice (Liebner et al., 2004), while recent studies suggest that Notch signaling plays a role upstream of TGF- β s during murine endocardial cushion transformation (Timmerman et al., 2004). Normal cushion tissue formation has also been shown to be controlled by regulators of the Ras signaling pathway (Lakkis and Epstein, 1998; Camenisch et al., 2002b; Gitler et al., 2003b). Recently, dynamic temporal and spatial changes in calcineurin signaling and NFATc1/VEGF expression were demonstrated to play key roles in endocardial EMT and in valve development (Chang et al., 2004). However, detailed signaling mechanisms as well as crosstalk between different signaling pathways remain largely unknown.

Several studies using a three-dimensional explant culture model in conjunction with antisense oligonucleotides and neutralizing antibodies have shown that TGF- β s, particularly TGF- β 2 and -3, as well as BMPs are essential growth factors for initiation and regulation of EMT (Potts and Runyan, 1989; Potts et al., 1991; Runyan et al., 1992; Markwald et al., 1996; Nakajima et al., 2000). In addition, studies using neutralizing antibodies in the chick explant culture model have suggested that the prototypical TGF- β type I receptor, ChALK5, is not involved in the cushion EMT, while inhibition of a related type I receptor, ChALK2, prevents the transdifferentiation (Lai et al., 2000). Subsequently, it was shown that constitutively active ALK2

alone is sufficient to stimulate EMT (Desgrosellier et al., 2005). Mice deficient both in BMP5 and BMP7 display defective cushion development (Solloway and Robertson, 1999), and it was recently reported that a tissue-specific deletion of the BMP type I receptor *Alk3* in myocardial cells leads to hypoplastic endocardial cushions and decreased *Tgf- β 2* expression (Gaussin et al., 2002). In fact, mice deficient in SMAD6, an inhibitory intracellular signal mediator of TGF- β s, display endocardial cushion hyperplasia (Galvin et al., 2000). However, single TGF- β null mutants do not demonstrate impaired endocardial EMT (Shull et al., 1992; Kulkarni et al., 1993; Kaartinen et al., 1995; Proetzel et al., 1995; Sanford et al., 1997), while mice deficient in TGF- β 2 display defects in later stages of valve remodeling (Sanford et al., 1997; Bartram et al., 2001).

TGF- β superfamily members signal via heteromeric receptor complexes composed of two type II and two type I receptors. Different receptor complexes have been shown to bind TGF- β ligands with different affinities, which determine downstream signaling responses (Derynck and Feng, 1997). For instance, TGF- β s typically bind to a receptor complex composed of the TGF- β type II receptor and ALK5, while BMPs regularly signal via BMP type II receptor and ALK3 (Massague and Chen, 2000). The type I receptor ALK2 has been shown to display a more restricted signaling specificity for BMPs 5, -6 and -7 (Macias-Silva et al., 1998). However, it has been suggested that ALK2 might mediate TGF- β signals in specific cell types in vitro, such as mouse mammary epithelial cells (Miettinen et al., 1994).

Studies to unravel the in vivo roles of several BMP ligands and their receptors in mammals have often been hampered by the fact that mice deficient in *Bmps 2* or -4, as well as mice deficient in type I receptors (*Alk2* and *Alk3*) die at or immediately after gastrulation, before cardiac morphogenesis (Winnier et al., 1995; Mishina et al., 1995; Zhang and Bradley, 1996; Gu et al., 1999; Mishina et al., 1999). To define the role of *Alk2* in endocardial cell transformation in vivo, we used the *Tie2-Cre* transgenic mouse line to target *Cre* recombinase into endothelial cells (Kisanuki et al., 2001; Koni et al., 2001). *Tie2-Cre* transgenic mice heterozygous for the *Alk2* knockout allele were subsequently crossed with homozygous *Alk2^{Fx/Fx}* mice (Kaartinen and Nagy, 2001; Kaartinen et al., 2004; Dudas et al., 2004). The resulting *Alk2/Tie2-Cre* mutants display AV and ventricular septal defects, and show that ALK2 is required for successful endothelial cell to mesenchyme transdifferentiation in AV cushions.

Materials and methods

Mice and genotyping

Mice homozygous for the *Alk2^{Fx}* allele and mice heterozygous for the *Alk2^{KO}* allele were generated and genotyped as described previously (Kaartinen and Nagy,

2001; Kaartinen et al., 2004; Dudas et al., 2004). *Tie2-Cre* and Rosa26R Cre (*R26R*) reporter mice were obtained from the Jackson Labs and genotyped by PCR (for detailed protocols see: <http://www.jax.org>). All mice were maintained on mixed genetic backgrounds. All studies were carried out at the Animal Care Facility of the Saban Research Institute of Childrens Hospital Los Angeles in accordance with institutional guidelines.

Histological analyses

Embryonic tissues were fixed with 4% buffered formaldehyde for 2–12 h, dehydrated and embedded in paraffin. Sections (5 μ m) were stained with hematoxylin and eosin. For cryostat sectioning, tissues were fixed in 4% buffered formaldehyde and equilibrated in 30% sucrose prior to freezing in HistoPrep (Fisher). Frozen sections were cut at 8 μ m. Embryos or sections were stained for β -galactosidase activity as described (Hogan et al., 1994). Briefly, the specimens were fixed in 4% buffered formaldehyde for 30 min at room temperature, washed 3 times for 10 min in the detergent wash and developed for 2 to 6 h in the X-gal staining solution.

Expression studies

Wholemound and section in situ hybridization on embryos was carried out as described (Hogan et al., 1994; Moorman et al., 2001). Probes specific for *Msx1* (Furuta et al., 1997), *Snail* (Cano et al., 2000), *Hey2* (Donovan et al., 2002), *Pdgfra* (Gitler et al., 2003a) and *Tgf- β 2* (Blavier et al., 2001) were used. To analyze phosphorylation of Smads, de-paraffinized sections were boiled for 10 min in 10 mM citrate buffer, pH 6.0 in a pressure cooker and immunostained using antibodies specific for phospho-Smad-1/5/8 or phospho-Smad-2 (Cell signaling). For immunohistochemistry, frozen sections were stained with monoclonal antibodies to CD31 (Sigma) or to NFATc1 (Developmental Studies Hybridoma Bank at the University of Iowa) according to standard procedures and counterstained with DAPI (Harlow and Lane, 1988).

Apoptosis and cell proliferation

Apoptotic cells were detected using the DeadEnd Fluorometric TUNEL system (Promega). Cell proliferation was analyzed using the BrdU incorporation assay (Zymed) or by immunostaining for phosphohistone H3 (Cell signaling).

Explant cultures

Collagen gels (1 mg/ml, type I rat tail collagen from BD) were prepared in OptiMEM supplemented with 1% fetal calf serum, 1 \times ITS (insulin, transferrin and selenium) and penicillin/streptomycin (1 \times) all from Invitrogen (Sugi et al., 2004). AV regions of the hearts were dissected from E10

embryos, cut longitudinally to expose the lumen and placed on the collagen gels. Additional media were added to the cultures 2 h later and incubation was continued under standard tissue culture conditions (37°C, 100% humidity, 8% CO₂). For some cultures, TGF- β 3 (10 ng/ml, Sigma) or BMP2 (50 ng/ml, Sigma) was added to the culture media.

Results

Alk2 is expressed in endothelial cells in the mouse AV canal

It has previously been shown that the *Alk1* gene (encoding the TGF- β /BMP type I receptor) is expressed in endothelial cells and that ALK1 mediates TGF- β signaling in concert with the prototypical TGF- β type I receptor ALK5 during the proliferative phase of angiogenesis (Goumans et al., 2003). Moreover, it has been suggested that in chick the type I receptor ALK2, which is closely related to ALK1, is involved in formation of AV-cushions during cardiac development by functioning in endothelial-to-mesenchymal transformation (Lai et al., 2000; Desgrosellier et al., 2005). Therefore, we were interested in whether ALK2 would also be involved in endothelial development in mammals, particularly during cardiac morphogenesis. As a first step, we analyzed expression of *Alk2* in the AV-canal at embryonic day 10 (E10), when the cushions are forming (Fig. 1A). *Alk2* mRNA was detected in endocardial cells, as well as in some underlying mesenchymal cells, as previously described by Gu et al. (1999). To analyze the function of *Alk2* in mammalian endothelial cells of the AV canal, we used the tissue-specific *Cre/loxP* gene targeting strategy. It has previously been shown that the *Tie2* promoter/enhancer will efficiently target transgene expression to the endothelium, and that mice expressing Cre recombinase under the control of the *Tie2* promoter can be used to abrogate a gene of interest efficiently and specifically in the endothelium (Kisanuki et al., 2001; Koni et al., 2001). To verify that this is indeed the case in the transgenic line that we opted to use (Koni et al., 2001), we followed the fate of cells recombined by the *Tie2-Cre* transgene by using the *ROSA26 Cre* reporter (*R26R*) assay as previously reported (Soriano, 1999; Chai et al., 2000; Jiang et al., 2000). The double transgenic embryos (*Tie2-Cre*^{+/-}; *R26R*^{+/-}) were harvested at E12, and stained for β -galactosidase activity. Positive staining was seen specifically in endothelial cells (Fig. 1B) and in mesenchymal cells derived from the endocardium, e.g., in the mesenchyme of endocardial cushions (Fig. 1C) confirming that this *Tie2-Cre* transgenic line could efficiently be used to induce recombination in the endothelial cell lineage. Therefore, we crossed the *Tie2-Cre* mice that also were heterozygous for the *Alk2* knockout allele with mice homozygous for the floxed *Alk2* (*Alk2*^{FX/FX}) allele (Kaartinen and Nagy, 2001; Kaartinen et al., 2004; Dudas et al., 2004). Efficiency of the *Cre*-induced recombination in the AV canal at E10 was verified by using the RT-PCR strategy as previously

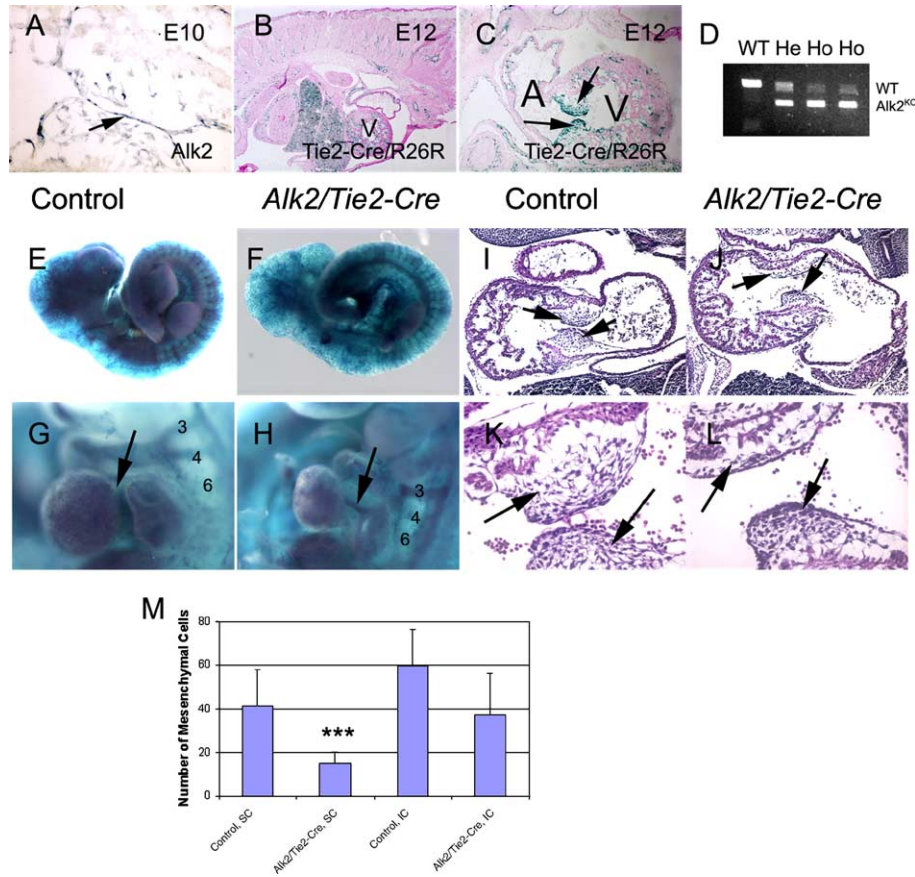


Fig. 1. Targeted deletion of *Alk2* in the endothelium. (A) *Alk2* is expressed in endocardial cushions at E10 (arrow points to the positive in situ hybridization signal [blue] in endothelial cells). (B–C) The *Tie2-Cre* transgene specifically induces recombination in endothelial cells (B) and in cells derived from them, i.e., in the mesenchyme of endocardial cushions (arrows in panel C) as demonstrated using *R26R* reporter assay and staining for β -galactosidase activity (Blue stain), counter stained with eosin. (D) The RT-PCR analysis of mRNA isolated from the AV-canal tissues at E10.0 in wild-type (WT = *Tie2-Cre*^{+/-}; *Alk2*^{WT/WT}), heterozygote (He = *Tie2-Cre*^{+/-}; *Alk2*^{KO/WT}) and homozygote (Ho = *Tie2-Cre*^{+/-}; *Alk2*^{FX/KO}) mutant embryos. Shorter amplification product (284 bp) was detected in heterozygote and homozygote embryos, confirming a successful recombination event in targeted tissues resulting in a truncated allele. (E–H) Wholemount staining of control (*Tie2-Cre*^{+/-}; *Alk2*^{FX/WT}; *R26R*^{+/-}) and *Alk2/Tie2-Cre* mutant (*Tie2-Cre*^{+/-}; *Alk2*^{FX/KO}; *R26R*^{+/-}) embryos for β -galactosidase activity (blue color); *R26R* lineage tracing assay. Comparable reporter activity can be seen in mutants (F, H) and controls (E, G), including the AV canal (arrows, G and H). Numbers (3, 4, 6) in panels G and H depict the aortic arch arteries. (I–L) Endocardial cushions (arrowheads in panels I–L) form in *Alk2/Tie2-Cre* mutants (J, L), but appear considerably smaller than in controls at E10.5 (I, K). (M) Quantification of mesenchymal cells in AV cushions at E10.5; mean \pm SD; **P* < 0.01 (*n* = 8, total number of mesenchymal cells were counted per parasagittal section; 3 sections analyzed per sample). A, Atrium; V, ventricle. Magnification: A, $\times 20$; B, $\times 5$; C, $\times 10$; D–F, $\times 3$; G–H, $\times 7$; I–J, $\times 10$; K–L, $\times 40$.

described (Dudas et al., 2004). This analysis showed that the *Alk2* gene was effectively recombined by the *Tie2-Cre* transgene (Fig. 1D). To compare the overall vascular phenotype between controls and *Alk2/Tie2-Cre* mutants early in development, we crossed *Alk2*^{KO/WT}; *Tie2-Cre* males with females that were homozygous both for the *Alk*^{FX} and *R26R* alleles (*Alk2*^{FX/FX}; *R26R*^{+/+}), harvested embryos at E9.5 and stained them for β -galactosidase activity (Figs. 1E–H). Blood vessels were strongly stained both in controls and mutants demonstrating that vascular development was not significantly affected by the loss of *Alk2* activity in endothelial cells.

Alk2/Tie2-Cre mutants display AV-canal defects

Genotype analysis of embryos from *Alk2*^{KO/WT}/*Tie2-Cre*^{+/-} \times *Alk2*^{FX/FX} crosses at E14.5 revealed that only

10% instead of the expected 25% of *Alk2/Tie2-Cre* mutants could be recovered at that time point (Table 1). In histological sections at E10, the endocardial cushions appeared smaller in mutants than in controls (Figs. 1I–L). A dramatic reduction in mesenchymal cell number was particularly evident in the superior cushion and differed significantly from that of controls (*P* < 0.01) (Fig. 1M).

Table 1

Genotype distribution of embryos from *Alk2*^{KO}; *Tie2-Cre*^{+/-} \times *Alk2*^{FX/FX} crosses

Genotype	E10–E11 (<i>n</i> = 307)	E14–E15 (<i>n</i> = 97)
<i>Alk2</i> ^{KO} / <i>Tie2-Cre</i> ⁺	89 (22.5%)	11 (10.2%)
<i>Alk2</i> ^{WT} / <i>Tie2-Cre</i> ⁺	108 (27.3%)	28 (28.8%)
<i>Alk2</i> ^{KO} / <i>Tie2-Cre</i> ⁻ and <i>Alk2</i> ^{WT} / <i>Tie2-Cre</i> ⁻	199 (50.2%)	58 (59.8%)

At E14.5, the surviving *Alk2/Tie2-Cre* embryos ($n = 17$) showed a range of cardiac defects (Fig. 2). Most of them (15 out of 17 studied) had a ventricular septal defect, of varying severity, where the secondary ventricular foramen has not closed. In addition, 11 had defective AV septation and valve development. The AV cushions were of variable size, shape and degree of fusion with one another, or with other septal structures. For example, the primary atrial foramen was still patent, allowing blood to shunt between right and left atria in 5 embryos. Outgrowth and formation of AV leaflets were also variable. In some, cushion tissue was sparse or absent altogether from most of the AV junction, especially on the right, although prominent trabecular structures, perhaps those that would normally support the developing leaflet cushions, were still evident. In contrast, outflow tract (OFT) septation and OFT leaflet formation appeared normal (Fig. 2 and data not shown).

Alk2 is not required in cardiac myocytes during heart organogenesis

Although our expression studies suggest that *Alk2* is not strongly expressed in the myocardium, we could not exclude the possibility that low level *Alk2* expression would still play a critical role in this cell type. Therefore, we deleted *Alk2* in the myocardium by using the cardiac

myocyte-specific *Cre* mouse line (α MHC-*Cre*) as previously described (Gaussin et al., 2002). *Alk2/\alpha*MHC-*Cre* mice are viable and failed to display any detectable altered cardiac phenotype (Fig. 2) demonstrating, that unlike *Alk3* (Gaussin et al., 2002), expression of *Alk2* in cardiac myocytes is not required during this developmental stage.

Endothelial cells deficient in Alk2 fail to populate the mesenchyme of the AV cushions in vivo

To follow the fate of *Alk2*-deficient endothelial cells during AV canal development, we applied the *R26R* reporter assay described above. Analysis of parasagittal sections of control embryos (at E10–E12) revealed that both the endocardium as well as the underlying mesenchyme of AV cushions stained positive for the β -galactosidase activity (Figs. 3A, E, G). In contrast, the mesenchyme of mutant AV cushions displayed only a few positively staining cells, while AV canal endocardial cells deficient in *Alk2* demonstrated strong positive β -galactosidase staining (Figs. 3B, F, H). Comparison of the cardiac phenotype between *Alk2/Tie2-Cre* and *Alk2/Tie2-Cre;R26R* mutants did not reveal any histological differences between these two backgrounds (data not shown). Consistent with the histological studies (Fig. 2), the cushions of *Alk2* mutants were overall smaller and less

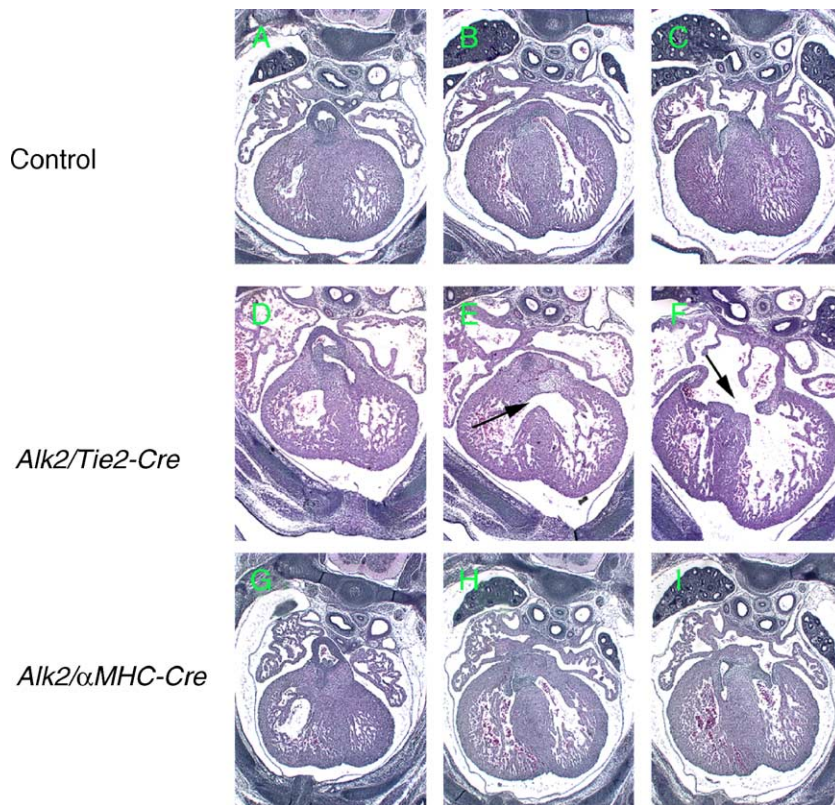


Fig. 2. Tissue-specific abrogation of *Alk2* in endothelial cells leads to septal and valvular defects. Transverse sections on 3 different levels from rostral to caudal in a control (A–C) and mutant (D–F) at E14.5. Defects can be seen both in ventricular (arrow in panel E) and atrioventricular (arrows in panel F) septation, as well as in AV valves (arrowhead in panel F). In contrast, tissue-specific abrogation of *Alk2* in cardiac myocytes does not impair cardiac development (G–I) at E14. Sections on comparable levels are shown. Magnification: $\times 5$.

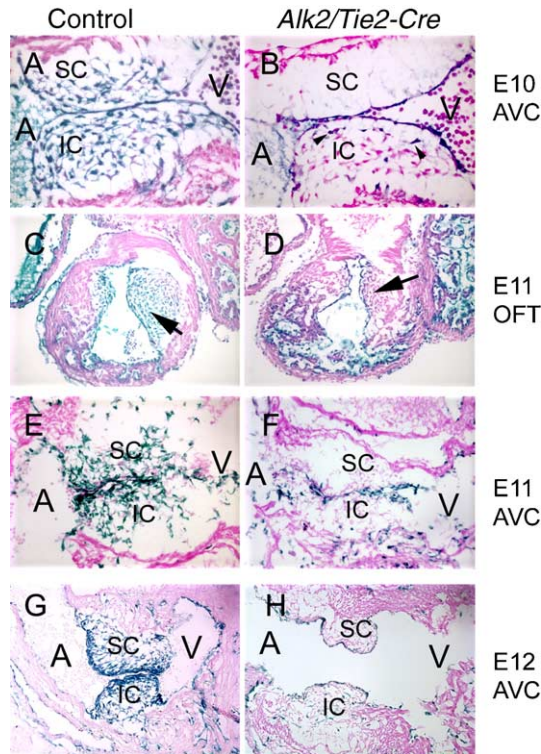


Fig. 3. Fate mapping of endothelial cells in *Alk2/Tie2-Cre* mutants. While endothelial cells covering endocardial AV-cushions stain positive both in controls (A, E, G) and *Alk2/Tie2-Cre* mutants (B, F, H) in the *R26R* reporter assay, only the wild-type mesenchyme demonstrates a large number of positively staining cells (parasagittal sections; arrowheads in panel B point to a few positively staining cells seen in the mesenchyme of *Alk2* mutants). In controls, the proximal outflow tract mesenchyme displayed a large number of positively staining cells at E11 (arrow in panel C), while in mutants, no positively staining cells could be seen (arrow in panel D) (C–D, transverse sections). In older mutant embryos, the AV canal mesenchyme displayed a larger number of cells, but still only a few of them stained positive in the lineage tracing assay (F and H), while about 90% of the mutant mesenchyme demonstrated positive reporter activity (E and G). (A–D) Samples (A–B, E10; C–D, E11) were harvested and stained as whole mounts and sectioned after post-fixation. (E–H) Samples (E–F, E11; G–H; E12) were cryostat sectioned, and subsequently frozen sections were stained for β -galactosidase activity. SC, superior endocardial cushion; IC, inferior endocardial cushion. A, atrium; V, ventricle. Magnification: A–B: $\times 40$; E–F: $\times 20$; C–D, G–H: $\times 10$.

cellular than those of controls (Figs. 3A,B). In addition to AV canal cushions, we also examined the contribution of the endocardially derived cells in the OFT mesenchyme. While mesenchymal cells of the proximal outflow tract from control embryos stained strongly positive for β -galactosidase at E11.0 (Fig. 3C) as previously reported (Kisanuki et al., 2001), no corresponding positively staining cells could be seen in the mesenchyme of *Alk2/Tie2-Cre* mutants (Fig. 3D). At E14, the mesenchyme of both the AV septum and AV valves was populated by positively staining cells derived from the endothelium in controls (Fig. 4). In contrast, the corresponding mesenchymal structures in *Alk2* mutants showed only a few positively staining cells. In addition, many mutants showed abnormal endocardial thickenings (Fig. 4F). Consistent with the histological analyses,

Alk2/Tie2-Cre mutants displayed ventricular septal defects (Fig. 4).

Altered gene expression and Smad activation in the AV canal of Alk2/Tie2-Cre mutants

The homeodomain protein *Msx1* is a well-known effector of BMP signaling (Suzuki et al., 1997), and its expression has been shown to be induced by BMPs (Bei and Maas, 1998). Moreover, it has been demonstrated that the *Msx1* gene is expressed in AV canal endocardial cells during EMT (Gitler et al., 2003a). Therefore, we investigated whether abrogation of *Alk2* in endothelial cells could influence *Msx1* expression. First, we isolated total RNA from the AV canal tissues harvested at E10 and analyzed them by RT-PCR. *Msx1* expression was clearly attenuated in *Alk2/Tie2-Cre* mutants when compared to controls (Fig. 5AA). Next, we compared the localization of *Msx1* mRNA by section in situ hybridization. In controls, *Msx1* was strongly expressed in endocardial cells in the AV canal as well as in the cushion mesenchyme at E10 (Fig. 5A). In contrast, in *Alk2/Tie2-Cre* mutants, the endothelium of the superior AV cushion did not display any detectable staining, and there were only a few positively staining cells in the AV-cushion mesenchyme (Fig. 5B). Similarly, a transcriptional repressor *Snail* has been shown to play a key role in EMT (Savagner et al., 1997; Batlle et al., 2000; Cano et al., 2000; Timmerman et al., 2004). In the control AV-canal, the pattern of *Snail* expression was very similar to that of *Msx1*:

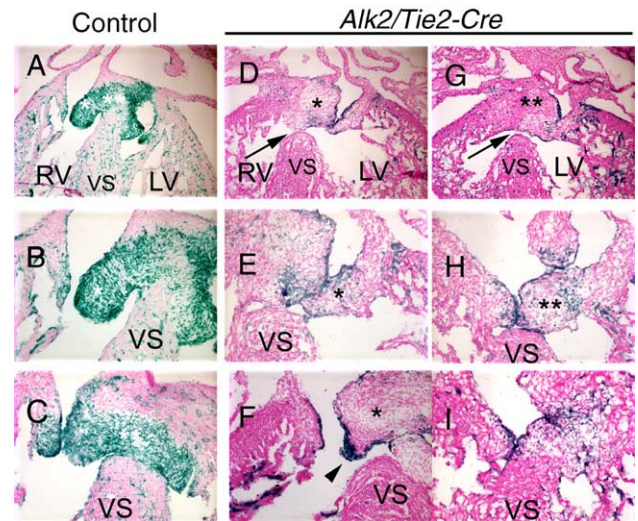


Fig. 4. Endothelial cells deficient in ALK2 fail to populate the valvular and septal mesenchyme. In controls (A–C), both the membrane portion of the interventricular septum and the valve mesenchyme are composed of cells derived from the endothelium (blue, denoted with white asterisks), while *Alk2/Tie2-Cre* mutants (D–I) do not demonstrate similar mesenchymal staining in corresponding locations (black asterisks). Arrows in panels D and G point to the ventricular septal defect. Arrowhead in panel F points to the abnormal epithelial thickening in a mutant. VS, ventricular septum; RV, right ventricle; LV, left ventricle. Magnification: A, D–G: $\times 10$; B–C, E–I: $\times 20$.

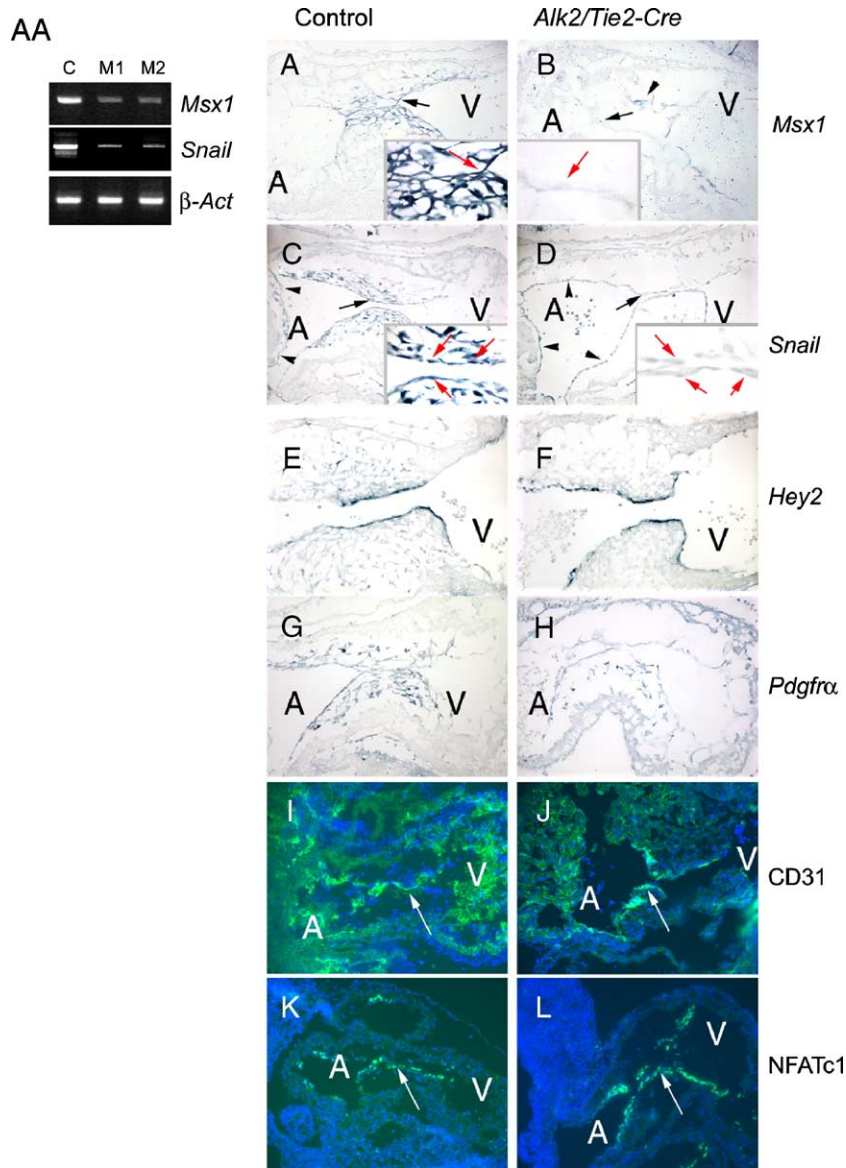


Fig. 5. *Msx1* and *Snail* are differentially expressed in *Alk2/Tie2-Cre* mutants. (AA) Semi-quantitative RT-PCR analysis of *Msx1* and *Snail* expression in the AV canal at E10; C, control, M1 and M2, two different mutant samples. β -actin was used as a quality and loading control (β -Act). *Msx1* is strongly expressed both in the endothelium (arrow) and in the underlying mesenchyme in controls (A). In *Alk2/Tie2-Cre* mutants (B), the AV canal endothelium shows no positive signal (arrow). Only a few positively staining cells can be seen in the mesenchyme (arrowhead). In controls, the transcriptional repressor *Snail* is expressed in both the AV canal endothelium (arrow), mesenchyme and in the atrial endothelium (arrowhead in panel C). Similar to controls, *Snail* is expressed in the mutant atrial endocardium (arrowheads in panel D), while the expression in the AV canal endothelium is notably reduced (arrow in panel D). Insets in panels A–D show the high magnification images ($\times 63$); red arrows depict the endocardial cells. Endocardial expression of *Hey2* (E–F) and *Pdgfra* (G–H) is comparable between controls (E, G) and mutants (F, H). Both control (I, K) and *Alk2/Tie2-Cre* mutant (J, L) samples display similar expression patterns of endothelial cell-specific proteins, CD31 and NFATc1. Parasagittal sections of either E10 hearts (A–D and G–K) or E11 hearts (E–F). A, atrium; V, ventricle. A–H, section in situ hybridization; I–L, immunohistochemistry. Magnification: $\times 20$.

strong expression both in the AV-canal endocardium and in the underlying mesenchyme (Fig. 5C). Moreover, *Snail* mRNA was detected in atrial endocardial cells, but not in those of the ventricles. Similar to controls, in *Alk2/Tie2-Cre* mutants, *Snail* was expressed in the atrial, but not in the ventricular endocardium. However, in the AV canal endothelium, expression of *Snail* was notably reduced when compared to controls or to the level of atrial expression in the same section (Fig. 5D). Recent studies have indicated

that *Notch* signaling promotes endocardial cell transformation (Timmerman et al., 2004). Because a basic helix–loop–helix transcription factor *Hey2* is an important mediator of *Notch* signaling (Donovan et al., 2002; Sakata et al., 2002), we compared the *Hey2* expression in *Alk2/Tie2-Cre* mutants and controls. We found that in both control and mutant samples *Hey2* was strongly expressed in the AV canal endocardium (Figs. 5E, F). In concordance with the earlier published studies, positive expression was

also found throughout the ventricular myocardium and the OFT endothelium (data not shown) (Donovan et al., 2002). In addition, we analyzed expression of *Pdgfra*, *Msx2*, *Slug* and *ErbB2*, all genes with a presumed role in the AV canal development; none of these genes displayed detectable differences in expression between controls and *Alk2/Tie2-Cre* mutants (Figs. 5G, H and data not shown).

To exclude the possibility that the loss of *Alk2* in the AV canal endocardium would cause a developmental delay in endocardial cell maturation, which would subsequently disturb cushion formation, we compared expression of CD31 (a well established endothelial cell marker) and NFATc1 (previously shown to be strongly and specifically expressed in endothelial cells of AV cushions at the time when the cushion mesenchyme is formed) (Ranger et al., 1998; de la Pompa et al., 1998), in the AV canal at E10 between controls and mutants. As can be seen in Figs. 5I–L, the expression of both of these markers was comparable between the genotypes suggesting that delayed endothelial maturation of the AV canal was not responsible for the observed phenotypes.

Endothelial cells deficient in Alk2 fail to transdifferentiate in vitro

AV endocardial explants from both mouse and chick embryos have been shown to transdifferentiate in vitro,

when placed on three-dimensional collagen gels (Bernanke and Markwald, 1984; Sugi and Markwald, 1996; Yamamura et al., 1997). We used this culture system and isolated AV canal tissues from controls and *Alk2/Tie2-Cre* mutants at E10.0. After dissection, explants were placed, endocardium down, onto collagen gels, and incubated under standard culture conditions for up to 50 h. The explants attached tightly on the gel and started to beat. After 24 h in culture, a large number of cells had migrated into the collagen gel from control explants (Fig. 6). About 90% of these cells showed a typical elongated fibroblastoid phenotype, while less than 10% of cells were of an intermediate rounded phenotype. These round cells presumably represent a population that is activated, but does not transdifferentiate, as seen in explants treated with the γ -secretase inhibitor, DAPT (Timmerman et al., 2004). In contrast, the number of migrating fibroblastoid cells in *Alk2* mutants was reduced to about 10% of that of controls. Exogenous addition of BMP2 (50 ng/ml) or TGF- β 3 (10 ng/ml) could not rescue the migration defect of *Alk2/Tie2-Cre* mutants (Fig. 6G).

Activation of BMP and TGF- β Smads is reduced in AV canal endocardial cells of Alk2/Tie2-Cre mutant mice

To establish how loss of *Alk2* affects downstream signaling, we examined phosphorylation of both BMP and TGF- β Smads using antibodies that specifically recognize

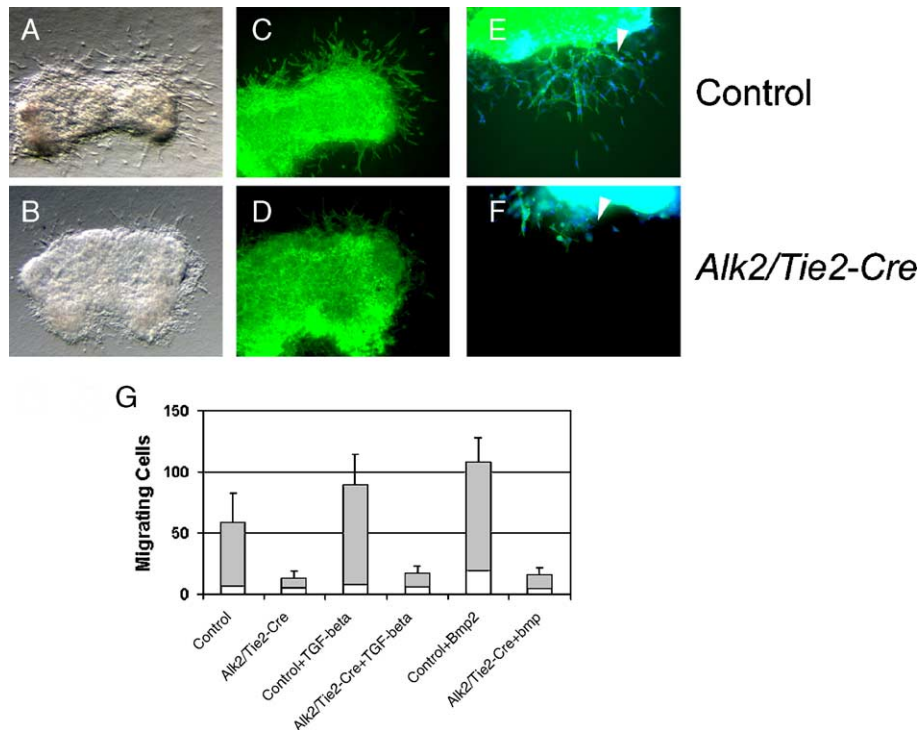


Fig. 6. Endothelial cells deficient in *Alk2* fail to transdifferentiate in vitro. (A–F) AV-canal explants were harvested at E10 and cultured for 24 h on 3-D collagen gels. Phase contrast images of control (A) and *Alk2/Tie2-Cre* (B) explants. (C–D) FITC-phalloidin staining ($\times 10$) and (E–F) FITC-Phalloidin staining ($\times 20$); counterstaining with DAPI. Transformed cells invading the collagen gel are indicated by arrowheads. The number of transformed cells is significantly reduced in *Alk2/Tie2-Cre* mutants when compared to controls. (G) Quantitative analysis of explants. Treatment of mutant explants with TGF- β 3 (10 ng/ml) or BMP2 (50 ng/ml) stimulated transdifferentiation of endothelial cells in control explants, but was not able to rescue the *Alk2*-mutant phenotype.

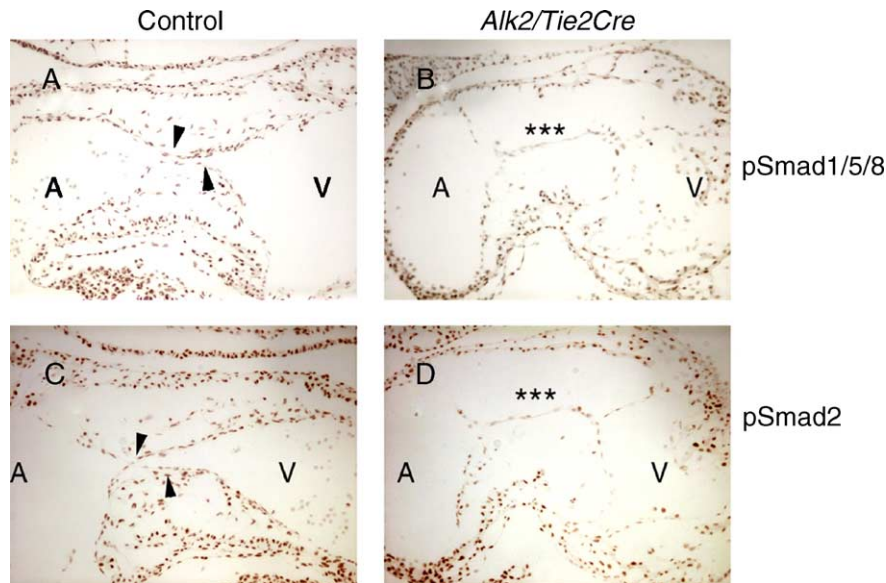


Fig. 7. Smad activation is affected in *Alk2/Tie2-Cre* mutants. Both Smad1/5/8 phosphorylation (A–B) and Smad2 (C–D) phosphorylation are notably reduced in the AV-canal endocardium of *Alk2/Tie2-Cre* mutants (***) at E10 when compared to those of controls (arrowheads in panels A and C). A, atrium; V, ventricle. Magnification: $\times 20$.

their phosphorylated, active forms. While the myocardium displayed a large number of the nuclei staining positive for phospho-Smad 1/5/8 both in controls and in mutants, the AV canal endocardium demonstrated positive staining only in controls (Figs. 7A–B). Surprisingly, the staining pattern of phospho-Smad2 was also affected in *Alk2* mutants when compared to controls (Figs. 7C–D). These findings suggest that loss of *Alk2* in AV canal endocardial cells affects activation not only of BMP Smads, which are directly phosphorylated by ALK2, but also that of related TGF- β Smads.

Discussion

Formation of endocardial cushions during cardiac morphogenesis is required for normal septal and valvular morphogenesis. A critical step in this process is transformation of endocardial cells into invasive mesenchymal cells (EMT). As outlined above, the key role of TGF- β superfamily member signaling in induction of EMT is well established, particularly in chick models. However, substantial interspecies differences have been detected in cell signaling between avian and mouse AV canal explants (Camenisch et al., 2002a), and very little is known about differential roles of type I receptors in mammalian endocardial cells. To better elucidate individual functions of the type I receptors during mouse AV canal morphogenesis, we deleted the *Alk2* gene specifically in endothelial cells. These *Alk2/Tie2-Cre* mice demonstrate severe cardiac defects in structures derived from endocardial cushions.

Our in vivo lineage tracing experiments demonstrated that in *Alk2/Tie2-Cre* mutants endothelial cells fail to

transdifferentiate to the mesenchyme. While at E10 the mutant cushions were noticeably smaller and less cellular than those of controls, at later time points, mutant cushions contained a surprising number of mesenchymal cells that did not stain positive for the β -galactosidase activity in a lineage tracing assay. This could indicate that, in *Alk2/Tie2-Cre* mutants, other cell types, e.g., epicardial cells, which have been implicated in formation of the endocardial cushion mesenchyme (Gittenberger-de Groot et al., 1998), can substitute for endothelial cells formation of the mesenchyme. We also considered the possibility that the *Tie2-Cre* transgene is transcriptionally regulated by ALK2-mediated signaling, since TGF- β superfamily members have been shown to be able to upregulate *Tie2* expression in cultured endothelial cells (Mandriota and Pepper, 1998). However, this scenario is unlikely, since the lineage tracing experiments demonstrated comparable staining intensity at E9.5 between control and mutant AV-canal endothelial cells. Conversely, it is possible that there were still some endothelial cells that failed to express *Cre* in sufficient quantities for efficient gene recombination, and that, in *Alk2* mutants, these cells could partially compensate the phenotype resulting from a total lack of endocardial cell transformation. This is supported by our findings that at E10 some isolated endothelial cells (both in controls and mutants) failed to stain positive for the β -galactosidase activity in a lineage-tracing assay. Moreover, the resulting morphologies are consistent with a depletion of size and cell number in the AV cushions. The mutant embryos recovered at E14.5 might have survived because incomplete removal of the *Alk2* gene allowed some endocardial cells to undergo EMT: the resulting structures, although dysmorphic, would be otherwise normal and hence able to fuse where size and alignment allowed, extend along

trabecular myocardium and become infiltrated by myocardium, all features observed among the surviving *Alk2/Tie2-Cre* embryos.

Previous studies have shown that mesenchymal cells in the proximal or conal OFT cushions are derived from the endothelium (Kisanuki et al., 2001), and that proximal OFT septation is mediated via the BMP type II receptor (Delot et al., 2003). Therefore, we were interested in determining whether ALK2 would also be involved in this process. While our analysis demonstrated that ALK2 function indeed is necessary for EMT in the proximal OFT, as well as in the AVC, it also pointed out that despite the lack of ALK2-mediated EMT, normal OFT septation or semilunar valve development can still occur. However, based on these experiments, we cannot exclude the possibility that defective EMT in conal cushions together with defective AV cushions may contribute to the formation of VSDs observed in *Alk2/Tie2-Cre* mutants. We have previously shown that mice lacking *Alk2* in neural crest cells display persistent truncus arteriosus as a result of defective outflow tract septation (Karttinen et al., 2004). Moreover, mice lacking *Alk3* in the neural crest display similar, albeit not identical outflow tract defects (Stottmann et al., 2004), indicating that these two related type I receptors play distinct non-redundant functions. As the function of *Alk3* in endothelial cells, if any, is currently unknown, we cannot determine whether *Alk2* and *Alk3* also display non-redundant functions in the endothelium during vascular or cardiac development.

Although ligands signaling via ALK2 in vivo are currently unknown, it is likely that they include BMPs, such as BMP2, -5, -6 and -7 (Macias-Silva et al., 1998). In the AV canal, a spectrum of BMPs is expressed by the myocardium, and BMP signaling via ALK3 was recently shown to upregulate mesenchymal expression of TGF- β 2 (Gaussin et al., 2002). Subsequently, it was suggested that BMP2 plays also a critical role in induction of autocrine TGF- β pathways in endocardial cells (Sugi et al., 2004). However, our observations that in *Alk2/Tie2-Cre* mutants the defect in EMT cannot be rescued by exogenous addition of TGF- β s, and that activation of both BMP Smads and TGF- β Smads is affected in *Alk2/Tie2-Cre* mutants, suggests that the role of BMP signaling in the endocardium is not solely the induction of TGF- β expression. Our current results are more consistent with a model in which BMP signaling via ALK2 is required to render endothelial cells permissive to TGF- β signaling (see a schematic model in Fig. 8). Alternatively, it can be argued that ALK2 mediates TGF- β signaling to induce EMT, as suggested by Miettinen et al. (1994) using a mouse mammary epithelial cell line model. However, this paradigm has been challenged by recent studies demonstrating that activation of TGF- β Smads is absolutely required, both in human and mouse epithelial cell lines, for TGF- β -induced EMT (Piek et al., 1999; Valcourt et al., 2005), whereas signaling leading to activation of BMP Smads (i.e., action mediated via ALK2)

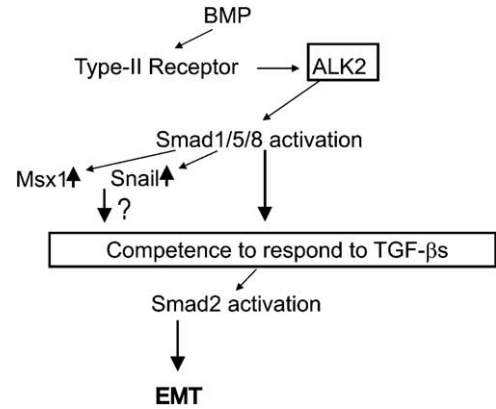


Fig. 8. Hypothetical model for induction of EMT by ALK2-mediated signaling. BMP binds to a type II receptor, which recruits ALK2. ALK2 becomes phosphorylated, which leads to subsequent phosphorylation of BMP Smads (1/5/8). Expression of genes encoding the putative downstream effectors, *Msx1* and *Snail*, is induced, and endothelial cells become competent to respond to TGF- β s. This in turn leads to activation of TGF- β Smads, which is prerequisite for EMT.

alone is neither necessary nor sufficient for EMT induction. A third possibility is that heteromeric TGF- β receptor complexes may be formed between different type I and type II receptors. Indeed, this type of signaling mechanism involving TGF β RII, ALK5 and ALK1 has been demonstrated to play an important regulatory role in endothelial cell proliferation (Goumans et al., 2003).

In summary, the role of TGF- β s and BMPs in endocardial cushion formation has been extensively studied in chick, using in vitro techniques. In contrast, much less is known about signaling mechanisms of these growth factors during mammalian development in vivo. Our present results demonstrate that signaling via the type-I receptor ALK2 is required for appropriate activation of BMP Smads and induction of EMT during formation of mouse endocardial cushions in vivo, and for subsequent morphogenetic steps that lead to development and maturation of AV-valve leaflets and septa. Our present results suggest that *Alk2*-mediated signaling maybe part of a critical pathway involved in pathogenesis of AV septal and valve malformations, which are among the most common congenital cardiac birth defects in humans.

Acknowledgments

We thank Saverio Bellusci, and Anil Bhushan for probes, and Ivan Vesely for support during the study. This work was supported by grants from the NIH (HL074862 and DE013085) to VK and by the AHA and NIH to JE.

References

- Anderson, R.H., Webb, S., Brown, N.A., Lamers, W., Moorman, A., 2003. Development of the heart: (2) Septation of the atriums and ventricles. *Heart* 89, 949–958.

- Bartram, U., Molin, D.G., Wisse, L.J., Mohamad, A., Sanford, L.P., Doetschman, T., Speer, C.P., Poelmann, R.E., 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.
- Battle, E., Sancho, E., Franci, C., Dominguez, D., Monfar, M., Baulida, J., Garcia, D.H., 2000. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat. Cell Biol.* 2, 84–89.
- Bei, M., Maas, R., 1998. FGFs and BMP4 induce both Msx1-independent and Msx1-dependent signaling pathways in early tooth development. *Development* 125, 4325–4333.
- Bernanke, D.H., Markwald, R.R., 1984. Effects of two glycosaminoglycans on seeding of cardiac cushion tissue cells into a collagen-lattice culture system. *Anat. Rec.* 210, 25–31.
- Blavier, L., Lazaryev, A., Groffen, J., Heisterkamp, N., DeClerck, Y.A., Kaartinen, V., 2001. TGF-beta3-induced palatogenesis requires matrix metalloproteinases. *Mol. Biol. Cell* 12, 1457–1466.
- Camenisch, T.D., Molin, D.G., Person, A., Runyan, R.B., Gittenberger-de Groot, A.C., McDonald, J.A., Klewer, S.E., 2002a. Temporal and distinct TGFbeta ligand requirements during mouse and avian endocardial cushion morphogenesis. *Dev. Biol.* 248, 170–181.
- Camenisch, T.D., Schroeder, J.A., Bradley, J., Klewer, S.E., McDonald, J.A., 2002b. Heart-valve mesenchyme formation is dependent on hyaluronan-augmented activation of ErbB2–ErbB3 receptors. *Nat. Med.* 8, 850–855.
- Cano, A., Perez-Moreno, M.A., Rodrigo, I., Locascio, A., Blanco, M.J., del Barrio, M.G., Portillo, F., Nieto, M.A., 2000. The transcription factor snail controls epithelial–mesenchymal transitions by repressing E-cadherin expression. *Nat. Cell Biol.* 2, 76–83.
- Chai, Y., Jiang, X., Ito, Y., Bringas Jr., P., Han, J., Rowitch, D.H., Soriano, P., McMahon, A.P., Sucov, H.M., 2000. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development* 127, 1671–1679.
- Chang, C.P., Neilson, J.R., Bayle, J.H., Gestwicki, J.E., Kuo, A., Stankunas, K., Graef, I.A., Crabtree, G.R., 2004. A field of myocardial–endocardial NFAT signaling underlies heart valve morphogenesis. *Cell* 118, 649–663.
- de la Pompa, J.L., Timmerman, L.A., Takimoto, H., Yoshida, H., Elia, A.J., Samper, E., Potter, J., Wakeham, A., Marengere, L., Langille, B.L., Crabtree, G.R., Mak, T.W., 1998. Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. *Nature* 392, 182–186.
- de Lange, F.J., Moorman, A.F., Anderson, R.H., Manner, J., Soufan, A.T., Gier-de Vries, C., Schneider, M.D., Webb, S., van den Hoff, M.J., Christoffels, V.M., 2004. Lineage and morphogenetic analysis of the cardiac valves. *Circ. Res.* 95, 645–654.
- Delort, E.C., Bahamonde, M.E., Zhao, M., Lyons, K.M., 2003. BMP signaling is required for septation of the outflow tract of the mammalian heart. *Development* 130, 209–220.
- Derynck, R., Feng, X.H., 1997. TGF-beta receptor signaling. *Biochim Biophys. Acta* 1333, F105–F150.
- Desgrosellier, J.S., Mundell, N.A., McDonnell, M.A., Moses, H.L., Barnett, J.V., 2005. Activin receptor-like kinase 2 and Smad6 regulate epithelial–mesenchymal transformation during cardiac valve formation. *Dev. Biol.* 280, 201–210.
- Donovan, J., Kordylewska, A., Jan, Y.N., Utset, M.F., 2002. Tetralogy of fallot and other congenital heart defects in Hey2 mutant mice. *Curr. Biol.* 12, 1605–1610.
- Dudas, M., Sridurongrit, S., Nagy, A., Okazaki, K., Kaartinen, V., 2004. Craniofacial defects in mice lacking BMP type I receptor Alk2 in neural crest cells. *Mech. Dev.* 121, 173–182.
- Eisenberg, L.M., Markwald, R.R., 1995. Molecular regulation of atrioventricular valvuloseptal morphogenesis. *Circ. Res.* 77, 1–6.
- Furuta, Y., Piston, D.W., Hogan, B.L., 1997. Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. *Development* 124, 2203–2212.
- Galvin, K.M., Donovan, M.J., Lynch, C.A., Meyer, R.I., Paul, R.J., Lorenz, J.N., Fairchild-Huntress, V., Dixon, K.L., Dunmore, J.H., Gimbrone Jr., M.A., Falb, D., Huszar, D., 2000. A role for smad6 in development and homeostasis of the cardiovascular system. *Nat. Genet.* 24, 171–174.
- Gaussin, V., Van de, P.T., Mishina, Y., Hanks, M.C., Zwijsen, A., Huylebroeck, D., Behringer, R.R., Schneider, M.D., 2002. Endocardial cushion and myocardial defects after cardiac myocyte-specific conditional deletion of the bone morphogenetic protein receptor ALK3. *Proc. Natl. Acad. Sci. U. S. A.* 99, 2878–2883.
- Gitler, A.D., Lu, M.M., Jiang, Y.Q., Epstein, J.A., Gruber, P.J., 2003a. Molecular markers of cardiac endocardial cushion development. *Dev. Dyn.* 228, 643–650.
- Gitler, A.D., Zhu, Y., Ismat, F.A., Lu, M.M., Yamauchi, Y., Parada, L.F., Epstein, J.A., 2003b. Nfl has an essential role in endothelial cells. *Nat. Genet.* 33, 75–79.
- Gittenberger-de Groot, A.C., Vrancken Peeters, M.P., Mentink, M.M., Gourdie, R.G., Poelmann, R.E., 1998. Epicardium-derived cells contribute a novel population to the myocardial wall and the atrioventricular cushions. *Circ. Res.* 82, 1043–1052.
- Goumans, M.J., Valdimarsdottir, G., Itoh, S., Lebrin, F., Larsson, J., Mummery, C., Karlsson, S., ten Dijke, P., 2003. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. *Mol. Cell* 12, 817–828.
- Gu, Z., Reynolds, E.M., Song, J., Lei, H., Feijen, A., Yu, L., He, W., MacLaughlin, D.T., van den Eijnden-van Raaij, Donahoe, P.K., Li, E., 1999. The type I serine/threonine kinase receptor ActRIA (ALK2) is required for gastrulation of the mouse embryo. *Development* 126, 2551–2561.
- Harlow, E., Lane, D., 1988. *Antibodies. A Laboratory Manual.* CSH Press, New York.
- Hoffman, J.I., Kaplan, S., 2002. The incidence of congenital heart disease. *J. Am. Coll. Cardiol.* 39, 1890–1900.
- Hogan, B., Bedington, R., Costantini, F., Lacy, E., 1994. *Manipulating the Mouse Embryo. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.
- Hurlstone, A.F., Haramis, A.P., Wienholds, E., Begthel, H., Korving, J., Van Eeden, F., Cuppen, E., Zivkovic, D., Plasterk, R.H., Clevers, H., 2003. The Wnt/beta-catenin pathway regulates cardiac valve formation. *Nature* 425, 633–637.
- Jiang, X., Rowitch, D.H., Soriano, P., McMahon, A.P., Sucov, H.M., 2000. Fate of the mammalian cardiac neural crest. *Development* 127, 1607–1616.
- Kaartinen, V., Nagy, A., 2001. Removal of the floxed neo gene from a conditional knockout allele by the adenoviral Cre recombinase in vivo. *Genesis* 31, 126–129.
- Kaartinen, V., Voncken, J.W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N., 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.
- Kaartinen, V., Dudas, M., Nagy, A., Sridurongrit, S., Lu, M.M., Epstein, J.A., 2004. Cardiac outflow tract defects in mice lacking ALK2 in neural crest cells. *Development* 131, 3481–3490.
- Kisanuki, Y.Y., Hammer, R.E., Miyazaki, J., Williams, S.C., Richardson, J.A., Yanagisawa, M., 2001. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev. Biol.* 230, 230–242.
- Koni, P.A., Joshi, S.K., Temann, U.A., Olson, D., Burkly, L., 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.
- Kulkarni, A.B., Huh, C.G., Becker, D., Geiser, A., Lyght, M., Flanders, K.C., Roberts, A.B., Sporn, M.B., Ward, J.M., Karlsson, S., 1993. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc. Natl. Acad. Sci. U. S. A.* 90, 770–774.
- Lai, Y.T., Beason, K.B., Brames, G.P., Desgrosellier, J.S., Clegggett, M.C., Shaw, M.V., Brown, C.B., Barnett, J.V., 2000. Activin receptor-like

- kinase 2 can mediate atrioventricular cushion transformation. *Dev. Biol.* 222, 1–11.
- Lakkis, M.M., Epstein, J.A., 1998. Neurofibromin modulation of ras activity is required for normal endocardial–mesenchymal transformation in the developing heart. *Development* 125, 4359–4367.
- Liebner, S., Cattelino, A., Gallini, R., Rudini, N., Iurlaro, M., Piccolo, S., Dejana, E., 2004. Beta-catenin is required for endothelial–mesenchymal transformation during heart cushion development in the mouse. *J. Cell Biol.* 166, 359–367.
- Macias-Silva, M., Hoodless, P.A., Tang, S.J., Buchwald, M., Wrana, J.L., 1998. Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. *J. Biol. Chem.* 273, 25628–25636.
- Mandriota, S.J., Pepper, M.S., 1998. Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. *Circ. Res.* 83, 852–859.
- Markwald, R., Eisenberg, C., Eisenberg, L., Trusk, T., Sugi, Y., 1996. Epithelial–mesenchymal transformations in early avian heart development. *Acta Anat. (Basel)* 156, 173–186.
- Massague, J., Chen, Y.G., 2000. Controlling TGF-beta signaling. *Genes Dev.* 14, 627–644.
- Miettinen, P.J., Ebner, R., Lopez, A.R., Derynck, R., 1994. TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. *J. Cell Biol.* 127, 2021–2036.
- Mishina, Y., Suzuki, A., Ueno, N., Behringer, R.R., 1995. Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. *Genes Dev.* 9, 3027–3037.
- Mishina, Y., Crombie, R., Bradley, A., Behringer, R.R., 1999. Multiple roles for activin-like kinase-2 signaling during mouse embryogenesis. *Dev. Biol.* 213, 314–326.
- Moorman, A.F., Houweling, A.C., de Boer, P.A., Christoffels, V.M., 2001. Sensitive nonradioactive detection of mRNA in tissue sections: novel application of the whole-mount in situ hybridization protocol. *J. Histochem. Cytochem.* 49, 1–8.
- Nakajima, Y., Yamagishi, T., Hokari, S., 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.
- Piek, E., Westermark, U., Kastemar, M., Heldin, C.H., van Zoelen, E.J., Nister, M., ten Dijke, P., 1999. Expression of transforming-growth-factor (TGF)-beta receptors and Smad proteins in glioblastoma cell lines with distinct responses to TGF-beta1. *Int. J. Cancer* 80, 756–763.
- Potts, J.D., 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.
- Potts, J.D., Dagle, J.M., Walder, J.A., Weeks, D.L., Runyan, R.B., 1991. Epithelial–mesenchymal transformation of embryonic cardiac endothelial cells is inhibited by a modified antisense oligodeoxynucleotide to transforming growth factor beta 3. *Proc. Natl. Acad. Sci. U. S. A.* 88, 1516–1520.
- Proetzel, G., Pawlowski, S.A., Wiles, M.V., Yin, M., Boivin, G.P., Howles, P.N., Ding, J., Ferguson, M.W., Doetschman, T., 1995. Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat. Genet.* 11, 409–414.
- Ranger, A.M., Grusby, M.J., Hodge, M.R., Gravallese, E.M., de la Brousse, F.C., Hoey, T., Mickanin, C., Baldwin, H.S., Glimcher, L.H., 1998. The transcription factor NF-ATc is essential for cardiac valve formation. *Nature* 392, 186–190.
- Runyan, R.B., Potts, J.D., Weeks, D.L., 1992. TGF-beta 3-mediated tissue interaction during embryonic heart development. *Mol. Reprod. Dev.* 32, 152–159.
- Sakata, Y., Kamei, C.N., Nakagami, H., Bronson, R., Liao, J.K., Chin, M.T., 2002. Ventricular septal defect and cardiomyopathy in mice lacking the transcription factor CHF1/Hey2. *Proc. Natl. Acad. Sci. U. S. A.* 99, 16197–16202.
- Sanford, L.P., Ormsby, I., Gittenberger-de Groot, A.C., Sariola, H., Friedman, R., Boivin, G.P., Cardell, E.L., Doetschman, T., 1997. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* 124, 2659–2670.
- Savagner, P., Yamada, K.M., Thiery, J.P., 1997. The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial–mesenchymal transition. *J. Cell Biol.* 137, 1403–1419.
- Schroeder, J.A., Jackson, L.F., Lee, D.C., Camenisch, T.D., 2003. Form and function of developing heart valves: coordination by extracellular matrix and growth factor signaling. *J. Mol. Med.* 81, 392–403.
- Shull, M.M., Ormsby, I., Kier, A.B., Pawlowski, S., Diebold, R.J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., Doetschman, T., 1992. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 359, 693–699.
- Solloway, M.J., Robertson, E.J., 1999. Early embryonic lethality in Bmp5/Bmp7 double mutant mice suggests functional redundancy within the 60A subgroup. *Development* 126, 1753–1768.
- Soriano, P., 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* 21, 70–71.
- Srivastava, D., 2001. Genetic assembly of the heart: implications for congenital heart disease. *Annu. Rev. Physiol.* 63, 451–469.
- Stottmann, R.W., Choi, M., Mishina, Y., Meyers, E.N., Klingensmith, J., 2004. BMP receptor IA is required in mammalian neural crest cells for development of the cardiac outflow tract and ventricular myocardium. *Development* 131, 2205–2218.
- Sugi, Y., Markwald, R.R., 1996. Formation and early morphogenesis of endocardial endothelial precursor cells and the role of endoderm. *Dev. Biol.* 175, 66–83.
- Sugi, Y., Yamamura, H., Okagawa, H., 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.
- Suzuki, A., Ueno, N., Hemmati-Brivanlou, A., 1997. *Xenopus* msx1 mediates epidermal induction and neural inhibition by BMP4. *Development* 124, 3037–3044.
- Timmerman, L.A., Grego-Bessa, J., Raya, A., Bertran, E., Perez-Pomares, J.M., Diez, J., Aranda, S., Palomo, S., McCormick, F., Izpisua-Belmonte, J.C., de la Pompa, J.L., 2004. Notch promotes epithelial–mesenchymal transition during cardiac development and oncogenic transformation. *Genes Dev.* 18, 99–115.
- Valcourt, U., Kowanetz, M., Niimi, H., Heldin, C.H., Moustakas, A., 2005. TGF-beta and the Smad signaling pathway support transcriptomic reprogramming during epithelial–mesenchymal cell transition. *Mol. Biol. Cell* 16, 1987–2002.
- Winnier, G., Blessing, M., Labosky, P.A., Hogan, B.L., 1995. Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev.* 9, 2105–2116.
- Yamamura, H., Zhang, M., Markwald, R.R., Mjaatvedt, C.H., 1997. A heart segmental defect in the anterior–posterior axis of a transgenic mutant mouse. *Dev. Biol.* 186, 58–72.
- Zhang, H., Bradley, A., 1996. Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. *Development* 122, 2977–2986.