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Signaling via the Tgf-β type I receptor Alk5 in heart development

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ABSTRACT

Trophic factors secreted both from the endocardium and epicardium regulate appropriate growth of the myocardium during cardiac development. Epicardially-derived cells play also a key role in development of the coronary vasculature. This process involves transformation of epithelial (epicardial) cells to mesenchymal cells (EMT). Similarly, a subset of endocardial cells undergoes EMT to form the mesenchyme of endocardial cushions, which function as primordia for developing valves and septa. While it has been suggested that transforming growth factor-βs (Tgf-β) play an important role in induction of EMT in the avian epi- and endocardium, the function of Tgf-βs in corresponding mammalian tissues is still poorly understood. In this study, we have ablated the Tgf-β type I receptor Alk5 in endo-, myo- and epicardial lineages using the *Tie2-Cre, Nkx2.5-Cre,* and *Gata5-Cre* driver lines, respectively. We show that while Alk5-mediated signaling does not play a major role in the myocardium during mouse cardiac development, it is critically important in the endocardium for induction of EMT both *in vitro* and *in vivo*. Moreover, loss of epicardial *Alk5*-mediated signaling leads to disruption of cell–cell interactions between the epicardium and myocardium resulting in a thinned myocardium. Furthermore, epicardial cells lacking *Alk5* fail to undergo Tgf-β-induced EMT *in vitro*. Late term mutant embryos lacking epicardial *Alk5* display defective formation of a smooth muscle cell layer around coronary arteries, and aberrant formation of capillary vessels in the myocardium suggesting that Alk5 is controlling vascular homeostasis during cardiogenesis. To conclude, Tgf-β signaling via Alk5 is not required in myocardial cells during mammalian cardiac development, but plays an irreplaceable cell-autonomous role regulating cellular communication, differentiation and proliferation in endocardial and epicardial cells.

Introduction

The heart is the first functional organ to develop in vertebrates. During gastrulation, cardiogenic mesodermal cells form the so called cardiogenic field, which subsequently gives rise to a linear heart tube composed of outer myocardial and inner endothelial layers (Anderson et al., 2003). After cardiac looping, cardiac neural crest cells migrate to the base of the aortic sac and form the aortico-pulmonary septum, which gradually separates the aorta from the pulmonary trunk (Hutson and Kirby, 2003). At the same time (around embryonic days 9.0–9.5 [E9.0–E9.5]), a region between the developing atria and ventricles is specified to form an atrio-ventricular canal (AVC), an important structure making up the heart-valve inducing field (Eisenberg and Markwald, 1995). First the extracellular matrix rich in hyaluronic acid is deposited by the AVC myocardium followed by epithelial-to-mesenchymal transformation (EMT) of a subset of endocardial cells. The formed endocardial cushions will be further refined to form the AV valves and septa.

Soon after cardiac looping, a separate cell population derived from the hepatic primordium gives rise to the proepicardial cells near a venous pole of the developing heart (Bernanke and Velkey, 2002). Epicardial progenitor cells then dislodge as cellular vesicles, which spread out, and gradually cover the entire developing heart to form a coherent epicardium. A subpopulation of epicardial cells undergoes EMT and migrates into the subepicardial space rich in extracellular matrix proteins. It has been suggested that these transformed mesenchymal cells will produce cardiac fibroblasts and the vascular smooth muscle of the adult heart (Reese et al., 2002; Wada et al., 2003). Formation of coronary vasculature is essential once the myocardium becomes so thick that diffusion is not able to supply enough nutrients and oxygen to the heart.

A crucial function of Tgf-beta ligands during murine heart development was first suggested by the conventional knockout mice studies (Sanford et al., 1997). While *Tgfb1*$^{-/-}$ and *Tgfb3*$^{-/-}$ mice show no obvious signs of congenital heart defects (Kaartinen et al., 1995; Proetzel et al., 1995; Shull et al., 1992), *Tgfb2*$^{-/-}$ embryos display multiple cardiac defects(Sanford et al., 1997). These include the double-outlet right ventricle (DORV), which is known to derive from an error in development of the second heart field that contributes to

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formation of the outflow tract (OFT) myocardium. *Tgfb2*$^{-/-}$ mice display a defective myocardialization that is associated with a deregulation of neural crest cell apoptosis (Bartram et al., 2001).

Tgf-β signaling, which is predominantly mediated via a heterotrameric receptor complex composed of two Tgf-β type II (TgfβRII) and two type I (Alk5) receptors, has also been implicated in induction of both endocardial and epicardial EMT (Brown et al., 1996; Jiao et al., 2006). Experiments on chick proepicardial organ and epicardial explant cultures have suggested that Tgf-β signaling via Alk5 is required for the loss of epithelial cell character of epicardial cells, while another type I receptor Alk2, known to mediate mainly Bmp signals, was shown to stimulate earlier proepicardial (PE) activation events (Compton et al., 2006; Olivey et al., 2006). Moreover, a recent study demonstrated that Tgf-β type III receptor (β-glycan), which binds Tgf-β2 with high affinity, is required for appropriate coronary vessel development in mouse embryos (Compton et al., 2007). Yet, the role of Tgf-βs in epicardial EMT, even in avians, is still controversial, since other studies have suggested that Fgfs are responsible of inducing epicardial EMT, while Tgf-βs would restrain their function (Morabito et al., 2001).

The Tgf-β type I receptor Alk5 was also recently shown to mediate endocardial transformation in the chick (Mercado-Pimentel et al., 2007). Interestingly, the murine Tgf-β type II receptor (TgfβRII, a prototypical binding partner of Alk5) was shown to be required for EMT *in vitro*, but not *in vivo* (Jiao et al., 2006). This discrepancy suggests that there is a mechanistic difference in Tgf-β signaling between avian and murine AVC transformation. Alternatively, it is possible that Alk5 can also interact with other members of the type II receptor family as recently suggested (Dudas et al., 2006). Other studies have demonstrated that Bmp2 is required both for specification of the AV canal myocardium and for endocardial EMT (Ma et al., 2005; Rivera-Feliciano and Tabin, 2006). However, it is currently not clear whether Bmp2-induced EMT in the AVC is mediated via Alk2 or Alk3, or whether they are both synergistically involved, since endothelial-specific abrogation of either *Alk2* or *Alk3* leads to a failure in EMT and severe defects in endocardial cushions *in vitro* and *in vivo* (Ma et al., 2005; Park et al., 2006; Song et al., 2007; Wang et al., 2005). Interestingly, deletion of Alk2 in endothelial cells not only attenuates phosphorylation of Bmp Smads 1/5/8, but also affects the activation of Tgf-beta Smads 2/3 (Wang et al., 2005). Based on these studies, it is likely that Smad2/3 activation, and thus Alk5 signaling is also important for endocardial EMT in mammals.

In the present study we have analyzed the role of *Alk5* during mouse heart development *in vivo*. Specifically, we deleted *Alk5* in the endocardium, myocardium and in epicardium by using the *Tie2-Cre*, *Nkx2.5-Cre* and *Gata5-Cre* transgenic driver lines, respectively (Koni et al., 2001; Merki et al., 2005; Moses et al., 2001). We discovered that while Alk5 is redundant in cardiomyocyte development, it is needed in the endocardium for appropriate EMT both *in vitro* and *in vivo*, and for the subsequent endocardial cushion development. We show that in the epicardium *Alk5* is required for epicardial to mesenchymal transformation *in vitro*, and for normal epicardial attachment and function *in vivo*. Moreover, our data indicate that disturbances in epicardial Alk5-mediated signaling lead to attenuated myocardial growth, defective formation of a smooth muscle cell layer surrounding coronary vessels and dramatic increase in a number of capillary vessels in the myocardium during late cardiac development.

Materials and methods

Mice, genotyping, timed-matings and embryo isolation

All mice were maintained on mixed genetic backgrounds, and all studies were carried out at the Animal Care Facility of the Saban Research Institute in accordance with national and institutional guidelines. To generate endothelial-specific *Alk5* mutants, *Alk5*$^{FX/FX}$ mice were crossed with *Tie2-Cre* driver mice (Koni et al., 2001), which were also heterozygous for the *Alk5*^{KO} allele. The resulting compound heterozygotes for the *Alk5*^{FX} and *Alk5*^{KO} alleles, which also carry the *Cre* transgene (*Alk5*$^{FX/KO}$/*Tie2-Cre*$^{+/WT}$), have *Alk5* specifically inactivated in endothelial cells (herein termed *Alk5/Tie2-Cre*), while the littermates with incomplete combinations (*Alk5*$^{FX/KO}$/*Tie2-Cre*$^{WT/WT}$, *Alk5*$^{FX/WT}$/*Tie2-Cre*$^{WT/WT}$ and *Alk5*$^{FX/WT}$/*Tie2-Cre*$^{+/WT}$) of these alleles (which were all phenotypically indistinguishable from wild-type embryos) serve as controls. A similar breeding strategy was used to generate mouse mutants lacking Alk5 in the myocardium (*Alk5/Nkx2.5-Cre*) (Moses et al., 2001) and the epicardium (*Alk5/Gata5-Cre*) (Merki et al., 2005). Oligonucleotides for PCR-genotyping of the *Alk5*^{FX} and for *Alk5*^{KO} alleles as well as for *Cre* have been described elsewhere (Dudas et al., 2006). *Tie2-Cre* and *Rosa26*-reporter (*R26R*) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA); for detailed PCR-genotyping, see http://www.jax.org. *Nkx2.5-Cre* and *Gata5-Cre* mice have been previously described (Merki et al., 2005; Moses et al., 2001).

Histological analyses, R26 reporter assay and immunostaining

For histology, embryos were fixed with 4% formaldehyde for 2–14 h, dehydrated and embedded in paraffin wax. Sections (7–8 μm) were stained with Hematoxylin and Eosin. Embryos and sections were stained for β-galactosidase activity as described (Hogan et al., 1994). For immunohistochemistry, fixed sections were stained with antibodies for WT1 (Santa Cruz Biotech.), *N*-cadherin (Invitrogen), ZO1 (Invitrogen), vascular smooth muscle myosin heavy chain (Biomedical Technologies Inc.) and Claudin-5 (Invitrogen). For VE-cadherin, β-galactosidase and PECAM-1 immunostaining, tissues were processed for frozen sectioning followed by staining with corresponding antibodies (antibodies for VE-cadherin, β-galactosidase and PECAM-1 were from BD Pharmingen, MP Biochemicals and BD Pharmingen, respectively).

Apoptosis and cell proliferation

Apoptotic cells were detected in paraffin sections as a green fluorescence using DeadEnd Fluorometric TUNEL system (Promega). Cell proliferation was immunodetected using *BrdU* incorporation assay (Amersham). Briefly, pregnant females were injected intraperitoneally with 200 μl of Amersham *BrdU* labeling reagent. After 40 min, the female mice were euthanized with CO_2, embryos were harvested, and processed for *BrdU* immunostaining according to the manufacturer's instructions. *BrdU*-positive cells were detected with Alexa Fluor 488-conjugated (green) secondary antibodies. Slides were counterstained with Propidium Iodide. For *BrdU/MF20* double labeling, cells were first stained for *BrdU* as outlined above followed by labeling for sarcomeric myosin heavy chain using mouse monoclonal MF20 primary antibody followed by Alexa Fluor 594-conjugated anti-mouse IgG2b secondary antibody (Invitrogen) (Redfield et al., 1997). Slides were counterstained with DAPI. Positively stained cells were counted manually in defined areas of tissues. Statistical analysis of cell counts in serial sections and comparison of mutant specimens with controls was performed using a nonparametric Wilcoxon Rank Sum Test.

Epicardial and AVC canal explant cultures

Epicardial cultures were established as described (Chen et al., 2002). Some mutant and control cultures were treated with 10 ng/ml of human recombinant hrTgf-β3 (Sigma Chemical Co., St Louis, MO) for 24 h. Both treated and untreated cultures were fixed with 2% formaldehyde for 15 min, and stained with anti-ZO1 antibody (Invitrogen) for the presence of the tight junction protein, zonula occludens and with FITC-phalloidin (Sigma Chemical Co.) for the presence of filamentous actin (f-actin). For AVC explant cultures, collagen gels (1 mg/ml, type I rat tail collagen from BD) were prepared in OptiMEM supplemented with 1% fetal calf serum, 1xITS (insulin, transferrin and selenium) and penicillin/streptomysin (1×) all from Invitrogen (Sugi et al., 2004). AV regions of the hearts were dissected from E10 embryos, cut longitudinally to expose the lumen and