Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 301 (2007) 276–286
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Essential functions of *Alk3* during AV cushion morphogenesis in mouse embryonic hearts

Lanying Song [a], Reinhard Fässler [b], Yuji Mishina [c], Kai Jiao [a,*], H. Scott Baldwin [d,*]

[a] *Division of Genetic and Translational Medicine, Department of Genetics, University of Alabama at Birmingham, Birmingham, AL 35294, USA*
[b] *Department of Molecular Medicine, Max Planck Institute for Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany*
[c] *Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA*
[d] *Division of Pediatric Cardiology, Department of Pediatrics, Vanderbilt University Medical Center, Nashville, TN 37232, USA*

Received for publication 24 April 2006; revised 11 July 2006; accepted 1 August 2006
Available online 9 August 2006

Abstract

Accumulated evidence has suggested that BMP pathways play critical roles during mammalian cardiogenesis and impairment of BMP signaling may contribute to human congenital heart diseases (CHDs), which are the leading cause of infant morbidity and mortality. *Alk3* encodes a BMP specific type I receptor expressed in mouse embryonic hearts. To reveal functions of *Alk3* during atrioventricular (AV) cushion morphogenesis and to overcome the early lethality of $Alk3^{-/-}$ embryos, we applied a Cre/loxp approach to specifically inactivate *Alk3* in the endothelium/endocardium. Our studies showed that endocardial depletion of *Alk3* severely impairs epithelium–mesenchymal–transformation (EMT) in the atrioventricular canal (AVC) region; the number of mesenchymal cells formed in *Tie1-Cre;*$Alk3^{loxp/loxp}$ embryos was reduced to only ~20% of the normal level from both *in vivo* section studies and *in vitro* explant assays. We showed, for the first time, that in addition to its functions on mesenchyme formation, *Alk3* is also required for the normal growth/survival of AV cushion mesenchymal cells. Functions of *Alk3* are accomplished through regulating expression/activation/subcellular localization of multiple downstream genes including Smads and cell-cycle regulators. Taken together, our study supports the notion that *Alk3*-mediated BMP signaling in AV endocardial/mesenchymal cells plays a central role during cushion morphogenesis.

Keywords: *Alk3*; Atrioventricular cushion; BMP; Cardiogenesis; Congenital heart diseases; Epithelial–mesenchymal transformation (EMT)

Introduction

CHDs are the leading cause of infant morbidity and mortality, occurring in as many as 1% of newborns (Hoffman, 1995; Hoffman and Kaplan, 2002). The most common CHDs are caused by maldevelopment of septation and valvulogenesis. EMT in the AVC and outflow tract (OFT) regions is a critical process regulating initial valve formation where a subpopulation of endocardial cells invade the extracellular matrix as the result of regional interaction between the myocardium and endocardium (Armstrong and Bischoff, 2004; Barnett and Desgrosellier, 2003; Schroeder et al., 2003). These mesenchymalized cushions serve as the primordia of valves and septa and are developed into mature structures through complicated remodeling processes. The molecular and cellular mechanisms underlying cushion morphogenesis and their potential clinical significance have received great attention.

Results from numerous studies have established that EMT relies on proper responses of the underlying endocardial cells to the stimulating signal molecules released from the overlying myocardium in the AVC region. Many signaling molecules including TGFβ, Notch, Wnt, EGF and VEGF have been shown with experimental evidence to be involved in EMT (Armstrong and Bischoff, 2004; Barnett and Desgrosellier, 2003; Schroeder et al., 2003). More recent studies have suggested that *Bmp2* plays a critical role in activating EMT in the AVC region. *In vitro* collagen gel assays with avian AVC explants showed that *Bmp2* can substitute for the overlying myocardium and is sufficient to

* Corresponding authors. H.S. Baldwin is to be contacted at Division of Pediatric Cardiology, Department of Pediatrics, Vanderbilt University Medical Center, Nashville, TN 37232, USA. Fax: +1 615 322 6541. K. Jiao, Division of Genetic and Translational Medicine, Department of Genetics, the University of Alabama at Birmingham, Birmingham, AL 35294, USA. Fax: +1 205 975 5689.
E-mail addresses: kjiao@uab.edu (K. Jiao), scott.baldwin@Vanderbilt.edu (H.S. Baldwin).

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.08.004

activate AV endocardial cells, and blocking *Bmp2* with its antagonist, noggin, inhibits EMT (Sugi et al., 2004). Furthermore, the most recent mouse genetic studies showed that myocardial inactivation of *Bmp2* blocks formation of AV cushion mesenchymal cells (Ma et al., 2005; Rivera-Feliciano and Tabin, 2006).

After mesenchymal cells are formed through EMT, AV cushions undergo complicated remodeling processes, which include cell migration, proliferation, apoptosis and differentiation, to form mature septal and valvular structures. Compared to the process of EMT, fewer studies have aimed at revealing the molecular and cellular mechanisms underlying cushion remodeling after EMT. Some published studies (i.e. Gaussin et al., 2005; Gaussin et al., 2002; Tevosian et al., 2000) showed that disruption of the integrity of myocardium also causes structural defects of septa and valves, suggesting that cushion remodeling at later stages also relies on proper communication between the myocardium and the mesenchyme/endocardium. We showed previously that myocardial *Bmp4* is dispensable for EMT, but is required for normal proliferation of AV cushion mesenchymal cells, as inactivation of *Bmp4* in the myocardium causes the atrioventricular canal defect (AVCD) (Jiao et al., 2003). However, it is unclear from this study whether Bmp4 released from the myocardium acts directly on AV cushion mesenchymal cells to stimulate their growth or if the function of Bmp4 is mediated through certain unknown signaling molecules released from the myocardium.

BMP ligands belong to the TGFβ superfamily of cytokines (Hogan, 1996; Kishigami and Mishina, 2005; von Bubnoff and Cho, 2001), and their activities are mediated through heterodimeric complexes of type I and type II serine/threonine kinase receptors (Kishigami and Mishina, 2005; Shi and Massague, 2003; von Bubnoff and Cho, 2001). After formation of the receptor/ligand complex, the type II receptor will phosphorylate the type I receptor, which in turn phosphorylates particular members of the Smad family of cytoplasmic proteins. The phosphorylated (activated) Smad proteins will then translocate into the nucleus to function as transcriptional modulators (Shi and Massague, 2003; von Bubnoff and Cho, 2001). Among the three type I receptors (*Alk2*, *3*, *6*) that can mediate BMP signals (de Caestecker, 2004; Kishigami and Mishina, 2005), *Alk6* is not expressed in embryonic hearts (Dewulf et al., 1995; Jiao et al., unpublished data), and is thus unlikely to play a direct role during cardiogenesis. A previous study showed that in *Tie2-Cre;* $Alk2^{loxp/loxp}$ embryonic hearts, the number of mesenchymal cells in the superior cushion is decreased to ~40% of the normal level, while that in the inferior cushion is ~65% of the normal level, indicating that *Alk2* is required for normal AV cushion morphogenesis (Wang et al., 2005). *Alk3* is another BMP type I receptor expressed in embryonic hearts. *Alk2* and *Alk3* appear to preferentially mediate different BMP ligands. The primary BMP ligands binding to *Alk2* are *Bmp5*, *6*, *7*, while *Alk3* primarily mediates *Bmp2*, *4* signaling although it may also mediate signaling through other BMP ligands (de Caestecker, 2004 and ref. therein). Therefore, *Alk3* may play a distinct role from that of *Alk2* during cardiogenesis. Consistent with this idea, myocardial inactivation of *Alk3* causes embryonic lethality due to heart failure (Gaussin et al., 2002), while myocardial *Alk2* appears to be dispensable for heart formation (Wang et al., 2005).

This report continues our study of functions of BMP signaling during AV cushion morphogenesis. To complement our previous approach using myocardial deletion of a gene encoding a BMP ligand (*Bmp4*) (Jiao et al., 2003), we specifically inactivated *Alk3* in endothelial cells by breeding *Tie1-Cre* and $Alk3^{loxp/loxp}$ mice. Detailed characterization of *Tie1-Cre;* $Alk3^{loxp/loxp}$ has led to the conclusion that endocardial/mesenchymal *Alk3* plays critical roles during AV cushion morphogenesis.

Materials and methods

Mouse and embryo manipulations

All procedures are approved by the Institutional Animal Care and Use Committee at Vanderbilt University and the University of Alabama. The *Tie1-Cre* mice (Gustafsson et al., 2001) were crossed with the $Alk3^{loxp/loxp}$ mice (Mishina et al., 2002) to specifically inactivate *Alk3* in endothelial cells (see Results for detailed breeding strategy). In performing conditional gene inactivation experiments, we always used the male *Tie1-cre;* $Alk3^{loxp/+}$ mice to cross with female $Alk3^{loxp/loxp}$ mice. To perform cell lineage assays, the $Alk3^{loxp/loxp}$ mice were crossed with the R26R mice (Soriano, 1999) (from the Jackson Lab) to acquire the $Alk3^{loxp/+}$*;R26R* mice, which were then intercrossed to generate the $Alk3^{loxp/loxp}$*;R26R/R26R* mice. Mouse genotyping, embryo dissection, sectioning, HE staining and X-gal staining were performed as described previously (Jiao et al., 2003; Jiao et al., 2002).

In vitro collagen gel assay

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

TUNEL assays and non-radioactive section in situ hybridization analysis

TUNEL assays were performed using the "Dead End Colormetric TUNEL system" (Promega) following manufacturer's instructions. Non-radioactive section *in situ* hybridization analysis was performed as described previously (Grapin-Botton et al., 2001).

Immunostaining studies

Immunofluorescence studies were performed as described previously (Jiao et al., 2003). cy2 conjugated secondary antibodies were used for visualization of the signal. Total nuclei were visualized with Propidiumiodide or DAPI staining. Samples were examined with the Leica HC fluorescent microscope equipped with a RT SLIDER digital camera. Immunohistochemistry studies were performed using the Envision+ system (DAKOcytomation) following manufacturer's instructions. Sections were counter stained briefly with Hematoxylin. The primary antibodies used in this study include antibodies recognizing cyclin D1 (BD Biosciences), α-smooth muscle actin (Sigma), Caspase 3 (Cell Signaling), p21 (Cell Signaling), phospho-H3 (UpState), phospho-Smad1,5,8 (Cell Signaling), phospho-Smad2,3 (Cell Signaling), NFATc1 (BD Biosciences) and Msx1/2 (Iowa Hybridoma Bank).

Results

A Tie1-Cre mouse line efficiently inactivates Alk3 in endothelial cells of mouse embryos

To overcome the early embryonic lethality of $Alk3^{-/-}$ mice (Mishina et al., 1995), and to reveal *in vivo* functions of *Alk3* during AV cushion morphogenesis, we decided to specifically inactivate *Alk3* in the endothelium/endocardium using *Tie1-Cre*