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Tbx18 regulates development of the epicardium and coronary vessels

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ABSTRACT

The epicardium and coronary vessels originate from progenitor cells in the proepicardium. Here we show that *Tbx18*, a T-box family member highly expressed in the proepicardium, controls critical early steps in coronary development. In $Tbx18^{-/-}$ mouse embryos, both the epicardium and coronary vessels exhibit structural and functional defects. At E12.5, the *Tbx18*-deficient epicardium contains protrusions and cyst-like structures overlying a disorganized coronary vascular plexus that contains ectopic structures resembling blood islands. At E13.5, the left and right coronary stems form correctly in mutant hearts. However, analysis of PECAM-1 whole mount immunostaining, distribution of $SM22\alpha^{lacZ/+}$ activity, and analysis of coronary vascular casts suggest that defective vascular plexus remodeling produces a compromised arterial network at birth consisting of fewer distributing conduit arteries with smaller lumens and a reduced capacity to conduct blood flow. Gene expression profiles of $Tbx18^{-/-}$ hearts at E12.5 reveal altered expression of 79 genes that are associated with development of the vascular system including sonic hedgehog signaling components *patched* and *smoothened*, *VEGF-A*, *angiopoietin-1*, *endoglin*, and *Wnt* factors compared to wild type hearts. Thus, formation of coronary vasculature is responsive to *Tbx18*-dependent gene targets in the epicardium, and a poorly structured network of coronary conduit vessels is formed in *Tbx18* null hearts due to defects in epicardial cell signaling and fate during heart development. Lastly, we demonstrate that Tbx18 possesses a SRF/CArG box dependent repressor activity capable of inhibiting progenitor cell differentiation into smooth muscle cells, suggesting a potential function of *Tbx18* in maintaining the progenitor status of epicardial-derived cells.

Introduction

Lineage mapping and genetic analysis studies have shown that proepicardial (PE) cells are progenitors for the epicardium, coronary smooth muscle cells (CoSMCs), perivascular and intermyocardial fibroblasts, and a subpopulation of cardiac myocytes (Mikawa and Fishman, 1992; Gittenberger-de Groot et al., 1998; von Gise and Pu, 2012; Zhou et al., 2008). In the mouse, the PE first appears around E8.5 on the surface of septum transversum mesenchyme near the sinoatrial junction (Viragh and Challice, 1981). PE cells reach the heart between E9.5 and E10.5, attach to the myocardium and migrate over it to produce a continuous sheet of mesothelial cells that forms the epicardium (Manner, 1992; Sengbusch et al., 2002). Coronary vessel development depends upon epicardial cells that undergo an epithelial to mesenchymal transition (EMT), followed by vasculogenesis in the subepicardial space, formation and remodeling of a capillary-like vascular plexus, and eventual recruitment of CoSMCs (Majesky, 2004; Olivery and Svensson, 2012). Development of a subepicardial coronary plexus occurs between E11.5 and E13.5 in the mouse, and is followed by formation of left and right coronary stems in the aortic valve sinuses. Unlike the systemic vasculature, which develops gradually along with formation of the heart, when the coronary plexus makes contact with the aortic lumen at E13.5, it establishes continuity with an already functional circulation. Therefore, formation of an optimal network of coronary arteries, veins and capillaries to ensure efficient perfusion in mature hearts requires a preformed vascular plexus in the subepicardium that is already well organized to receive blood flow from the aorta at E13.5.

T-box 18 (*Tbx18*) is a member of the T-box transcription factor family (Kraus et al., 2001; Naiche et al., 2005). Loss of function

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studies show that *Tbx1*, *Tbx2*, *Tbx3*, *Tbx5*, and *Tbx20* play critical roles in cardiac development (Greulich et al., 2011). Expression of *Tbx18* is first detected in presomitic mesoderm and cranial paraxial mesoderm, in E7.75 mouse embryos (Kraus et al., 2001; Bussen et al., 2004). Later, *Tbx18* is strongly expressed in the PE, epicardium, epicardial-derived cells (EPDC) in subepicardial mesenchyme, septum transversum, somites, limb buds, aorta-gonad-mesonephros region and a subset of myocardium (Bussen et al., 2004; Airik et al., 2006; Christoffels et al., 2009; Christoffels et al., 2006). Analysis of *Tbx18*-expressing cell fates in the mouse heart showed that epicardial cells, CoSMCs, atrioventricular valve interstitial cells, and a subpopulation of cardiac myocytes derive from *Tbx18*-positive progenitor cells (Cai et al., 2008; Christoffels et al., 2009; Mommersteeg et al., 2010). Loss of *Tbx18* function causes perinatal lethality with defects in skeletal, urogenital systems and pleuropericardial membranes, and with concomitant disruptions of Notch, Wnt and Hedgehog signaling pathways (Bussen et al., 2004; Airik et al., 2006; Norden et al., 2012). In the developing heart, *Tbx18* deficiency results in dysmorphogenesis of the sinus node (Wiese et al., 2009), while forced overexpression of *Tbx18* reprograms ventricular myocytes to pacemaker cells in rodents (Kapoor et al., 2012). Because *Tbx18* is highly expressed in the PE and epicardium, we sought to determine its role in coronary vessel formation during heart development. We found that *Tbx18*-deficient PE produces an epicardium and coronary vasculature with structural and functional defects and that remodeling of the disorganized subepicardial plexus in *Tbx18*-deficient hearts produced a mature coronary artery network with fewer distributing conduit vessels and smaller lumen profiles than that of wild type hearts.

Materials and methods

Generation of Tbx18 mutant mice

The *Tbx18*^{tm1mwm} allele was generated by replacing exon 1 sequences encoding the predicted translation initiation site and the N-terminal domain of the protein, and exon 2 sequences encoding the amino-terminal residues of the highly conserved T-box, with an IRES-nlacZ reporter as depicted in Fig. S1. All mice were maintained in a 129/C57BL6/J mixed background.

Histological analysis and immunofluorescence staining

Histological analysis was performed by using standard procedures (Landerholm et al., 1999).

Skeletal preparation

The skeletal preparation procedure was adopted from Mcleod (1980).

Whole mount ß-galactosidase activity

Embryos or tissues were whole mount-stained for *ß*-galactosidase activity as previously described (Passman et al., 2008).

Scanning electron microscopy (SEM)

Freshly harvested hearts were fixed in 2% PFA/2.5% glutaraldehyde in 0.15M Karlsson and Schultz solution at 4 °C overnight, postfixed in 1% buffered osmium tetroxide, dehydrated, critical point dried, sputter coated with a 60/40 Au/Pd alloy and examined on a Cambridge S-200 scanning electron microscope as described (Dong et al., 2008).

Whole mount immunofluorescence and confocal microscopy

Dissected hearts were placed in ice-cold PBS, fixed and permeabilized in methanol/DMSO (4:1) overnight at 4 °C, bleached in methanol/DMSO/H_2O_2 (4:1:1) for 4 h at room temperature, and preserved in methanol at −20 °C. Tissues were prepared, stained, and imaged by confocal microscopy as previously described (Passman et al., 2008; Dong et al., 2008).

Cell proliferation and apoptosis assays

Cell proliferation was assessed in whole mount or tissue sections using anti-phosphohistone-H3 antibody. Apoptosis was determined by TUNEL assay using the ApopTag Fluoroscein in situ Apoptosis Detection kit (Chemicon, Inc) according to the manufacturer's instructions.

Coronary vascular casts

Vascular casts were prepared as described (Adamson et al., 2002). Coronary casts were mounted on an SEM stub, with the apex of the ventricles oriented upward, and visualized by scanning electron microscopy (SEM).

Microarray analysis

Generation of labeled antisense RNA for microarray: total RNA was extracted from four pooled Tbx18-null hearts and three pooled wild type E12.5 hearts (ToTally RNA kit, Ambion, Inc.), and underwent one round of linear amplification (Amino Allyl MessageAmpII kit, Ambion, Inc.). The paired antisense RNA samples were hybridized to glass microarrays containing the Operon Mouse Aros 4.0 Oligo library (oligomers spotted by the Microarray Core Facility, Department of Systems Biology & Translational Medicine, Texas A&M University). After imaging, the acquired data were transformed, normalized and filtered using the GeneSpring v7.0 software package (Silicon Genetics, Agilent Technologies). Gene expression significance was assessed using multiple *t*-tests ($p < 0.05$) and the Benjamini–Hochberg false discovery rate multiple testing correction. Molecular function and pathway analysis was performed via Ingenuity Pathway Analysis.

Real-time quantitative RT-PCR analysis

cDNA was generated by M-MLV Reverse Transcriptase (Invitrogen) followed by Taqman real time PCR analysis on an ABI-7700 thermal cycler according to the manufacturer's instructions. Data were analyzed by the ΔΔCT method.

Transient transfection and reporter assays

COS7 and C3H10T1/2 cells were cultured in 24-well tissue culture plates at a starting density of 5×10^4 cells/well in DMEM supplemented with 10% fetal bovine serum and transfected with FuGENE6 (Roche) per the manufacturer's instructions. For reporter assays, the amount per well of plasmid DNA was kept constant at 50 ng for luciferase constructs, 50 ng for SRF and 5 ng for myocardin, while titering in Tbx18 (50–200 ng) or complimentary amounts of empty vector DNA to reach a constant 300 ng total amount of these two plasmid DNAs. Each well was co-transfected with pGL4-SV40-*Renilla* luciferase-reporter (Promega, with a constant ratio of *Firefly* luciferase-reporter: *Renilla* luciferase-reporter=50:1) for normalization. Luciferase assays were performed using a commercially available substrate kit (Promega).