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Inactivation of *Bmp4* from the *Tbx1* Expression Domain Causes Abnormal Pharyngeal Arch Artery and Cardiac Outflow Tract Remodeling

Xuguang Nie[a] Christopher B. Brown[b] Qin Wang[c] Kai Jiao[a]

[a]Division of Research, Department of Genetics, University of Alabama at Birmingham, Birmingham, Ala., [b]Department of Pediatrics, Vanderbilt University Medical Center, Nashville, Tenn., and [c]Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, Ala., USA

Key Words
Bmp4 · Outflow tract · Pharyngeal arch artery · Morphogenesis

Abstract
Maldevelopment of outflow tract and aortic arch arteries is among the most common forms of human congenital heart diseases. Both *Bmp4* and *Tbx1* are known to play critical roles during cardiovascular development. Expression of these two genes partially overlaps in pharyngeal arch areas in mouse embryos. In this study, we applied a conditional gene inactivation approach to test the hypothesis that Bmp4 expressed from the *Tbx1* expression domain plays a critical role for normal development of outflow tract and pharyngeal arch arteries. We showed that inactivation of *Bmp4* from *Tbx1*-expressing cells leads to the spectrum of deformities resembling the cardiovascular defects observed in human DiGeorge syndrome patients. Inactivation of *Bmp4* from the *Tbx1* expression domain did not cause patterning defects, but affected remodeling of outflow tract and pharyngeal arch arteries. Our further examination revealed that *Bmp4* is required for normal recruitment/differentiation of smooth muscle cells surrounding the PAA4 and survival of outflow tract cushion mesenchymal cells.

Introduction

Congenital heart diseases occur in as many as 1% of newborns, and are the leading cause of infant morbidity and mortality [Hoffman, 1995; Hoffman and Kaplan, 2002]. Maldevelopment of the cardiac outflow tract and great vessels are the most often observed forms of congenital heart diseases in human patients [Hoffman, 1995; Hoffman and Kaplan, 2002]. The genetic, molecular and cellular mechanisms underlying proper development of the outflow tract and aortic arch arteries have been extensively studied, and yet remain elusive.

Abbreviations used in this paper

BMP	bone morphogenic protein
DS	DiGeorge syndrome
EMT	epithelial-mesenchymal transformation
IAA-B	interrupted aortic arch artery type B
NCC	neural crest cell
SMA	smooth muscle actin
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

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Fax +41 61 306 12 34
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Dr. Kai Jiao
Division of Research, Department of Genetics
University of Alabama at Birmingham, 768 Kaul Building
720 20th St. South, Birmingham, AL 35294 (USA)
Tel. +1 205 996 4198, Fax +1 205 975 5689, E-Mail kjiao@uab.edu

The embryonic outflow tract is derived from the second heart field and is initially formed as a single tube connecting the primitive right ventricle with symmetric pharyngeal arch arteries [Eisenberg and Markwald, 2004; Buckingham et al., 2005; Kelly, 2005; Black, 2007; Dyer and Kirby, 2009; Nakajima, 2010]. Pharyngeal arch arteries reside in a set of temporal embryonic apparatus termed pharyngeal arches, and are surrounded by mesenchymal cells derived from both paraxial mesodermal cells and neural crest cells (NCCs). During midgestation, a septum is formed within the single tube of the outflow tract to divide it into pulmonary and aortic outlets, which are connected with the right and left ventricles, respectively. In coordination with outflow tract septation, the original symmetric pharyngeal arch arteries are remodeled into the mature asymmetric aortic arch arteries [Graham, 2001; Hiruma et al., 2002; Graham, 2003; Yamagishi and Srivastava, 2003].

DiGeorge syndrome (DS) is the most common chromosome microdeletion syndrome in humans, affecting 1:4,000 live births [Lindsay and Baldini, 1998]. About 75% of DS patients are born with cardiac malformations mainly affecting the outflow tract and aortic arch arteries [Epstein, 2001; Grossfeld, 2003; Yamagishi and Srivastava, 2003; Baldini, 2004; Scambler, 2010]. Most DS patients have an about 3-Mb deletion in the 22q11.2 region, containing about 30 genes including *TBX1*, which encodes a T box transcription factor [Ryan and Chin, 2003; Yamagishi and Srivastava, 2003; Baldini, 2004; Plageman and Yutzey, 2005; Scambler, 2010]. Direct evidence linking *TBX1* to DS first came from mouse genetic studies showing that heterozygosity of *Tbx1* causes aortic arch defects affecting 4th pharyngeal arch artery derivatives similar to DS, while homozygosity of *Tbx1* causes most cardiovascular defects (including outflow tract and aortic arch defects) and pharyngeal arch defects (including hypoplasia or aplasia of pharyngeal glands, craniofacial dysmorphism and ear defects) seen in DS patients [Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001]. The discovery of mutations in *TBX1* from patients with DS phenotypes but without chromosomal deletion provides the conclusive evidence supporting the critical role of *Tbx1* in the pathogenesis of DS [Yagi et al., 2003]. During midgestation of mouse embryos, *Tbx1* is expressed in pharyngeal endodermal and mesodermal cells, and acts in both cell autonomous and nonautonomous manners to regulate outflow tract and pharyngeal arch artery development [Chapman et al., 1996; Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001; Abu-Issa et al., 2002; Frank et al., 2002; Kochilas et al., 2002; Vitelli et al., 2002a, b; Brown et al., 2004; Hu et al., 2004; Xu et al., 2004; Zhang et al., 2005, 2006; Arnold et al., 2006a, b]. Therefore, the *Tbx1* expression domain defines a group of cells with critical functions for outflow tract development and pharyngeal arch artery remodeling.

Bmp4 belongs to the bone morphogenic protein (BMP) family of secreted cytokines. Bmp4, like other BMP ligands, exerts its activity by binding to the type 1 and 2 receptor complex on the surface of target cells. The ligand-receptor complex then activates downstream signaling cascades through the canonical Smad pathway and noncanonical kinase pathways [Datto and Wang, 2000; Shi and Massagué, 2003; de Caestecker, 2004; ten Dijke and Hill, 2004; Feng and Derynck, 2005; Massague et al., 2005; Moustakas and Heldin, 2005; Massagué and Gomis, 2006]. Previous studies have demonstrated that *Bmp4* plays an essential role during outflow tract and pharyngeal arch artery development [Jiao et al., 2003; Liu et al., 2005; McCulley et al., 2008]. Of particular significance, *Bmp4* is highly expressed in the pharyngeal mesenchyme, overlapping with *Tbx1* expression in this region [Vitelli et al., 2002a; Jiao et al., 2003; Liu et al., 2004; Xu et al., 2004]. In this study, we use a conditional gene inactivation approach to address whether Bmp4 expressed from the *Tbx1* expression domain is an essential source for normal cardiovascular development.

Materials and Methods

Mouse Maintenance and Genotyping

All procedures using mice were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. *Tbx1-Cre*, $Bmp4^{loxP\text{-}lacZ}$ and $Bmp4^{tm1}$ (*Bmp4* null allele) mouse lines have been previously described [Fujiwara et al., 2002; Kulessa and Hogan, 2002; Jiao et al., 2003; Brown et al., 2004]. *Tbx1-Cre* mice were crossed with $Bmp^{tm1/+}$ mice to generate *Tbx1-Cre*;$Bmp4^{tm1/+}$ male mice, which were then crossed with $Bmp4^{loxP\text{-}lacZ/loxP\text{-}lacZ}$ female mice to produce *Tbx1-Cre*;$Bmp^{tm1/loxP\text{-}lacZ}$ mutant embryos. Mouse genotypes were determined by PCR analysis using *Cre* and *Bmp4* primers.

Histology, in situ Hybridization, X-Gal and Ink Injection

For histological analysis, all samples were fixed with 4% paraformaldehyde (PFA) and processed into paraffin-embedded sections using routine procedures. Procedures for wholemount in situ hybridization, X-gal staining and Indian ink injection were the same as described in Nie et al. [2008]. The plasmids for generation of in situ probes against *AP-2* and *Crabp1* were originally described in Feng and Williams [2003] and Dolle et al. [1990], respectively.

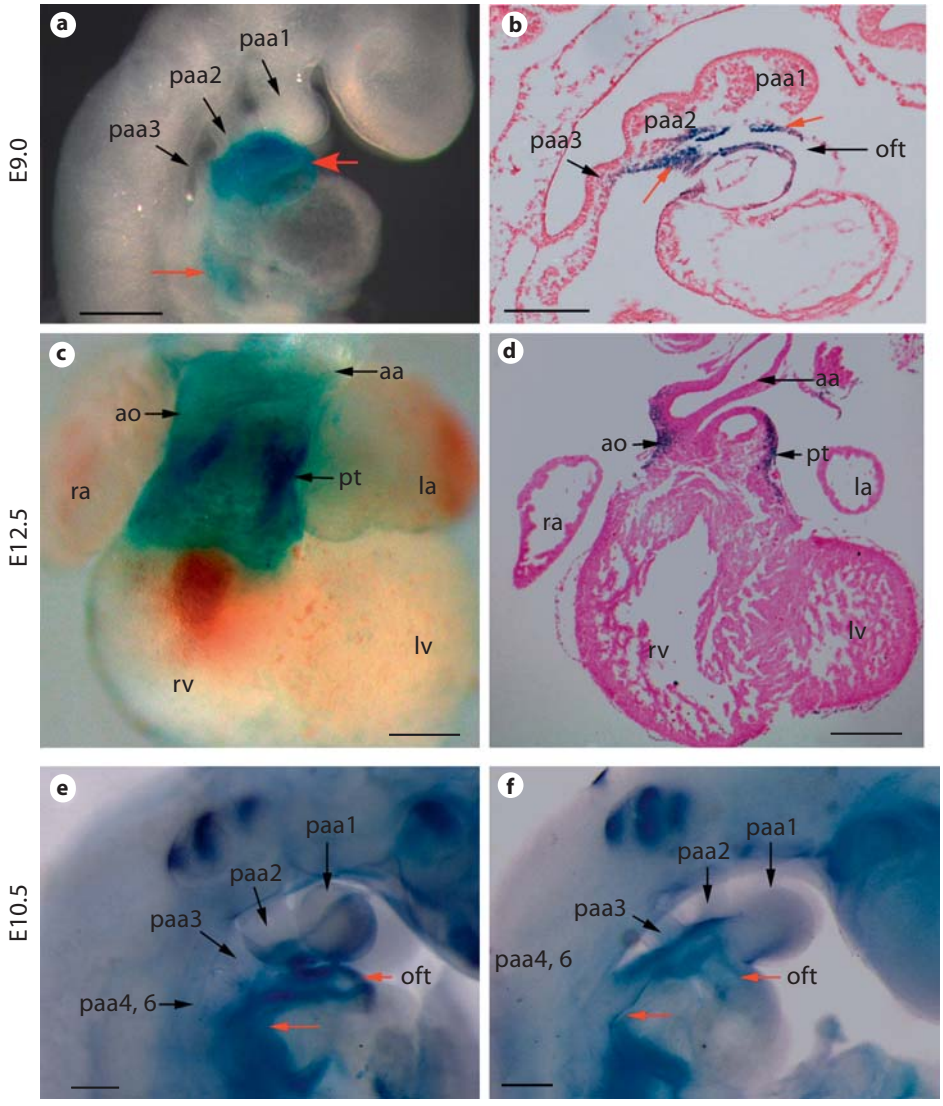

Fig. 1. Effective inactivation of *Bmp4* by *Tbx1-Cre*. **a–d** *Tbx1-Cre* male mice were crossed with $Bmp4^{loxP\text{-}lacZ/loxP\text{-}lacZ}$ female mice to obtain *Tbx1-Cre;-*$Bmp4^{loxP\text{-}lacZ/+}$ embryos at different stages. Cre-mediated recombination on the $Bmp4^{loxP\text{-}lacZ}$ allele will lead to *lacZ* knocked into the *Bmp4* locus, and therefore expression of the *lacZ* reporter will be under the control of endogenous *Bmp4* regulatory elements. An embryo at E9.0 was wholemount stained with X-gal (**a**), and was sagittally sectioned (**b**). A heart isolated from an E12.5 embryo was stained with X-gal (**c**) and further sectioned (**d**). The red arrows indicate examples of positively stained cells. **e**, **f** *Tbx1-Cre;*$Bmp4^{tm1/+}$ male mice were crossed with $Bmp4^{loxP\text{-}lacZ/loxP\text{-}lacZ}$ female mice to get mutant embryos (*Tbx1-Cre;-*$Bmp4^{tm1/loxP\text{-}lacZ}$) and their littermate controls at E10.5. Wholemount in situ hybridization analysis was performed using a probe corresponding to the exon 4 of *Bmp4*, which is expected to be removed upon Cre-mediated recombination. The red arrows indicate the region where the signal was dramatically reduced in the mutant embryo (**f**) compared to the control (**e**). Scale bar = 200 μm.

Abbreviations used in figures 1–6

aa	aortic arch artery	paa1–4, paa6	1st–4th and 6th pharyngeal arch artery derivatives
ao	aorta (or ascending aorta)	paa	pharyngeal arch artery
as	aortic sac	pt	pulmonary trunk
da	descending aorta	PTA	persistent truncus arteriosus
la	left atrium	ra	right atrium
lca	left carotid artery	rca	right carotid artery
lsa	left subclavian artery	rsa	right subclavian artery
lv	left ventricle	rv	right ventricle
oft	outflow tract	vsd	ventricular septal defect
pa	pulmonary artery	vs	ventricular septum

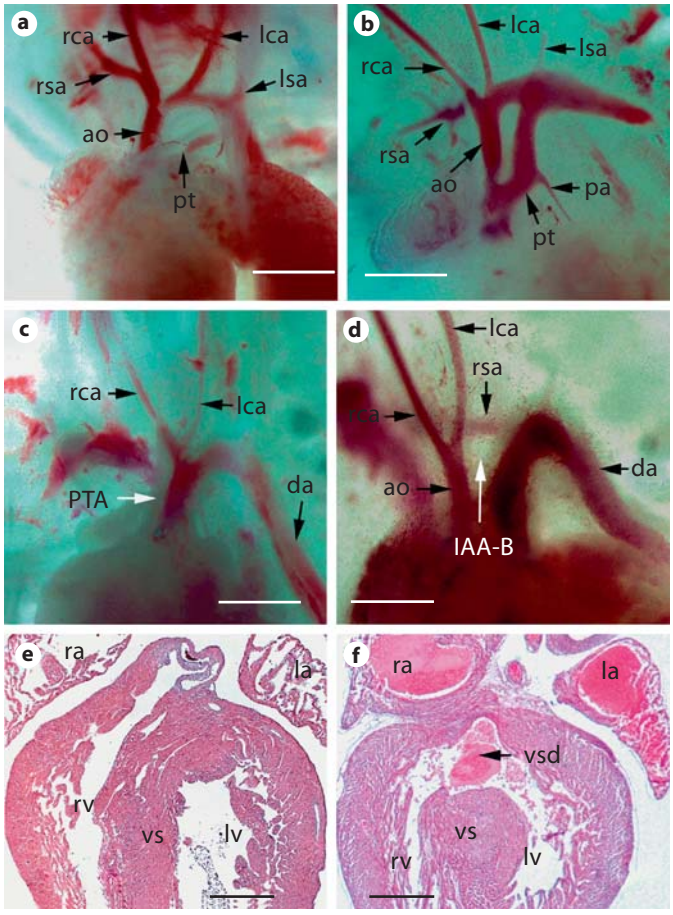

Fig. 2. Spectrum of cardiovascular defects in *Tbx1-cre;Bmp4*$^{tm1/loxP\text{-}lacZ}$ embryos. *Tbx1-Cre;Bmp4*$^{tm1/+}$ male mice were crossed with *Bmp4*$^{loxP\text{-}lacZ/loxP\text{-}lacZ}$ female mice to obtain mutant embryos *(Tbx1-Cre;Bmp4*$^{tm1/loxP\text{-}lacZ}$*)* and their littermate controls at different stages. **a–d** Gross examination of the outflow tract and pharyngeal arch artery regions of control (**a**) and mutant embryos (**c**, **d**) at E14.5. **b** Example of a mutant embryo with the outflow tract septation defect observed in the proximal region, but not in the distal region. **c** Example of a mutant embryo with complete persistent truncus arteriosus. **d** Embryo with IAA-B and retroesophageal right subclavian artery, which are commonly observed aortic arch artery defects in DS patients. All embryos were subsequently sectioned, revealing that they all possess a ventricular septum defect (data not shown). **e**, **f** A mutant and a control embryonic heart at E19.5 were sectioned and HE stained. A ventricular septal defect is identified in the mutant heart (**f**). Scale bars = 500 μm.

Apoptosis and Immunohistochemistry Assays

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed using DeadEnd Colorimetric TUNEL System (Promega) following the manufacturer's protocol. An anti-phosphorylated histone H3 polyclonal antibody (1:1,000, cat. No. 06-570, Upstate) and an anti-smooth muscle actin (SMA) monoclonal antibody (1:1,000, clone 1A4, Sigma) were used for immunohistochemical analysis. Signals were visualized using the Envision + system (DakoCytomation), and sections were counterstained with hematoxylin to visualize nuclei.

Table 1. Penetrance of different phenotypes observed in mutant embryos between E14.5 and E19.5

Phenotype	Penetrance
Persistent truncus arteriosus	43% (21/49)
Outflow tract septation defect at the distal region	57% (28/49)
Interrupted aortic arch artery type B	47% (23/49)
Retroesophageal right subclavian artery	8% (4/49)
Ventricular septal defect	100% (49/49)

Results

Efficient Inactivation of Bmp4 *in the Pharyngeal Region by* Tbx1-Cre

The *Tbx1-Cre* transgene has been shown previously to faithfully label *Tbx1* expressing cells in pharyngeal mesodermal and endodermal cells [Brown et al., 2004]. To determine whether *Tbx1-Cre* can efficiently inactivate *Bmp4*, we crossed *Tbx1-Cre* mice with *Bmp4*$^{loxP\text{-}lacZ/loxP\text{-}lacZ}$ mice to obtain *Tbx1-Cre;Bmp4*$^{loxP\text{-}lacZ/+}$ embryos, and performed X-gal staining on these embryos. As demonstrated in previous studies [Kulessa and Hogan, 2002; Jiao et al., 2003], Cre-mediated recombination in the *Bmp4*$^{loxP\text{-}lacZ}$ allele will lead to expression of lacZ under the control of endogenous *Bmp4* regulatory elements. Therefore, the lacZ-positive cells are derivatives of *Tbx1*-expressing cells that express endogenous *Bmp4*. As early as E9.0, lacZ-positive cells could be detected in the outflow tract myocardial cells and pharyngeal mesenchymal cells from both wholemount and section studies (fig. 1a, b). We also observed weak signals in cells adjacent to the venous pole of embryonic hearts from wholemount staining (fig. 1a). These results confirmed that *Bmp4* is expressed in *Tbx1* expression derivatives and demonstrated that *Tbx1-Cre* can mediate recombination at the *Bmp4* locus at E9.0 when cells from the anterior heart field actively join the outflow tract through the arterial pole. It was shown previously that *Tbx1-Cre* can inactivate target genes in the pharyngeal endoderm [Brown et al., 2004], where expression of *Bmp4* is also detected [Liu et al., 2005; McCulley et al., 2008]. However, we did not observe lacZ-positive endodermal cells in the pharyngeal region. This result suggests

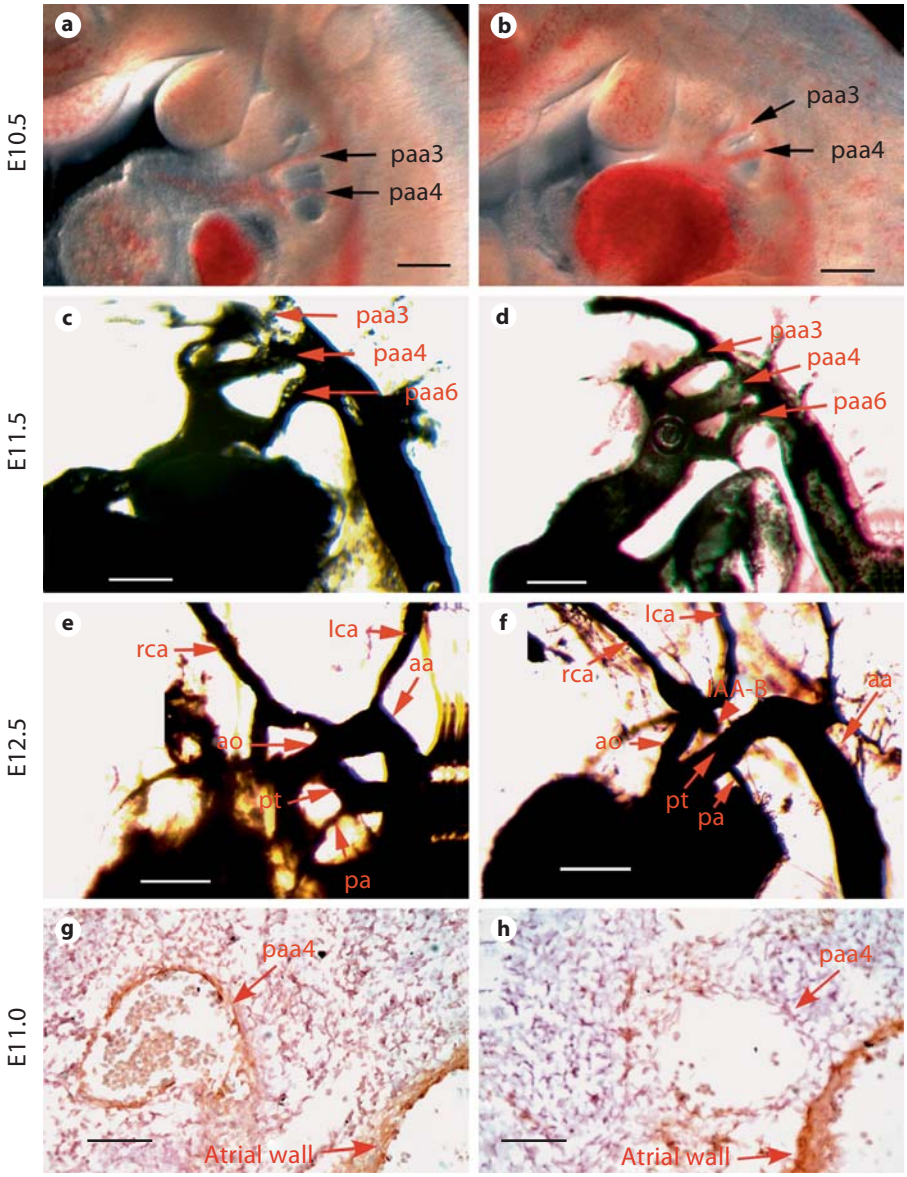

Fig. 3. Defective pharyngeal arch artery remodeling in *Tbx1-cre;Bmp4*$^{tm1/loxP\text{-}lacZ}$ embryos. **a**, **b** The 3rd and 4th pharyngeal arch artery derivatives were patent in both wild-type (**a**) and mutant (**b**) E10.5 embryos. **c–f** Cardiac ink injection was performed on E11.5 (**c**, **d**) and E12.5 (**e**, **f**) embryos. The remodeling of pharyngeal arch arteries was comparable between wild-type (**c**) and mutant (**d**) embryos at E11.5. While at E12.5, the remodeling defect became apparent in mutant embryos (**f**). The arrowhead in **f** indicates the IAA-B defect. **g**, **h** Sagittal sections of a wild-type (**g**) and a mutant (**h**) embryo at E11.0 were immunostained with an antibody against SMA. A group of SMA-positive cells surrounding the endothelial tube of the right 4th pharyngeal arch artery derivative were observed in the wild-type embryo but not in the mutant one. Expression of SMA was detected in the atrial wall of both control and mutant embryos. Scale bars = 200 μm (**a–f**); 30 μm (**g**, **h**).

that the group of cells expressing *Bmp4* do not overlap with those expressing Cre in the endoderm. At E12.5, lacZ-positive cells were mainly detected in the cardiomyocytes at the roots of the aorta and pulmonary trunk (fig. 1c, d).

To directly test the reduction of *Bmp4* at the mRNA level, we crossed *Tbx1-Cre;Bmp4*$^{tm1/+}$ mice with *Bmp4*$^{loxP\text{-}lacZ/loxP\text{-}lacZ}$ mice to acquire mutant embryos (*Tbx1-Cre;Bmp4*$^{tm1/loxP\text{-}lacZ}$). *Bmp4*^{tm1} is a null allele of *Bmp4* [Fujiwara et al., 2002]. Wholemount in situ hybridization was performed using a probe corresponding to the exon 4 of *Bmp4*. Exon 4 is flanked with two *LoxP* sites in the *Bmp4*$^{loxP\text{-}lacZ}$ allele and is expected to be deleted upon Cre-mediated recombination [Kulessa and Hogan, 2002]. As shown in figure 1e, f, expression of *Bmp4* was nearly eliminated from the developing outflow tract and was visibly reduced from the pharyngeal mesenchyme in mutant embryos compared with litter mate control embryos. Therefore, results from both reporter and in situ hybridization assays collectively indicate that *Tbx1-Cre* can efficiently inactivate expression of *Bmp4* from the pharyngeal mesenchymal and outflow tract myocardial cells.

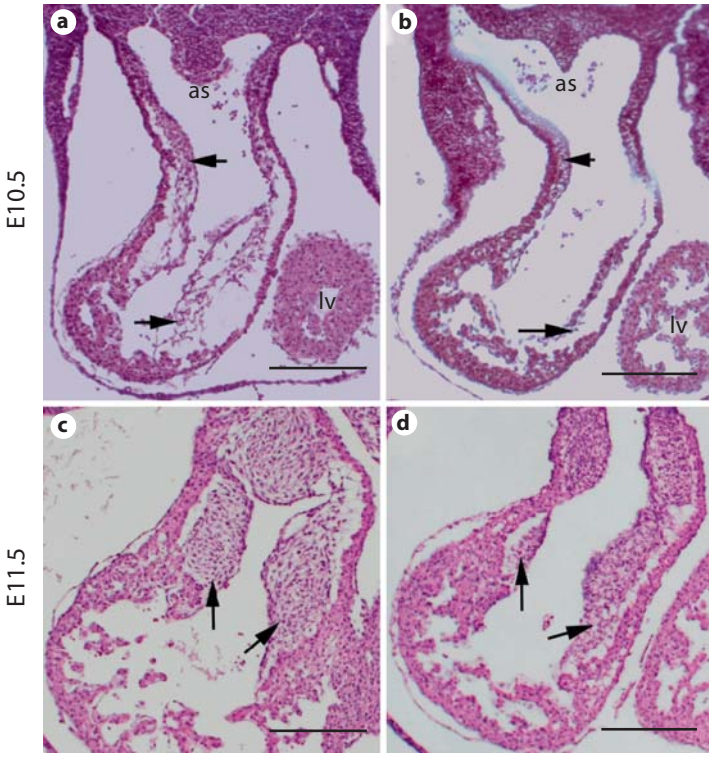

Fig. 4. Hypoplastic outflow tract cushion defect in *Tbx1-cre;Bmp4*$^{tm1/loxP\text{-}lacZ}$ embryos. Mutant (**b**, **d**) and littermate control (**a**, **c**) embryos were isolated at E10.5 (**a**, **b**) and 11.5 (**c**, **d**), and cross-sectioned followed by HE staining. The arrows indicate outflow tract cushions. Scale bars = 200 μm.

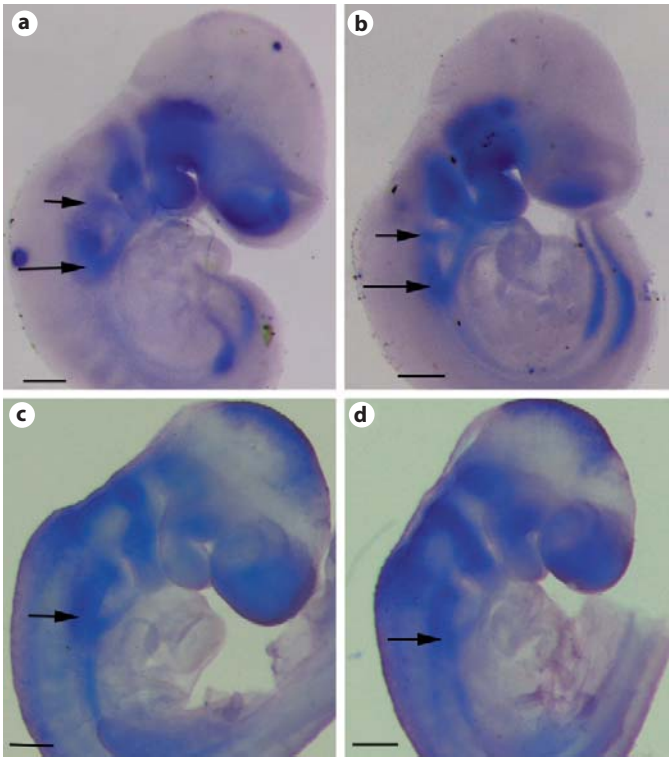

Fig. 5. Normal expression of NCC markers in the pharyngeal region. Mutant (**b**, **d**) and control (**a**, **c**) embryos were isolated at E9.5 and subjected to wholemount in situ hybridization analysis using *AP-2* (**a**, **b**) and *Crabp1* (**c**, **d**) probes. The arrows indicate NCCs between the 4th and 6th pharyngeal arch artery derivatives, where cardiac NCCs are localized. Scale bars = 200 μm.

Inactivation of Bmp4 *from the* Tbx1 *Expression Domain Leads to a Spectrum of Cardiovascular Defects Resembling Those of the Human DS*

Tbx1-Cre;Bmp$^{tm1/+}$ male mice were crossed with female *Bmp4*$^{loxP\text{-}lacZ/loxP\text{-}lacZ}$ mice to generate *Tbx1-Cre;Bmp4*$^{tm1/loxP\text{-}lacZ}$ mutant animals. The number of mutants found at birth was about 14% (19/137), which is lower than the expected Mendelian ratio. Newborn mutants were externally normal, but died shortly after birth presumably due to cardiovascular defects. The number of mutant embryos at E16.5 was about 24% (23/97), close to the expected Mendelian ratio, suggesting that many mutant embryos died between E16.5 and birth.

Gross and sectional examination showed that all the mutant embryos beyond E14.5 displayed outflow tract separation defect and ventricular septal defect (fig. 2, table 1). In severe cases, the outflow tract remained as a single vessel connecting to both left and right ventricles, a condition known as persistent truncus arteriosus. In less severe cases, the proximal portion of the outflow tract remained unseparated, whereas the distal portion was unaffected. Interrupted aortic arch artery type B (IAA-B), which is a characteristic vascular defect in DS patients, was frequently observed in mutant embryos. Retroesophageal right subclavian artery, another vascular defect associated with DS, was also occasionally seen in mutant embryos. In table 1, we summarized the frequency of various cardiovascular defects in embryos with *Bmp4* inactivated in the *Tbx1* expression domain.

Defective Pharyngeal Arch Remodeling and Abnormal Aortic Arch Morphogenesis in the Mutant Embryos

To determine whether these pharyngeal arch artery abnormities were due to early patterning defects, we examined embryos at E10.5, and found that pharyngeal arch arteries were well formed and patent in all mutant embryos examined (fig. 3a, b). We next performed cardiac ink injection on E11.5, E12.5 and E13.5 embryos to exam-

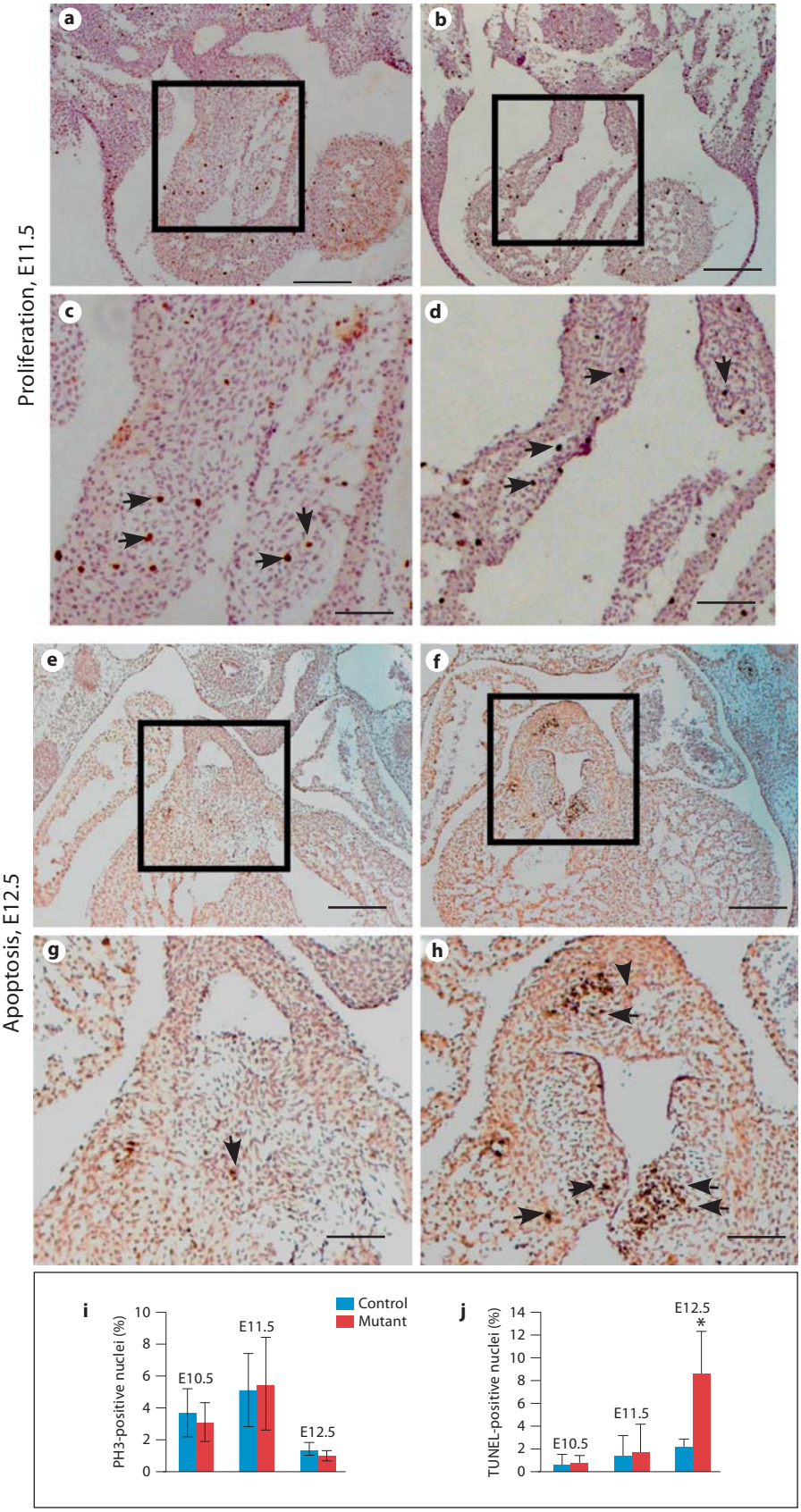

Fig. 6. Cell proliferation and apoptosis in the outflow tract cushion of control (**a**, **c**, **e**, **g**) and mutant (**b**, **d**, **f**, **h**) embryos. **a–d** E11.5 embryos were cross-sectioned and immunostained with an antibody against phospho-histone H3 (PH3), which stains cells at the M phase. **c** and **d** correspond to **a** and **b**, respectively. The arrows indicate examples of positively stained nuclei. No visible reduction in cell proliferation rate was detected. Similar results were observed at other stages (data not shown). **e–h** E12.5 embryos were cross-sectioned and subjected to the TUNEL assay. **g** and **h** correspond to **e** and **f**, respectively. Arrows indicate examples of apoptotic cells in outflow tract cushions. More apoptotic cells were observed in mutant outflow tract cushions. **i**, **j** Quantitative analysis of cell proliferation (**i**) and apoptosis (**j**) in outflow tract cushions of control and mutant embryos from E10.5 to E12.5. Data were averaged from at least 3 independent embryos with error bars indicating standard derivation. * $p < 0.01$ (Student's t test). Scale bars = 200 μm (**a**, **b**, **e**, **f**); 80 μm (**c**, **d**, **g**, **h**).

ine the remodeling status of the pharyngeal arch arteries. No pharyngeal arch artery defect was apparent in E11.5 embryos (fig. 3c, d). Starting from E12.5, we often detected complete interruption or regression of the aortic arch in the mutant embryos (4/7) (fig. 3e, f). These data suggest that inactivation of *Bmp4* from *Tbx1* domains does not affect the initial formation and patterning of pharyngeal arch arteries, but rather cause their abnormal remodeling. To better understand the cellular mechanism underlying the interruption of the 4th pharyngeal arch artery derivative, we performed immunohistochemistry analysis on sections of mutant and control embryos at E11.0 using an antibody against SMA (fig. 3g, h). We showed that the endothelial tube of the right 4th pharyngeal arch artery derivative in the control embryo is surrounded by a group of SMA-positive cells, while few SMA-positive cells are observed surrounding the 4th pharyngeal arch artery derivative of mutant embryos. Our results suggest that Bmp4 expressed in the *Tbx1* expression domain is required for normal recruitment and/or differentiation of smooth muscle cells surrounding the endothelial tube of the 4th pharyngeal arch artery derivative .

Bmp4 Is Required for Normal Cellularization of Outflow Tract Cushions

Outflow tract cushions are precursors of outflow tract septum and semilunar valves. We examined the outflow tract cushions at E10.5 and E11.5. Transverse sections of the embryos revealed that mutant outflow tract cushions were visibly hypoplastic and poorly cellularized compared with those of littermate controls (fig. 4). The outflow tract cushion mesenchymal cells are derived from both endocardial cells and incoming NCCs. We therefore further tested whether distribution of NCCs in the pharyngeal region was also impaired in mutant embryos. We examined expression of NCC markers including *Crabp1* and *AP-2* at E9.5, when cardiac NCCs have already colonized at the pharyngeal regions. Expression of *Crabp1* and *Ap-2α* in the mutant embryos was comparable to the controls in the pharyngeal region between the 3rd an 6th pharyngeal arch artery derivatives (fig. 5), implying that cardiac NCC migration to the pharyngeal arches and their distribution in the pharyngeal region was largely unaffected by disruption of *Bmp4*.

We next examined cell apoptosis/proliferation during outflow tract cushion morphogenesis. No obvious reduction in cell proliferation rate was observed in mutant outflow tract cushions at E11.5 (fig. 6a–d) or later stages (data not shown). We can clearly detect apoptotic cells in wild-type outflow tract cushions at E12.5, suggesting that active apoptosis is a normal process during outflow tract cushion remodeling. The number of apoptotic cells was visibly increased starting from E12.5 in mutant outflow tract cushions. Our observation is further confirmed with quantitative analysis (fig. 6i, j). Since the hypocellular outflow tract cushion defect was observed in E10.5 and E11.5 mutant embryos, the increased cell death at E12.5 cannot account for the initial cushion defect (also see Discussion).

Discussion

In this study, we provide convincing evidence demonstrating that inactivation of *Bmp4* from the *Tbx1* expression domain leads to a spectrum of cardiovascular defects resembling DS. No defect is observed in the myocardium of mutant embryos, even though expression of *Bmp4* from the outflow tract myocardium is visibly reduced in *Tbx1-Cre;Bmp4*$^{tm1/loxP\text{-}lacZ}$ mutant embryos (fig. 1). The lack of myocardial defect is consistent with previous studies using different Cre lines (*cTnt-Cre*, *Nkx2.5-Cre* and *Mef2c-AHF-Cre*) to conditionally inactivate *Bmp4* [Jiao et al., 2003; Liu et al., 2004; McCulley et al., 2008]. These studies collectively support the notion that Bmp4 expressed in the outflow tract myocardium and pharyngeal region is not essential for cardiomyocyte development, but is required for remodeling of outflow tract cushions and pharyngeal arch arteries. A previously published study demonstrated that inactivation of *Bmp4* specifically from the anterior heart field by *Mef2c-AHF-Cre* led to abnormal outflow tract development, while no defect in pharyngeal arch arteries was observed in mutant embryos [McCulley et al., 2008]. We noticed that *Tbx1-Cre* and *Mef2c-AHF-Cre* both inactivate *Bmp4* expression in similar pharyngeal regions including pharyngeal arch mesenchymal cells and the outflow tract myocardial cells, and yet defects in *Tbx1-Cre;Bmp4*$^{tm1/loxP\text{-}lacZ}$ embryos were not restricted to the outflow tract region. In addition to abnormal outflow tract morphogenesis, *Tbx1-Cre;Bmp4*$^{tm1/loxP\text{-}lacZ}$ embryos displayed severe pharyngeal arch artery defects including IAA-B and retroesophageal right subclavian artery, which are both characteristic vascular abnormalities associated with DS. We further showed that Bmp4 expressed from the *Tbx1* expression domain is essential for forming the smooth muscle cell wall surrounding the 4th pharyngeal arch artery derivative. The different results between these two studies are likely due to differences in Cre activity and/or the extent of target cell population between the two lines.

Pharyngeal arch arteries are initially formed as bilateral symmetric vessels. During remodeling, some pharyngeal arch artery segments degenerated, while other segments remained part of the major vessels. Asymmetric remodeling of outflow tract and pharyngeal arch artery is a complex process governed by a signaling network that remains poorly understood. Our data provide additional evidence that Bmp4 is an important participant within this network. Interestingly, tissue-specific disruption of *Bmp4* from *Tbx1*-expressing cells does not affect early morphogenesis of the outflow tract and pharyngeal arch arteries, but leads to abnormal pharyngeal arch artery and outflow tract remodeling. This is in contrast to *Tbx1* mutant embryos. Most *Tbx1*+/– embryos display hypoplastic fourth pharyngeal arch arteries at E10.5, and the failure of recovery from this early defect at least partially contributes to the pharyngeal arch artery defects at later developmental stages [Vitelli et al., 2002a]. Therefore, *Bmp4* may promote remodeling of outflow tract cushions and pharyngeal arch arteries through a pathway not directly associated with *Tbx1*. This idea is further supported by our observation that expression of *Tbx1* was not visibly altered in *Tbx1-Cre;Bmp4*$^{tm1/loxP\text{-}lacZ}$ embryos (data not shown). However, these results do not exclude the potential crosstalk between *Tbx1*- and *Bmp4*- mediated pathways. A recent study showed that *Tbx1* can modulate Bmp-signaling activities by binding BMP receptor-activated Smads, which are nuclear mediators of BMP signaling, to prevent them from forming a complex with the Smad4 transcriptional coactivator [Fulcoli et al., 2009]. In addition, several previous studies have demonstrated that *Tbx1* is required for normal expression during development [Raft et al., 2004; Moraes et al., 2005; Arnold et al., 2006a; Aggarwal et al., 2010]. In *Tbx1*–/– embryos, the expression level and pattern of *Bmp4* was disturbed in otocysts and mandibular arch [Raft et al., 2004; Moraes et al., 2005; Aggarwal et al., 2010]. The requirement of *Tbx1* for normal *Bmp4* expression in otic vesicle was also demonstrated through tissue-specific gene ablation studies [Arnold et al., 2006a]. These results suggest that *Tbx1* acts upstream of *Bmp4* to regulate its expression; however, it is currently unclear whether *Bmp4* is a direct downstream target gene of Tbx1. Our immunohistochemical assay showed that *Bmp4* is required for proper recruitment and/or differentiation of smooth muscle cells surrounding the endothelial tube of the 4th pharyngeal arch artery derivative on the right side (fig. 3). The absence of an intact smooth muscle cell layer may cause fragility of pharyngeal arch artery 4 and result in interruption during remodeling. Our result is consistent with the previous report in which a smooth muscle reporter mouse line was used to show that inactivation of *Bmp4* by *Nkx2.5-Cre* leads to a deficiency in recruiting smooth muscle cells to the pharyngeal arch artery region [Liu et al., 2004].

We observed the severe hypocellular abnormality in both proximal and distal regions of mutant outflow tract cushions as early as E10.5 and E11.5, and yet no obvious defect in cushion mesenchyme proliferation/death was detected at these stages (fig. 6). Therefore, the initial cushion defect in mutant embryos is not caused by aberrant cell proliferation and apoptosis. The mesenchymal cells within the proximal truncus arteriosus region of the outflow tract are mainly derived from endocardial cells through epithelial-mesenchymal transformation (EMT) [Jiang et al., 2000; Person et al., 2005]. Thus, the hypocellular defect of the proximal outflow tract region is likely caused by impaired EMT in mutant embryos. The role of BMP signaling in promoting EMT has been well documented in atrioventricular cushions [Ma et al., 2005; Wang et al., 2005; Park et al., 2006; Rivera-Feliciano and Tabin, 2006; Song et al., 2007]. Our data suggest that Bmp4 expressed from the *Tbx1* expression domain is required for normal EMT in the outflow tract; however, confirmation of this hypothesis will require further vigorous experimental testing. In addition to endocardial cell-derived mesenchyme, a large portion of mesenchymal cells within the distal conus arteriosus region of the outflow tract are derived from cardiac NCCs, which have migrated into outflow tract cushions from the dorsal neural tube through PA3, 4 and 6. Our in situ hybridization analysis using probes against *Crabp1* and *Ap-2α* showed normal distribution of NCCs in the pharyngeal region (fig. 5), suggesting that NCCs can properly migrate into pharyngeal arches from the neural crest. We therefore infer that migration of cardiac NCCs from pharyngeal arches into outflow tract cushions is impaired by depletion of *Bmp4* and contributes to the hypocellular outflow tract cushion defect in the distal region of mutant embryos. A previous study has demonstrated that Fgf signaling promotes *Bmp4* expression in the mesodermal cells of second heart field in an autocrine manner, and disruption of Fgf signaling in the second heart field significantly reduced *Bmp4* expression in the outflow tract and led to outflow tract cushion defects [Park et al., 2008]. Our study further suggests that *Bmp4* in pharyngeal mesodermal cells acts on endocardial cells, outflow tract cushion mesenchymal cells and NCCs to promote normal outflow tract development.

Several lines of evidence suggest that outflow tract morphogenesis is differently regulated along the proximal-distal segments [Kim et al., 2001; Liu et al., 2004]. Although *Bmp4* is highly expressed throughout the entire outflow tract myocardial tube, *Bmp4* deficiency from the outflow tract most severely affected the proximal portion. All *Tbx1-Cre;Bmp4*$^{tm1/loxP\text{-}lacZ}$ embryos examined displayed septation defects in the proximal portion whereas 57% of them were well separated in the distal portion. Previous studies showed that the distal portion of the outflow tract can also be regulated by other Bmp ligands, such as *Bmp6* and *Bmp7* [Kim et al., 2001; Liu et al., 2004]. Double knockout of *Bmp6* and *Bmp7* leads to separation defects in the distal outflow tract [Kim et al., 2001]. Therefore, functional redundancy among Bmp ligands may explain the incomplete penetrance of separation defects on the distal portion of the mutant outflow tract.

In summary, our data highlight the importance of the Bmp4 ligand produced from the *Tbx1* expression domain in regulating outflow tract and pharyngeal arch artery remodeling. This study provides a unique mouse model for further studying the Bmp4 downstream regulatory targets in the pharyngeal arch mesenchymal cells and the outflow tract cardiomyocytes that are critical for cardiovascular development.

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