

Cardiac outflow tract defects in mice lacking ALK2 in neural crest cells

Vesa Kaartinen^{1,*}, Marek Dudas¹, Andre Nagy¹, Somyoth Sridurongrit¹, Min Min Lu² and Jonathan A. Epstein²

¹Developmental Biology Program, The Saban Research Institute of Childrens' Hospital Los Angeles, Departments of Pathology and Surgery, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

²Cardiovascular Division, Department of Medicine and the Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA, USA

*Author for correspondence (e-mail. vkaartinen@chla.usc.edu)

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Summary

Cardiac neural crest cells are multipotent migratory cells that contribute to the formation of the cardiac outflow tract and pharyngeal arch arteries. Neural crest-related developmental defects account for a large proportion of congenital heart disorders. Recently, the genetic bases for some of these disorders have been elucidated, and signaling pathways required for induction, migration and differentiation of cardiac neural crest have emerged. Bone morphogenetic proteins comprise a family of secreted ligands implicated in numerous aspects of organogenesis, including heart and neural crest development. However, it has remained generally unclear whether BMP ligands act directly on neural crest or cardiac myocytes during cardiac morphogenesis, or function indirectly by activating other cell types. Studies on BMP receptor signaling during organogenesis have been hampered by the fact that receptor knockouts often lead to early embryonic lethality.

We have used a Cre/loxP system for neural crest-specific deletion of the type I receptor, ALK2, in mouse embryos. Mutant mice display cardiovascular defects, including persistent truncus arteriosus, and abnormal maturation of the aortic arch reminiscent of common forms of human congenital heart disease. Migration of mutant neural crest cells to the outflow tract is impaired, and differentiation to smooth muscle around aortic arch arteries is deficient. Moreover, in *Alk2* mutants, the distal outflow tract fails to express *Msx1*, one of the major effectors of BMP signaling. Thus, the type I BMP receptor ALK2 plays an essential cell-autonomous role in the development of the cardiac outflow tract and aortic arch derivatives.

Key words: Bone morphogenetic proteins, ALK2, Outflow tract development, Mouse

Introduction

Conotruncal cardiovascular malformations are a major cause of neonatal mortality (Creazzo et al., 1998). These conditions can be isolated or they can be associated with craniofacial, thymic and parathyroid birth defects as a part of the velocardiofacial/DiGeorge syndrome that is caused by a deletion of the DiGeorge critical region (DGCR) on chromosome 22q11 (Scambler, 2000; Epstein, 2001). Within this region, *TBX1* has emerged as a crucial gene for cardiovascular development, and mutations in *TBX1* have recently been described in individuals with the DiGeorge phenotype, but without the DGCR deletion (Yagi et al., 2003). Other genes within the DGCR have also been implicated in the DiGeorge phenotype including *HIRA* and *CRKL* (Farrell et al., 1999b; Guris et al., 2001). Neural crest cells (NCC) are known to migrate to the arterial pole of the heart and to differentiate into vascular smooth muscle, and outflow tract (OFT) and aortic arch defects, such as those seen in DiGeorge syndrome, can be reproduced in animal models with defects in neural crest. However, *Tbx1* is not expressed in NCCs, leading to the hypothesis that crucial signaling pathways between *Tbx1*-expressing cells and migrating NCCs orchestrate specific

aspects of cardiovascular development. Members of the FGF, PDGF and TGF β family of signaling molecules have been implicated in this process (Lindsay and Baldini, 2001; Abu-Issa et al., 2002; Frank et al., 2002; Bachiller et al., 2003; Tallquist and Soriano, 2003; Brown et al., 2004). Aberrant signaling through these pathways may contribute to some forms of genetic and environmentally induced congenital heart disease.

The NC is a multipotent population of cells that originates from the dorsal neural tube at all axial levels (LaBonne and Bronner-Fraser, 1999). A subpopulation of NCCs called the cardiac neural crest (CNC) delaminates from the rhombencephalon between the mid-otic placode and the third somite (Kirby et al., 1983; Kirby and Waldo, 1995), and migrates lateroventrally along the pharyngeal arch arteries into pharyngeal arches 3, 4 and 6. Some CNC cells participate in patterning of pharyngeal arch arteries, whereas others continue to migrate to the conotruncus, where they contribute to formation of the aorticopulmonary septum (Knecht and Bronner-Fraser, 2002). BMPs have been shown to play an important role together with other soluble factors, such as WNTs and FGFs, in NC specification, possibly by serving a

maintenance role in the induction process (Gammill and Bronner-Fraser, 2003). Misexpression of the BMP antagonist noggin (Nog) in the premigratory NC disrupts early specification, although it is unclear if this is a cell-autonomous effect (Ohnemus et al., 2002). A direct role for BMP signaling in later stages of cardiac neural crest development has not been demonstrated.

Members of the TGF β superfamily, including BMP and TGF β proteins signal through a heteromeric receptor complex of type I and type II transmembrane Ser/Thr kinase receptors (Massague, 1998). Upon ligand binding, type II receptors, which are constitutively active kinases, phosphorylate and activate type I receptors (also called ALKs) (Massague, 2000; Derynck and Zhang, 2003). It has been shown that individual BMPs elicit distinct cellular responses and bind to different type I receptors with different binding affinities. For example, ALK3 and ALK6 are able to bind and transduce signaling from structurally distinct BMPs (ten Dijke et al., 1994; Jamin et al., 2002). ALK2, however, is more specific and binds preferentially the 60A subgroup of BMPs, i.e. BMP5, BMP6 and BMP7 in vitro (Macias-Silva et al., 1998). Simultaneous inactivation of *Bmp6* and *Bmp7* in mice leads to cardiac OFT, valve and septation defects (Kim et al., 2001). Interestingly, *Tgfb2* knockout mice display a plethora of phenotypes including cardiac defects, such as double outlet right ventricle (Sanford et al., 1997), while mice deficient in *Tgfb1* or *Tgfb3* do not display any cardiac phenotypes (Shull et al., 1992; Kulkarni et al., 1993; Kaartinen et al., 1995; Proetzel et al., 1995). ALK2 was originally cloned as a TGF β type I receptor (Ebner et al., 1993), and, indeed, it appears that in specific cell types, such as mouse mammary epithelial cells and chick endocardial cushion cells, ALK2 can also mediate TGF β signals (Miettinen et al., 1994; Lai et al., 2000). Although ALK2 is also able to bind activin in vitro (Attisano et al., 1993; Tsuchida et al., 1993), it cannot transduce activin-like signals under physiological conditions (Chen et al., 1997; Suzuki et al., 1997a; Macias-Silva et al., 1998).

Type I receptors are the primary determinants of the downstream signaling specificity and therefore understanding their function is key to uncovering molecular signaling mechanisms regulated by BMPs during embryogenesis. Unfortunately, the early embryonic lethality at gastrulation has prevented the use of conventional *Alk2*-knockout mice in assessing the role of ALK2 later in development (Gu et al., 1999; Mishina et al., 1999). To circumvent this hurdle, we used the Cre-loxP strategy to specifically abrogate *Alk2* function in NCCs. The resulting ALK2 mutants display aortic arch and cardiac OFT defects reminiscent of common forms of human congenital heart disease. Based on the presented results we conclude that ALK2 plays an essential cell-autonomous role in the later stages of cardiac neural crest development and differentiation.

Materials and methods

Alk2/Wnt1-Cre mice

The *Alk2* targeting vector was generated by inserting a *loxP-pgkneo-loxP* cassette in intron 7, and an additional loxP site in intron 6 as described (Kaartinen and Nagy, 2001; Dudas et al., 2004). Mice homozygous for the *Alk2^{Flox}* allele (*Alk2^{Flox/Flox}*) were generated by removing the *PGK-Neo* selection marker as described (Kaartinen and

Nagy, 2001). Cre-mediated recombination of the *Alk2^{Flox}* allele generates the *Alk2^{KO}* allele. Mice heterozygous for the *Alk2^{KO}* allele are phenotypically normal, whereas mice homozygous for the *Alk2^{KO}* allele die in gastrulation with a phenotype identical to that previously described for a related null allele (Gu et al., 1999; Mishina et al., 1999). *Wnt1-Cre* mice were kindly provided by A. McMahon (Harvard University) and R26R reporter mice were obtained from the Jackson Laboratories.

Histological analyses

Embryonic tissues were fixed with 4% formaldehyde for 12 hours, dehydrated and embedded in paraffin wax. Sections (5 μ m) were stained with Hematoxylin and Eosin. Embryos or dissected hearts were stained for β -galactosidase activity as described (Hogan et al., 1994). Briefly, the specimens were fixed in 4% formaldehyde for 30 minutes at room temperature, washed three times for 10 minutes in the detergent wash, and developed for 2-6 hours in the X-gal staining solution.

Intracardiac ink injections

Indian ink was injected intracardially with custom made glass pipettes (12 μ m opening) at E10.5 and E11.5. After injections, embryos were fixed in 4% formaldehyde for 12 hours, dehydrated and cleared in benzyl benzoate:benzyl alcohol (2:1).

In situ hybridization and immunohistochemistry

Radioactive in situ hybridization was performed as described (Wawersik and Epstein, 2000). Protocols are available at www.uphs.upenn.edu/mcrc. Whole-mount in situ hybridization on embryos was carried out as described (Hogan et al., 1994). Probes specific for *Bmp2* (Kim et al., 2001), *Bmp4* (Kim et al., 2001), *Bmp5* (Solloway and Robertson, 1999), *Bmp6* (Kim et al., 2001), *Bmp7* (Kim et al., 2001), *Tgfb2* (Blavier et al., 2001), *Tgfb3* (Blavier et al., 2001), *Msx1* (Furuta et al., 1997), *Foxd3* (Labosky and Kaestner, 1998), *Sox10* (Lioubinski et al., 2003), Plexin A2 (*Plxna2* – Mouse Genome Informatics) (Brown et al., 2001) and *Ednra* (Feiner et al., 2001) were used. For immunohistochemistry, fixed sections were stained with monoclonal α -smooth muscle actin antibody (DAKO) using Histomouse kit (Zymed) according to the manufacturer's instructions.

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). The proliferation index was calculated as number of positively staining nuclei divided by the total number of nuclei per cross-section of the OFT.

Results

Alk2 is expressed in the cardiac outflow tract at E11.5

Although *Alk2* expression has previously been localized to the developing heart (Dewulf et al., 1995), detailed information about its cardiac expression pattern has been lacking. Therefore, we examined *Alk2* mRNA expression using in situ hybridization in the cardiac OFT region during mid-gestation, when conotruncal septation is occurring (Fig. 1). At embryonic day 11.5 (E11.5), *Alk2* was expressed in the OFT (Fig. 1A). This expression resembled that of fate-mapped neural crest derivatives (Fig. 1B) as well as the pattern of *Plxna2* and *Ednra* (Fig. 1C,D), which have been previously shown to be expressed in migrating NCCs (Brown et al., 2001; Charite et al., 2001). Expression patterns of BMP and TGF β proteins

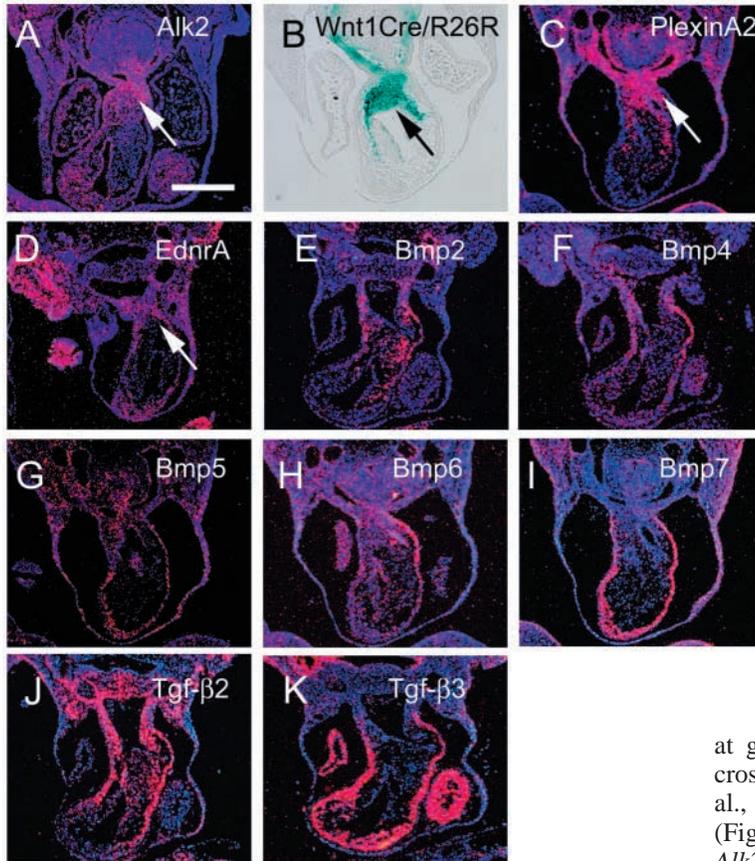


Fig. 1. Expression of *Alk2* and its putative ligands during cardiac development at E11.5. Radioactive in situ hybridization analysis of transverse sections through the cardiac OFT. *Alk2* mRNA (A) displays a very similar expression pattern to *Plxna2* (C) and *Ednra* (D), indicating expression by cardiac neural crest cells (Brown et al., 2001; Charite et al., 2001). Arrows indicate the positive hybridization signal (A,C,D). For comparison, cardiac neural crest cells were fate mapped using the WNT1-Cre/R26R reporter assay (Jiang et al., 2000) (B). An arrow indicates NCCs in the OFT region (blue signal). *Bmp4* (F), *Bmp5* (G), *Bmp6* (H), *Bmp7* (I) and *Tgfb3* (K) are expressed in the myocardium, while *Bmp2* (E) and *Tgfb2* (J) are also expressed in the mesenchyme of OFT cushions. Scale bar: 200 μ m.

Mice lacking *Alk2* in NCCs display cardiovascular defects

To analyze the functional role of *Alk2* in NCCs during cardiac morphogenesis, we generated a floxed allele of *Alk2* (*Alk2^{Flox}*) (Fig. 2A) to allow for tissue-specific gene inactivation (Kaartinen and Nagy, 2001; Dudas et al., 2004). *ALK2^{Flox/Flox}* mice survive to adulthood, breed normally and appear unaffected, whereas mice homozygous for the *Alk2* knockout allele (*Alk2^{KO}*) die at gastrulation (Gu et al., 1999; Mishina et al., 1999). We crossed *Alk2^{Flox/Flox}* mice with *Wnt1-Cre* mice (Danielian et al., 1998), which were also heterozygous for the *Alk2^{KO}* allele (Fig. 2B). The resulting compound heterozygotes for the *Alk2^{Flox}* and *Alk2^{KO}* alleles, which also carry the *Wnt1-Cre* transgene have *Alk2* specifically inactivated in NCCs (herein termed *Alk2/Wnt1-Cre*) (Dudas et al., 2004), while the littermates with incomplete combination of these alleles serve as controls (Fig. 2B). We could not detect any toxic effects of the Cre recombinase (Loonstra et al., 2001) in Cre-positive control mice (*Alk2^{Flox}; Alk2^{WT}; Wnt1-Cre* mice) compared with other controls (see Fig. 2B). Only 60% (13 out of 22) of the expected *Alk2/Wnt1-Cre* mutants were recovered at birth. However, 17 out of the expected 20 (85%) were identified at E14, indicating that there was a significant lethality between E14 and birth (Table 1). Hearts of *Alk2/Wnt1-Cre* newborn mice are abnormal (Fig. 2C). Normally, the aorta arises from the left ventricle while the pulmonary artery arises ventrally from the right ventricle. In every *Alk2/Wnt1-Cre* mutant studied ($n=13$), the heart was significantly enlarged, and displayed a persistent truncus arteriosus (PTA) (Table 2); a distinct aorta and pulmonary artery was not present (Fig. 2C). However, thymic development, which is impaired in individuals with DiGeorge syndrome, is not affected in *Alk2* mutants (Fig. 2E). Next, we examined hearts of *Alk2* mutants and control littermates during mid-gestation. At E14.5,

have been extensively studied during mouse cardiac development (reviewed by Delot, 2003). To gain a better understanding of the sources of putative ligands for ALK2 during OFT morphogenesis, we analyzed their expression during the crucial period when OFT septation takes place. We show that at E11.5, *Bmp7* and *Tgfb3* are broadly expressed in the OFT myocardium (Fig. 1I,K). In addition, *Bmp5* is broadly expressed in the myocardium, although quite weakly compared with *Bmp7* (Fig. 1G), whereas *Bmp4* expression is more localized to the distal OFT myocardium (Fig. 1F). In concordance with the study of Kim and co-workers (Kim et al., 2001), *Bmp6* expression is much stronger on the left side of the OFT myocardium (Fig. 1H). *Bmp2* and *Tgfb2* are expressed both in the distal myocardium and also in the cushion mesenchyme of the OFT (Fig. 1E,J). Furthermore, ALK2 is expressed by CNC cells during crucial stages of OFT septation and aortic arch artery remodeling. Moreover, many BMP/TGF β ligands, particularly *Bmp2* and *Tgfb2* are expressed in close proximity to cells expressing *Alk2*.

Table 1. Genotype distribution of offspring from *Alk2^{KO/+}; Wnt1-Cre^{+/-} \times *Alk2^{Flox/Flox}* crosses*

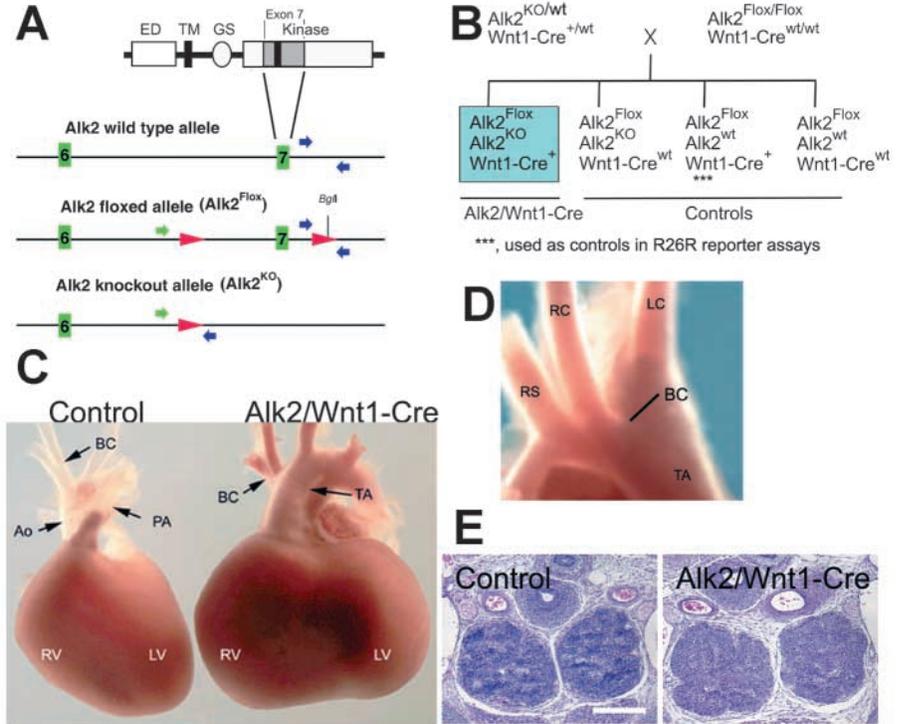
Genotype	E14 ($n=80$)	E18 ($n=87$)
<i>Alk2^{KO}/Wnt1-Cre+</i>	18 (23%)	13 (15%)
<i>Alk2^{WT}/Wnt1-Cre+*</i>	23 (29%)	23 (26%)
<i>Alk2^{KO}/Wnt1-Cre-*</i>	19 (24%)	27 (31%)
<i>Alk2^{KO}/Wnt1-Cre-*</i>	20 (25%)	24 (27%)

*Normal phenotype; referred to in text as controls.

Table 2. Summary of phenotypes in late term (E18) *Alk2/Wnt1-Cre* mutants ($n=13$)

Phenotype	n	Penetrance (%)
Persistent truncus arteriosus	13	100
Hyperplastic right ventricle	11	85
Retroesophageal right subclavian artery	2	15
Short/missing brachiocephalic artery	10	77

Fig. 2. Cardiac OFT defects in newborn *Alk2/Wnt1-Cre* mice. (A) Functional domains of the ALK2 protein and schematic presentation of the *Alk2* locus, the floxed and knockout alleles. Cre-mediated recombination results in excision of exon 7 flanked by the *loxP* sites (*Alk2^{Flox}* allele; red arrowheads), which encodes the Smad interacting domain (the L45 loop) and a crucial part of the kinase domain. This recombination results in generation of a null allele (*Alk2^{KO}*), which is biochemically inactive (Dudas et al., 2004). Blue arrows depict primers used to identify the *Alk2^{Flox}* allele. Intron 6 and intron 7 specific primers (green and blue arrows, respectively) were used to identify the *Alk2^{KO}* allele. (B) Homozygous floxed *Alk2* (*Alk2^{Flox/Flox}*) mice were bred with *Wnt1-Cre* mice that are heterozygous for the *Alk2* knockout allele (*Alk2^{KO}*) to produce a NC-specific deletion of exon 7. (C) Normal anatomy is seen in the control heart with the aorta (Ao) arising to the left and dorsal to the main pulmonary artery (PA) (left). The *Alk2* mutant displays an abnormal single OFT, persistent truncus arteriosus (TA) (right). Moreover, the right ventricle (RV) of the mutant heart (right) is significantly larger than that of the control (left), and the brachiocephalic artery (BC) is short in the *Alk2* mutant (C, frontal view and D, lateral view). Atria have been removed to facilitate better visualization of the OFTs. LV, left ventricle; RV, right ventricle. RS, right subclavian artery; RC, right common carotid artery; LC, left subclavian artery. (E) Hematoxylin and Eosin staining shows that the thymus is unaffected in *Alk2/Wnt1-Cre* mutants (right) compared with controls at E14.5 (left). Scale bar: 100 μ m.



Alk2/Wnt1-Cre embryos ($n=12$) displayed a complete failure of OFT septation (persistent truncus arteriosus type A2) associated with a ventricular septation defect (Fig. 3). Moreover, *Alk2/Wnt1-Cre* mutants that were born alive displayed fully penetrant craniofacial phenotypes, as described in detail elsewhere (Dudas et al., 2004), and died during the first postnatal day.

In addition to PTA, *Alk2/Wnt1-Cre* mutants displayed aortic arch defects, which are often associated with neural crest deficiencies (Table 2). The brachiocephalic artery was consistently either missing or remarkably short so that the right common carotid artery directly branched from the truncus arteriosus (Fig. 2D). Moreover, *Alk2/Wnt1-Cre* mutants displayed retroesophageal right subclavian artery with variable penetrance (two out of 13 examined).

***Alk2/Wnt1-Cre* mutants display regression of the pharyngeal arch arteries**

We examined pharyngeal arch arterial anatomy and patency at E10.5 and E11.5 using intracardiac India ink injections. At E10.5, the 3rd, 4th and 6th pharyngeal arch arteries were indistinguishable between mutants and controls (Fig. 4A,B). By contrast, at E11.5, the 3rd and particularly 6th arteries of mutant embryos displayed bilaterally inappropriate regression, while the 4th artery appeared relatively normal (Fig. 4C,D). Recent studies have demonstrated that NCCs populate the pharyngeal arch arteries and differentiate into vascular smooth muscle cells (Waldo et al., 1996; Kochilas et al., 2002). Moreover, it has been shown that NCCs in a mouse model of DiGeorge syndrome fail to differentiate appropriately in a

process associated with aberrant regression of pharyngeal arch arteries (Lindsay et al., 2001; Kochilas et al., 2002). Therefore, we examined smooth muscle differentiation using an α -smooth muscle actin (α SMA) antibody in *Alk2/Wnt1-Cre* embryos prior to the time when inappropriate regression was apparent (E11.0). In controls, α SMA-positive cells formed a characteristic immunopositive ring around the 3rd, 4th and 6th pharyngeal arch arteries (Fig. 4E,G), while in *Alk2* mutants we consistently observed only weak and diffuse staining around the 3rd and particularly 6th arteries (Fig. 4F,I). Thus, the pharyngeal arch arteries develop normally in *Alk2/Wnt1-Cre* mutants, but the 3rd and 6th arteries regress inappropriately associated with defective neural crest-derived smooth muscle cell differentiation.

Fate mapping of cardiac NCCs in *Alk2* mutants

As we have demonstrated before, the overall migration of NCCs deficient in *Alk2* did not differ from that of controls at E8.5–11 (Dudas et al., 2004). To verify and complement this finding, we analyzed expression of NCC markers that detect premigratory NCCs (*Msx1*; expressed also in the neural plate) and migratory NCCs (*Sox10* and *Foxd3*) at E9.0. As shown in Fig. 5, the expression patterns of these genes were very similar between *Alk2* mutants and controls.

We then analyzed the fate of *Alk2*-deficient NCCs during cardiac OFT morphogenesis. We crossed *Alk2^{Flox/Flox}* mice with the *ROSA26 Cre* reporter (*R26R*) mice (Chai et al., 2000; Jiang et al., 2000). Subsequently, *Alk2^{Flox/Flox}; R26R(+/+)* female mice were crossed with *Alk2^{KO/WT}; Wnt1-Cre* males (Dudas et al., 2004). The resulting embryos have the NCC

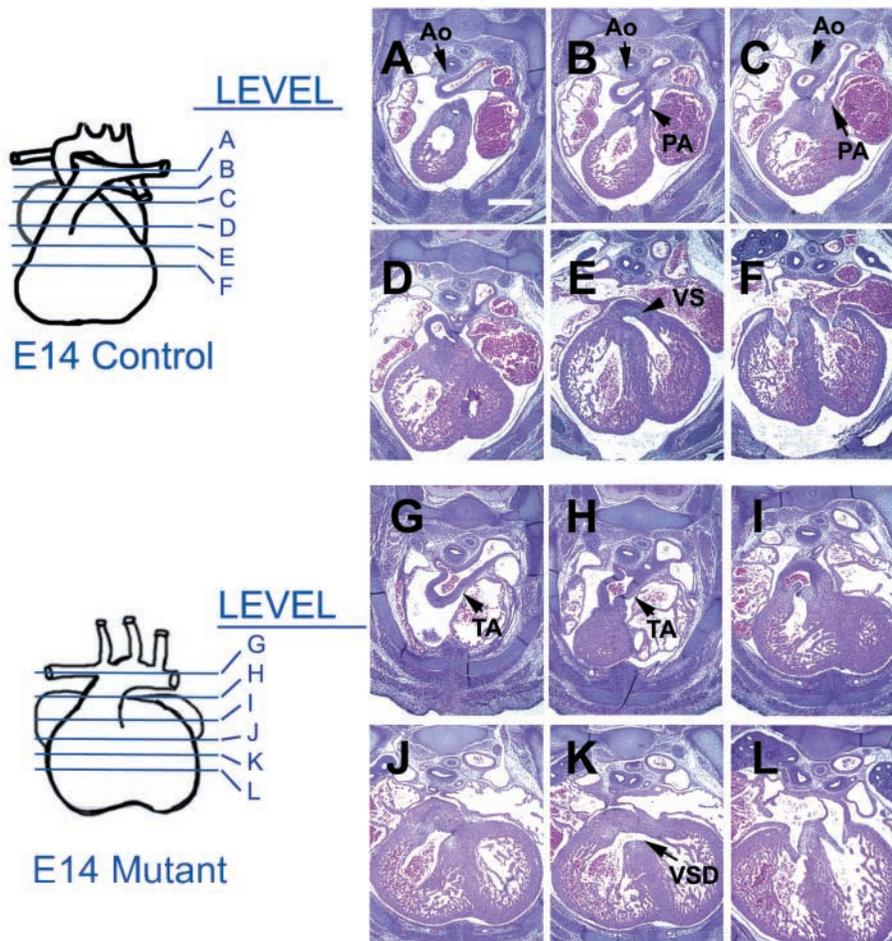


Fig. 3. Abrogation of *Alk2* in neural crest cells leads to persistent truncus arteriosus. Hematoxylin and Eosin-stained sections of control (A-F) and *Alk2* mutant (G-L) littermate embryos at E14. In controls (A-F), pulmonary artery (PA) and the ascending aorta (Ao) are separated by the conotruncal (aortico-pulmonary) septum, and the right and left ventricles by the interventricular septum (VS). In *Alk2* mutants (G-L), the conotruncal septum fails to form, i.e. they display persistent truncus arteriosus (TA; G,H), which is always associated with a ventricular septal defect (VSD, K). Both the atrioventricular and semilunar valves appear normal. An approximate plane of the shown sections is indicated on the schematic drawings (left). Scale bar: 200 μ m.

lineage permanently marked with β -galactosidase expression. To show that the presence of an additional Cre target does not increase the severity of the detected OFT phenotype (perhaps by inducing Cre-mediated chromosomal rearrangement), we compared the cardiac phenotypes of *Alk2/Wnt1-Cre; R26R* mutants to that of *Alk2/Wnt1-Cre* mutants (without the R26R reporter). This analysis did not reveal any phenotypic differences between these two backgrounds (data not shown). Nor could we see any detectable phenotypes in control mice positive for both the WNT1-Cre transgene and the R26R locus (*ALK2^{Flox/wt}; WNT1-Cre⁺; R26R⁺*) (Fig. 2B). Thus, potential Cre-induced chromosomal rearrangements between loxP sites on different chromosomes did not play a significant role in the expression of cardiovascular phenotypes in these mice.

The detailed analysis of *R26R* reporter mice at E10.5 and E11.5 revealed that, although NCCs deficient in ALK2 populated the pharyngeal arch mesenchyme and surrounded the forming aortic arch arteries (Fig. 4H,J; Fig. 6A-F) (Dudas et al., 2004), the *Alk2* mutants failed to demonstrate progression of NCCs into the proximal OFT when compared with control littermates (Fig. 6A-F). This finding was verified by sectioning the specimens in the frontal plane (Fig. 6G-L). In controls, the mesenchyme of the OFT cushions was well formed and contained a large number of β -galactosidase-positive cells at all three levels. By contrast, the OFT cushions in *Alk2/Wnt1-Cre* mutants were reduced in size. Moreover, the proximal OFT was essentially devoid of NCCs, while the distal

OFT displayed a dramatic decrease in the amount of positively staining cells. At E13, when OFT septation is complete, intense blue staining could be seen both in the aorta and pulmonary trunk of control embryos, indicating neural crest derivatives. Positively staining cells were also present in similar regions of the 7 *Alk2* mutants studied, but the numbers of labeled cells were smaller.

In addition to defects in migration, decrease in NCC numbers in the OFT region could also result from reduced cell proliferation or from a local increase in apoptosis, both of which have been associated with cardiac OFT defects (Kim et al., 2001; Abu-Issa et al., 2002; Frank et al., 2002). Therefore, we studied cell proliferation in *Alk2/Wnt1-Cre* mutants at E11.5 by using BrdU and phospho-histone H3 staining. *Alk2* mutants and controls at E10 displayed a similar high number of proliferating BrdU-positive cells in the mesenchyme of pharyngeal arches 3-6 (data not shown). Moreover, although the mesenchyme of conotruncal cushions at E11.0 was smaller in *Alk2* mutants than in controls, it still contained comparable numbers of BrdU-positive cells ($17.6 \pm 7\%$ in *Alk2/Wnt1-Cre* mutants; $14.9 \pm 6\%$ in controls; $n=3$) (Fig. 7A,B). The results of these experiments were verified by immunostaining for the mitotic marker phospho-histone H3 (data not shown). The pattern and number of TUNEL-positive cells in the pharyngeal arches is comparable between *Alk2/Wnt1-Cre* mutants and controls (Dudas et al., 2004). Likewise, we could not detect significant differences in the number of dying TUNEL-positive cells in the mesenchyme of the OFT cushions between the controls and *Alk2/Wnt1-Cre* mutants at E11.0 (data not shown).

Since the fate-mapping experiments indicated that NCCs deficient in *Alk2* failed to appropriately populate the OFT region, we compared the expression pattern of the cardiac NC marker, *Plxna2* between ALK2 mutants and controls at E11.0. As shown in Fig. 7C,D, *Alk2/Wnt1-Cre* mutants displayed a significant reduction of *Plxna2*-positive cells in the OFT when compared with the controls. A homeobox gene *Msx1* has a well-documented role as an effector of BMP signaling (Suzuki

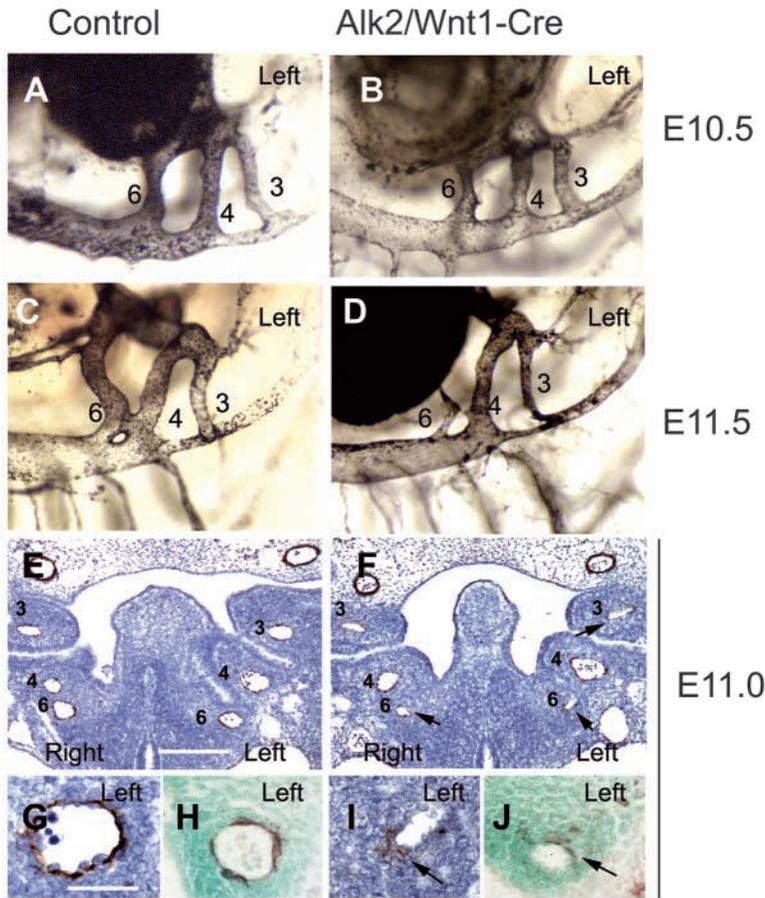


Fig. 4. Abnormal pharyngeal arch arteries in *Alk2/Wnt1-Cre* mutants. Left lateral view after intracardiac ink injection to visualize developing pharyngeal arch arteries at E10.5 (A, B) and at E11.5 (C,D) in controls (A,C) and *Alk2* mutants (B,D). The pharyngeal arch arteries are numbered. Note regression of arteries 3 and 6 in the *Alk2* mutant at E11.5. Immunohistochemistry for the smooth muscle cell marker, α -smooth muscle actin at E11.0 in control (E,G) and *Alk2* mutant samples (F,I). The high-power pictures show the 6th pharyngeal arch artery in the control (G,H) and mutant (I,J). There is diffuse discontinuous brown staining around the 3rd and 6th pharyngeal arch arteries at E11.0 in mutants (I, arrows in J) when compared with controls (G,H). Neural crest cells were fate mapped using the WNT1-Cre/R26R reporter assay (H,J; green) to show that in controls (H) and in mutants (J) the aortic arch arteries are surrounded by NCCs. Scale bars: 200 μ m in E,F; 50 μ m in G-J.

et al., 1997b). Moreover, it has been shown that *Msx1* expression can be induced by BMPs (Bei and Maas, 1998) and repressed by TGF β s (Ito et al., 2003). Interestingly, in *Alk2/Wnt1-Cre* mutants, the pattern of *Msx1* expression in the OFT region was significantly different from that of controls (Fig. 7E,F). In controls, *Msx1* expression continued from the proximal (conotruncus) to the distal (truncus arteriosus) OFT. In mutants, positive staining was limited to the most proximal

conotruncal region only (Fig. 7E,F). This difference was reproducible and consistent ($n=3$).

The results of the present study imply that while cardiac NCCs deficient in *Alk2* are capable of populating the conotruncus, they do not reach the OFT in sufficient numbers or do not otherwise function properly during the crucial period around E11.0 for OFT septation to occur normally. Thus, *Alk2* is required cell-autonomously for successful NCC migration to the distal cardiac OFT and for appropriate remodeling of the aortic arch arteries.

Discussion

The important role of NCCs in cardiac OFT morphogenesis has been known for two decades (Kirby et al., 1983; Kirby et al., 1985), and many signaling proteins, including BMPs have been implicated in their specification and function (Ohnemus et al., 2002; Bachiller et al., 2003). However, it is currently unclear whether BMPs influence NCC functions directly or indirectly. In the present study, we unequivocally demonstrate for the first time that BMP/TGF β signaling transduced via ALK2 is cell-autonomously required for specific NCC functions during cardiovascular morphogenesis.

Bmp6 and *Bmp7* are both expressed in the cardiac OFT, and *Bmp6*^{-/-}; *Bmp7*^{-/-} mutants display a delay in OFT and ventricular septation (Kim et al., 2001). Therefore, it is possible that these ligands transduce their signals via ALK2 to induce OFT septation. However, defects displayed by double knockouts are relatively mild compared with those seen in our *Alk2/Wnt1-Cre* mutants. Hence, additional or distinct BMPs, such as *Bmp2* or *Bmp5*, are likely to be involved in ALK2

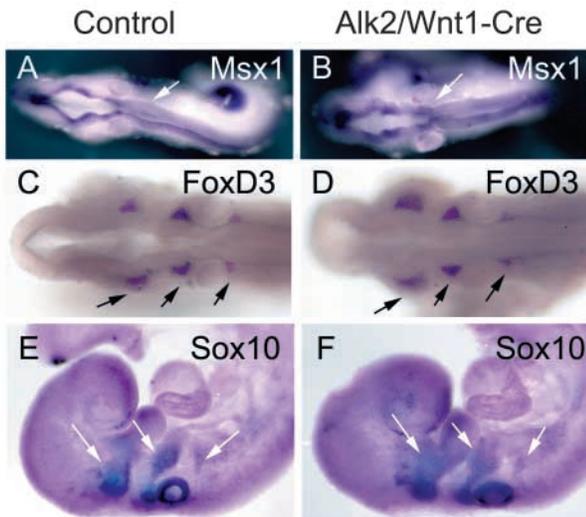


Fig. 5. Early migration of NCCs in *Alk2/Wnt1-Cre* mutants. Expression of NCC markers *Msx1* (A,B), *Foxd3* (C,D) and *Sox10* (E,F) in controls (A,C,E) and *Alk2* mutants (B,D,F) at E9.0. The expression pattern of *Msx1* (A,B; arrows; dorsal view) in the dorsal neural tube is indistinguishable between controls (A) and *Alk2* mutants (B). Both *Foxd3* (C,D; dorsal view) and *Sox10* (E,F; lateral view) are expressed in all three trajectories of migrating NCCs (arrows in C-F) and show no differences between the *Alk2* mutants and controls.

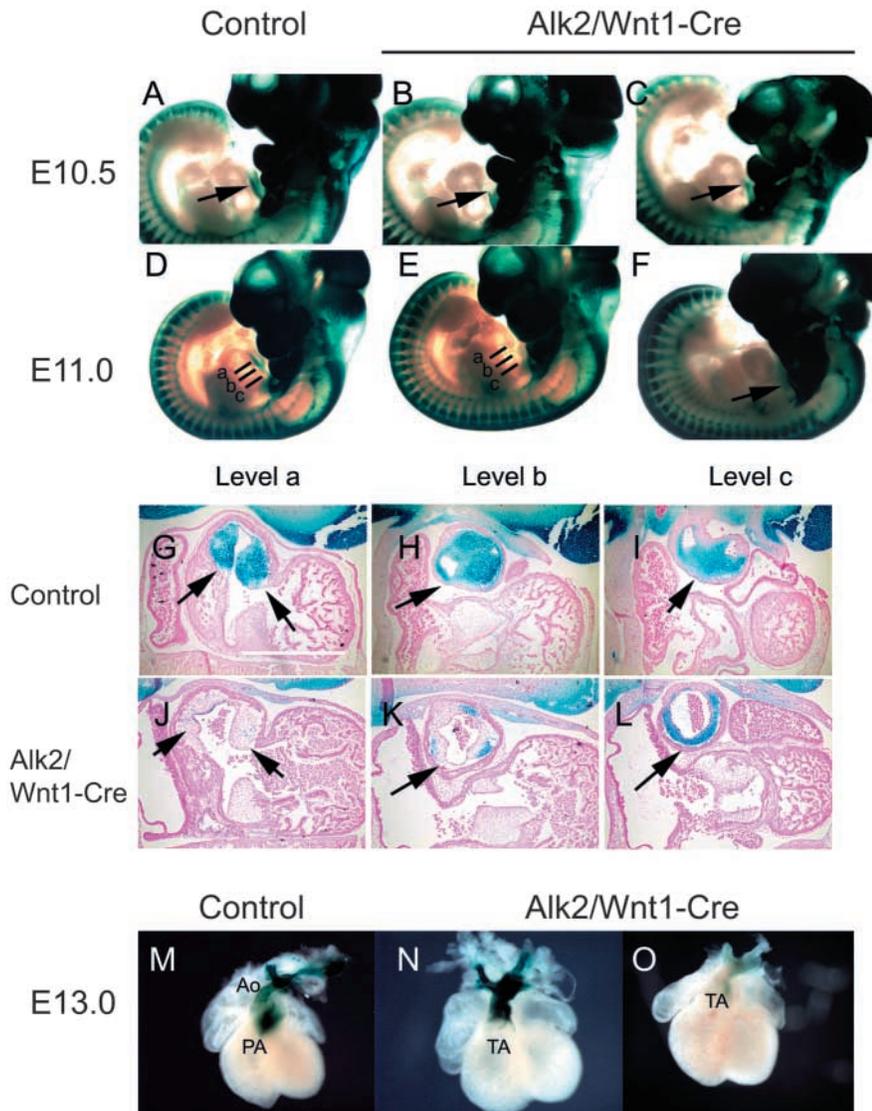


Fig. 6. Fate mapping of cardiac NCCs in *Alk2/Wnt1-Cre* mutants. Embryos carrying the *R26 Cre* reporter were stained for the β -galactosidase activity at E10.5 (A-C) and at E11.5 (D-F) to identify cells derived from the NC (A,D, controls; B,C and E,F, *Alk2* mutants). Arrows indicate the OFT. In the control, blue staining is more proximal than in the mutant. Frontal sections through the conotruncal region of control (G-I) and *Alk2* mutant (J-L) at E11.5. Level and orientation of each section has been indicated in D and E. In control sections, arrows indicate positively staining NC-derived cells that contribute to formation of conotruncal cushions. In mutants, arrows highlight near-complete absence of NC-derived cells in the more proximal region (level a) and significant reduction of positively staining cells in more distal regions (levels b and c). M-O, the whole-mount staining of the control (M) and *Alk2* mutant (N,O) hearts at E13. Both the aorta and pulmonary trunk stain positive for cells derived from NCCs (blue staining) in the control (M). In *Alk2* mutants, the persistent truncus arteriosus shows positively staining cells, albeit with variable intensity (N,O). Scale bars: 200 μ m in G-L.

signaling during cardiac OFT development. In addition, *Bmp4* is expressed in the OFT region and its abrogation specifically in cardiomyocytes has been shown to lead to OFT abnormalities, such as double-outlet-right-ventricle and atrioventricular canal defects (Jiao et al., 2003). Moreover, Delot and co-workers demonstrated that mice homozygous for a hypomorphic BMP2 receptor (*Bmpr2*) display a failure in aortico-pulmonary septation (Delot et al., 2003). However, these experiments did not involve a tissue-specific gene inactivation approach, so it remains unclear whether this defect reflects disruption of the same signaling pathway, as that reported herein for ALK2. Interestingly, the defect in *Bmpr2* hypomorphs was detectable only below the valve level and was therefore distinctly different from the defect seen in *Alk2/Wnt1-Cre* mutants. In addition to BMP genes, TGF β genes, particularly *Tgfb2* and *Tgfb3*, are also strongly expressed in the cardiac OFT (Fig. 1). Moreover, *Tgfb2* null mutants display aortic arch artery and OFT septation defects (Sanford et al., 1997; Molin et al., 2002). Based on the phenotypic similarities between *Tgfb2*^{-/-} and *Alk2/Wnt1-Cre* mutants, we cannot exclude the possibility that ALK2 is also

involved in TGF β signaling during cardiac OFT development. Interestingly, recent studies suggest that in endothelial cells, the TGF β type I receptor (ALK5) mediates TGF β -dependent recruitment of ALK1, which is closely related to ALK2, into a TGF β receptor complex (Goumans et al., 2003). Whether similar heteromeric complexes between ALK2 and other type I receptors occur in NCCs remains to be shown. However, our finding that expression of *MSX1* (a known BMP target) is significantly reduced in the distal OFT region suggests that ALK2 probably mediates BMP signaling, but does not rule out (additional) signaling through TGF β s. Alternatively, it is possible that the differences in *Msx1* expression are secondary and result from the altered anatomy of the OFT.

It has been previously demonstrated that ALK2 is involved in cardiac morphogenesis in amphibians and avians (Ramsdell and Yost, 1999; Lai et al., 2000). Here, we demonstrate that *Alk2* is expressed in cardiac NCCs. This expression pattern overlaps with another BMP1 receptor *Alk3*, which is ubiquitously expressed, and required for atrioventricular cushion morphogenesis (Mishina et al., 1995; Dewulf et al., 1995; Gaussin et al., 2002). Therefore, it is notable that ALK2 function in CNC cells during OFT morphogenesis is essential and cannot be substituted by other type I receptors, such as ALK3. It remains to be shown whether this reflects differences in ligand preference, level of expression, or divergence in downstream signaling mechanisms.

In mouse models of DiGeorge syndrome, the 4th and 6th arch arteries often show inappropriate regression (Jerome and Papaioannou, 2001; Kochilas et al., 2002). During normal

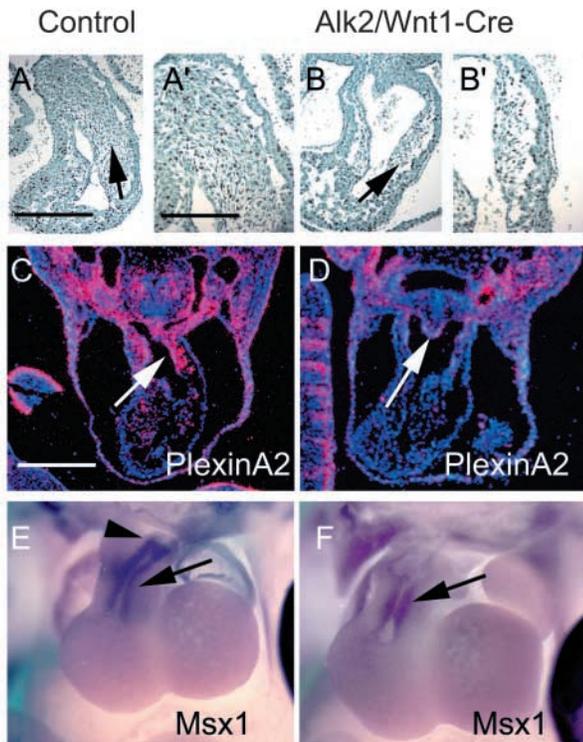


Fig. 7. Cell proliferation and marker gene expression in the cardiac OFT. Cell proliferation was analyzed in transverse sections of controls (A) and *Alk2/Wnt1-Cre* mutants (B) by using BrdU incorporation assay. The mesenchyme of OFT cushions (arrows) is significantly reduced in size in *Alk2* mutants (B) when compared with the corresponding tissues in controls (A). (A',B') High-magnification views of the OFT cushions indicated with arrows in A and B. Comparison of the proliferation indices for OFT cushion cells indicated that cell proliferation did not significantly differ between these two genotypes ($n=3$). In *Alk2/Wnt1-Cre* mutants, the level of *PlxnA2* (arrows, marker for NCCs) expression is diminished at E11.0 (D) compared with controls (C). *Msx1*, an effector of BMP signaling, is expressed in the proximal OFT mesenchyme (arrows) both in controls (E) and *ALK/WNT1-Cre* mutants (F). However, only the control samples display *Msx1* expression in the distal OFT mesenchyme (arrowhead in E). Scale bars: in A, 200 μ m for A,B; in A', 100 μ m for A',B'; in C, 200 μ m for C-F.

development, the 3rd arch arteries give rise to common carotid arteries, the 4th arch arteries contribute to the formation of the distal part of the aortic arch, the brachiocephalic artery and a proximal part of the right subclavian artery, while the sixth arch arteries contribute to the ductus arteriosus and the proximal parts of the pulmonary arteries. In *Alk2/Wnt1-Cre* mutants we detect inappropriate regression of the 3rd and particularly the 6th arch arteries, whereas the 4th arch arteries appear relatively normal. Despite this regression pattern, the lungs and head appear to develop normally. This may reflect the substantial capacity of embryos to overcome a localized arterial growth impairment as previously suggested (Lindsay and Baldini, 2001; Tallquist and Soriano, 2003).

The fate of migrating NCCs is specified by the environment through which they migrate (Trainor et al., 2002). Consequently, the pharynx is a likely source of important instructive signals for the migrating CNC cells. Interestingly,

TBX1, a gene associated with DiGeorge syndrome, is not expressed in the CNC, but in the adjacent mesendoderm of the pharyngeal arches (Garg et al., 2001). Recent studies have shown that expression of a secreted growth factor *Fgf8* is diminished in *Tbx1*-expressing cells from *Tbx1* mutant mice (Vitelli et al., 2002), and that mice deficient in FGF8 display abnormal apoptosis of CNC cells and a typical DiGeorge syndrome phenotype (Abu-Issa et al., 2002; Brown et al., 2004). However, it is not currently known whether CNC cells are direct targets of FGF8 or whether its effect is indirect. Although downregulation of BMP signaling in pharyngeal endoderm seems to be a prerequisite for CNC cell survival (Garg et al., 2001; Tiso et al., 2002; Bachiller et al., 2003), our present results demonstrate that signals transduced via ALK2 are required for normal CNC cell differentiation and for successful colonization of the distal OFT. Therefore, it is possible that FGF and TGF β superfamily signaling pathways converge to control CNC cell fate during cardiac OFT morphogenesis. Convergence of FGF and BMP signaling to orchestrate cell fate specification has been demonstrated during limb and tooth development, and may be a common theme during organogenesis (Peters and Balling, 1999; Zuniga et al., 1999; Capdevila and Izpisua Belmonte, 2001).

In addition to NCCs, the myocardium of the OFT is populated by cells from the presumptive anterior (secondary) heart-forming field (AHF), which also gives rise to the majority of cells in the right ventricle (Kelly et al., 2001; Yelbuz et al., 2002; Kelly and Buckingham, 2002). These two distinct populations of cells have been shown to be in close apposition in the pharyngeal arch mesenchyme at E9, and therefore signaling between them may play an important role in OFT morphogenesis (Kelly and Buckingham, 2002). In accordance with this model, it has been demonstrated in the chick that NCCs in the caudal pharyngeal arches are required for differentiation and function of the myocardium, and it has been suggested that NCCs may regulate availability of factors, such as FGF8, in the pharynx that control addition of myocardium from the AHF (Waldo et al., 1999; Farrell et al., 1999a). In the present study, we show that *Alk2/Wnt1-Cre* mice display OFT septation defects and significant lethality between E14 and E18, and demonstrate remarkable hyperplasia of the right ventricle, a phenotype not commonly seen in other mouse mutants with PTA. Therefore, it is possible that signaling via ALK2 in NCCs is required for proper regulation of cells in the AHF, and that disturbances in this process lead to defects not only in the OFT, but also in the right ventricle. Alternatively, the hyperplastic right ventricle may result from hemodynamic changes secondary to PTA, although a similar degree of right ventricular hyperplasia is not seen in other mouse PTA models.

In summary, tissue-specific knockout of the *Alk2* gene in NCCs results in cardiac malformations reminiscent of common congenital heart defects seen in human newborns. As many newborns with aortic arch and/or outflow defects do not display characteristic deletions in the *DGCR* or mutations in *TBX1*, our results open the possibility that BMP signaling transduced via ALK2 in NCCs is part of a critical pathway involved in conotruncal development in humans. Moreover, the phenotypic characteristics of the *Alk2/Wnt1-Cre* mouse make it a valuable experimental model for the study of human conotruncal birth defects.

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