Published in final edited form as:
Dev Dyn. 2007 February ; 236(2): 512–517.

BMP Type I Receptor ALK2 Is Essential for Proper Patterning at Late Gastrulation During Mouse Embryogenesis

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Abstract

Bone morphogenetic proteins (BMPs) have multiple functions during vertebrate development. Previously, it was shown that BMP type I receptor ALK2 (also known as ACVRI, ActRI, or ActRIA) was important for normal mouse gastrulation by deleting exon 4 or exon 5 of *Alk2*. Recently, flanking exon 7 by loxP sites generated a conditional allele for *Alk2*. To assess whether the deletion of exon 7 causes functional null of ALK2, and does not produce a dominant negative form or a partially functional form of ALK2, we performed a comparative analysis between *Alk2* homozygous mutant embryos with an exon 5 deletion ($Alk2^{\Delta 5/\Delta 5}$) and embryos with an exon 7 deletion ($Alk2^{\Delta 7/\Delta 7}$). Both $Alk2^{\Delta 5/\Delta 5}$ and $Alk2^{\Delta 7/\Delta 7}$ mutants showed identical morphological gastrulation defects. Histological examinations and molecular marker analyses revealed identical abnormal gastrulation phenotypes in $Alk2^{\Delta 5/\Delta 5}$ and $Alk2^{\Delta 7/\Delta 7}$ mutants. Although *Fgf8* was expressed in the primitive streak of $Alk2^{\Delta 5/\Delta 5}$ and $Alk2^{\Delta 7/\Delta 7}$ mutants, *Brachyury*, *Wnt3a*, and *Tbx6* were dramatically downregulated in $Alk2^{\Delta 5/\Delta 5}$ and $Alk2^{\Delta 7/\Delta 7}$ mutants. These results indicate that deletion of exon 7 for *Alk2* leads to a functionally null mutation in vivo, and *Alk2* is crucial for sustaining the proper gastrulation events in early mouse embryogenesis.

Keywords

Alk2; bone morphogenetic protein; gastrulation; mouse

INTRODUCTION

Genetic studies for the transforming growth factor-beta (TGF-β) superfamily have revealed various functions of family members in mammalian development (Roberts and Sporn, 1993; Wall and Hogan, 1994; ten Dijke et al., 2000). TGF-β superfamily signals including bone morphogenetic proteins (BMPs) are mediated through membrane-bound heteromeric complexes of type I and type II serine/threonine kinase receptors (Heldin et al., 1997; Kretzschmar and Massague, 1998; Whitman, 1998; Miyazono et al., 2000). Upon binding to a ligand, the type II receptors phosphorylate and activate associated type I receptors, which in

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Grant sponsor: NIH; Grant number: HL074862; Grant number: DE013085.

turn transduce the signal by phosphorylating signaling pathway using SMADs, especially SMAD1/5/8 for BMP signaling cascades (Heldin et al., 1997; Whitman, 1998).

ALK2 (known as ACVRI, ActRI, or ActRIA) is one of the type I BMP receptors to bind BMPs in conjunction with corresponding type II receptors (Attisano et al., 1993; He et al., 1993; ten Dijke et al., 1993, 1994; Mishina, 2003; Kishigami and Mishina, 2005). During mouse embryogenesis, *Alk2* is expressed in visceral endoderm at embryonic day (E) E6.5, and in both the visceral endoderm and mesoderm at E7.5 (Roelen et al., 1994; Gu et al., 1999). To understand the role of BMP signaling mediated by ALK2 in mammalian embryogenesis, two different types of conventional knockout mice were generated previously (Gu et al., 1999; Mishina et al., 1999). Exon 4, which encodes a transmembrane domain, or exon 5, which encodes a GS-domain (rich in Gly and Ser residues), were eliminated (Gu et al., 1999; Mishina et al., 1999). Despite different targeting strategies for *Alk2*, both *Alk2*-deficient mice showed a similar phenotype of early embryonic lethality with severe disruption of mesoderm formation (Gu et al., 1999; Mishina et al., 1999). The mutant embryos start the gastrulation, however their development is arrested at late streak stages. In addition, results of chimeric studies suggested that *Alk2* is essential in the extraembryonic tissue at gastrulation for normal mesoderm formation (Gu et al., 1999; Mishina et al., 1999). Furthermore, it is reported that BMP signaling mediated by ALK2 in the visceral endoderm is necessary for the generation of primordial germ cells in the mouse embryo (de Sousa Lopes et al., 2004).

Recently, to address how ALK2-mediated BMP signaling is associated with later stages of mammalian development, an *Alk2* conditional mouse line was generated by floxing exon 7 (Kaartinen and Nagy, 2001). Using this line, the importance of *Alk2* for the normal cranial, cardiac, and neuronal development was revealed (Dudas et al., 2004; Kaartinen et al., 2004; Wang et al., 2005; Israelsson et al., 2006). However, exon 7 encodes the Smad interacting domain (L45 loop) and a part of the kinase domain (Kaartinen and Nagy, 2001). This raises concerns about the deletion of exon 7 and whether it might produce a dominant negative form, or a partially functional form of ALK2. Although in vitro analysis showed no induction for phosphorylation of Smad1 when co-transfected with Smad1 cDNA and a construct lacking sequences encoded by exon 7 into CHO cells (Dudas et al., 2004), it is still necessary to analyze the exon 7 deletion mutant in more detail in vivo. Therefore, we carefully compared *Alk2* homozygous mutant embryos with an exon 5 deletion ($Alk2^{\Delta 5/\Delta 5}$) to embryos with an exon 7 deletion ($Alk2^{\Delta 7/\Delta 7}$).

Both $Alk2^{\Delta 5/\Delta 5}$ and $Alk2^{\Delta 7/\Delta 7}$ mutants showed similar gastrulation defects at the late streak stage. To examine the molecular cascade initiated by *Alk2* in mouse gastrulation, we analyzed the expression patterns of anteroposterior axis marker genes. Interestingly, while *Fgf8* was detected in the primitive streak of $Alk2^{\Delta 5/\Delta 5}$ and $Alk2^{\Delta 7/\Delta 7}$embryos, *Brachyury*, *Wnt3a*, and *Tbx6* were dramatically downregulated in both $Alk2^{\Delta 5/\Delta 5}$ and $Alk2^{\Delta 7/\Delta 7}$ mutants, suggesting that *Alk2* is involved in *Brachyury* and *Wnt3a* signaling cascades. These results indicate that deletion of exon 7 for *Alk2* leads to the functionally null allele in vivo and, that *Alk2* is essential for the mouse gastrulation procedures.

RESULTS AND DISCUSSION

The mouse *Alk2* gene is encoded by 10 exons (Schmitt et al., 1995; Fig. 1A). We previously used conventional gene targeting to delete exon 5 of *Alk2*, which encodes a GS domain that is critical for ALK2 kinase activity (Mishina et al., 1999; Fig. 1B). In the present study, exon 7, which is a part of the kinase domain of ALK2, was floxed and removed as previously described (Kaartinen and Nagy, 2001). Genotype was confirmed by polymerase chain reaction (PCR) for both *Alk2* exon 5 and exon 7 deletion (Fig. 1C).

At the onset of gastrulation at E6.5, *Alk2*$^{\Delta 5/\Delta 5}$ and *Alk2*$^{\Delta 7/\Delta 7}$ mutant embryos appeared morphologically indistinguishable from normal littermates (data not shown). However, we started to recover abnormal embryos around E7.0–E7.5 (Fig. 1D). Both *Alk2*$^{\Delta 5/\Delta 5}$ and *Alk2*$^{\Delta 7/\Delta 7}$ mutant embryos showed morphologically consistent phenotypes (N = 31/31 for *Alk2*$^{\Delta 5/\Delta 5}$ mutant embryos, N = 30/30 for *Alk2*$^{\Delta 7/\Delta 7}$ mutant embryos). *Alk2*$^{\Delta 5/\Delta 5}$ and *Alk2*$^{\Delta 7/\Delta 7}$ mutant embryos were much smaller and had formed empty sacs composed of parietal endoderm (Fig. 1D, arrowheads). Histological examination confirmed the presence of the thicker primitive streak forcing the posterior epiblast into the proamniotic cavity in *Alk2*$^{\Delta 5/\Delta 5}$ mutants as previously reported (Gu et al., 1999;Mishina et al., 1999; Fig. 2F–H). As shown in Figure 2, *Alk2*$^{\Delta 7/\Delta 7}$ mutants displayed similar abnormalities suggesting that as seen in the *Alk2*$^{\Delta 5/\Delta 5}$ mutants mesoderm formation is initiated, but the subsequent development is arrested during the mid-late streak stages.

To confirm whether the mesoderm formation is initiated and develops normally in *Alk2*$^{\Delta 5/\Delta 5}$ and *Alk2*$^{\Delta 7/\Delta 7}$ mutants, we analyzed expression of markers for anterior tissues and primitive streak by in situ hybridization. *Shh* is normally expressed in the anterior mesendoderm (Lu and Robertson, 2004; Fig. 3A). However, the expression level of *Shh* was dramatically decreased in both *Alk2*$^{\Delta 5/\Delta 5}$ and *Alk2*$^{\Delta 7/\Delta 7}$ mutants (Fig. 3B,C). In contrast, *Cer1*, a marker for the anterior definitive endoderm (Shawlot et al., 1998; Fig. 3D), was expressed in the definitive endoderm with an expanded expression domain (Fig. 3E,F). An axial mesoderm marker, *Foxa2* was also examined (Sasaki and Hogan, 1993; Fig. 3G). *Foxa2* was expressed in ten of thirteen *Alk2*$^{\Delta 5/\Delta 5}$ mutants and in five of five *Alk2*$^{\Delta 7/\Delta 7}$ mutants. Interestingly, expression of *Foxa2* was restricted in primitive streak like wild-type embryos at E6.5 (Fig. 3H,I). This suggests that development of *Alk2*$^{\Delta 5/\Delta 5}$ and *Alk2*$^{\Delta 7/\Delta 7}$ mutants is arrested at late streak stages, and does not proceed to headfold stage where the *Foxa2* expression domain moves to anteriorly.

Next, to analyze the arrested phenotype of the primitive streak, we examined the expression of *Brachyury*, *Fgf8*, *Wnt3a*, and *Tbx6* in *Alk2*$^{\Delta 5/\Delta 5}$ and *Alk2*$^{\Delta 7/\Delta 7}$ mutants. *Brachyury* is one of the earliest mesoderm markers and it is strongly expressed in the primitive streak (Wilkinson et al., 1990). Genetic and vertebrate embryological studies have also revealed a conserved role of *Brachyury* for maintenance, axis elongation, and the specification of posterior mesoderm populations (Smith, 1997). In *Alk2*$^{\Delta 5/\Delta 5}$ mutants, *Brachyury* expression was repressed or undetectable (Fig. 4B and data not shown) as reported previously (Gu et al., 1999; Mishina et al., 1999). As shown in Figure 4C, *Brachyury* expression was not detected in *Alk2*$^{\Delta 7/\Delta 7}$ mutants either. Fibroblast growth factor (FGF) signaling is crucial for mesoderm cell fate specification and required for *Brachyury* expression at gastrulation (Griffin et al., 1995; Ciruna and Rossant, 2001). Therefore, another primitive streak and nascent mesoderm marker, *Fgf8* was analyzed (Sun et al., 1999). As shown in Figure 4E,F, *Fgf8* was expressed in both *Alk2*$^{\Delta 5/\Delta 5}$ and *Alk2*$^{\Delta 7/\Delta 7}$ mutants. At late streak stages, *Wnt3a* is expressed in the primitive streak, which is known as a direct regulator of *Brachyury* (Yamaguchi et al., 1999). *Tbx6* is also required for the specification of posterior paraxial mesoderm during late streak stages (Chapman et al., 1996). Interestingly, both *Alk2*$^{\Delta 5/\Delta 5}$ and *Alk2*$^{\Delta 7/\Delta 7}$ mutant embryos failed to express *Wnt3a* (Fig. 4H,I) and *Tbx6* (Fig. 4K,L). These results suggest that ALK2 signaling associates with the expression of *Brachyury*, *Wnt3a*, and *Tbx6* at late streak stages to complete appropriate gastrulation.

In this study, we analyzed *Alk2* mutant embryos homozygous for an exon 5 deletion (*Alk2*$^{\Delta 5/\Delta 5}$) and for an exon 7 deletion (*Alk2*$^{\Delta 7/\Delta 7}$). Both *Alk2*$^{\Delta 5/\Delta 5}$ and *Alk2*$^{\Delta 7/\Delta 7}$ mutants showed a similar categorized phenotype. Targeted disruption of exon 4 for *Alk2*, which encodes a transmembrane domain of ALK2, blocks BMP signal transfer into the intracellular region through ALK2, because mutated ALK2 is unable to remain in the plasma membrane (Gu et al., 1999). Another conventional mutant targeted exon 5, which encodes the GS box of ALK2, displayed an identical phenotype (Mishina et al., 1999). Exon 7 encodes the Smad interacting

domain (L45 loop) in part of the ALK2 kinase domain (Kaartinen and Nagy, 2001). Disruption of floxed exon 7 by Cre re-combinase is expected to produce a null allele of *Alk2*. However, it is possible that the deleted allele would produce a truncated ALK2, which still contains a ligand binding domain, a transmembrane domain, a GS domain, and a part of the kinase domain (Kaartinen and Nagy, 2001). This finding raises the possibility that deletion of exon 7 leads to a formation of a dominant negative form or a partially functional form of ALK2. Expression of mutant proteins that lack cytoplasmic regions are frequently used to block the specific signals, because these types of proteins can act as dominant negatives (Chen et al., 1998; Zhao et al., 2002). On the other hand, a mutation in the GS domain was reported recently to lead to a rare autosomal dominant disorder of skeletal malformations, fibrodysplasia ossificans progressiva (Shore et al., 2006). This finding would suggest that the truncated ALK2, which still has the GS domain, could act as a signaling molecule. Therefore, we believed that it was important to clarify whether the targeted mutation of exon 7 produces a functionally null allele as do the conventional *Alk2* mutations, or whether it produces a dominant-negative form or a partially functional form of ALK2 in vivo. Our present study demonstrates that *Alk2* targeted for exon 7 is functionally null, showing that the studies using the *Alk2* floxed mouse reveal phenotypes that result from the null mutation of *Alk2*.

In conclusion, the comparative analysis of two different types of *Alk2*-deficient mutants showed an identical phenotype. Although the targeted exon was different from conventional *Alk2* mutations, the *Alk2* floxed mouse can be used to make the conditional *Alk2* null mouse. The defects observed in $Alk2^{\Delta 5/\Delta 5}$ and $Alk2^{\Delta 7/\Delta 7}$ mutants reveal intriguing links between the *Alk2*, *Brachyury*, *Wnt3a*, and *Tbx6* genetic pathways in primitive streak development. Because BMP signaling through ALK2 is involved in the function of extraembryonic region (Gu et al., 1999; Mishina et al., 1999; de Sousa Lopes et al., 2004), one possibility is that ALK2 signaling in the extraembryonic region induces unknown factors that act on the embryonic region to regulate the expression of *Brachyury*, *Wnt3a*, and *Tbx6*. Further analysis for the downstream target genes of *Alk2* in the extraembryonic region will provide more detailed information about BMP signaling mediated by ALK2 in proper mouse gastrulation.

EXPERIMENTAL PROCEDURES

Animals

Generation of *Alk2* exon 5 or exon 7 mutant mice were reported previously (Mishina et al., 1999; Kaartinen and Nagy, 2001). Both $Alk2^{\Delta 5/\Delta 5}$ and $Alk2^{\Delta 7/\Delta 7}$ mutants were maintained on a mixed background of 129/SvEv and C57BL/6J. All mouse experiments were performed in accordance with National Institute of Environmental Health Sciences (NIEHS) guidelines covering the humane care and use of animals in research.

Genotyping Analysis

The DNA from mouse embryos was analyzed by PCR. Primer sequences for exon 5-specific *Alk2* mutant were 5′-ATG CTA GAC CTG GGC AGC CAT A-3′, 5′-CAT GCT AGC AGC TCG GAG AAA C-3′, 5′-GAG ACT AGT GAG ACG TGC TAC T-3′. The conditions for genotyping PCR were 94°C for 20 sec, 65°C for 20 sec, 72°C for 20 min, repeated 40 cycles for detecting of exon 5-specific *Alk2* mutant allele. Genotyping PCR reaction yielded 371 bp for wild-type, or 333 bp for *Alk2* exon 5 mutant DNA fragment. Primer sequences for exon7-specific *Alk2* mutant were 5′-CCC CCA TTG AAG GTT TAG AGA GAC-3′, 5′-TGA GAT TGT TCT AGC ACT GCC C-3′, 5′-GAA TTG CTA GAA GCC CAT AGG C-3′. The conditions for genotyping PCR were 94°C for 20 sec, 60°C for 20 sec, 72°C for 20 min, repeated 40 cycles for detecting of exon 7-specific *Alk2* mutant allele. Genotyping PCR reaction yielded 530 bp for wild-type or 625 bp for *Alk2* exon 7 mutant DNA fragment.

Histological Analysis and In Situ Hybridization

Embryos were fixed in 4% paraformal-dehyde, embedded in paraffin, and stained with hematoxylin and eosin. In situ hybridization was performed with a digoxigenin-labeled RNA probe by standard procedures (Wilkinson and Nieto, 1993). *Brachyury*, *Cer1*, *Fgf8*, *Foxa2*, *Shh*, *Tbx6*, and *Wnt3a* probes were kindly provided by Drs. B.G. Herrmann, W. Shawlot, G.R. Martin, H. Sasaki, A.P. McMahon, D.L. Chapman, and T.P. Yamaguchi, respectively.

Acknowledgements

We thank Drs. B.G. Herrmann, W. Shawlot, G.R. Martin, H. Sasaki, A.P. McMahon, D.L. Chapman, and T.P. Yamaguchi for in situ probes. We also thank Ms. M. Kamikawa-Miyado for advice on histological analysis, Dr. C. Kimura-Yoshida for suggestion of in situ hybridization, and Drs. E.M. Eddy and M.K. Ray for critical comments. We are grateful to Ms. Li He and Ms. Chiaki Komatsu for encouragement. V.K. was funded by a grant from the NIH and Y.M. was funded by the Intramural Research Program of the National Institutes of Health, National Institute of Environmental Health Sciences (NIH/NIEHS).

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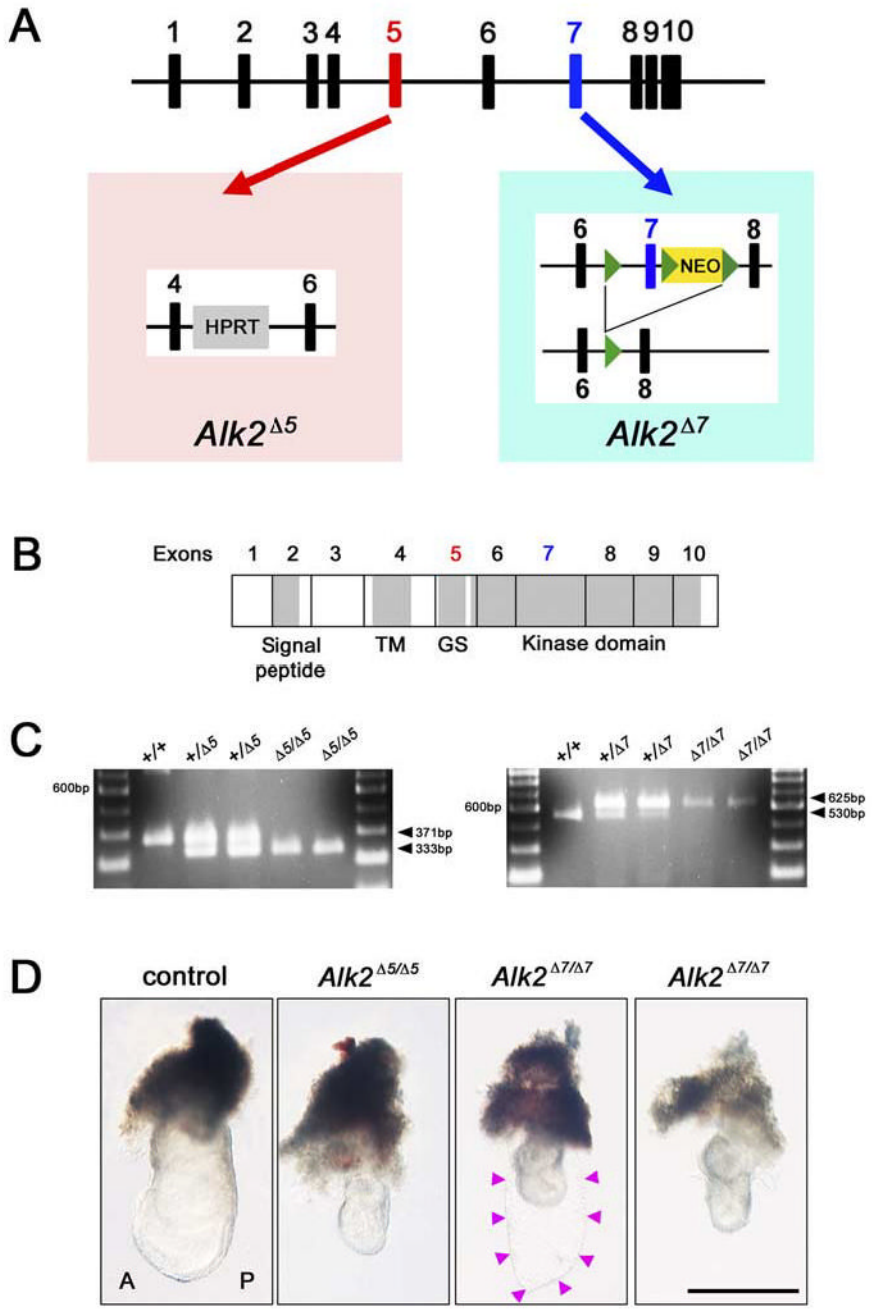

Fig. 1.

Targeted disruption of the *Alk2* exon 5 and exon 7. **A:** Schematic illustration of the mouse *Alk2* gene. The left panel shows the conventional *Alk2* targeted exon 5 allele ($Alk2^{\Delta 5}$), and the right panel shows the targeted mutation of the *Alk2* exon 7 allele ($Alk2^{\Delta 7}$). Black boxes represent *Alk2* exons. Red and blue boxes show *Alk2* exon 5 and exon 7, respectively. HPRT, hypoxanthine-guanine phosphori-bosyltransferase; NEO, neomycin. **B:** A schematic presentation of ALK2 protein and organization of *Alk2* exons 5 and 7. Exon 1 is noncording. TM, transmembrane domain; GS, GS-domain rich in Gly and Ser. **C:** Genotyping results of the disruption for *Alk2* exon 5 (wild-type; 371 bp, mutant; 333 bp) or *Alk2* exon 7 (wild-type; 530 bp, mutant; 625 bp) by polymerase chain reaction analysis. **D:** Whole-mount view of

control (wild-type or *Alk2* heterozygous) and *Alk2* homozygous for exon 5 ($Alk2^{\Delta 5/\Delta 5}$) or exon 7 ($Alk2^{\Delta 7/\Delta 7}$) embryos at embryonic day (E) 7.5. A, anterior; P, posterior. Scale bar = 500 μm.

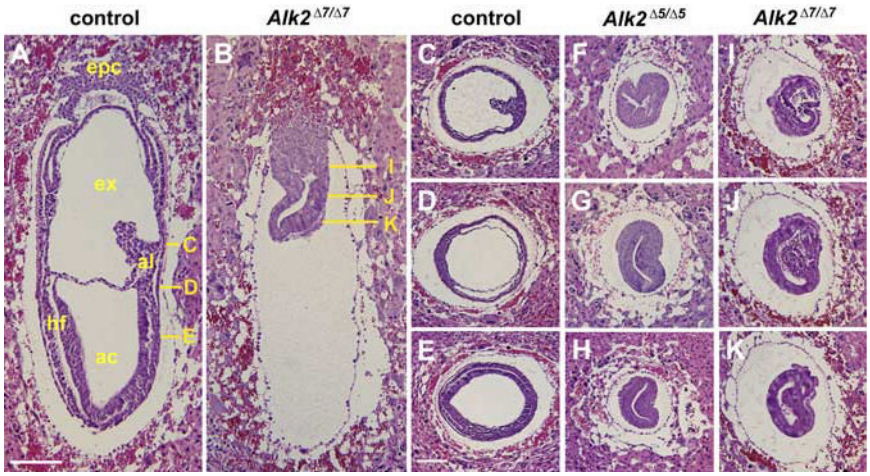

Fig. 2.

Histological analysis of *Alk2*$^{\Delta 5/\Delta 5}$ and *Alk2*$^{\Delta 7/\Delta 7}$ mutant embryos. **A,B:** Sagittal sections of embryonic day (E) 7.5 control (A) and *Alk2*$^{\Delta 7/\Delta 7}$ mutant (B) embryos. **C–K:** Histological comparison of the control (C–E), *Alk2*$^{\Delta 5/\Delta 5}$ mutant (F–H), and *Alk2*$^{\Delta 7/\Delta 7}$ mutant (I–K) specimen by transverse section at E7.5. The solid yellow lines in A and B indicate the approximate position of the transverse sections in C–E and I–K, respectively. ac, amniotic cavity; al, allantois; epc, ectoplacental cone; ex, exocoelomic cavity; hf, head fold. Scale bar = 200 μm.

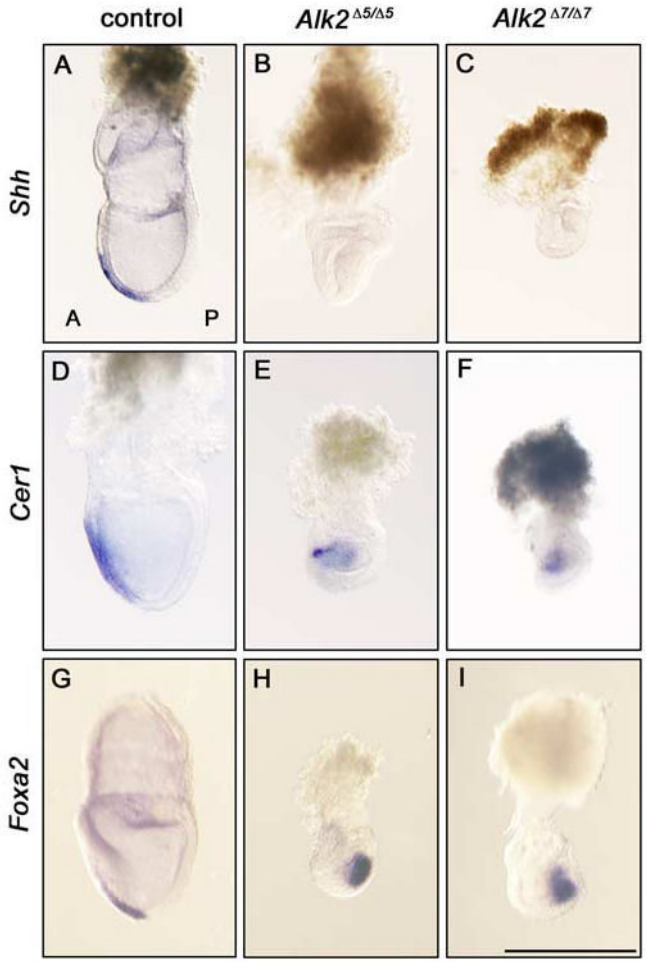

Fig. 3.

Anterior marker gene expression analysis of *Alk2*$^{\Delta 5/\Delta 5}$ and *Alk2*$^{\Delta 7/\Delta 7}$ mutant embryos. **A–I:** Marker gene expression pattern of *Shh* (A–C), *Cer1* (D–F), and *Foxa2* (G–I) at E7.5 in control (A,D,G), *Alk2*$^{\Delta 5/\Delta 5}$ mutant (B,E,H), and *Alk2*$^{\Delta 7/\Delta 7}$ (C,F,I) mutant embryos, respectively. A, anterior; P, posterior. Scale bar = 500 μm.

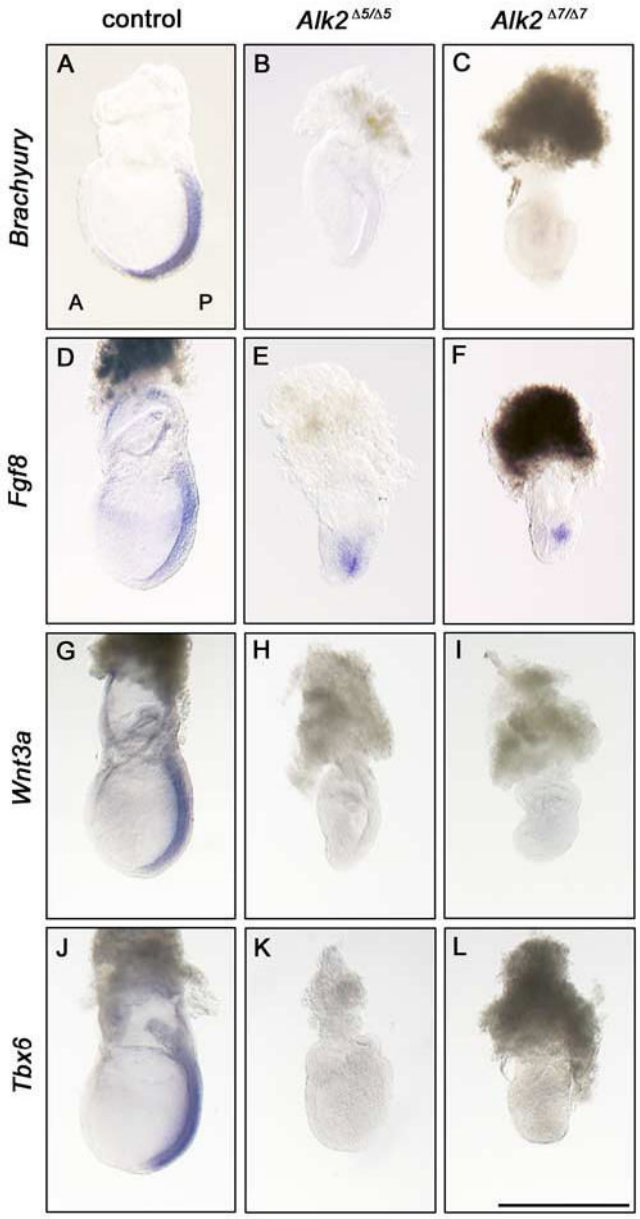

Fig. 4.

Posterior marker gene expression analysis of *Alk2*$^{\Delta 5/\Delta 5}$ and *Alk2*$^{\Delta 7/\Delta 7}$ mutant embryos. **A–L:** Marker gene expression pattern of *Brachyury* (A–C), *Fgf8* (D–F), *Wnt3a* (G–I), and *Tbx6* (J–L) at embryonic day (E) 7.5 in control embryos (A,D,G,J), *Alk2*$^{\Delta 5/\Delta 5}$ mutant embryos (B,E,H,K), and *Alk2*$^{\Delta 7/\Delta 7}$ (C,F,I,L) mutant embryos, respectively. A, anterior; P, posterior. Scale bar = 500 μm.