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Deficient Alk3-mediated BMP signaling causes prenatal omphalocele-like defect

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Abstract

BMP signaling plays important roles in many embryonic developmental processes. Alk3 is one of two BMP type I receptors that transduces BMP signal from the cell surface into cell. Conventional knockout of Alk3 resulted in early embryonic lethality around E7.5–E9.5. In this study, we have generated embryonic mesoderm-specific Alk3 conditional knockout by crossing Dermo1-Cre and floxed Alk3 mice. Abrogation of Alk3-mediated BMP signaling in this mouse resulted in severe defect of secondary ventral body wall formation, replicating the omphalocele phenotype in human. Our finding suggests that Alk3 plays an essential role in the formation of embryonic ventral abdominal wall, and abrogation of BMP signaling activity due to gene mutations in its signaling components could be one of the underlying causes of omphalocele at birth.

Keywords: BMP; Alk3; Omphalocele; Ventral body wall

Ventral abdominal wall defects, including gastroschisis and omphalocele, are congenital diseases that affect neonates [1]. Omphalocele is characterized by the absence of abdominal muscles, fascia, and skin; and in place of the abdominal wall covering is a membrane consisting of peritoneum and amnion [2]. The abdominal contents, including liver, stomach, and gut, often extrude ventrally through this abnormally enlarged membranous sac, or called umbilical ring. The incidence of omphalocele is reported to be 1 in 300–4000 births. Multiple genetic mutations and environmental factors have been identified to be associated with this defect. Moreover, omphalocele frequently occurs in conjunction with other abnormalities, including cardiac or genitourinary abnormalities, neural tube or skeletal defects, as well as chromosomal anomalies, such as trisomy 13 and 18. Omphalocele may also be associated with Beckwith–Wiedemann Syndrome [3,4]. However, the pathogenic mechanism of omphalocele is still poorly understood. Animal models of omphalocele are therefore important to help identifying the key genetic mutations and understanding the molecular pathogenic mechanisms.

During mouse embryonic development, following folding and turning of embryo, the ventral surface of the embryo is covered by a primary body wall, consisting of a thin epithelial membrane and loose mesenchyme, at early embryonic stage of E9.5 [2]. This primary body wall persists until E12, when a secondary body wall starts to form to replace the membrane wall by somite-derived cells migrating towards the ventral midline. Thus, the secondary abdominal wall is composed of skin, muscle, abdominal band derivatives and an inner epithelium. Disruption of this secondary body wall formation results in defects including omphalocele. The abnormal ventral body wall

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phenotype that mimics omphalocele has been observed in several different gene knockout mice. These genes include growth factors (IGF-II, Ephrin-B1, and TGF-β2/3) [5–7], receptors (IGF-II receptor and EphB2/B3) [5,8], and transcription factors (AP-2α, Zic3, Hic1, Pax3, and Msx1/2) [9–13]. The involvement of Msx genes, which are known downstream targets of BMP signal pathway, in ventral body wall formation suggests that Bone Morphogenetic Protein (BMP) signaling cascade may be intimately engaged in coordinating the development of ventral body wall. However, direct evidence for BMP signal in regulating secondary ventral body wall formation has not been shown.

BMPs, with more than 20 family members, have been shown to regulate many fundamental biological processes including cell proliferation, differentiation, apoptosis, migration, and adhesion [14]. Furthermore, they are involved in the development of almost all tissues and organs, as well as the specification of the basic embryonic body plan, such as dorso-ventral patterning, left–right asymmetric axis, and proximal–distal axis formation [15]. As extracellular growth factors, BMPs bind to heteromeric complexes of BMP serine/threonine kinase type I and type II receptors [16,17]. Upon ligand-induced aggregation of the receptors, constitutively activated BMP type II receptor kinase phosphorylates and activates the type I receptor, which subsequently phosphorylates and activates BMP specific Smad proteins (Smad1, Smad5, and Smad8). These activated Smads then form complexes with Smad4, translocate into the nucleus, and act as transcriptional co-modulators to induce or repress BMP-target gene expression [18]. In addition to this canonical pathway, Smad-independent BMP pathways have also been reported [19]. Three cognate BMP type I receptors (Alk2, Alk3, and Alk6) have been identified. Alk3, also called BMP receptor type IA (BMPR-IA), plays an essential role during early embryonic development, particularly in mesoderm formation and gastrulation. The conventional Alk3 gene null mutation causes early embryonic lethal in mice (E7.5–9.5) prior to the formation of secondary body wall [20]. Herein, we generated a mesoderm-specific Alk3 conditional knockout mouse using a Dermo1-Cre knockin driver line to dissect Alk3-mediated BMP signaling in promoting mesoderm-derived tissue development and its related pathogenic role in birth defects.

Methods

Mouse strains and breeding. The floxed Alk3 ($Alk3^{fx/fx}$) mice were generated in Dr. Yujii Mishina's Lab [21]. In $Alk3^{fx/fx}$, the exon 2 of Alk3 gene was flanked with two *loxP* DNA elements. Deletion of exon 2 will cause frameshift and eliminate functional Alk3 protein expression. Dermo1-Cre heterozygous knockin ($Dermo1\text{-}Cre^{+/-}$) mice was generated in Dr. David Ornitz's Lab [22]. Timed mating between $Alk3^{fx/fx}$ and $Alk3^{+/fx}$/$Dermo1\text{-}Cre^{+}$ mice generated mesoderm-specific Alk3 conditional knockout (Alk3 CKO) mice ($Alk3^{fx/fx}$/$Dermo1\text{-}Cre^{+}$), heterozygous Alk3 knockout (HT) mice ($Alk3^{fx/+}$/$Dermo1\text{-}Cre^{+}$), and control mice ($Alk3^{fx/+}$, or $Alk3^{fx/fx}$) (Fig. 1). All mice were bred in C57BL/6 strain background. Mice used in this study were housed in pathogen-free condition according to the protocol approved by IACUC at the Saban Research Institute of Children Hospital Los Angeles.

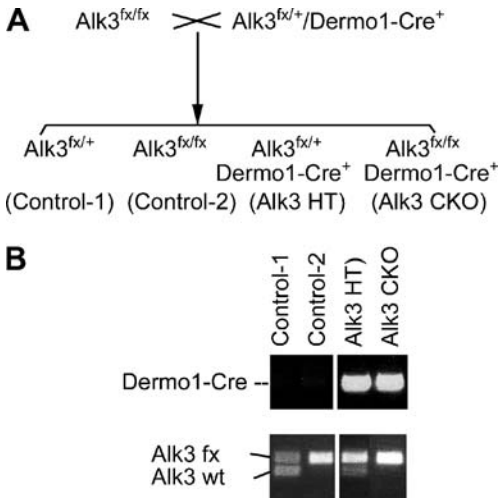

Fig. 1. Generation of mesoderm-specific Alk3 conditional knockout mice. (A) Breeding scheme for Alk3 CKO mice by crossing $Alk3^{fx/fx}$ and Dermo1-Cre mice. (B) Genotypes of fetal mice by tail genomic DNA PCR.

Mouse genotyping. Genotypes of mice were determined by tail tissue genomic DNA PCR. The PCR primers for Alk3 genotyping are 5′-GCA GCT GCT GCT GCA GCC TCC-3′ and 5′-TGG CTA CAA TTT GTC TCA TGC-3′. The PCR primers used to detect Cre knocking in 5′ of exon 1 of Dermo1 genomic structure are 5′-TGC CAC GAC CAA GTG ACA GCA ATG-3′ and 5′-AGA GAC GGA AAT CCA TCG CTC G-3′. GoTaq® Flexi DNA Polymerase (Promega) was used for PCR reaction.

Histology. Mouse embryos at different stages were obtained under dissecting microscope, and photographed with Spot digital camera. Samples were then fixed with 4% buffered paraformaldehyde at 4 °C overnight, dehydrated and embedded in paraffin. About 5 μm sections were stained with hematoxylin and eosin (HE), as reported previously [23]. Microscopic images were taken as described above.

Results and discussion

Mesoderm specific Alk3 conditional knockout (CKO) mice were generated by crossing floxed Alk3 ($Alk3^{fx/fx}$) and Dermo1-Cre heterozygous knock-in ($Dermo1\text{-}Cre^{+}$) mice (Fig. 1A). Dermo1 expression in mouse embryos from E10.5 to E12.5 has been detected in many mesodermal tissues, including somite derived dermatome, myotome, sclerotome, and limb [24]. Therefore, the Cre transgene under the control of the endogenous Dermo1 promoter has the identical expression pattern as the endogenous Dermo1 [22], which would result in mesoderm-specific deletion of floxed Alk3 gene and subsequent abrogation of Alk3 protein function. To demonstrate Cre-mediated recombination of floxed Alk3 allele, genomic DNA isolated from the tail of mouse fetus was subjected to PCR-based genotype analysis (Fig. 1B). Since $Alk3^{fx/fx}$ and $Alk3^{fx/+}$ embryos are morphologically indistinguishable from wild-type ($Alk3^{+/+}$) embryos, they are all classified as normal wild type (WT) controls in this study.

In Alk3 CKO ($Alk3^{fx/fx}$/ $Dermo1\text{-}Cre^{+}$) mice, embryonic development seems normal until approximately

E12.5, when secondary ventral body wall development has just begun (Fig. 2). By gross view, protrusion of liver and intestine from peritoneal cavity was not obviously discernible in E12.5 Alk3 CKO mouse embryos. However, ventral extrusion of stomach and liver covered with only a transparent membrane was clearly seen in E13.5 Alk3 CKO embryos, which suggested that formation of the secondary ventral body wall was disrupted and closure of umbilical ring was defective. In addition, the length of both forelimb and hind limb in E13.5 Alk3 CKO fetuses was shorter than that in control mice, suggesting a retardation of limb development. These phenotypes of abnormal ventral wall and limb formation were more obvious at E14.5 (Fig. 3). From both side and top views of Alk3 CKO fetuses, liver and part of intestines were protruded outside peritoneal cavity, which was covered only with a transparent membrane, while control fetuses already had very well formed secondary abdominal wall at this age. Interestingly, abnormal patterning of distal digits were also observed in both forelimb and hind limb, suggesting that defective Alk3-mediated BMP signaling in mesoderm may be one of the important contributing factors to a group of congenital diseases called limb-body wall complex [25].

The defect of ventral body wall formation from E12.5 to E13.5 was then examined histologically. Although development of the thoracic wall in Alk3 CKO embryos was not affected, as seen in the sagittal section of E12.5 embryo, the secondary abdominal wall failed to develop. The abdominal organs including stomach and liver were not fully engulfed in the coelom. In E13.5 wild-type embryos, secondary ventral body wall, including skin, muscle, connective tissue, and inner epithelia, was fully formed, and the transition from the body wall to the amnion covering the umbilical cord constitutes a typical umbilical ring and

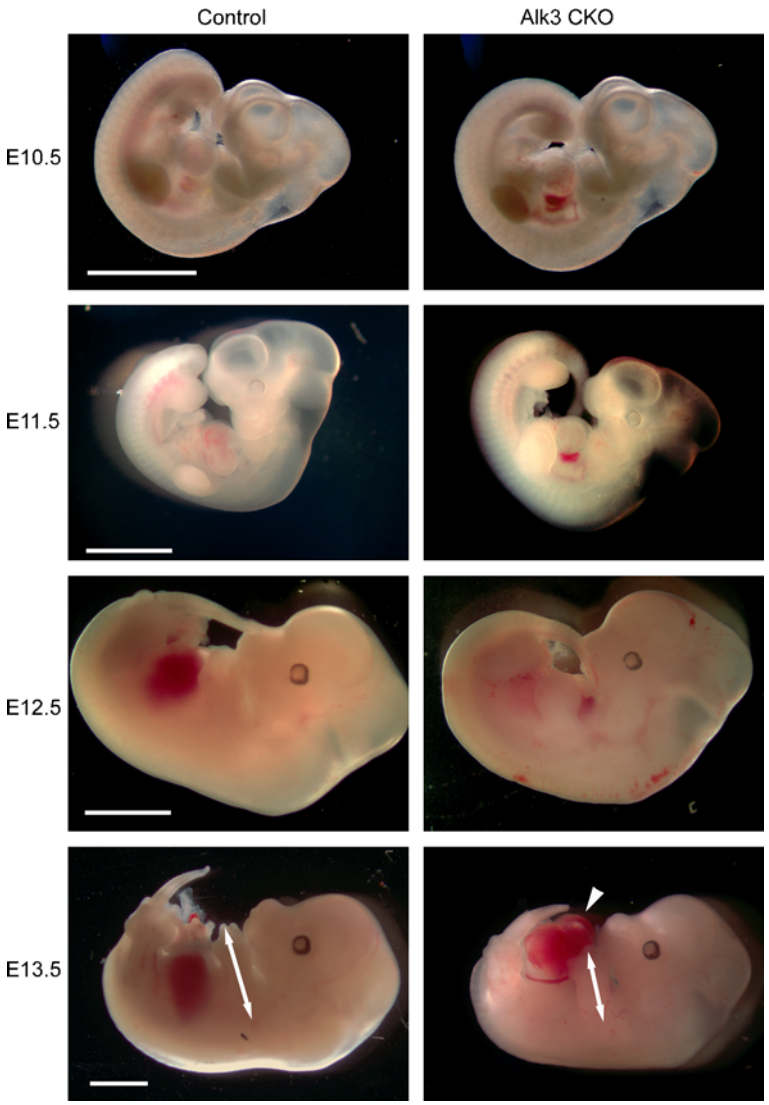

Fig. 2. Gross view of mouse embryos from E10.5 to E13.5. Ventral extrusion of liver was clearly seen in E13.5 Alk3 conditional knockout mice (Alk3 CKO, pointed with arrow head), compared to normal littermate control embryos at the same stage. Scale bar: 2 mm. The length of the forelimb at E13.5 was indicated by bi-directional arrow.

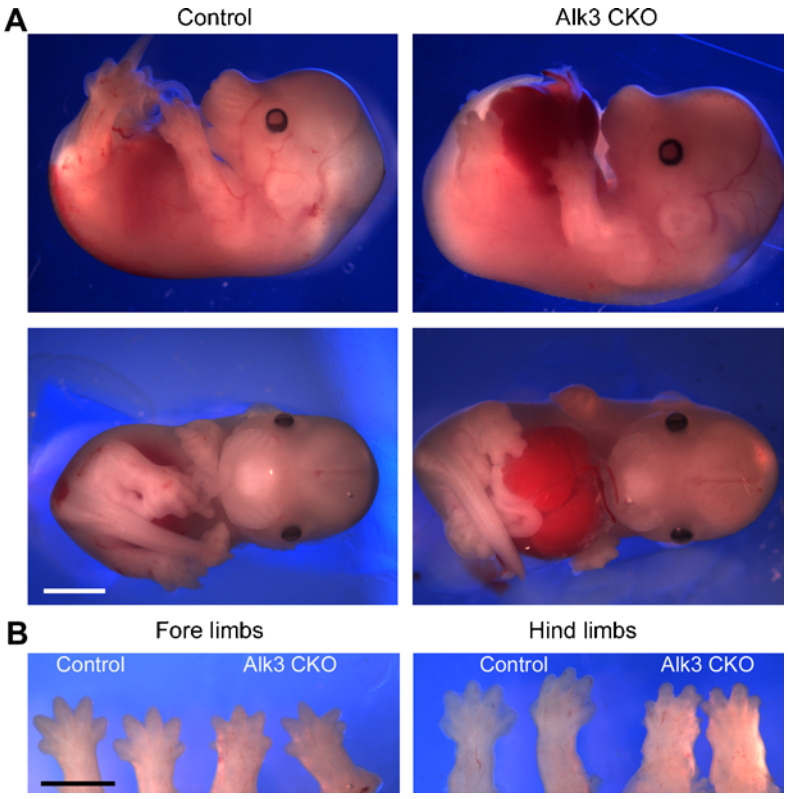

Fig. 3. Alk3 CKO mouse embryos at E14.5. (A) In normal control, a ventral abdominal wall covered with normal skin was very well formed, so that abdominal contents were engulfed into peritoneal cavity, and not visible from both side and top. In contrast, only a thin membrane covered peritoneal cavity in Alk3 CKO mice, and the abdominal contents were clearly seen through this transparent membrane. Extrusion of liver was obvious from both side and top views. Scale bar: 2 mm. (B) Abnormal patterning of distal digits in both forelimb and hind limb of Alk3 CKO mice at E14.5. Scale bar: 1 mm.

the related umbilical hernia (Fig. 4). A part of gut was still seen within the physiological umbilical hernia until its return to the peritoneal cavity through the umbilical ring around E16 in mice. In contrast, the secondary ventral body wall formation did not occur in Alk3 CKO mouse embryos. The peritoneal cavity was only covered by a thin membrane consisting of single layer of epithelia and loose mesenchyme, with ventral extrusion of liver, stomach, and gut. In particular, the musculature failed to grow into ventral midline (Fig. 4). Therefore, Alk3-mediated BMP signaling is essential for somite-derived tissue growth to form secondary ventral body wall. Deficient Alk3-mediated BMP signaling in these tissues at this stage resulted in a severe defect in the ventral body wall, reminiscent of omphalocele in human.

Defective mammalian ventral body wall formation can be caused by chromosomal anomalies in humans and targeted gene mutations in mice. However, the underlying mechanisms of pathogenesis in ventral body wall abnormalities are still poorly understood. Mouse models with a variety of gene knockouts provide an effective approach to identify and finally understand key cell signaling pathways that regulate abdominal wall morphogenesis. Although several genes, including TGF-β2/β3, AP-2α, Msx1/2, and Pax3, have been associated with ventral body wall closure defects [7,9,13,26], components of BMP signaling pathway with the exception of Msx genes have not been identified as a key regulator for secondary ventral body wall formation. The conventional knockout of BMP receptor Alk3 or downstream specific Smad1 or Smad5 resulted in early embryonic lethality around E7.5–11.5 prior to the secondary ventral body wall formation [20,27,28]. In order to determine BMP signaling in mesoderm-derived organ/tissue morphogenesis, we have crossed Dermo1-Cre and floxed Alk3 mice to selectively inactivate Alk3 in these tissues. A severe abnormality with omphalocele-like abdominal wall defect plus distal digit malformation was found in the Alk3 CKO, suggesting that Alk3-mediated BMP signaling is essential for ventral body wall formation. In addition, an abnormal limb development phenotype, represented by shortened limb, was similar to that seen in Prx1-Cre/Alk3 conditional knockout mice [29].

The deletion of Alk3 may have disrupted signal transduction cascade that is normally initiated by the binding of BMP4, one of ligands for BMP receptor, to Alk3. BMP4 has been found to express in the developing primary body wall and regulate MyoD, Myf5 and myogenin expression which are transcriptional factors essential for myoblast differentiation in hypaxial myotomes [30]. Interestingly, abnormal body wall formation was also observed in BMP2/BMP4 double heterozygous

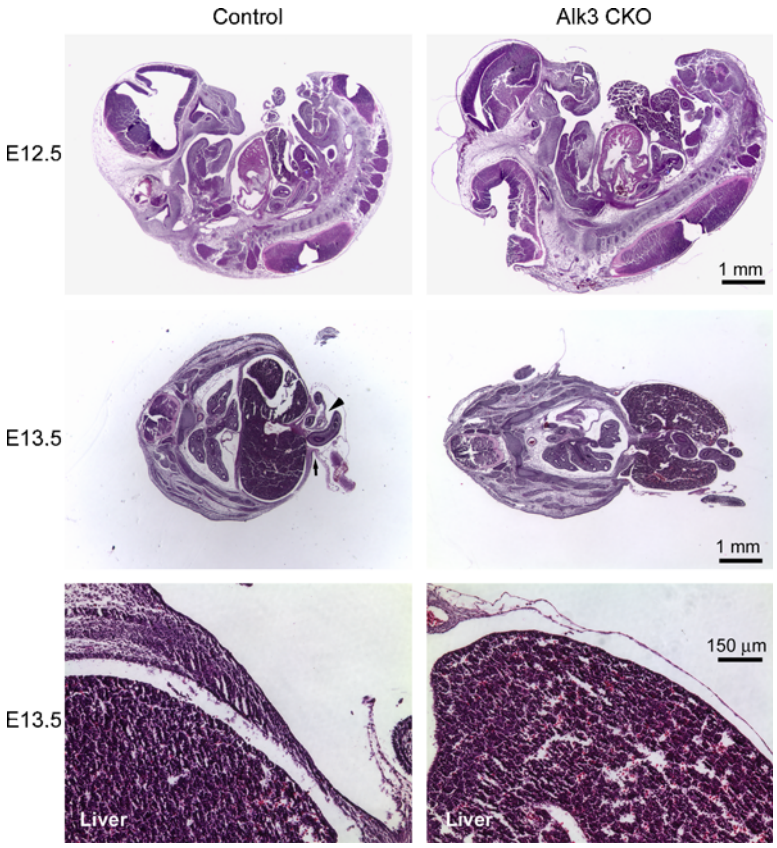

Fig. 4. Histology of Alk3 CKO mouse embryos from E12.5 to E13.5. At E12.5, thoracic wall formation was observed in both Alk3 CKO and littermate control mouse embryos in the sagittal sections. However, only partial engulfment of abdominal organs including liver and stomach was observed in Alk3 CKO. In horizontal tissue section of E13.5 embryos, secondary abdominal wall was formed in control embryos, with a typical umbilical ring (arrow) and umbilical hernia. Only part of gut remained in the umbilical hernia (arrow head). In contrast, a thin membrane covered abdominal wall in Alk3 CKO embryos. Ventral extrusion of abdominal organs (liver, stomach, and gut) occurred. Under high magnification, a single layer of cells was detected in the membrane of Alk3 CKO ventral wall, while a typical secondary ventral body wall, including skin, muscle, connective tissue, and inner epithelia, was detected in the control.

knockout mice with less than 5% penetrancy (Yuji Mishina, unpublished observation). Moreover, BMP signaling was required to maintain a pool of *Pax-3*-expressing proliferating muscle precursors in somites by restraining Pax-3 expressing myotome from differentiating [31]. In addition, Msx1/2, whose knockout resulted in defective abdominal wall formation, have been indicated as BMP-regulated downstream target genes in a variety of experimental conditions [32]. Therefore, further investigation on interaction between BMP signal pathway and other candidate genes that were known to be involved in secondary ventral body wall formation will be needed to understand the pathogenic mechanisms for ventral body wall defect, and possibly to aid in future design of genetic screen and therapeutic strategies to these congenital diseases.

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