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Smad1/Smad5 signaling in limb ectoderm functions redundantly and is required for interdigital programmed cell death

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Abstract

Bone morphogenetic proteins (BMPs) are secreted signals that regulate apical ectodermal ridge (AER) functions and interdigital programmed cell death (PCD) of developing limb. However the identities of the intracellular mediators of these signals are unknown. To investigate the role of Smad proteins in BMP-regulated AER functions in limb development, we inactivated *Smad1* and *Smad5* selectively in AER and ventral ectoderm of developing limb, using *Smad1* or/and *Smad5* floxed alleles and an $En1^{Cre/+}$ knock-in allele. Single inactivation of either *Smad1* or *Smad5* did not result in limb abnormalities. However, the *Smad1/Smad5* double mutants exhibited syndactyly due to a reduction in interdigital PCD and an increase in interdigital cell proliferation. Cell tracing experiments in the *Smad1/Smad5* double mutants showed that ventral ectoderm became thicker and the descendents of ventral $En1^{Cre/+}$ expressing ectodermal cells were located at dorsal interdigital regions. At the molecular level, *Fgf8* expression was prolonged in the interdigital ectoderm of embryonic day (E) 13 *Smad1/Smad5* double mutants, suggesting that the ectopic *Fgf8* expression may serve as a survival signal for interdigital epithelial and mesenchymal cells. Our result suggests that Smad1 and Smad5 are required and function redundantly as intracellular mediators for BMP signaling in the AER and ventral ectoderm. Smad1/Smad5 signaling in the AER and ventral ectoderm regulates interdigital tissue regression of developing limb. Our mutants with defects in interdigital PCD could also serve as a valuable model for investigation of PCD regulation machinery.

Keywords

Receptor Smads; Programmed cell death; Limb development; AER; Conditional inactivation

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Introduction

The developing limb has easily identified discrete pattern and structure formation and is conveniently accessible for experimental manipulation. It has therefore long been an excellent model for investigating how different signals interact to control different cellular activities during pattern formation (Towers and Tickle, 2009). The mouse limbs develop from the embryonic limb buds, coming out from either side of body wall at the appropriate positions along the body axis. Early limb bud is made up of a mass of undifferentiated mesenchymal cells covered with surface ectoderm. The growth and patterning of the limb bud along the proximal–distal, anterior–posterior and dorsal–ventral axes are coordinated by reciprocal interactions between specialized regions known as apical ectodermal ridge (AER), zone of polarizing activity and the non-ridge ectoderm of the limb bud respectively (Capdevila and Izpisua Belmonte, 2001; Martin, 1990). AER is a stratified columnar epithelial structure located at the apex of the forelimb and hindlimb bud starting from embryonic day (E) 10.5 and 11.5 respectively. It is a source of secreted factors required for limb outgrowth (Dudley et al., 2002). Alternatively, this structure is also involved in regulating programmed cell death (PCD) in the developing limb (Maatouk et al., 2009; Pajni-Underwood et al., 2007). PCD is an important genetically controlled process that regulates development and homeostasis inmulticellular organisms (Fadeel and Orrenius, 2005; Jacobson et al., 1997). PCD is mainly observed in the interdigital undifferentiated mesenchymal cells and the AER (Fernandez-Teran et al., 2006; Zuzarte-Luis and Hurle, 2005). This process has to be controlled precisely so that the developing limb is sculpted into a particular shape and structure that is particular to an organism (Zuzarte-Luis and Hurle, 2005). Interdigital region of developing limb is of particular interest because the fate of interdigital mesenchymal cells to undergo cell death or not or chondrogenesis is subjected to precise genetic control and appropriate signaling between the ectoderm and underlying mesenchyme (Bandyopadhyay et al., 2006; Hernández-Martínez and Covarrubias, 2011; Hurle and Ganan, 1986). Dying cells located in the mesenchyme between the forming digits of the developing limbs are referred as interdigital PCD that is responsible in restricting interdigital tissue growth and promoting tissue regression in order to separate digits (Hernández-Martínez and Covarrubias, 2011; Macias et al., 1997; Montero et al., 2001). Defects in patterning and interdigital PCD in the developing limb are correlated with various types of congenital limb malformations (Al-Qattan et al., 2009; Goodman, 2002).

Interdigital PCD in developing limb is tightly controlled under the effects of different signaling factors like fibroblast growth factors (FGFs) (Delgado et al., 2008; Montero et al., 2001; Pajni-Underwood et al., 2007; Salas-Vidal et al., 2001), BMPs (Ganan et al., 1996; Guha et al., 2002; Macias et al., 1997; Pajni-Underwood et al., 2007; Salas-Vidal et al., 2001; Zou and Niswander, 1996), transforming growth factor-beta (TGF-β) (Ganan et al., 1996), Msx-2 (Marazzi et al., 1997; Salas-Vidal et al., 2001), Wnts (Grotewold and Ruther, 2002), Shh (Buscher et al., 1997) and retinoic acid (Ali-Khan and Hales, 2006; Galdones et al., 2006). BMP signaling in the AER has been shown to regulate interdigital PCD but the identity of the intracellular mediator remains unclear (Maatouk et al., 2009; Pajni-Underwood et al., 2007; Zuzarte-Luis and Hurle, 2005). One of the pathways involves receptor-regulated Smad proteins (Zuzarte-Luis and Hurle, 2005; Zuzarte-Luis et al., 2004).

Binding of BMP ligand to membrane-bound serine/threonine kinase receptors results in receptor phosphorylation and subsequent activation of receptor-regulated Smad proteins (R-Smads) Smads1/5/8 by phosphorylation. Activated Smad1, 5 or 8 form complexes with common partner Smad (CoSmad), Smad4 and translocate into the nucleus where they regulate the transcription of target genes (Itoh et al., 2000).

In this study, we investigated the identity of the Smad proteins involved in BMP-regulated AER functions during limb development. Knockout of either *Smad1* or *Smad5* results in early embryonic lethality, hindering the studies of Smad function in limb formation (Chang et al., 1999; Lechleider et al., 2001). Therefore, Cre/*loxP* approach was used to inactivate *Smad1* and/or *Smad5* in the developing limb ectoderm in a tissue-specific manner by the use of *Smad1* and/or *Smad5* conditional null (floxed) alleles (Huang et al., 2002; Umans et al., 2003) and an *Engrailed1*-Cre-recombinase knock-in allele ($En1^{Cre/+}$) (Kimmel et al., 2000). $En1^{Cre/+}$ first expresses in the ventral ectoderm and the ventral part of the developing AER of forelimb bud at E9.5 (Kwan et al., 2004). After E11.5, $En1^{Cre/+}$ expressing lineage is present throughout the dorsal–ventral extent of the AER (Kimmel et al., 2000). Our data showed that conditional inactivation of either *Smad1* or *Smad5* in the limb AER and ventral ectoderm does not result in limb abnormalities. However, double inactivation of both *Smad1/Smad5* resulted in syndactyly due to a reduction in interdigital PCD and an increase in interdigital cell proliferation. Our data suggest that Smad1 and Smad5 act as the intracellular mediator downstream of the BMP receptor Ia (BmprIa) to transduce BMPs signal in the AER. The two proteins are required and function redundantly to regulate AER functions. The Smad1/Smad5 signaling in AER indirectly regulates interdigital PCD and cell proliferation in developing limb. Furthermore, we have investigated on the possible molecular mechanism of the Smad1/Smad5 signaling in regulating the interdigital tissue regression.

Materials and methods

Mouse strain generation and genotyping

The generation and genotyping of conditional (floxed) alleles of *Smad1* ($Smad1^{f}$) and *Smad5* ($Smad5^{f}$) have been described previously (Huang et al., 2002; Pangas et al., 2008; Umans et al., 2003). The $En1^{Cre}$ knock-in allele has been described previously (Kimmel et al., 2000; Kwan et al., 2004). To generate the AER and ventral ectoderm conditional *Smad1/Smad5* knockout mutant, we crossed $En1^{Cre/+}$ mice with $Smad1^{f/f}$; $Smad5^{f/f}$ mice. Their $En1^{Cre/+}$; $Smad1^{f/+}$; $Smad5^{f/+}$ female offspring were then crossed with $Smad1^{f/f}$; $Smad5^{f/f}$ male to produce $En1^{Cre/+}$; $Smad1^{f/+}$; $Smad5^{f/f}$ males. To generate the $En1^{Cre/+}$; $Smad1^{f/f}$; $Smad5^{f/f}$ mutant embryos for analysis, $Smad1^{f/f}$; $Smad5^{f/f}$ females were then crossed with $En1^{Cre/+}$; $Smad1^{f/+}$; $Smad5^{f/f}$ males. Their littermates, $En1^{Cre/+}$; $Smad1^{f/+}$; $Smad5^{f/f}$, which developed normally, were used as controls. Since the $En1^{Cre}$ is a knock-in allele, we used $En1^{Cre/+}$; $Smad1^{f/+}$; $Smad5^{f/f}$ mice as the controls to eliminate the possible effect of the *En1* heterozygous null genotype on the phenotypic analysis of the double *Smad1/Smad5* conditional mutants. All mouse strains were on a mixed genetic background. All animal procedures were conducted with the approval of the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong.

Immunofluorescence, cell death and cell proliferation analysis, section and whole mount RNA in situ hybridization

Embryos or embryonic limbs were isolated, fixed in 4% paraformaldehyde (PFA) at 4 °C overnight, paraffin-embedded and sectioned using standard techniques. Antibodies against Smad1 (Invitrogen) at a dilution of 4 μg/ml, Smad5 at a dilution of 1:50 (Santa Cruz), phospho-Smad1/5/8 at a dilution of 1:100 (Cell Signaling Technology) and CD44 at a dilution of 1:50 (Cell Signaling Technology) were used for immunofluorescence as described previously (Andl et al., 2004; Flanders et al., 2001; Maatouk et al., 2009). To detect cell death, the TUNEL assay (Roche) and anti-cleaved caspase3 antibody at a 1:150 dilution (Cell Signaling Technology) were performed according to the manufacturers' protocol. BrdU labeling (Roche) was used to detect cell proliferation level. For BrdU labeling, pregnant females at 13.5 dpc were injected with BrdU (1 mg per 10 g body weight) and sacrificed after 2 h. Embryonic limbs were isolated, fixed in 4% PFA at 4 °C overnight, paraffin-embedded and sectioned. BrdU incorporation was detected by antibody against BrdU (Chemicon) at dilution of 1:500. The number of cleaved caspase3 positive and BrdU positive cells were counted from a fixed area circle within the interdigital regions between digit2/digit3, digit3/digit4 and digit4/digit5 under the ectoderm from 7 μm sections from each embryo. Percentage of BrdU positive cells were calculated by dividing number of BrdU positive cells by the number of Hoechst-stained nuclei in fixed area circle within the interdigital area. Section *in situ* hybridization was performed according to the protocol previously described (He et al., 2001; Kwan et al., 2004). Whole mount RNA *in situ* hybridization was performed according to protocol previously described (Wilkinson, 1992). Probes used to detect *Bmp2*, *Bmp4*, *Bmp7*, *En1*, *Fgf8*, *Gremlin*, *Lmx1b*, *Msx1*, *Msx2 and Shh* have been described previously (Maatouk et al., 2009; Pajni-Underwood et al., 2007). At least three embryos for each genotype were examined in the aforementioned experiments. Embryos from the same litter were used for comparisons between the mutant and control embryos.

Cell tracing and skeletal preparations

To trace the limb ectodermal cells and their descendents whose *Smad1/Smad5* were inactivated by $En1^{Cre/+}$, we stained for the Cre dependent β-gal activity of the *R26R* reporter allele in $En1^{Cre/+}$; $Smad1^{f/f}$; $Smad5^{f/f}$; $Rosa26^{R26R/+}$ embryos as previously described (Soriano, 1999). Stained embryos were postfixed in 4% PFA at 4 °C overnight, paraffin-embedded and stained with eosin for histological analysis of the limbs. Skeletal staining using Alcian blue and Alizarin red was performed as previously described (Ovchinnikov, 2009).

Results

$En1^{Cre/+}$ inactivation of Smad1 and Smad5 conditional alleles in the AER and limb ventral ectoderm

To investigate the functions of Smad1 and/or Smad5 signaling in limb ectoderm and AER, *Smad1* and/or *Smad5* were inactivated in the AER and ventral ectoderm, using *Smad1* and/or *Smad5* conditional null alleles and an $En1^{Cre/+}$ knock-in allele. Previous reports have shown that $En1^{Cre/+}$ knock-in allele is active in the ventral ectoderm and the ventral part of

the developing AER in the forelimb buds as early as E9.5 (Kimmel et al., 2000; Kwan et al., 2004). At later stages, from E11.5, *En1*$^{Cre/+}$ expressing cells are also found throughout the dorsal–ventral extent of the AER (Kimmel et al., 2000). To determine whether the inactivation of *Smad* genes by *En1*$^{Cre/+}$ knock-in allele followed this pattern; we performed immunofluorescence on the forelimb buds of E10.5 *Smad1/Smad5* double conditional knockout mutant (hereafter referred as *Smad1/5* mutant) embryo using anti-Smad1 and anti-Smad5 antibodies. Smad1 and Smad5 were absent in the ventral ectoderm and ventral AER of the *Smad1/5* mutants (Fig. 1A–D), suggesting *En1*$^{Cre/+}$ knock-in allele successfully recombined *Smad1* and *Smad5* conditional alleles in the limb ventral ectoderm of the mutant. Smad1 and Smad5 were present in the dorsal ectoderm. When using antibody against pSmad1/5/8 for performing immunofluorescence, the signal was detected in the AER and throughout ectoderm of both the control and *Smad1/5* mutant suggesting that the expression of another BMPR-Smad, Smad8, was still present in the limb AER and ectoderm of the *Smad1/5* mutants (Fig. 1E, F).

Inactivation of both Smad1/Smad5 signaling in the limb AER and ventral ectoderm results in interdigital tissue regression defects

Ectodermal BMP receptor BmprIa and the ligands Bmp2/4 are important for regulating interdigital PCD (Maatouk et al., 2009; Pajni-Underwood et al., 2007). However, it remains unclear whether canonical BMP signaling through R-Smads are involved and what are their roles in regulating the interdigital PCD. To investigate which of the ectodermal R-Smads signaling is essential for the interdigital PCD, single *Smad1* or *Smad5* and double conditional knockout mutants were examined. Single inactivation of either *Smad1* (*En1*$^{Cre/+}$; *Smad1*$^{f/f}$) or *Smad5* (*En1*$^{Cre/+}$; *Smad5*$^{f/f}$) does not result in limb development abnormalities (Fig. 2A–D). Embryos harboring only one functional allele of either *Smad1* allele (*En1*$^{Cre/+}$; *Smad1*$^{f/+}$; *Smad5*$^{f/f}$) (Fig. 2E, G) or *Smad5* allele (*En1*$^{Cre/+}$; *Smad1*$^{f/f}$; *Smad5*$^{f/+}$) (data not shown) also had normal limbs. Thus, inactivation of three alleles of the *Smad1/5* genes in the AER and ventral ectoderm did not result in a visible limb abnormality. Embryos of *En1*$^{Cre/+}$; *Smad1*$^{f/+}$; *Smad5*$^{f/f}$ genotype were used as control throughout the study. Interestingly, combined deletion of both *Smad1* and *Smad5* in limb AER and ventral ectoderm resulted in limb abnormalities similar to embryos lacking BmprIa or BMP2/BMP4 in the AER (Maatouk et al., 2009; Pajni-Underwood et al., 2007). Both the forelimbs and hindlimbs of our *Smad1/5* mutants were severely affected at E18.5. Interdigital tissue was retained and syndactyly was observed in the mutant limbs (Fig. 2F, H). The defect of interdigital tissue regression could be observed at E13.5 when forming digits started to separate. (Fig. 2I–L). In controls, the forming digits started to separate at this stage and individual digits could be observed in both forelimbs and hindlimbs (Fig. 2I, K). However, the mutant limb autopods at E13.5 remained as a plate-like structure and the interdigital tissue was maintained between the forming digits of both forelimbs and hindlimbs (Fig. 2J, L). The autopods of *Smad1/5* mutant were expanded slightly along the anterior to posterior axis. This demonstrates that Smad1 and Smad5 in the ectoderm and AER function redundantly to regulate the interdigital mesenchymal tissue regression.

Alcian blue/alizarin red skeletal staining revealed no major differences of limb skeletal elements along the proximal to distal axis between the control and the *Smad1/5* mutant

limbs at P10 (Fig. 2M–P). Skeletal elements along the proximal to distal axis were formed properly in *Smad1/5* mutants. Inactivation of both *Smad1* and *Smad5* in the AER and ventral ectoderm did not result in defects in proximal–distal patterning of limb. Interestingly, our *Smad1/5* mutants exhibited skeletal alteration in the anterior to posterior axis. Postaxial polydactyly was observed at E18.5 *Smad 1/5* mutants (four out of four *Smad1/5* mutants analyzed) (Suppl. Fig. 1F). The ectopic rudiment attaches to the base of the distal phalange of the digitus minimus.

Smad1/Smad5 signaling in the limb AER and ventral ectoderm is required for regulating interdigital cell death and cell proliferation

To examine whether the syndactylyl phenotype in the *Smad1/5* mutants is caused by a decrease in apoptosis in the interdigital regions, the TUNEL assay was employed to mark the apoptotic cells in the E13.5 limb. Prominent apoptosis was observed in the interdigital regions of the control embryos (Fig. 3A). However, there was marked decrease in apoptosis in the interdigital regions of the *Smad1/5* mutants (Fig. 3B). This suggests that a reduction in interdigital cell death leads to the retention of interdigital tissue. Dying cells may activate caspase3 to execute interdigital apoptosis. To investigate whether there was reduction in caspase3 activation, immunofluorescence was employed to detect activated cleaved caspase3 in limbs from the *Smad1/5* mutants at E13.5. Interdigital mesenchyme of mutant limbs showed a drastic decrease in cleaved caspase3 positive cells compared with controls ($p<0.005$) (Fig. 3C–E), suggesting that activation of caspase3 in interdigital mesenchyme may be compromised after both *Smad1* and *Smad5* are inactivated in the limb ectoderm. These results demonstrate that Smad1/5 signaling in the AER and ventral ectoderm is required to regulate activation of caspase3 in interdigital mesenchyme and hence interdigital PCD.

Apart from the reduction in apoptosis, it is possible that ectopic cell proliferation in the interdigital mesenchyme can also contribute to the syndactyly in the *Smad1/5* mutants. Thus, BrdU labeling was employed to measure cell proliferation in the limbs of the mutant embryos at E13.5 (Fig. 3F–J). There was an increase in percentage of BrdU positive cells in the distal interdigital mesenchymal regions of the mutant limbs when comparing to the controls. This result demonstrates that Smad1/5 signaling in the AER and ventral ectoderm also regulates cell proliferation in the interdigital mesenchyme.

Fgf8 is persisted at the interdigital distal ectoderm and serves as survival signal for interdigital mesenchyme upon inactivation of the Smad1/Smad5 signaling in AER and ventral ectoderm

AER is an important signaling center in the developing limb bud which secretes various factors regulating limb development (Dudley et al., 2002; Pajni-Underwood et al., 2007). To analyze the roles of Smad1/5 signaling in regulating AER structure and function, the expression of *Fgf8* and *En1*, markers for AER, was examined in the mutant limbs. At E10.5, similar pattern of *Fgf8* expression was observed at the AER of the *Smad1/5* mutant and the control (Fig. 4A–B). At E11.5, similar pattern and level of *Fgf8* and *En1* expressions within the AER were observed in the *Smad1/5* mutant and control limb buds as shown by the section *in-situ* hybridization on sections along the dorsal–ventral axis (Fig. 4C–F). This

result showed that *En1* expression was maintained in the absence of Smad1/5 signaling in the AER and ventral ectoderm while the morphology of the AER was not altered in the *Smad1/5* mutant at E11.5. At E13, *Fgf8* expression was expressed in the AER overlying the developing digits but ceased over the interdigital region when interdigital cell death was initiated in the controls (Fig. 4G, I). However, we found that *Fgf8* expression was maintained at the AER over the interdigital region in the E13 *Smad1/5* mutant limbs, consistent with the previous findings in the *BmprIa* mutants (Pajni-Underwood et al., 2007). The expression of *Fgf8* was punctate throughout the mutant AER (Fig. 4H, J). The *Fgf8* expression in AER over the interdigital region ceased by E13.5 in the *Smad1/5* mutant (Fig. 4L). This suggests that FGF signaling in the *Smad1/5* mutant limbs may be elevated. The ectopic AER-specific *Fgf8* expression over the interdigital region may serve as survival signal for interdigital cells in *Smad1/5* the mutants (Macias et al., 1996; Martin, 1998; Pajni-Underwood et al., 2007; Weatherbee et al., 2006).

Mesenchymal BMP signals are not altered in the developing autopod of the Smad1/5 mutants

BMP signals acting on the interdigital mesenchyme are suggested to be the triggering factors for interdigital PCD. *Bmp2*, *Bmp4* and *Bmp7* are expressed in the interdigital regions at E12.5 and suggested to be the candidate ligands in these regions (Lyons et al., 1990, 1995; Zou and Niswander, 1996). To examine if the decrease in interdigital PCD in our *Smad1/5* mutants was related to a decrease in mesenchymal BMP signals, expression of the BMP signaling components was examined in E12.5 autopod. The expression of the three BMP ligands, *Bmp2*, *Bmp4* and *Bmp7*, in the interdigital regions was similar between the *Smad1/5* mutants and the controls at E12.5 (Fig. 5A–F). Interestingly, there were enhanced expressions of *Bmp2* (Fig. 5B) and *Bmp4* (Fig. 5D and Supp. Fig. 1B) in the AER of the *Smad1/5* mutants. Gremlin is known to be antagonist of BMP signaling (Khokha et al., 2003; Zuniga et al., 1999). No significant difference in interdigital *Gremlin* expression was found between the *Smad1/5* mutant and control (Fig. 5G, H). Msx1 and Msx2 are downstream targets of BMP signaling in the interdigital regions (Guha et al., 2002; Marazzi et al., 1997; Zou and Niswander, 1996). No significant decrease in the expression of *Msx1* and *Msx2* was detected in the *Smad1/5* mutant interdigital mesenchyme. Taken together, these results indicate that the mesenchymal BMP signaling does not alter in our *Smad1/5* mutants and the defect of interdigital PCD in the mutants is not caused by the decrease in mesenchymal BMPs signaling levels. Interestingly, the ectopic *Bmp2* and *Bmp4* expression in the distal ectoderm in the *Smad1/5* mutants suggests that there may be an auto-regulatory loop of BMP signaling and BMP signals in the limb AER (Pajni-Underwood et al., 2007).

Smad1/Smad5 inactivation in the limb ventral ectoderm resulted in ventral ectoderm thickening and ectopic En1-expressing cells and their descendents in the dorsal interdigital ectoderm

The fate of mesenchymal cells of the developing limb bud has been shown to be regulated by signaling from the ectoderm (Hurle and Ganan, 1986; Maatouk et al., 2009; Pajni-Underwood et al., 2007). Interdigital mesenchyme showed a reduction in apoptosis and increase in cell proliferation after *Smad1/5* inactivation in the AER and ventral ectoderm. However, the fate of the ventral ectodermal cells after *Smad1/Smad5* inactivation remains

unclear. To investigate the possible changes in the ventral ectoderm after *Smad1/5* inactivation, a *R26R lacZ* reporter system was employed to trace the ventral ectodermal cells. The morphology of the ventral ectoderm was examined in E15.5 limbs. Transverse sections of the control limbs showed that the ventral ectoderm was a thin structure consisting of 1–2 layers of cells (Figs. 6E and 7E). However, the ventral ectoderm of the *Smad1/5* mutant became thicker and consisted of 8–10 layers of cells (Figs. 6F and 7F). To determine if this change occurs in the earlier stage limb buds, we examined an ectodermal marker CD44 at E13.5. CD44 immunostaining showed that the ectoderm of E13.5 control limb was thin and made up of 1–2 layers of cells (Fig. 6K). However, the ectoderm of the mutant was thicker, which was made up of multiple layers of cells (Fig. 6L). These results suggest that Smad1/5 signaling regulates the number of cell layers formation at the ventral ectoderm. When *Smad1* and *Smad5* were inactivated, the ventral limb ectoderm became thickened with multiple layers of cells. Very interestingly, some ventral *En1*-expressing cells and their descendents were located at the interdigital webbing of the developing limb of the *Smad1/5* mutants at E14.5 (Fig. 6G, H) and E15.5 (Fig. 6A, B) while the digits of the controls were separated. The border of interdigital blue streaks extend more proximally at E15.5 compared to E14.5 (Fig. 6B, H). Transverse sections of the E15.5mutant limbs showed that some *En1*-expressing cells and their descendents were located ectopically at the dorsal interdigital ectoderm (Fig. 6D). The boundary of ventral ectoderm was shown to displace dorsally slightly in E13.5 mutant limb buds (Fig. 6I, J).

Inactivation of both Smad1 and Smad5 in ventral limb ectoderm and AER does not cause defects in dorsal–ventral patterning

Previous report shows that BMP signaling in the preAER/AER is required for dorsal–ventral patterning. Inactivation of *BmprIa* in limb ectoderm by *Brn4*-Cre would result in double dorsal structures in the limb and absence of *En1* expression (Ahn et al., 2001). To investigate if the inactivation of ectodermal *Smad1* and *Smad5* would affect dorsal–ventral patterning, the expressions of *En1* and *Lmx1b* were examined. *En1* was expressed normally in the AER and ventral ectoderm of E11.5 forelimb buds of the *Smad1/5* mutants. The expression pattern of *En1* in the mutants was comparable to the control limbs (Figs. 4E, F and 7A, B). *Lmx1b* is expressed at the dorsal mesenchyme which promotes dorsal structure differentiation. *Lmx1b* was expressed normally at the dorsal mesenchyme of the *Smad1/5* mutant limb buds at E11.5. No ectopic *Lmx1b* expression was observed at the ventral mesenchyme of mutant limbs (Fig. 7C, D). Histological sections across the limb dorsal–ventral axis at E15.5 revealed that ventral mesenchyme structure such as tendon were formed and located properly in the ventral positions of the *Smad1/5* mutant limbs, which is in contrast with loss of ventral mesenchyme structure in the conditional *BmprIa* mutant (Ahn et al., 2001). Also, no ectopic dorsal ectodermal structure was observed at the ventral side of our *Smad1/5* mutant limbs (Fig. 7G, H), contrasting with the conditional *BmprIa* mutant.

Smad1/Smad5 inactivation in the limb AER and ventral ectoderm resulted in postaxial polydactyly and expanded Shh expression in the posterior limb mesenchyme

Shh is expressed at the posterior mesenchyme of the developing limb bud to regulate anterior–posterior axis formation (Chang et al., 1994; Riddle et al., 1993). Anterior

expansion of *Shh* signaling within the limb mesenchyme is suggested to be one of the mechanisms underlying the polydactyly (Selever et al., 2004). Alcian blue/alizarin red skeletal staining revealed that our *Smad1/5* mutants exhibited postaxial polydactyly (Suppl. Fig. 1F). *Shh* expression was expanded in the posterior mesenchyme of the *Smad1/5* mutant forelimb buds when compared with the control at E10.5 (Suppl. Fig. 1C, D). Thus, the lost of ventral ectodermal Smad1/5 signaling may lead to the expanded *Shh* expression that induce the postaxial polydactyly.

Discussion

Previous reports have shown that BMP signaling in the AER plays an essential role in regulating limb patterning and interdigital PCD (Ahn et al., 2001; Guha et al., 2002; Maatouk et al., 2009; Pajni-Underwood et al., 2007). However, these studies do not address the intracellular mechanism downstream of the receptor BmprIa mediating the BMP signals in the AER. In addition, complete inactivation of *Smad1* or *Smad5* or heterozygous inactivation of both *Smad1* and *Smad5* would lead to early embryonic lethality (Chang et al., 1999; Lechleider et al., 2001). Thus, the functions and the interactions of ectodermal R-Smads in AER and ventral ectoderm were investigated by conditional inactivation approach in this study. First, we discovered that Smad1 and Smad5 are employed as intracellular mediators of BMP signaling in the limb AER and ventral ectoderm. Second, the AER and ectoderm *Smad1* and *Smad5* function redundantly to regulate interdigital PCD and cell proliferation of developing limb indirectly. Third, the interaction between the limb ectoderm and mesenchyme is required to regulate the aforementioned processes. Smad1/Smad5 signaling regulates AER *Fgf* expression to control cell death and survival of the interdigital mesenchyme of limb. Finally, apart from the interdigital mesechyme, the development of ectoderm is also regulated by ectodermal Smad1/Smad5 signaling.

Functional redundancy between Smad1 and Smad5 in BMP-regulated AER and ventral ectoderm functions

The syndactyly phenotype of our mutant embryos in which both *Smad1* and *Smad5* have been inactivated is similar to the mice lacking *BmprIa* or *Bmp2/Bmp4* in the ectoderm of developing limb bud (Maatouk et al., 2009; Pajni-Underwood et al., 2007). This suggests that R-Smad1 and R-Smad5 act as the intracellular mediator downstream of BmprIa to transduce BMP signals in the AER and ventral ectoderm to regulate interdigital PCD and digit separation. Since single inactivation of either *Smad1* or *Smad5* does not result in limb abnormality, we suggest that there is functional redundancy between the two R-Smads in the AER and ventral ectoderm. Smad1 and Smad5 can compensate the functions of one another to regulate digit separation and even one allele of either *Smad1* or *Smad5* is sufficient to transduce BMP signals in the AER for regulating interdigital PCD. In addition, this study provides further evidence that functional redundancy between *Smad1* and *Smad5* is not restricted to cancer development (Pangas et al., 2008) and bone development (Retting et al., 2009) but also in the limb AER function and PCD. However, the expression of Smad8 alone, another member of BMP receptor-regulated Smad, cannot compensate for the function of Smad1 and Smad5 in AER and ventral ectoderm. This may be explained by the early divergence of *Smad1/5* and *Smad8* during vertebrate evolution (Arnold et al., 2006).

The syndactyly defect of the *Smad1/5* mutant is caused by reduction of interdigital PCD as suggested by TUNEL assay. The interdigital mesenchymal cells fail to undergo normal PCD and are maintained in the interdigital regions of the mutant to form the webbing. This result suggests that the ectodermal Smad1/Smad5 signaling in the AER indirectly regulate interdigital mesenchymal cell death. On the other hand, controversies remain regarding whether the dying cells activate caspase3 to execute interdigital apoptosis (Zuzarte-Luis and Hurle, 2005). Caspase3 activation is compromised in our *Smad1/5* mutant as detected by anti-cleaved caspase3 antibody. This suggests that caspase3 activation is involved in interdigital PCD and Smad1/Smad5 signaling in the ectoderm regulates activation of caspase3 in interdigital mesenchyme and hence interdigital apoptosis. Interdigital mesenchyme of our *Smad1/5* mutants also undergoes ectopic cell proliferation that contributes to the formation of the webbing. Since the AER and ventral ectodermal inactivation of both *Smad1* and *Smad5* leads to the decrease in cell death and ectopic cell proliferation in mesenchyme, this suggests that appropriate interactions between the ectoderm and the mesenchyme are required to regulate cell death and cell proliferation in the mesenchyme.

Molecular mechanisms of limb AER and ventral ectodermal Smad1/Smad5 signaling to control cell death and survival in the interdigital regions

Previous studies suggested that removal of BMP signaling components in the AER would result in failure in AER maturation and expansion of *Fgf8* expression. The prolonged expression of *Fgf8* at the AER would maintain interdigital cell survival in the *Bmpr1a* (Pajni-Underwood et al., 2007) and *Bmp2/4* (Maatouk et al., 2009) conditional mutants. The BMPs expressed in AER control Fgf activity of the AER to regulate interdigital cell death. In our *Smad1/5* mutants, *Fgf8* was ectopically expressed in the AER over the interdigits and was punctate throughout the AER at E13, which is consistent with the previous findings. We propose that *Smad1* and *Smad5* are employed as intracellular mediator to transduce BMPs signal acting on the AER and ventral ectoderm to repress *Fgf* expression in the AER over interdigits. Interdigital PCD is triggered by the reduction in Fgf signaling in AER over interdigital regions and this leads to separation of digits (Macias et al., 1996; Martin, 1998). *Smad1* and *Smad5* function redundantly to repress the *Fgf*8 expression in AER over interdigital region. Simultaneous inactivation of both *Smad1* and *Smad5* in the AER and ventral ectoderm would result in prolonged *Fgf8* expression. This prolonged expression of *Fgf8* in the AER over interdigital region serves as survival signal to the interdigital mesenchyme, leading to decrease in cell death and increase in cell proliferation in this tissue (Fig. 8). In addition, our data showing the unaltered expression of mesenchymal BMP signaling components in our *Smad1/5* mutants further support the idea that the BMP signaling within the AER and ectoderm does not regulate the mesenchymal BMP signaling of developing limbs (Pajni-Underwood et al., 2007). The Smad1/5 signaling in the AER and ventral ectoderm only indirectly regulates interdigital PCD through regulating the ectodermal FGF expression. On the other hand, previous report showed that knockout of BMP ligands in the mesenchyme resulted in expanded Fgf8 expression in AER of E11.5 limb bud (Bandyopadhyay et al., 2006). The question of whether mesenchymal BMP signaling can regulate BMP signaling within AER and ectoderm awaits to be elucidated.

Interestingly, the expression of *Fgf8* in our E10.5 *Smad1/5* mutants did not expanded as previously reported for the *Bmpr1a* and *Bmp2/4* conditional mutants, suggesting that the AER of our *Smad1/5* mutants instead undergoes maturation properly. The difference in *Fgf8* expression of our *Smad1/5* mutants at E10.5 could possibly be due to the different Cre mouse line used in our study. At E10.5, the *En1*-Cre knockin allele is only active in the ventral half of the developing AER and the ventral ectoderm (Kwan et al., 2004) while the *Msx2*-Cre employed in previous reports is active in the entire AER of the early limb bud and the ectoderm (Barrow et al., 2003; Maatouk et al., 2009; Pajni-Underwood et al., 2007). Our results suggest that BMP signaling in the dorsal and ventral AER at E10.5 may be responsible for differential functions during limb development. Dorsal ectodermal BMP signaling may be required for the maturation of the AER and restricting the expression of *Fgf8* at this earlier stage of limb bud development. Our experiments give insights into the possible differential functions of different parts of limb ectoderm in AER development.

The role of Smad1/Smad5 signaling in limb ectoderm structure formation

The epidermis is an important organ throughout the body that provides protection against dehydration, injury and infection. During development, the epidermis is a well-organized structure which consists of 2–3 layers of cells at E15.5. The inner layer is the stratum germinativum and contains proliferating cells that divide continuously to replace the outer layer. The outer layer gives rise to periderm which is a temporary covering of the skin and will shed continuously. The balance between proliferation of the stratum geminativum and the shedding of the outer layer is critical for the maintenance of the structure of the epidermis and its protection function (Simpson et al., 2011). When both *Smad1* and *Smad5* were inactivated in the ventral ectoderm of the developing limb, the ectoderm lost the well-organized structure of 2–3 layers of cells. The ectoderm of the *Smad1/Smad5* mutants became a thick structure densely packed with multiple layers of cells. These multiple layers of cells are descendents of the *Smad1/Smad5*-inactivated ventral ectodermal cells. Furthermore, the ventral *En1*-expressing cells and their descendents were found ectopically at the dorsal interdigital ectoderm. Our data suggests that Smad1/Smad5 signaling in the ventral ectoderm is important in regulating its own structural development and hence the functions of the epidermis. We propose that Smad1/Smad5 signaling may also function redundantly in the ventral ectoderm to limit ectodermal cells in the ventral position. When both *Smad1* and *Smad5* are inactivated in the ectodermal cells at the distal end of the developing limb, these ectodermal cells may undergo ectopic proliferation and migrate to the dorsal position as suggested by our cell tracing experiment and the thickening of ventral ectoderm in the *Smad1/5* mutants.

The role of ventral ectodermal Smad1/5 signaling in dorsal–ventral patterning

Previous report showed that preAER/AER BMP signaling is required for dorsal–ventral patterning. Inactivation of the receptor *BmprIa* in limb ectoderm by *Brn4*-Cre would result in double dorsal defects including the absence of ventral mesenchymal structures such as flexor digitorum profundus tendon and sesamoid process and the loss of *En1* expression (Ahn et al., 2001). Moreover, knockout of BMP ligands Bmp2/Bmp4 in the AER would result in ectopic nail plate on the ventral side (Maatouk et al., 2009). In contrast, expression of *En1* and *Lmx1b* in the developing limb buds of our *Smad1/5* mutants was comparable to

the controls. No visible dorsal–ventral defects were observed in the *Smad1/5* mutants. Ventral mesenchyme structures are formed and located properly in the *Smad1/5* mutant limbs, which is in contrast with loss of ventral mesenchyme structure in the conditional *BmprIa* mutant (Ahn et al., 2001). No ectopic dorsal ectodermal structure was observed at the ventral side of the mutant limb, contrasting with the conditional *Bmp2/4* mutant (Maatouk et al., 2009). The lack of defects in the dorsal–ventral patterning of our *Smad1/5* mutant could possibly due to the different Cre mouse line used in our study. The *Brn4*-Cre and *Msx2*-Cre show their activities in the entire ectoderm during limb bud formation while the *En1*-Cre is only active in the AER and ventral ectoderm. The signaling through the Smad1/Smad5 in the dorsal ectodermal region of our *Smad1/5* mutant limb may be sufficient to maintain the *En1* expression and the patterning of dorsal–ventral axis. It is also possible that the AER-expressed BMP ligands may function through alternative pathways, other than R-Smad, upstream of *En1* to regulate dorsal–ventral patterning.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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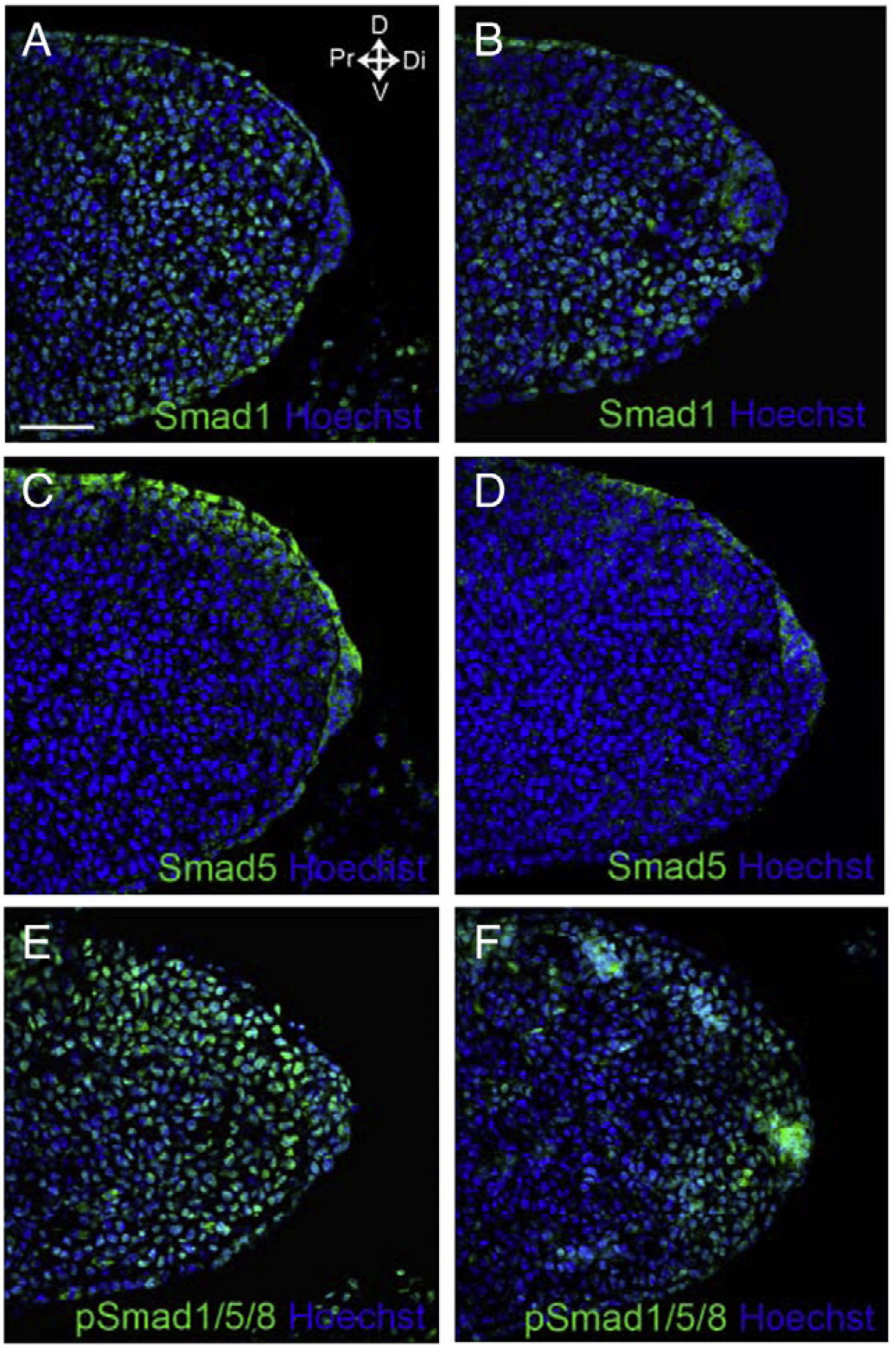

Fig. 1.

En1-Cre inactivated *Smad1* and *Smad5* conditional null alleles in ventral ectoderm and ventral AER of forelimb buds. (A–D) Immunofluorescence detecting Smad1 (A, B) and Smad5 (C, D) in E10.5 control (A, C) (Smad1$^{f/+}$; Smad5$^{f/f}$) and *Smad1/5* mutant (B, D) (En1$^{Cre/+}$; Smad1$^{f/f}$; Smad5$^{f/f}$) forelimb buds respectively. *Smad1* and *Smad5* were detected in the dorsal ectoderm but not in the ventral ectoderm and ventral half of AER of *Smad1/5* mutant. (E, F) Immunofluorescence detecting pSmad1/5/8 in the forelimb buds of E10.5 control (E) (*En1*$^{Cre/+}$; *Smad1*$^{f/+}$; *Smad5*$^{f/f}$) and *Smad1/5* mutant (F) (*En1*$^{Cre/+}$*; Smad1*$^{f/f}$*;*

Smad5$^{f/f}$). pSmad1/5/8 signal was detected throughout AER and ectoderm in both control and *Smad1/5* mutant. The histological samples are sectioned saggitally along the dorsal–ventral axis of the limb buds. Arrows indicate the major axes: D, dorsal; V, ventral; Di, distal; Pr, proximal. Scale bar: 50 μm.

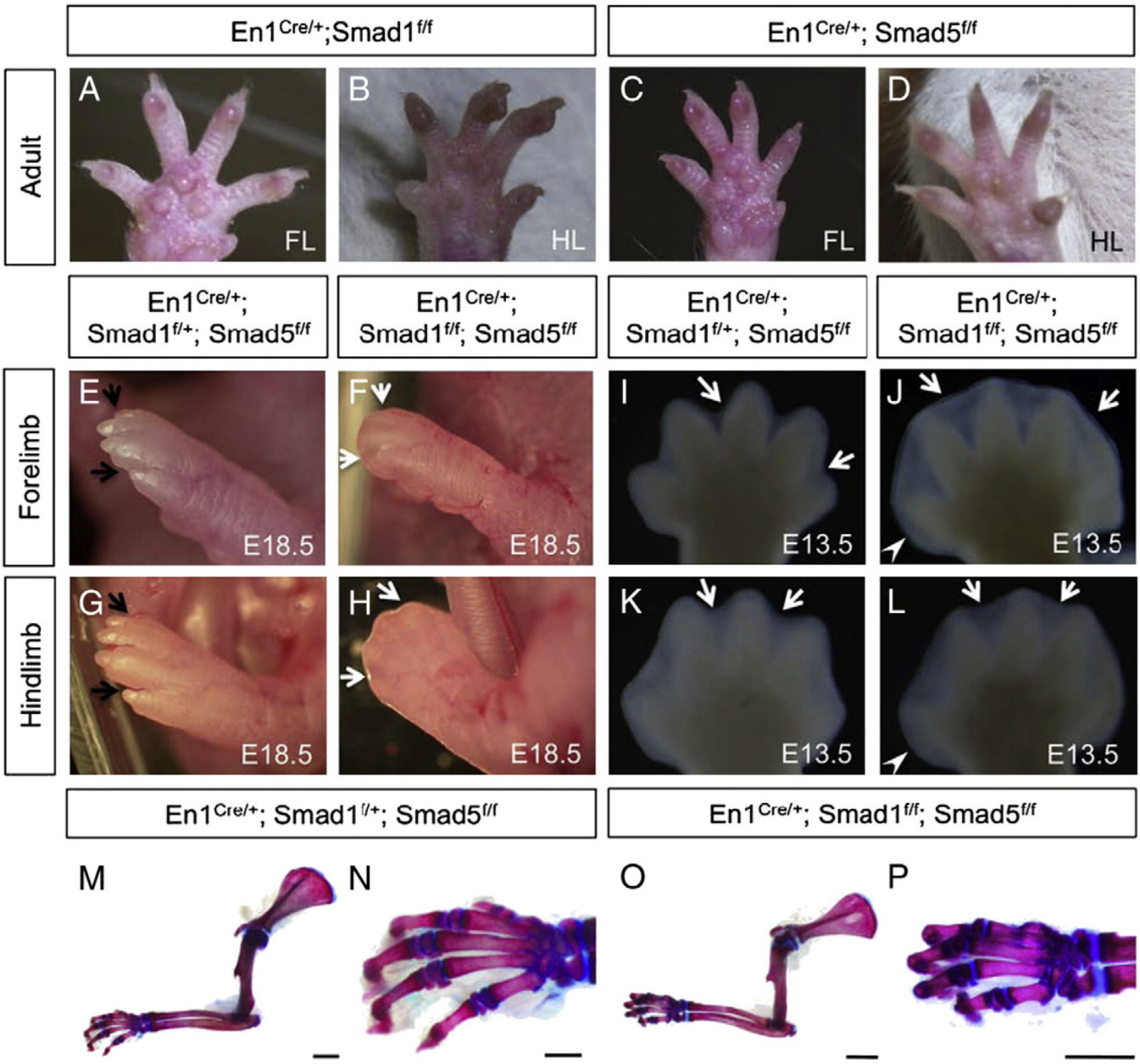

Fig. 2.

Inactivation of *Smad1* and *Smad5* in the AER and ventral ectoderm by *En1*$^{Cre/+}$ knock-in allele resulted in syndactyly and retention of interdigital tissue. Inactivation of either *Smad1* (A, B) or *Smad5* (C, D) in the AER and ventral ectoderm did not result in visible phenotypes in both forelimbs (FL) and hindlimbs (HL) of the individuals at adult stage. (E–H) Fore- and hindlimb from the control (E, G) and *Smad1/5* mutant fetuses (F, H) at E18.5. Black arrows indicate separated digits in the control, while white arrows indicate the retained interdigital tissue in the mutants. (I–L) The defect in interdigital tissue regression was observed in both fore- and hindlimbs of the *Smad1/5* mutants starting from E13.5. Syndactyly (webbing) is observed in the double mutants (J, L). White arrows indicate interdigital tissue. White arrowheads indicate that the posterior end of the autopod of Smad1/5 mutant is broadened at

E13.5. (M–P) Skeletal preparations of the forelimbs from the control (M) and *Smad1/5* mutants (O) at postnatal day 10. Higher magnification view of the autopod of the controls (N) and the mutants (P). Inactivation of *Smad1* and *Smad5* in the AER and ventral ectoderm did not result in skeletal defects in the autopod and along proximal–distal axis. A–D are the ventral views of the limb with anterior to the right. E–L are dorsal views of the limb plates with anterior to the right. Scale bars: M, O=5 mm; N, P=500 μm.

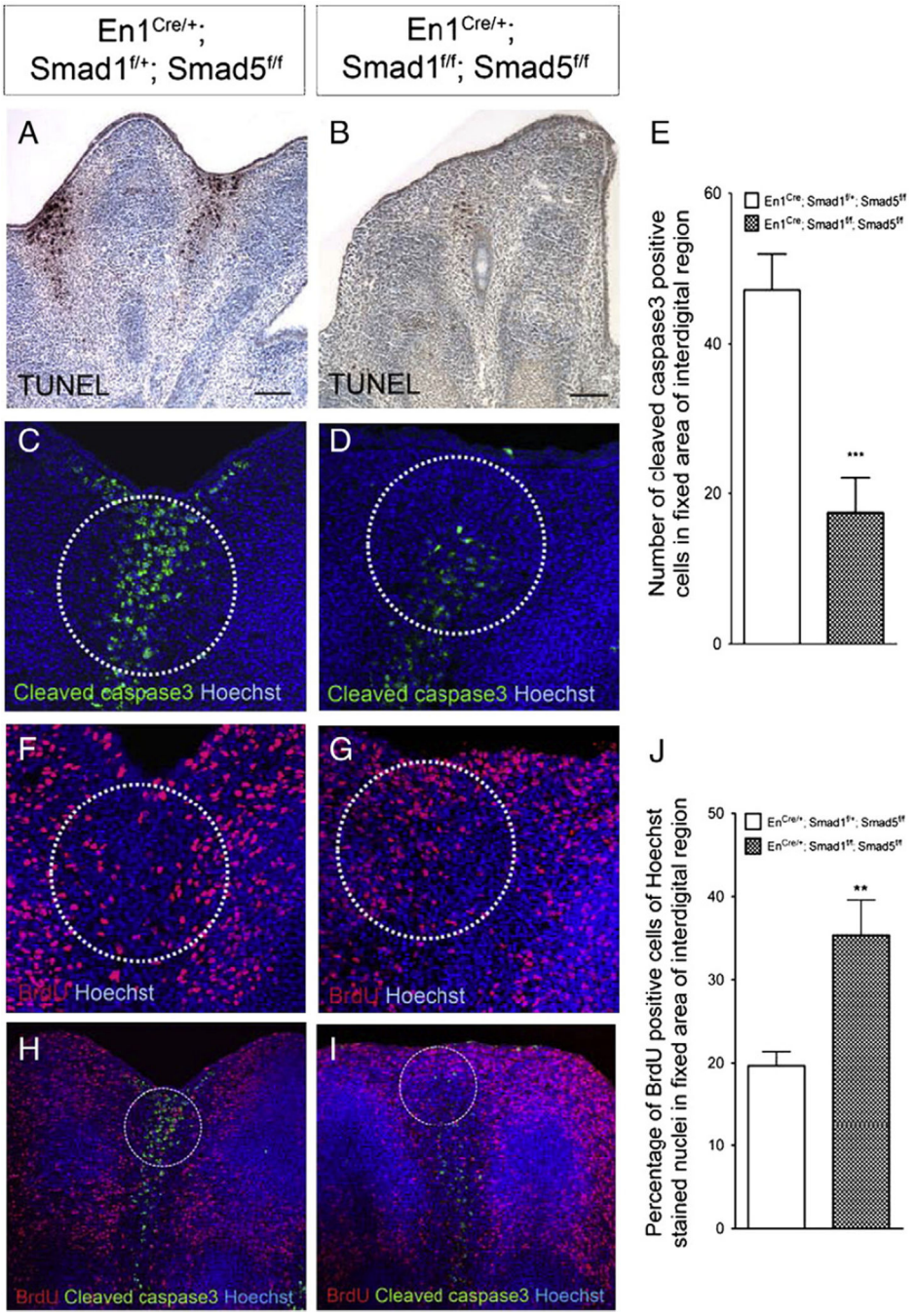

Fig. 3.

Smad1/Smad5 signaling in the AER and ventral ectoderm is required for regulating cell death and cell proliferation in interdigital regions. (A, B) TUNEL assay and (C, D) immunofluorescence detecting cleaved caspase3 to measure cell death in E13.5 forelimbs. Inactivation of *Smad1* and *Smad5* resulted in a decrease in cell death. (E) Quantification of the number of anti-cleaved caspase3 positive cells demonstrated that there was a significantly decrease in the number of cells undergoing cell death in the *Smad1/5* mutant limbs (***p<0.005). (F, G) Smad1/5 signaling in the AER and ventral ectoderm is required

for regulating cell proliferation in the underlying mesenchyme. BrdU labeling assay showed that inactivation of *Smad1* and *Smad5* resulted in an increase in cell proliferation in distal interdigital mesenchymal regions. (H, I) Cleaved caspase3 and BrdU co-immunostaining indicated that distal interdigital areas (white circle) of *Smad1/5* mutant are abundant in BrdU positive cells. (J) Quantification of the percentage of BrdU positive cells with Hoechst stained nuclei demonstrated that there was a significant increase in the percentage of proliferating cells in the distal interdigital areas of the *Smad1/5* mutant limbs (**$p<0.005$). Scale bars: A–B=100 μm.

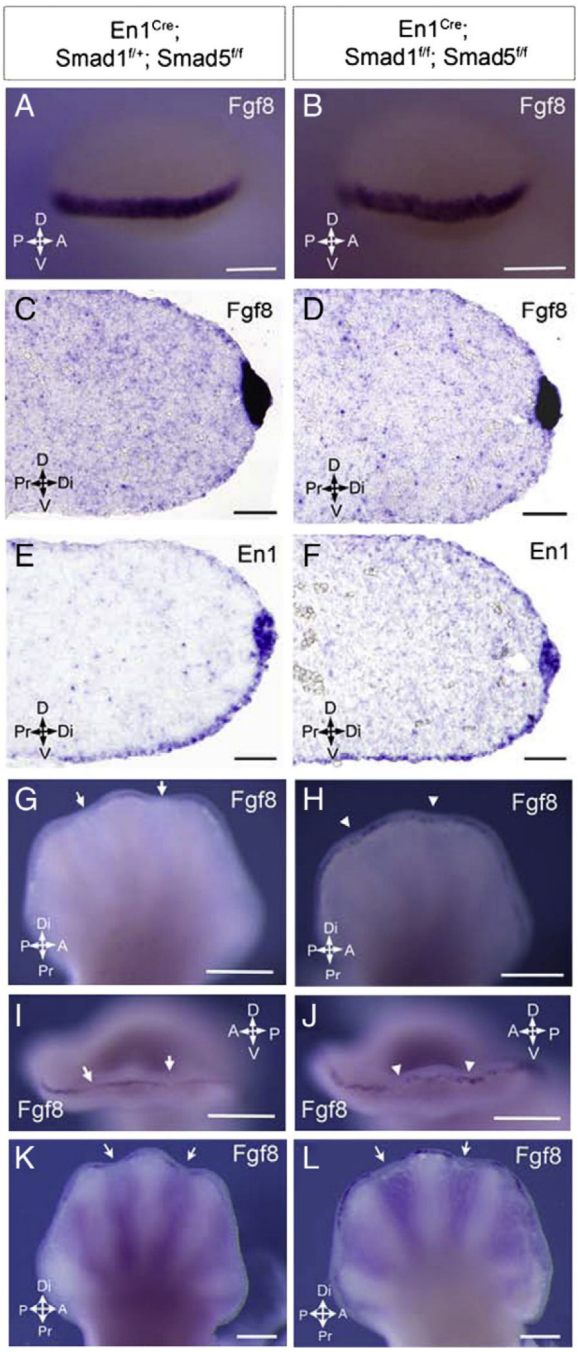

Fig. 4.

AER and ventral ectoderm Smad1/Smad5 signaling is required to repress expression of *Fgf8* in the interdigital distal ectoderm. (A, B) Whole mount *in situ* hybridization of E10.5 mutant forelimb buds showed no expansion of *Fgf8* expression along dorsal–ventral or anterior–posterior axis. (C–F) Section *in situ* hybridization of E11.5 mutant forelimb buds showed that the expression of AER marker *Fgf8* (C, D) and *En1* (E, F) was restricted in AER and ventral ectoderm along the dorsal–ventral axis that was comparable with the control. The morphology of AER in the *Smad1/5* mutant did not show any abnormality. (G–J) Ectopic

Fgf8 expression was observed in the forelimb interdigital ectoderm of the *Smad1/5* mutants at E13 (white arrowhead) and the *Fgf8* expression was punctuate throughout the distal ectoderm of the mutants. (K, L) *Fgf8* expression faded in AER over interdigit of Smad1/5 mutants at E13.5 indicated by white arrows. Arrows indicate the major axes: A, anterior; P, posterior; D, dorsal; V, ventral; Di, distal; Pr, proximal. Scale bars: A–B=500 μm; C–F=50 μm; G–L=1 mm.

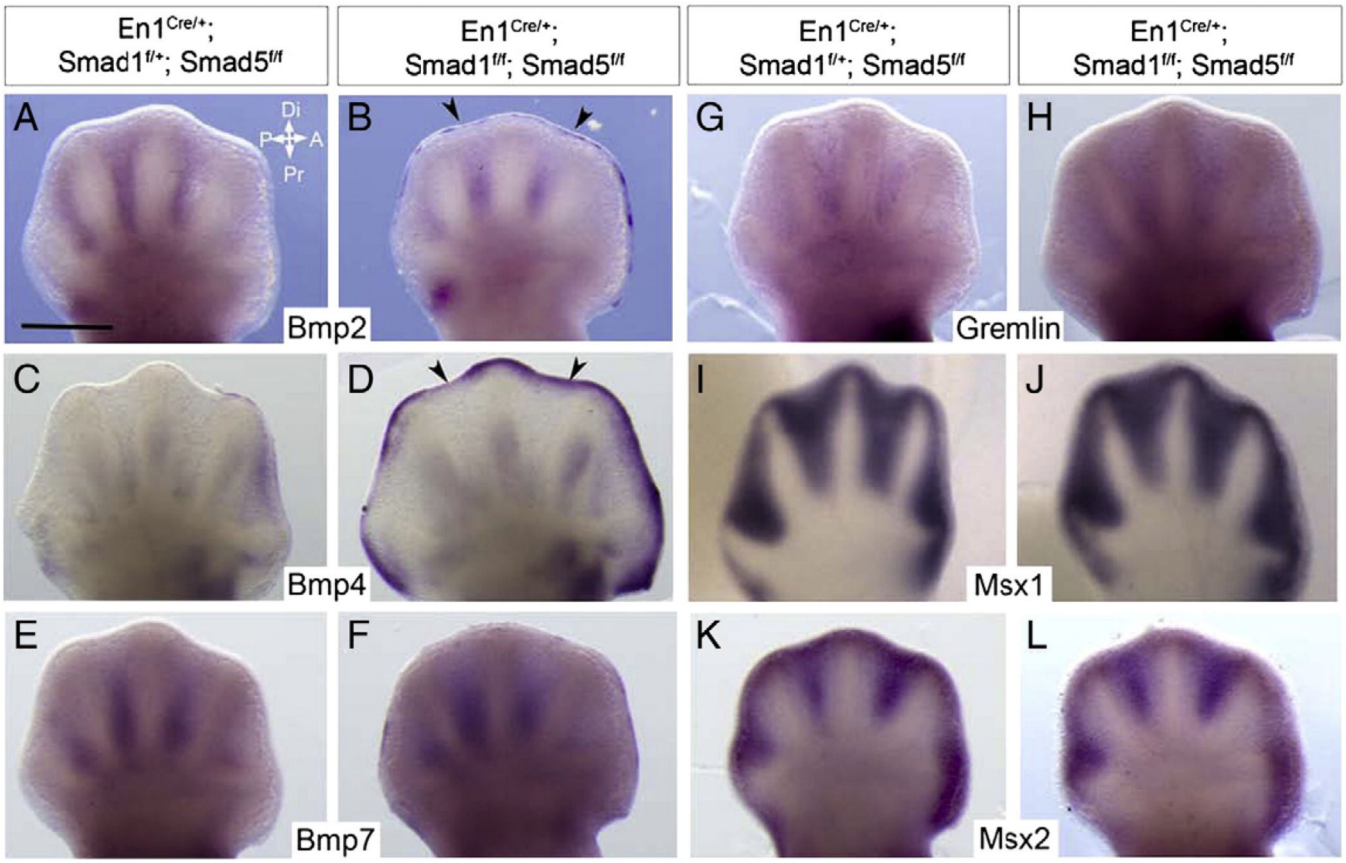

Fig. 5.

Syndactyly phenotype of the *Smad1/5* mutants is not caused by changes in mesenchymal BMP signaling of the developing autopod. Whole mount *in situ* hybridization of E12.5 controls (A, C, E, G, I and K) and mutants (B, D, F, H, J and L) forelimb buds using the probes as indicated. All are dorsal views. Arrowheads indicated ectopic expression of the genes indicated in the distal ectoderm. Arrows indicate the major axes: A, anterior; P, posterior; Di, distal; Pr, proximal. Scale bars = 500 μm.

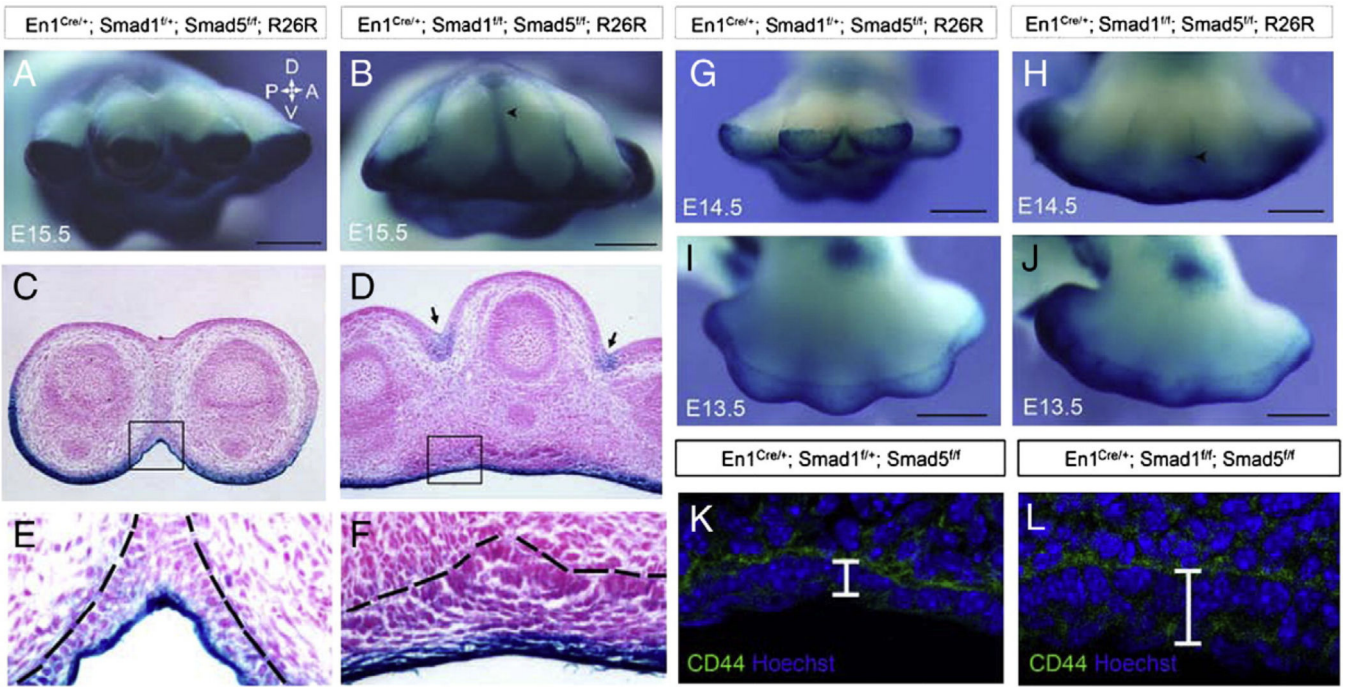

Fig. 6.

Smad1/5 signaling in the ventral AER and ectoderm is required for regulating the cell fate of ventral ectodermal cells. The Cre/loxP system was employed to map the ventral ectodermal cells and their descendenst after *Smad1/5* inactivation in ventral AER and ectoderm. (A–B, G–J) Whole-mount X-gal staining of forelimb plates at E15.5 (A, B), E14.5 (G, H) and E13.5 (I, J) to identify *En1*-expressing cells and their descendents after *Smad1/Smad5* inactivation. The border of the interdigital blue streak (arrowheads) extended more proximally at E15.5 (B) compared to E14.5 (H). (C–F) Transverse sections of the X-gal stained E15.5 forelimb plates showing β-galactosidase activity. (D, F) Ventral ectoderm was thickened in the *Smad1/5* mutant. Blue lacZ staining was observed in the ventral ectoderm and interdigital ectoderm in the dorsal regions of the *Smad1/5* mutant (arrows). (E, F) Higher magnification views of the areas indicated by the squares in (C and D). Ventral ectoderm layers of the *Smad1/5* mutants became thicker and consisted of multiple layers of cells at E15.5 (compare E and F). (K–L) Immunostaining using ectodermal marker CD44 showed that ectoderm thickening was observed in mutant forelimb plates at E13.5. Dotted lines (E and F) indicated the basement membrane of the epithelium. Arrows indicate the major axes: A, anterior; P, posterior; D, dorsal; V, ventral. Scale bars: A–B, G–J=500 μm.

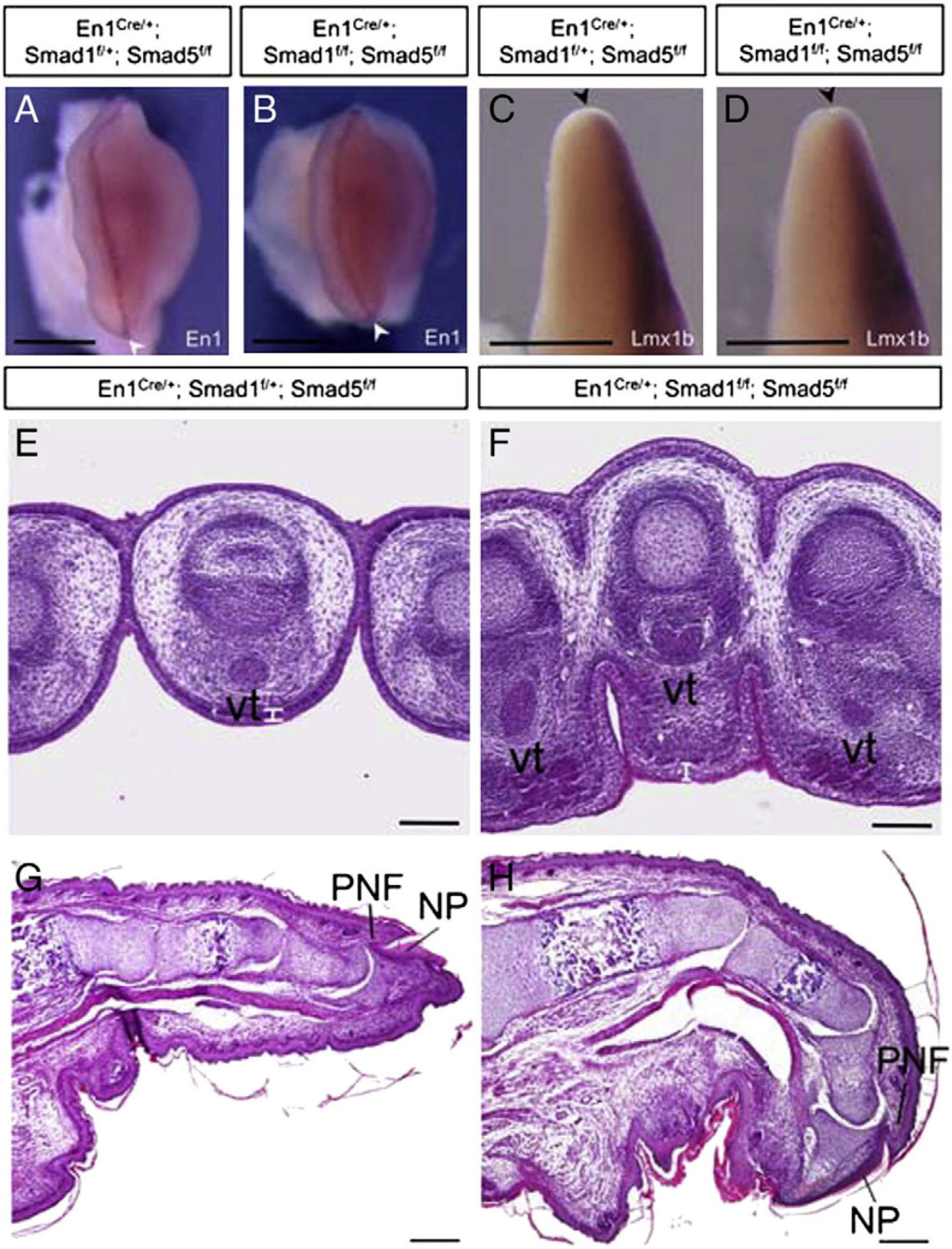

Fig. 7.

Smad1 and Smad5 signaling in the AER and ventral ectoderm are not required for dorsal–ventral patterning of the limb. (A, B) *En1* expression in the AER (white arrowheads) and the ventral ectoderm of the forelimb buds from control (A) and *Smad1/5* mutant (B) at E11.5. (C, D) *Lmx1b*, which is a dorsal limb mesoderm marker, showed restricted expression in the dorsal mesoderm of the mutant forelimb bud at E11.5 (D), similar to the control (C). Black arrowheads mark the location of AER. For panel A–D, dorsal side of limb buds towards the right. (E, F) Transverse sections through the E15.5 forelimb hand-plates are shown. The

tendons, which are a ventral mesodermal structure, were formed and located properly at the ventral side of the mutant limbs. Aberrant thickening of ventral epidermal structure is observed in the *Smad1/5* mutant limb (white bars). (G, H) Sagittal section *Smad1/5* mutant limbs at E18.5 does not show ectopic nail plate on the ventral side. NP, nail plate; PNF, Proximal nail fold; Vt, ventral tendon. Scale bars: A–D=1 mm; E–F=100 μm; G–H=200 μm.

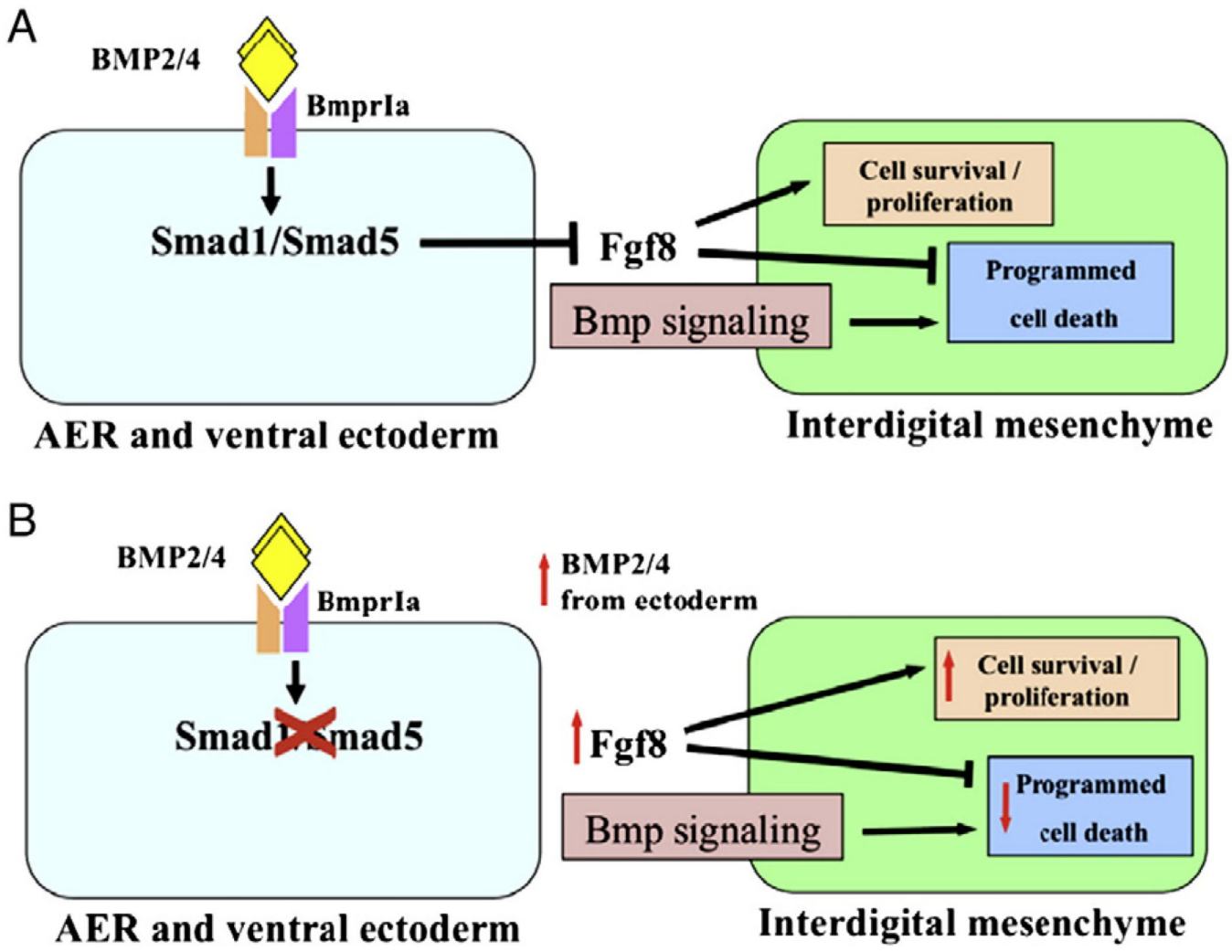

Fig. 8.

Our proposed model for the roles and interactions of Smad1/Smad5 in AER and ventral ectoderm of developing limbs. (A) Smad1 and Smad5 functions redundantly as the intracellular mediator of the BMP signaling downstream of BmprIa in the AER and ventral ectoderm (Maatouk et al., 2009; Pajni-Underwood et al., 2007). *Fgf8* expression at the AER serves as cell survival signal to the interdigital mesenchyme (arrow). Smad1/5 signaling at the AER and ventral ectoderm inhibits *Fgf8* expression. The termination of *Fgf8* expression initiates programmed cell death in the intedigital mesenchyme. BMP signaling within the mesenchyme was shown to regulate interdigital PCD (Bandyopadhyay et al., 2006). (B) Inactivation of *Smad1/Smad5* in the AER and ventral ectoderm would result in persisted *Fgf8* expression. *Fgf8* is expressed ectopically in the AER over interdigital region that leads to inhibition of PCD and ectopic cell survival and proliferation in the interdigital mesenchyme. In addition, there is an auto-regulatory loop of BMP signaling and BMP signals in the limb AER. In the absence of Smad1/5 signaling, BMP signals in the AER is elevated.