Antagonism between Smad1 and Smad2 signaling determines the site of distal visceral endoderm formation in the mouse embryo

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The anterior–posterior axis of the mouse embryo is established by formation of distal visceral endoderm (DVE) and its subsequent migration. The precise mechanism of DVE formation has remained unknown, however. Here we show that bone morphogenetic protein (BMP) signaling plays dual roles in DVE formation. BMP signaling is required at an early stage for differentiation of the primitive endoderm into the embryonic visceral endoderm (VE), whereas it inhibits DVE formation, restricting it to the distal region, at a later stage. A Smad2-activating factor such as Activin also contributes to DVE formation by generating a region of VE positive for the Smad2 signal and negative for Smad1 signal. DVE is thus formed at the distal end of the embryo, the only region of VE negative for the Smad1 signal and positive for Smad2 signal. An inverse relation between the level of phosphorylated Smad1 and that of phosphorylated Smad2 in VE suggests an involvement of antagonism between Smad1- and Smad2-mediated signaling.

Introduction

The anterior–posterior (A-P) axis is established early in mouse development. In this process, distal visceral endoderm (DVE) located at the distal tip of the embryo migrates toward the future anterior side and becomes anterior visceral endoderm (AVE; Beddington and Robertson, 1998, 1999). Several signals are necessary for A-P axis formation. For example, Nodal signaling from the epiblast induces DVE formation at embryonic day (E) 5.5 (Lu and Robertson, 2004). Removal of the extraembryonic ectoderm (ExE) leads to expansion of DVE at the pregastrulation stage (Rodriguez et al., 2005; Mesnard et al., 2006). Asymmetrical expression of *Lefty1* and *Cerl* in DVE along the future A-P axis results in asymmetrical inhibition of Nodal signaling and thus determines the future anterior

side (Yamamoto et al., 2004). Inhibition of Wnt signaling by Dkk1 is also necessary for the anterior shift of DVE (Kimura-Yoshida et al., 2005). In addition, signaling from AVE has been proposed to induce anterior and suppress posterior identity in the epiblast (Kimura et al., 2000; Perea-Gomez et al., 2002). However, the molecular mechanism of DVE formation has remained unknown.

Nodal, a secreted member of the TGF-β superfamily of ligands (Zhou et al., 1993), is required for DVE formation. ALK4 and ALK7 function as type 1 receptors for Nodal, whereas ActR2A and ActR2B function as type 2 receptors for this ligand. Nodal signaling is modulated by members of the EGF-CFC protein family and it is transduced by intracellular molecules including Smad2 and Smad3. With regard to formation of the A-P axis, *Nodal*^{−/−} embryos manifest a failure of DVE formation at E5.5 (Brennan et al., 2001). However, a

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Figure 1. **DVE formation requires BMP signaling in the extraembryonic region.** Expression of *Lefty1* (A and A'), *Cerl* (B and B'), *Dkk1* (C and C'), *Lim1* (D and D'), *Hex* (E, E', I, and I'), *Hnf4* (J and J'), *Pem* (K and K'), *Bmp2* (L and L'), and *Fgf8* (M, M', and M") was examined by in situ hybridization in wild-type (*Bmpr2*^{+/+}) and *Bmpr2*^{−/−} mouse embryos at E5.5 or the indicated stages. The DVE was absent (A'–E') in the mutant embryos. The primitive endoderm (I') and extraembryonic VE (J' and K') were formed, whereas embryonic VE was absent (L' and M') or impaired (M"). Arrowheads in J' indicate the junction between the extraembryonic and embryonic regions. Green ES FM260 cell (*Bmpr2*^{+/+})←→*Bmpr2*^{+/+} tetraploid (F–H) and green ES FM260 cell←→*Bmpr2*^{−/−} tetraploid (F'–H') chimeric embryos were recovered at E6.5 and examined for EGFP fluorescence (F and F') or for expression of *Hex* (G and G') and *Lefty1* (H and H'). The expression of *Hex* and *Lefty1* was absent in the green ES FM260 cell←→*Bmpr2*^{−/−} tetraploid chimeric embryos. (N) The level of p-Smad1 staining and the level of expression of DVE markers are compared between *Bmpr2*^{−/−} and *Bmpr2*^{−/−},*Actr2b*^{+/−} embryos. Dark blue, light blue, orange, and red bars indicate normal expression, expression at a moderately reduced level, expression at a severely reduced level, and no expression, respectively. The numbers of embryos showing each expression pattern are indicated. Bars, 50 µm.
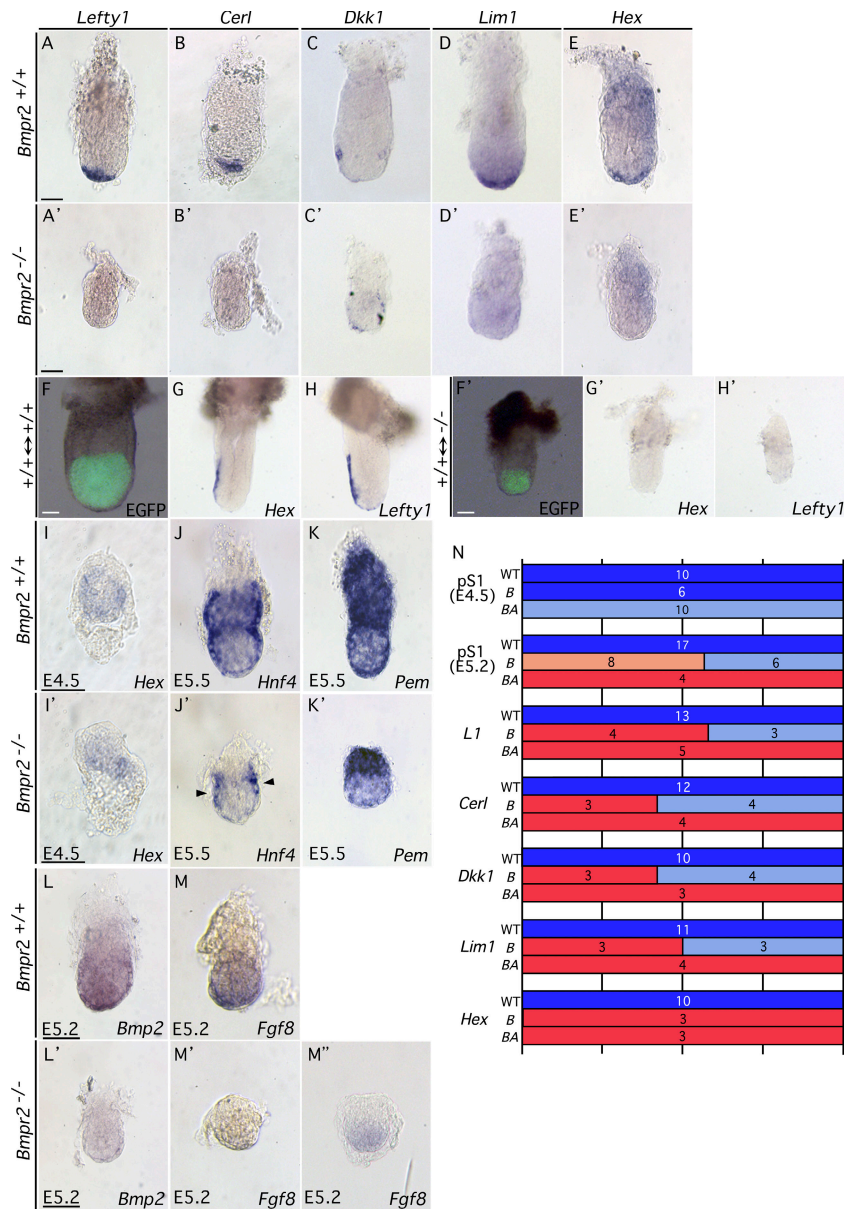
recent study (Mesnard et al., 2006) showed that the visceral endoderm (VE) of *Nodal*^{−/−} embryos is abnormally specified before DVE formation. The primary role of Nodal in DVE formation is therefore to define an embryonic VE compartment before DVE formation.

The ExE is the source of another signal that regulates DVE formation (Rodriguez et al., 2005). Embryonic explants that lack ExE generate ectopic DVE, suggesting that a signal derived from ExE inhibits DVE formation (Rodriguez et al., 2005; Mesnard et al., 2006).

Bone morphogenetic proteins (BMPs) are also secreted ligands of the TGF-β superfamily and potential regulators of A-P patterning. BMPs function by binding to ALK1, ALK2, ALK3, or ALK6 as a type 1 receptor or to BMPR2, ActR2A, or ActR2B as a type 2 receptor (Zhao, 2003; Kishigami and Mishina, 2005). Gene targeting in the mouse has shown that BMP signaling is required for mesoderm formation (Mishina et al., 1995, 1999; Gu et al., 1999; Beppu et al., 2000; Lechleider et al., 2001;

Tremblay et al., 2001). In the mouse embryo, anterior identity is established in the extraembryonic endoderm before formation of the primitive streak (Thomas et al., 1998; Beddington and Robertson, 1999). Some mutants that show a defect in mesoderm formation also manifest defective anterior patterning (Conlon et al., 1994; Brennan et al., 2001; Mesnard et al., 2006). Indeed, depletion of BMP4 has been shown to affect AVE formation (Soares et al., 2005). These various observations thus suggest that BMP signaling might regulate formation of the A-P axis, and that of DVE in particular.

To investigate the mechanism of DVE formation, we have now examined mutant mice that lack BMPR2, ActR2B, Lefty1, or Nodal and performed experiments with embryonic explants. Our data demonstrate that DVE formation is regulated by an antagonism between BMP-Smad1 and Activin/Nodal-Smad2 signaling. The DVE is thus formed at the distal region of the embryo where Smad2-mediated signal is present and Smad1-mediated signal is absent.
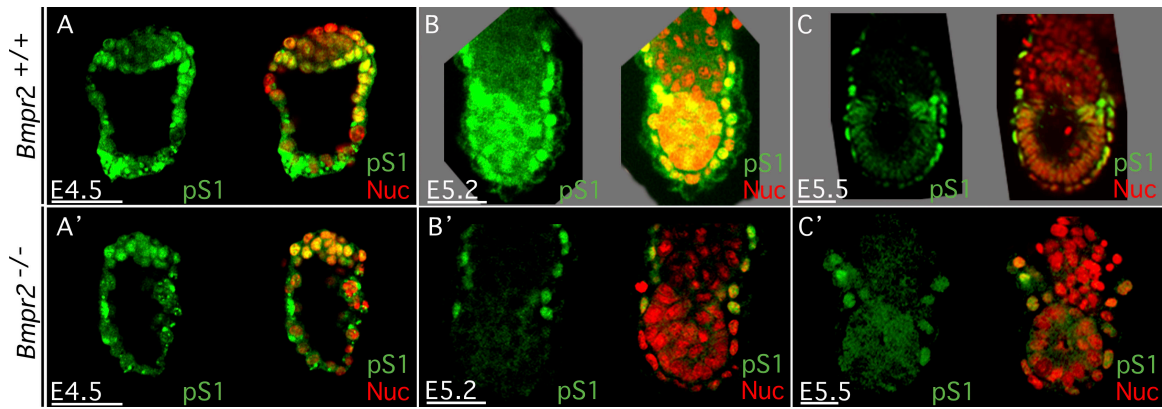
Figure 2. **p-Smad1 in wild-type and *Bmpr2*$^{-/-}$ embryos.** Wild-type (A–C) or *Bmpr2*$^{-/-}$ (A'–C') embryos at the indicated stages of development were subjected to immunohistofluorescence staining with antibodies to p-Smad1 (pS1; green); merged images with staining of nuclei by YOYO-1 (Nuc; red) are also shown. Staining for p-Smad1 was decreased in *Bmpr2*$^{-/-}$ embryos. Bars, 50 µm.

Results

DVE formation is impaired in *Bmpr2*$^{-/-}$ embryos

Formation of the primitive streak is impaired in *Bmpr2*$^{-/-}$ embryos (Beppu et al., 2000). To determine whether formation of the A-P axis occurs normally in these mutant embryos, we first examined the expression of AVE or DVE marker genes at E6.5 and E5.5, respectively. In wild-type embryos at E5.5, five DVE marker genes, *Lefty1*, *Cerl*, *Dkk1*, *Lim1*, and *Hex*, are expressed in VE at the distal tip (Fig. 1, A–E). Expression of *Hex*, *Hesx1*, and *Cerl* is absent at E5.2 but is apparent at E5.5 (Fig. S1, A–C and E–G, available at http://www.jcb.org/cgi/content/full/jcb .200808044/DC1), whereas *Lefty1* expression is maintained between E4.0 and E5.5 (Takaoka et al., 2006; Fig. S1, D and H), indicating that cells positive for a full range of DVE markers are formed between E5.2 and E5.5. In *Bmpr2*$^{-/-}$ embryos, however, expression of AVE marker genes at E6.5 was absent or reduced compared with that in wild-type embryos (Fig. S2, A–D, A'–D', and A''–D''). *Dkk1* expression was lost (Fig. S2 C') or remained relatively normal (Fig. S2 C''). At E5.5, expression of *Lefty1*, *Cerl*, *Dkk1*, and *Lim1* was absent (4/7, 3/7, 3/7, and 3/6 embryos, respectively) or markedly reduced (3/7, 4/7, 4/7, and 3/6 embryos, respectively), and that of *Hex* was also lost (3/3 embryos; Fig. 1, A'–E' and N; and Fig. S2, I and I').

To determine the region of the embryo in which BMP signaling exerts the observed effects, we examined expression of DVE marker genes in green embryonic stem (ES) FM260 cell←→*Bmpr2*$^{-/-}$ tetraploid chimeric embryos, which were generated by aggregation of ES cells expressing EGFP with *Bmpr2*$^{-/-}$ tetraploid embryos. In such chimeras, expression of *Hex* (*n* = 3) and *Lefty1* (*n* = 3) was absent at E6.5 (Fig. 1, F–H and F'–H'). This phenotype was indistinguishable from that of *Bmpr2*$^{-/-}$ embryos, suggesting that BMPR2 in the extraembryonic region is required for DVE formation.

We next examined whether VE is formed normally in *Bmpr2*$^{-/-}$ embryos. VE, which is composed of embryonic VE and extraembryonic VE at E5.5, is derived from the primitive endoderm of the E4.0–4.5 embryo. Expression of *Hex*, which is a marker of the primitive endoderm, was maintained in *Bmpr2*$^{-/-}$

embryos at E4.5 (*n* = 3; Fig. 1, I and I'). Expression of *Hnf4* (*n* = 13), *Gata4* (*n* = 4), and *Pem* (*n* = 7) was maintained in the extraembryonic VE of *Bmpr2*$^{-/-}$ embryos at E5.5 (Fig. 1, J' and K'; and Fig. S2 O'). However, expression of *Bmp2* (*n* = 4), *Fgf8* (*n* = 7), and *Hnf4* (*n* = 13) in the embryonic VE was down-regulated in the mutant embryos at E5.2 and E5.5 (Fig. 1, J, J', L, L', M, M', and M''). Staining for phosphorylated ERK and expression of the ExE marker genes *Eomes*, *Bmp4*, and *Mash2* were normal, whereas that of *Wnt7b* was slightly decreased, in the mutant embryos (Fig. S2, Q–W and Q'–W'). These results suggested that BMPR2 is not essential for formation of the primitive endoderm or extraembryonic VE, but rather is specifically required for specification of embryonic VE. The failure of DVE formation in *Bmpr2*$^{-/-}$ embryos is thus likely caused by the impaired differentiation of the primitive endoderm into embryonic VE.

Smad1-mediated signaling is reduced in *Bmpr2*$^{-/-}$ embryos

To evaluate how the lack of BMPR2 affects BMP signaling in the embryo, we examined the distribution of phosphorylated Smad1/5 (p-Smad1) in *Bmpr2*$^{-/-}$ embryos by immunohistofluorescence staining. In wild-type embryos, p-Smad1 was found in the nuclei of epiblast and primitive endoderm cells at E4.5 and of epiblast and VE cells at E5.2 (Fig. 1 N and Fig. 2, A and B). The distribution of p-Smad1 changed quickly between E5.2 and E5.5 and had shifted to the proximal epiblast and VE, excluding DVE, at E5.5 (Fig. 2 C and Fig. S3, A–J, available at http://www.jcb.org/cgi/content/full/jcb.200808044/DC1). In *Bmpr2*$^{-/-}$ embryos, the distribution of p-Smad1 was similar to that in wild-type embryos at E4.5 but showed two distinct patterns at later stages (Fig. 1 N and Fig. 2, A'–C'). In severely affected mutant embryos (8/14 embryos at E5.2 and 8/15 embryos at E5.5), p-Smad1 was apparent only in the proximal VE at E5.2 (8/8 embryos; Fig. 2 B') and was barely detected at E5.5 (8/8 embryos; Fig. 2 C'). In mildly affected embryos (6/14 embryos at E5.5 and 7/15 embryos at E5.5), p-Smad1 was found in the same regions as in wild-type embryos at E5.2, but its abundance was lower than that in the wild type (6/6 embryos; Fig. 1 N and Fig. S2). It was not detected in the epiblast and there were fewer positive cells in the VE of the mildly affected embryos at E5.5

Figure 3. **BMP signaling is required for embryonic VE differentiation independently of Nodal signaling.** Expression of *Bmpr2* (A–C) and *Actr2b* (D–F) in wild-type (WT) embryos as well as expression of p-Smad1 (pS1; green) and merged images of p-Smad1 with nuclear staining (Nuc; red) for wild-type (G and H) and *Bmpr2⁻/⁻,Actr2b⁺/⁻* (G' and H') embryos at the indicated stages are shown. Expression of *Lefty1* (I, I', S, and S'), *Cerl* (J and J'), *Dkk1* (K and K'), *Lim1* (L and L'), *Hex* (M, M', R, and R'), *Hnf4* (N and N'), *Pem* (O and O'), *Bmp2* (P and P'), *Fgf8* (Q and Q'), *Nodal* (T and T'), *Foxh1* (U and U'), and *Cripto* (V and V') in wild-type or *Bmpr2⁻/⁻,Actr2b⁺/⁻* embryos at E5.5 or the indicated stages was determined by in situ hybridization. Expression of p-Smad2 (pS2; green) and merged images of p-Smad2 with nuclear staining (Nuc; red) are shown for wild-type (W), *Bmpr2⁻/⁻,Actr2b⁺/⁻* (W'), or *Bmpr2⁻/⁻* (X) embryos at E5.5. Bars, 50 μm.
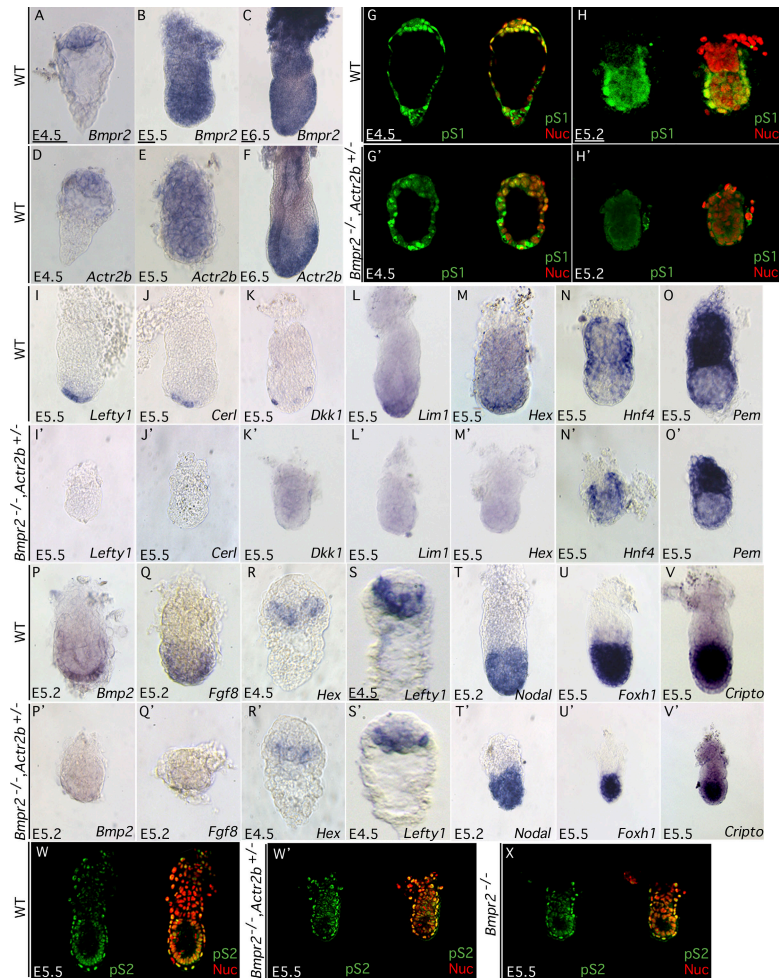
(7/7 embryos; Fig. S2). These data suggested that BMP signaling is not completely lost but is reduced in *Bmpr2⁻/⁻* embryos. Other type 2 receptors may thus play a redundant role in this mutant. Variability in the phenotype of *Bmpr2⁻/⁻* embryos is most likely caused by the variable level of BMP signaling that remains.

BMP signals via BMPR2 and ActR2B in the mouse embryo

The residual level of p-Smad1 in *Bmpr2⁻/⁻* embryos suggested that additional type 2 receptors may compensate for the lack of BMPR2. ActR2A and ActR2B were potential candidates for such receptors because they transduce the BMP signal as well as that of other TGF-β superfamily members (Massague and Chen, 2000; Zhao, 2003). Expression of *Bmpr2* was detected in both embryonic and extraembryonic regions of the wild-type conceptus up to E6.5 (Fig. 3, A–C; Roelen et al., 1997; Beppu et al., 2000). Expression of *Actr2b* was also apparent in the same regions up to E5.5 as well as in the epiblast and overlying VE at E6.5 (Fig. 3 D–F; Beppu et al., 2000), suggesting that ActR2B and BMPR2 may play redundant roles. We therefore generated *Bmpr2⁻/⁻,Actr2b⁺/⁻* double mutant mice.

Removal of one copy of *Actr2b* from *Bmpr2⁻/⁻* mice had pronounced effects. Staining for p-Smad1 was reduced at E4.5 and was not detected at E5.2 in the double mutant embryos (*n* = 4; Fig. 1 N and Fig. 3, G, G', H, and H'). A similar pattern of

p-Smad1 staining was observed in green ES FM260 cell⟵→ *Bmpr2⁻/⁻,Actr2b⁺/⁻* tetraploid chimeric embryos (Fig. S3, K and L). These results suggested that both BMPR2 and ActR2b act as receptors for BMP in VE. We next examined DVE markers in *Bmpr2⁻/⁻,Actr2b⁺/⁻* embryos at E5.5 to determine whether BMP signaling is required for DVE formation. Expression of DVE markers was detected in some of the *Bmpr2⁻/⁻* embryos examined (Fig. S2). However, expression of *Lefty1* (*n* = 5), *Cerl* (*n* = 4), *Dkk1* (*n* = 3), *Lim1* (*n* = 4), and *Hex* (*n* = 3) was always lost in *Bmpr2⁻/⁻,Actr2b⁺/⁻* embryos (Fig. 3, I–M and I'–M'). We also examined endoderm markers in *Bmpr2⁻/⁻,Actr2b⁺/⁻* embryos from E4.5 to E5.5. *Hnf4* (*n* = 5) and *Pem* (*n* = 3) were expressed normally at E5.5 (Fig. 3, N, N', O, and O'), whereas expression of *Bmp2* (*n* = 4) and *Fgf8* (*n* = 4) was abolished at E5.2 (Fig. 3, P, P', Q, and Q'), in the double mutant embryos. At E4.5, expression of *Hex* (*n* = 3) and *Lefty1* (*n* = 4) was maintained (Fig. 3, R, R', S, and S'), suggesting that the primitive endoderm is correctly formed at E4.5 in the double mutant embryos.

We then examined the expression of genes for Nodal signaling components and the distribution of phosphorylated Smad2/3 (p-Smad2) in *Bmpr2⁻/⁻,Actr2b⁺/⁻* embryos (Whitman, 2001). *Nodal*, *Foxh1*, and *Cripto* (*n* = 7, 4, and 4, respectively) were all expressed normally in the double mutant embryos at E5.5 (Fig. 3, T–V and T'–V'). The distribution of p-Smad2 in the double mutant embryos remained similar to that
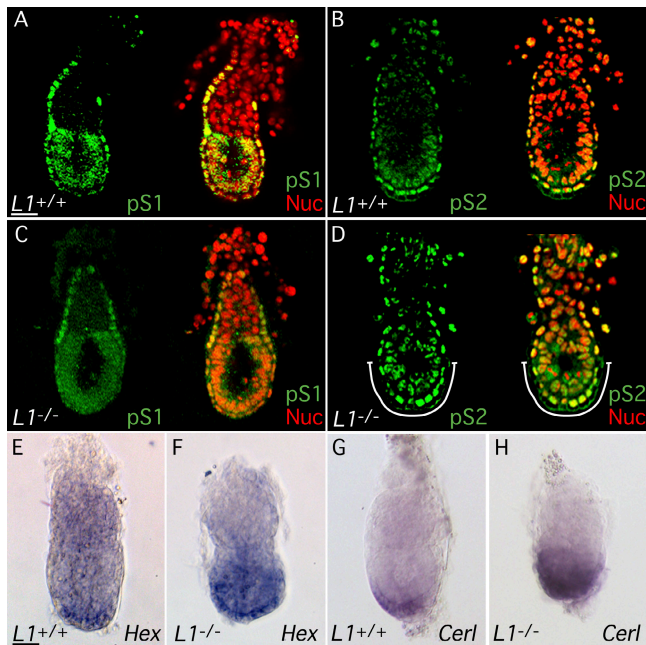
Figure 4. **Expansion of the region positive for DVE markers and negative for p-Smad1 in *Lefty1* mutant embryos.** Wild-type (A and B) or *Lefty1*$^{-/-}$ (C and D) embryos at E5.5 were subjected to immunohistofluorescence staining with antibodies to p-Smad1 (pS1; A and C, green) or to p-Smad2 (pS2; B and D, green). Merged images with nuclear staining (Nuc; red) are also shown. Green color in merged images indicates increased level of p-Smad1 or p-Smad2 staining. Bracket in D indicates the region (the epiblast and VE in the distal region) with increased p-Smad2 staining. Wild-type ($L1^{+/+}$) or *Lefty1*$^{-/-}$ ($L1^{-/-}$) embryos at E5.5 were also examined for expression of *Hex* (E and F) and *Cerl* (G and H). Bars, 50 μm.

in wild-type or *Bmpr2*$^{-/-}$ embryos at E5.5 (Fig. 3, W, W', and X). Unlike *Bmpr2*$^{-/-}$,*Actr2b*$^{+/-}$ embryos in which expression of DVE markers was always absent, it was detected in some of the *Bmpr2*$^{-/-}$,*Nodal*$^{+/-}$ embryos examined (Fig. S4, A–M, available at http://www.jcb.org/cgi/content/full/jcb.200808044/DC1). Furthermore, p-Smad1 and p-Smad2 staining patterns of the *Bmpr2*$^{-/-}$,*Nodal*$^{+/-}$ embryos were similar to those of the *Bmpr2*$^{-/-}$ embryos (Fig. S4, N–P). These results (summarized in Fig. 1 N) thus indicated that both BMPR2 and ActR2B function as type 2 receptors for BMP signaling during formation of embryonic VE and DVE.

Smad1 signaling is absent when and where DVE is newly formed

Staining for p-Smad1 was apparent throughout the primitive endoderm and VE until E5.2 (Fig. 2, A and B). At E5.5, however, p-Smad1 had disappeared from DVE while it was still apparent in the proximal portion of VE (Fig. 2 C and Fig. S3). In *Lefty1*$^{-/-}$ embryos ($n = 5$), the region of VE negative for p-Smad1 expanded (Fig. 4, A and C) along with the expansion of the region positive for DVE markers (Fig. 4, E–H). Thus, p-Smad1 is lost when and where DVE is formed.

We next examined the distribution of p-Smad1 in embryo explants lacking ExE. An inhibitory signal derived from ExE has been shown to restrict induction of DVE to the distal tip of the E5.5 embryo (Rodriguez et al., 2005). Ectopic expression of a *Hex-Venus* transgene was thus induced in VE when ExE was
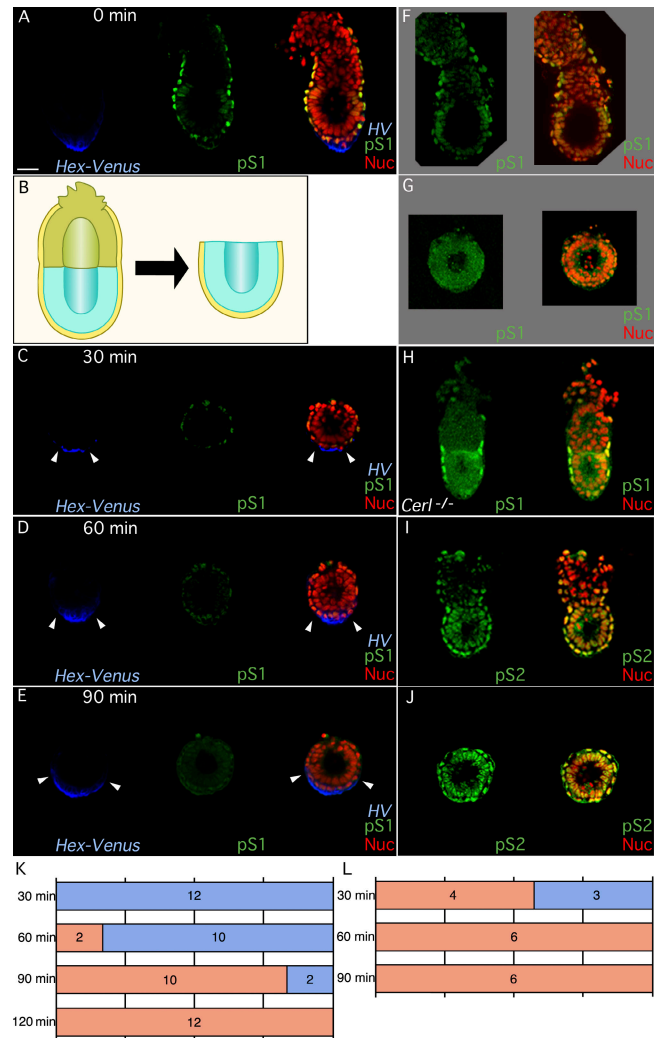

Figure 5. **BMP signaling is lost when and where DVE is newly formed.** The DVE region was monitored by detection of the expression of a *Hex-Venus* transgene (blue) before (A) or at the indicated times after (C–E) removal of ExE. Wild-type (A, F, and I) or *Cerl*$^{-/-}$ (H) embryos or wild-type embryo explants stripped of ExE (C–E, G, and J) at E5.5 were subjected to immunohistofluorescence staining with antibodies to p-Smad1 (pS1; A and C–H, green) or to p-Smad2 (pS2; I and J, green). Merged images with nuclear staining (Nuc; red) are also shown. Green color in merged images indicates increased level of p-Smad1 or p-Smad2 staining. The explants shown in G and J were examined after culture for 6 h. Arrowheads in C–E indicate the boundaries of *Hex-Venus* expression. The experimental strategy for explant experiments is shown in B. The ExE was separated from the epiblast and embryonic VE by a cut along the embryonic–extraembryonic junction at E5.5. The epiblast and embryonic VE were then cultured alone. The effects of ExE removal on expression of *Hex-Venus* (K) and p-Smad1 staining (L) at the indicated times of culture are summarized. Blue and orange bars indicate no change and expansion of the Venus positive domain (K) or no change and down-regulation of p-Smad1 (L), respectively. The numbers of embryos showing each pattern are indicated. Bars, 50 μm.

removed at E5.5 (Fig. 5, A–E and K; Rodriguez et al., 2005). Removal of ExE also induced the complete loss of p-Smad1 (Fig. 5, A–G and L) and up-regulation of p-Smad2 (Fig. 5, I and J). Several observations suggested that the BMP signal disappears from the prospective DVE before, not after, DVE formation (between E5.2 and E5.5 in the wild-type embryo). First, loss of the BMP signal was not caused by expression of the DVE-specific

gene *Cerl*, whose product can inhibit BMP signaling (Belo et al., 2000), because the distribution of p-Smad1 appeared normal in *Cerl*$^{-/-}$ embryos (Fig. 5 H). Second, in explants lacking ExE, expansion of DVE begins ∼90 min after removal of ExE, whereas p-Smad1 staining is lost from VE much earlier, ∼30 min after ExE removal (Fig. 5, C–E, K, and L). These results suggested that loss of the BMP signal from prospective DVE, which coincides with the onset of DVE formation, may be required for DVE formation.

BMP is the ExE-derived inhibitory signal that restricts DVE formation

The loss of p-Smad1 immediately before DVE formation suggests that the ExE-derived signal that inhibits DVE formation may be the BMP signal itself. Previous observations support this notion. First, expression of *Bmp4* and *Bmp8b* is apparent in ExE (Coucouvanis and Martin, 1999; Ying et al., 2000). Second, the DVE region expands in response to removal of ExE (Rodriguez et al., 2005; Mesnard et al., 2006), although such DVE expansion was not observed in a third study (Georgiades and Rossant, 2006). The knockout serum replacement used for culture in this latter study is known to contain a high level of BMP signaling activity (Xu et al., 2005), however, which may explain why DVE failed to expand.

To examine whether the ExE-derived inhibitory signal is indeed the BMP signal, we examined the response of DVE to BMP or a BMP inhibitor. When wild-type embryos were cultured from E5.2 to E5.5 in the presence of BMP4, expression of *Hex*, *Lefty1*, and *Cerl* was lost (Fig. S5, A–F, available at http://www.jcb.org/cgi/content/full/jcb.200808044/DC1). Whereas staining for p-Smad1 was lost in VE in response to removal of ExE at E5.5 (Fig. 6 A), p-Smad1 staining was maintained in VE if the explants lacking the ExE were cultured with BMP4 (Fig. 6 C). Moreover, expansion of the DVE region in such explants was inhibited by BMP4 (Fig. 6, B and D; and Fig. S5, T–W). In contrast, the region positive for DVE markers expanded and the level of expression of DVE markers increased when whole embryos at E5.2 were cultured with Noggin (Fig. 6, E–J), which induced down-regulation of p-Smad1 (Fig. 6, K and L).

Finally, we generated chimeric explants composed of an embryonic portion harboring the *Hex-Venus* transgene and an extraembryonic portion treated with Noggin or with BSA as a control (Fig. 6, O–X). The extraembryonic portion treated with BSA inhibited expansion of the DVE region (Fig. 6, P–R), showing typical ExE activity. However, the extraembryonic portion treated with Noggin allowed expansion of DVE, indicating that the inhibitory ExE activity was abolished by Noggin (Fig. 6, S–U). If the Noggin-treated ExE was attached to the side of a whole embryo harboring the *Hex-Venus* transgene (Fig. 6 O), DVE formation was not affected (Fig. 6, V–X). These results indicate that the BMP signal is the inhibitory signal derived from ExE. We therefore conclude that DVE is formed in a portion of embryonic VE that is negative for BMP signaling and positive for Activin signaling.

Antagonism between Smad2- and Smad1-mediated signaling in VE

A recent study showed that induction of DVE depends on Nodal and proprotein convertase activities (Mesnard et al., 2006). To detect Nodal activity in DVE, we first examined the distribution of p-Smad2 in wild-type embryos at E5.5. Staining for p-Smad2 was apparent in the epiblast (especially on the distal side) and VE including DVE (Fig. 3 W, Fig. 4 B, and Fig. 7 A). We also examined the distribution of p-Smad2 in *Nodal*$^{-/-}$ embryos at E5.5. Unexpectedly, however, staining for p-Smad2 was still apparent in the VE, including DVE, of these mutant embryos (Fig. 7, B and C), suggesting that the appearance of p-Smad2 in DVE is induced by another TGF-β–related factor. Activin is the most likely candidate because Activin-βA and Activin-βB are expressed in the decidual zone of the dam, and recombinant Activin is able to induce ectopic DVE (Jones et al., 2006; Mesnard et al., 2006). GDF1 and GDF3 may be other candidates that trigger Smad2-mediated signaling because DVE formation is impaired in a portion of the mutant mice lacking *Gdf3* or *Gdf3* and *Gdf1* (Chen et al., 2006; Andersson et al., 2007). However, recent evidence (Andersson et al., 2007; Tanaka et al., 2007) has shown that the native form of GDF1 and GDF3 is incapable of inducing downstream signaling. GDF1 and GDF3 act as coligands of Nodal, which interact with Nodal and stimulate Nodal activity (Tanaka et al., 2007).

To examine further whether Activin signaling contributes to the appearance of p-Smad2 in DVE, we cultured wild-type embryos at E5.5 with follistatin, which inhibits Activin signaling but not Nodal signaling. Staining for p-Smad2 was maintained in the epiblast, but was lost in most part of the VE of follistatin-treated embryos (7/10 embryos; Fig. 7, D and E), indicating that Activin activates Smad2 in DVE. Moreover, expression of the DVE marker genes *Hex*, *Cerl*, and *Lefty1* was reduced in the follistatin-treated embryos compared with that in control embryos (Fig. 7, M–R and V). *Fgf8* expression was maintained in follistatin-treated embryos, suggesting that follistatin does not impair embryonic VE formation (Fig. S5, G and H). Although follistatin has been shown to inhibit BMP signaling (Yamashita et al., 1995), p-Smad1 staining in follistatin-treated embryos was increased or normal in DVE (Fig. 7, G–I). Conversely, treatment of embryos with Activin resulted in up-regulation of p-Smad2 and down-regulation of p-Smad1 on the distal side (Fig. 7, D, F, G, and L). Moreover, expression of DVE maker genes was markedly expanded and/or up-regulated in the Activin-treated embryos (Fig. 7, S–V). Treatment of E5.5 embryos with SB431542, a drug that inhibits Activin and Nodal signaling, resulted not only in loss of p-Smad2 and up-regulation of p-Smad1 in DVE but also in down-regulation of the expression of DVE markers (Fig. S5, K–R; Mesnard et al., 2006). These results suggested that Smad2 signaling, most likely triggered by Activin, plays an essential role in DVE formation. Furthermore, an inverse relation between the level of p-Smad1 and that of p-Smad2 suggests that antagonism between Smad1 and Smad2 signaling operates in VE.

Antagonism between Smad1 and Smad2 signaling is also apparent in the mutant embryo lacking Lefty1, an inhibitor of Nodal signaling. The distribution of p-Smad2 in *Lefty1*$^{+/-}$ embryos at E5.5 was similar to that in the wild type (unpublished data). However, the level of p-Smad2 was increased on the distal side of *Lefty1*$^{-/-}$ embryos at this stage (*n* = 5; Fig. 4, B and D). In contrast, the level of p-Smad1 was decreased on the distal side of *Lefty1*$^{-/-}$ embryos (*n* = 5; Fig. 4, A and C). In addition,
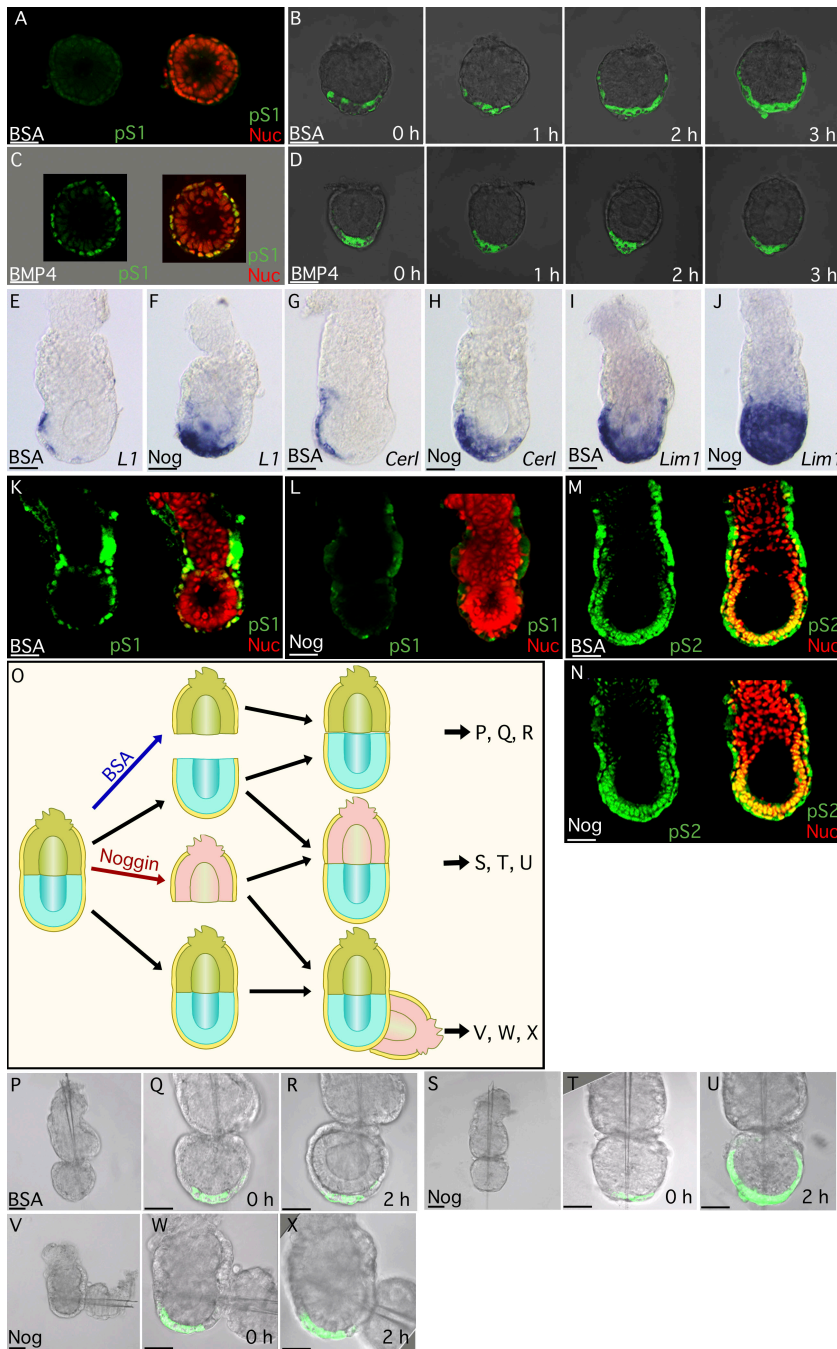
Figure 6. **Identification of BMP as the inhibitory signal from ExE that restricts DVE formation.** The DVE region was monitored by detection of the expression of a *Hex-Venus* transgene (B, D, Q, R, T, U, W, and X, green) at the indicated times after removal of ExE (B and D) or after ExE attachment (Q, R, T, U, W, and X) at E5.5. Embryo explants stripped of ExE (A and C) or normal embryos (K–N) at E5.5 were subjected to immunohistofluorescence staining with antibodies to p-Smad1 (pS1; green) or to p-Smad2 (pS2; green), as indicated. Merged images with nuclear staining (Nuc; red) are also shown. Green color in merged images indicates increased level of p-Smad1 or p-Smad2 staining. The explants or embryos were cultured with BSA (A, B, K, and M), BMP4 (C and D), or Noggin (Nog; L and N) for 6 h or the indicated times. Expression of *Lefty1* (*L1*), *Cerl*, and *Lim1* was determined in E5.2 embryos cultured for 8 h with either BSA (E, G, and I) or Noggin (F, H, and J). The experimental strategy for the explant experiments involving removal and attachment of ExE (P–X) is shown in O. Chimeric explants were composed of an embryonic portion harboring the *Hex-Venus* transgene and an extraembryonic portion treated with BSA or Noggin, as indicated. Bright-field images alone are shown in P, S, and V. Fluorescence images superimposed on bright-field images (B, D, Q, R, T, U, W, and X) are also shown. Bars, 50 µm.

the expression domains of *Hex* ($n = 5$) and *Cerl* ($n = 5$) were markedly expanded in the distal region of *Lefty1*$^{-/-}$ embryos (Fig. 4, E–H).

ActR2A and ActR2B are rate-limiting factors involved in the antagonistic balance

We next examined the molecular basis of the antagonism between BMP and Activin–Nodal signaling. Activin–Nodal and BMP share common signaling components, extracellular components such as ActR2A and ActR2B as well as intracellular components including Smad4. If the amount of a common component is limited, an increase in one signal would result in a decrease in the other. To test this possibility, we introduced a *Smad4* or *Actr2a+Actr2b* expression vector into a p-Smad1–positive cell in the distal region of E5.2 embryo and examined if such cells would maintain or lose p-Smad1 12 h later (equivalent to E5.7; Fig. 8). Normally, such p-Smad1–positive cells at E5.2 (Fig. 2 B) lose p-Smad1 by E5.5 (Fig. 2 C). However, if a limiting factor commonly used for both signaling is overexpressed into such a cell, it would prevent the antagonism and the cell would remain positive for p-Smad1 at E5.5. This is what we observed when *Actr2* was overexpressed (Fig. 8 A).

Expression of *EGFP* alone (10/10 embryos) or *EGFP+Smad4* (8/8 embryos) did not influence the level of p-Smad1. Thus, the cell in the distal region of the E5.7 embryo that received *EGFP* or *EGFP+Smad4* lost p-Smad1 (Fig. 8, B and C). In contrast, the

Figure 7. **Smad2 signaling in VE is essential for specification of DVE.** (A–L) Expression of p-Smad2 (pS2; green) or p-Smad1 (pS1; green), and merged images with nuclear staining (Nuc; red), for wild-type (WT; A and J) or $Nodal^{-/-}$ (B, C, and K) embryos at E5.5 as well as for E5.5 wild-type embryos treated with BSA (D and G), follistatin (Fol; E, H, and I), or Activin (Act; F and L) for 7 h. Arrowheads indicate loss of p-Smad2 fluorescence from DVE of the follistatin-treated embryo (E). Green color in merged images indicates increased level of p-Smad1 or p-Smad2 staining. Bracket in F indicates the region with increased p-Smad2 staining. (M–U) Expression of *Hex* (M, P, and S), *Cerl* (N, Q, and T), and *Lefty1* (*L1*; O, R, and U) in E5.5 wild-type embryos treated with BSA, follistatin, or Activin, as indicated, for 7 h. (V) Summary of the effects of treatment with BSA, follistatin, or Activin on the expression of p-Smad1 in DVE, p-Smad2 in DVE, and the indicated genes. Light blue, dark blue, orange, and red bars indicate normal expression, expanded expression, no expression, and expanded no expression portion, respectively. The numbers of embryos showing each expression pattern are indicated. Bars, 50 µm.
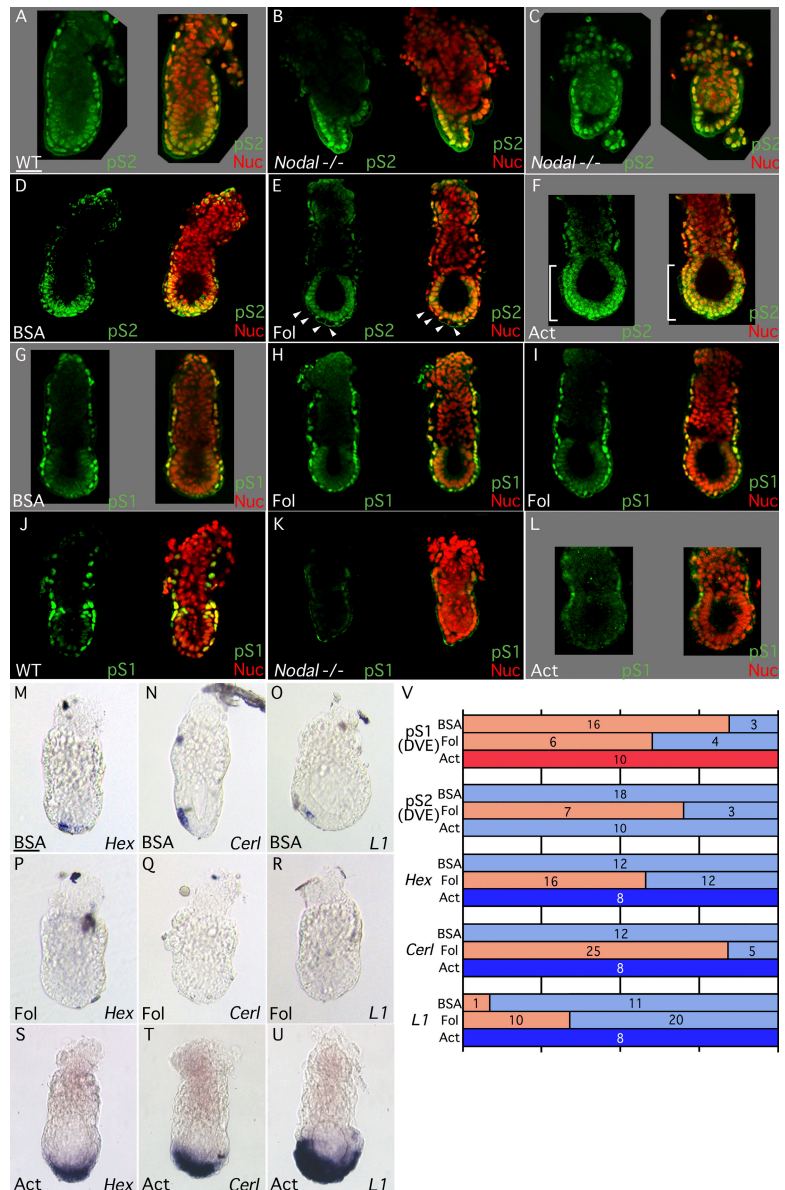
cells that received *Actr2a+Actr2b* expression vectors remained positive for p-Smad1 (25/25 embryos; Fig. 8, D and E). These results suggest that the limited level of ActR2A and ActR2B is responsible for the antagonistic balance between Smad2 and Smad1 signaling.

Discussion

Dual roles of BMP signaling in DVE formation

Four different types of endoderm are formed from the primitive endoderm before gastrulation: parietal endoderm, extraembryonic VE, embryonic VE, and DVE (Fig. 9). Primitive endoderm cells that maintain contact with the ectoderm differentiate into VE, whereas those that migrate away from the ectoderm along the inner surface of the trophectoderm form parietal endoderm (Nadijcka and Hillman, 1974; Enders et al., 1978). VE cells give rise to both extraembryonic VE and embryonic VE, and the

distal portion of embryonic VE becomes DVE. We have now shown that BMP signaling plays dual roles in DVE formation.

At early stages of development (until E5.2), BMP signaling is required for formation of embryonic VE. BMP signaling, together with Nodal signaling, thus promotes the differentiation of VE into embryonic VE rather than into extraembryonic VE. This conclusion is supported by previous observations (Mesnard et al., 2006) as well as by our present data. First, extraembryonic VE markers remain ectopically expressed at high levels in the embryonic region and embryonic VE markers fail to be induced in $Nodal^{-/-}$ embryos (Mesnard et al., 2006). In addition, given that the p-Smad1 staining observed in wild-type embryos at E5.5 is lost in $Nodal^{-/-}$ embryos (Fig. 7), Nodal signaling is necessary for the appearance of BMP signaling. Nodal signaling may therefore induce the BMP signal in VE. Second, embryonic VE markers also failed to be induced in $Bmpr2^{-/-}$,$Actr2b^{+/-}$ embryos, in which p-Smad1 staining was absent at E5.2 (Fig. 3). However, extraembryonic VE markers were maintained in such
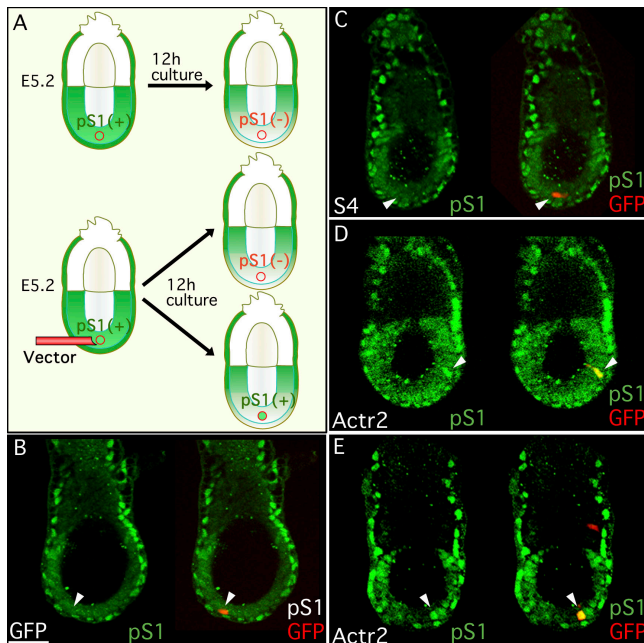
Figure 8. **A limited level of type II Activin receptors is responsible for antagonistic balance between Smad1 and Smad2 signaling in the mouse embryo.** (A) Experimental strategy. An effector gene, together with a GFP expression vector, was introduced into epiblast cells on the distal side of E5.2 mouse embryos. The embryos were cultured for 12 h and were examined for the distribution of p-Smad1 by immunohistofluorescence staining. (B–E) Localization of p-Smad1 (pS1; green) and merged images of p-Smad1 with GFP-positive cells (GFP; red). Arrowhead indicates the GFP-positive cell. S4, *Smad4*; Actr2, *Actr2a* plus *Actr2b*. Bars, 50 µm.

embryos at E5.5. Embryonic VE markers were also down-regulated in *Bmpr2*$^{-/-}$ embryos, in which p-Smad1 staining was reduced at E5.2 (Fig. 2). These results indicate that BMP signaling is essential until E5.2 for formation of embryonic VE, whereas it is dispensable for formation of extraembryonic VE.

At E5.5, DVE cells arise from a portion of embryonic VE that lacks the BMP signal. Whereas embryonic VE cells that have lost the BMP signal appear to be induced to differentiate into DVE, extraembryonic VE is not competent to become DVE even if it loses the BMP signal. First, in wild-type embryos at E5.2, before DVE formation, the entire embryonic VE is positive for the BMP signal. At E5.5, however, the BMP signal is lost specifically from DVE, with the remainder of the embryonic VE remaining positive for this signal (Fig. 2). Second, in *Lefty1*$^{-/-}$ embryos, the BMP-negative region of embryonic VE expands to the proximal side, toward which DVE formation also expands (Fig. 4). Third, the portion of embryonic VE that is converted to DVE was found to be increased in Noggin-treated embryos (Fig. 6). Although the BMP signal disappeared in both embryonic VE and extraembryonic VE of such embryos, extraembryonic VE was not converted to DVE. Fourth, in embryo explants stripped of ExE, in which *Bmp4* and *Bmp8b* are expressed (Coucouvanis and Martin, 1999; Ying et al., 2000), the loss of BMP signaling precedes DVE expansion, suggesting that the former may be responsible for the latter. Expansion of *Hex* expression thus begins ∼90 min after the removal of ExE, whereas the BMP signal is lost from VE much earlier, ∼30 min after ExE removal (Fig. 5). Finally, in ExE-stripped embryos treated with BMP4

(Fig. 6) or with knockout serum replacement that contains BMP activity (Georgiades and Rossant, 2006), the *Hex*-expressing domain fails to expand. These results indicate that loss of the BMP signal from embryonic VE results in its conversion to DVE.

How is the BMP/Smad1 signal down-regulated in the region of DVE formation?

Although it is not clear how BMP signaling is down-regulated in the DVE region at E5.5, several mechanisms are possible. First, a BMP antagonist (or antagonists), such as Cerl, may be responsible. However, p-Smad1 was also absent in DVE of *Cerl*$^{-/-}$ embryos (Fig. 5 H), suggesting that Cerl is either not involved in or alone is not sufficient for the down-regulation of BMP signaling. Second, *Bmp4* and *Bmp8b*, which encode the major BMP ligands at this stage, are expressed in ExE (Coucouvanis and Martin, 1999; Ying et al., 2000); elongation of the embryo at the egg cylinder stage along the proximodistal axis between E5.2 and E5.5 may therefore prevent BMPs from reaching the distal end of the embryo. Also, a low level of *Bmp2* expression that can be detected in the distal region of the E5.2 embryo disappears by E5.5 (Fig. S5, I and J). Finally, an antagonism between Smad2 and Smad1 signaling may play the major role in down-regulation of the BMP signal. An inverse relation between the level of p-Smad1 and p-Smad2 was thus repeatedly observed in E5.2–5.5 embryos. In the wild-type embryo at E5.5, for instance, BMP signaling via p-Smad1 was detected predominantly in the proximal epiblast, a distribution opposite to that of p-Smad2 (Fig. 2 C vs. Fig. 4 B; and Fig. 7, A vs. J and D vs. G). Furthermore, p-Smad1 was lost from the distal half of VE in E5.5 *Lefty1*$^{-/-}$ embryos (Fig. 4 C) and Activin-treated wild-type embryos (Fig. 7 L), whereas the level of p-Smad2 was increased in this region. Staining for p-Smad1 was also lost from the entire VE, whereas that for p-Smad2 was increased in embryo explants stripped of ExE (Fig. 5) and in Noggin-treated embryos (Fig. 6, L and N). Staining for p-Smad2 was completely lost, whereas that for p-Smad1 was increased in embryos treated with SB431542 (Fig. S5, K–N). The antagonism between Smad1 and Smad2 signaling may convert a small shift of the balance to a robust difference.

Antagonism between Smad1 and Smad2 signaling

Our data suggest a previously unknown role for Activin in mouse embryonic patterning. Inhibition of Activin action by follistatin (Fig. 7, P–R) or SB431542 (Fig. S5, P and R) thus abolished DVE formation. Such treatment not only down-regulated p-Smad2 in DVE but also slightly up-regulated p-Smad1 on the distal side of the embryo. Conversely, treatment with Activin expanded the site of DVE formation by up-regulating p-Smad2 and down-regulating p-Smad1 on the distal side of the embryo (Fig. 7). These observations are again indicative of an antagonism between Smad1 and Smad2 signaling during DVE formation. Treatment of explants lacking ExE with SB431542 induced the loss of both p-Smad1 and p-Smad2. DVE formation was abolished in such explants (Mesnard et al., 2006; this study), suggesting that DVE formation requires both the absence of Smad1 signal and the presence of Smad2 signal. The balance between the ratio of p-Smad1 and p-Smad2 may determine whether or

Figure 9. **The location of DVE formation is determined by the concerted action of Smad1- and Smad2-mediated signals.** (A) BMP signaling promotes the differentiation of primitive endoderm (PrE) into VE until E4.5. (B) VE differentiates into embryonic VE as a result of the concerted action of BMP and other signals until E5.2. (C) DVE arises from a region of embryonic VE in which Activin signaling is present and BMP signaling is absent at E5.5. (D and E) VE gives rise to extraembryonic and embryonic VE until E5.2. (F) Summary of the relation between endoderm development and signaling by multiple TGF-β superfamily members from E4.5 to E5.5. a, Activin; b, BMP; Epi, epiblast; ICM, inner cell mass; l, Lefty1; n, Nodal; PE, parietal endoderm; PrE, primitive endoderm; s1^p, phosphorylated Smad1/5; s2^p, phosphorylated Smad2/3; s4, Smad4; TE, trophectoderm. Broken arrows indicate the mechanism is obscure.
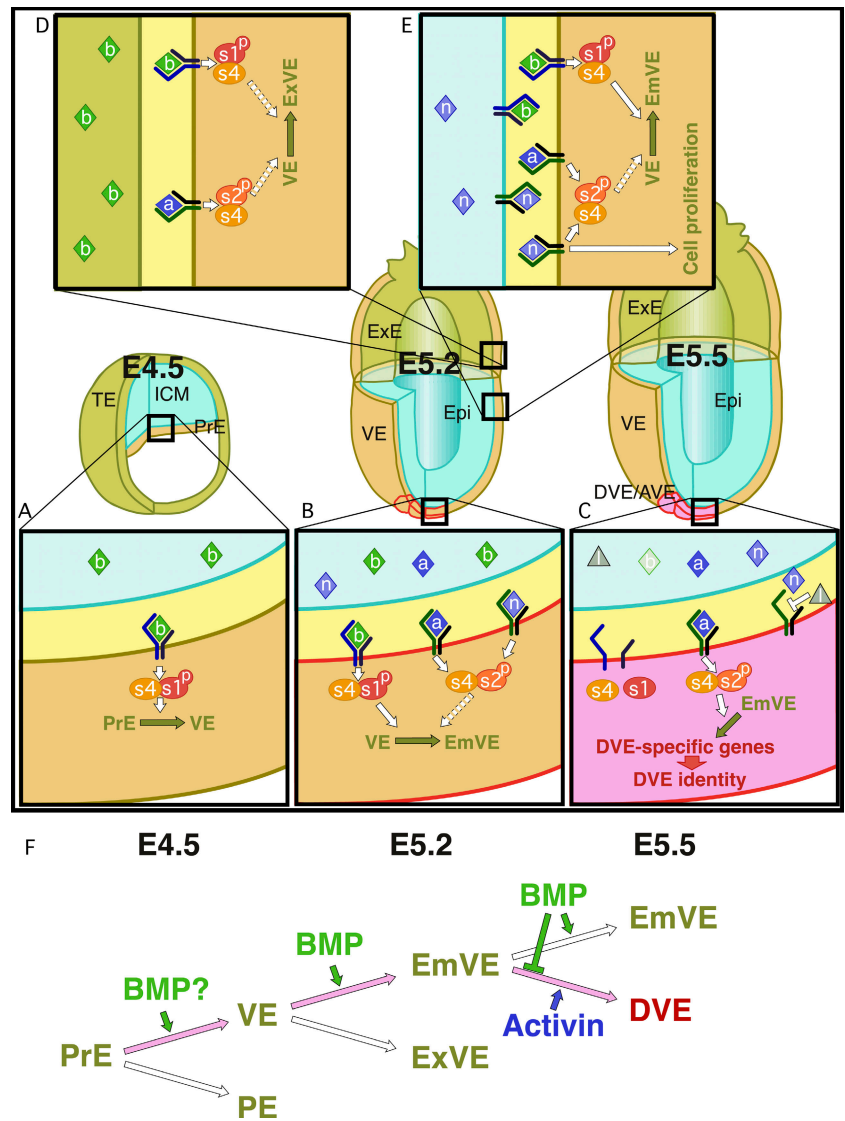
not a cell in the VE will become DVE. To test this, it would be necessary to quantify the amounts of p-Smad1 and p-Smad2 in individual cells and to monitor their interaction with Smad4, such as by FRET analysis.

Mutant embryos lacking Activin establish a normal A-P axis, most likely because Activin is provided by the decidual zone of the dam, not by the embryo (Albano et al., 1994; Jones et al., 2006). Maternally derived molecules of a large molecular weight can cross Reichert membrane and be transferred to the embryo. For example, maternal immunoglobulins (Bernard et al., 1977) and maternal and DiI-labeled high density lipoprotein intravenously injected into a pregnant mother (Smith et al., 2006) have been found in the embryo. In fact, maternally derived Activin proteins have been detected within the embryo including the VE between E3.5 and E6.5 (Jones et al., 2006).

Antagonism between Smad2 and Smad1 signaling may play a role in vertebrate development more generally. For example, BMP4 blocks the dorsal mesoderm-inducing activity of Activin in *Xenopus laevis* (Dale et al., 1992; Jones et al., 1992; Piccolo et al., 1996; Zimmerman and Mathews, 1996). Also in

Xenopus, Chordin and Noggin prevent BMPs from interacting with their cognate receptors and thus allow underlying Activin signaling in the dorsal region of the embryo to induce dorsal mesoderm (Piccolo et al., 1996; Zimmerman and Mathews, 1996). Expression of *Nodal* is observed in the deep region of the involuting marginal zone, which is equivalent to AVE in mouse, before the onset of gastrulation in *Xenopus* embryos (Jones et al., 1995). Whereas p-Smad2 is present in these cells, p-Smad1 is absent (Schohl and Fagotto, 2002). In the mouse, *Chordin* and *Noggin*, in addition to *Nodal*, are expressed in the future node region (Conlon et al., 1994; Bachiller et al., 2000), and they may similarly shift the balance toward Activin–Nodal signaling. Indeed, the reduction in the level of Nodal signaling in *Foxh1*$^{-/-}$ embryos prevents node formation (Hoodless et al., 2001; Yamamoto et al., 2001). These observations suggest that the antagonism between Activin–Nodal and BMP signaling may play a role in organizer formation in vertebrates.

What is the molecular basis of the antagonism between BMP and Activin–Nodal signaling? Our result suggests that common signaling components, ActR2a and ActR2B, are rate-limiting

factors in the E5.5 mouse embryo (Fig. 8). However, these results obtained by overexpression experiments need to be confirmed by other approaches because overexpressed type II receptor molecules may bind to type I receptor easily, which is often sufficient to activate signaling, or may interact with nonphysiological type I receptors. In *Xenopus* animal caps, Activin and BMP signaling have been shown to antagonize each other through the intracellular component Smad4 (Candia et al., 1997). However, the level of Smad4 was not low enough to cause the antagonism at least in the E5.5 mouse embryo. Different mechanisms may underlie the antagonism between BMP and Activin–Nodal depending on the cell type.

Materials and methods

In situ hybridization and immunohistofluorescence
Embryos were carefully staged on the basis of their morphology. Whole-mount in situ hybridization was performed according to a standard procedure or a method designed for peri-implantation embryos (Strumpf et al., 2005; protocol is available at http://www.sickkids.ca/rossant/protocols/doublefluor.asp). Plasmids to generate probes for in situ hybridization were provided by R. Behringer (University of Texas, Houston, TX), R.S. Beddington (National Institute for Medical Research, London, England, UK), J.E. Darnell Jr. (The Rockefeller University, New York, NY), B. Hogan (Duke University Medical Center, Durham, NC), C.L. MacLeod (University of California, San Diego, La Jolla, CA), A. McMahon (Harvard University, Cambridge, MA), C. Nihers (Deutsches Krebsforschungszentrum, Heidelberg, Germany), M.S. Parmacek (University of Pennsylvania, Philadelphia, PA), J. Rossant (The Hospital for Sick Children, Toronto, Canada), and E. De Robertis (University of California, Los Angeles, CA). Immunostaining was performed with rabbit polyclonal antibodies to p-Smad1 generated in response to the phosphorylated C-terminal region of mouse Smad1 (Persson et al., 1998; a gift from P. ten Dijke, Leiden University Medical Center, Leiden, Netherlands), rabbit monoclonal antibodies to p-Smad2 (Cell Signaling Technology), and Alexa Fluor 568–conjugated goat antibodies to rabbit immunoglobulin G (Invitrogen) used at 1:200, 1:25, and 1:2,000 dilutions, respectively. Nuclei were stained with YOYO-1 (Invitrogen). For immunostaining, embryos recovered in DME or PBS including phosphatase inhibitor cocktail containing 10 mM sodium fluoride, 2 mM sodium orthovanadate, and 50 mM β-glycerophosphate were fixed in 4% PFA for 10 min and were blocked in TSA blocking reagent (PerkinElmer). Methanol series is necessary for p-Smad1 staining before the blocking process. Washing solution is PBS including 0.1% Triton X-100. Confocal images were obtained with a laser scanning confocal microscope (LSM 510; Carl Zeiss, Inc.) mounted on an inverted microscope (Axiovert 100M; Carl Zeiss, Inc.), using Plan Apochromat 20×/0.75 NA and C-Apochromat 40×/1.2 NA objectives (Carl Zeiss, Inc.). Images were processed with Photoshop CS software (Adobe). Genomic DNA isolated from whole embryos was used for genotype analysis. Second antibody alone did not give rise to detectable staining of p-Smad1 and p-Smad2.

Generation and analysis of chimeric embryos
Chimeras were generated by ES cell tetraploid embryo aggregation. Two-cell embryos were collected from intercrosses between $Bmpr2^{+/-}$ or $Bmpr2^{+/-},Actr2b^{+/-}$ mice and were aggregated with green ES FM260 cells expressing EGFP (provided by M. Okabe, Osaka University, Osaka, Japan). Chimeric embryos were recovered at the indicated stages, fixed, and both examined by fluorescence microscopy (MZ FLIII; Leica) using PlanApo 1.0× WILD objective (Leica) and processed for whole-mount in situ hybridization or for immunohistofluorescence analysis. The genotype of the host embryo was determined retrospectively with extraembryonic tissue. Images were obtained with a digital camera (DP11; Olympus) and processed with Photoshop CS software.

Embryo explant culture
For explant cultures, the ExE and epiblast overlying embryonic VE were removed from ICR mouse (SLC) or *Hex-Venus* transgenic mouse embryos dissected at E5.5 with fine forceps and a tungsten needle (Takaoka et al., 2006). Chimeric explants were generated with the use of a fine glass needle (Drummond Scientific Co.). The explants were cultured for 6 h or the indicated times under a humidified atmosphere of 5% CO_2 at 37°C in dishes or in gel matrix (Mebiol) containing DME supplemented with 75% rat serum.

Noggin, BMP4, follistatin, Activin (R&D Systems), and SB431542 (Sigma-Aldrich) were used at concentrations of 500 ng/ml, 50 ng/ml, 1.2 µg/ml, 50 µg/ml, and 10 µmol/l, respectively. Images were analyzed using a laser scanning confocal microscope (LSM 510) mounted on an inverted microscope (Axiovert 100M) using Plan Apochromat 20×/0.75 NA and C-Apochromat 40×/1.2 NA objectives (Carl Zeiss, Inc). Images were processed with Photoshop CS software.

Introduction of expression vectors into the epiblast
Introduction of expression vectors (a GFP expression alone or with various effectors; Mizushima and Nagata, 1990) into the epiblast was performed as described by Yamamoto et al. (2004). Embryos were cultured for 12 h under a humidified atmosphere of 5% CO_2 at 37°C in dishes containing DME supplemented with 75% rat serum.

Online supplemental material
Fig. S1 shows expression of DVE markers in the wild-type mouse embryos at E5.2 and E5.5. Fig. S2 shows phenotype of AVE, VE, and ExE in $Bmpr2^{-/-}$ embryos. Fig. S3 shows p-Smad1 staining in wild-type E5.5 embryos and in the chimeric embryo at E5.0. Fig. S4 shows phenotype of DVE in $Bmpr2^{-/-},Nodal^{+/-}$ embryos. Fig. S5 shows effects of SB431542, follistatin, and BMP4 on DVE formation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200808044/DC1.

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