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BMP signaling is required for cell cleavage in preimplantation-mouse embryos

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Abstract

The mechanisms regulating cell division during development of the mouse pre-implantation embryo are poorly understood. We have investigated whether bone morphogenetic protein (BMP) signaling is involved in controlling cell cycle during mouse pre-implantation development. We mapped and quantitated the dynamic activities of BMP signaling through high-resolution immunofluorescence imaging combined with a 3D segmentation method. Immunostaining for phosphorylated Smad1/5/8 shows that BMP signaling is activated in mouse embryos as early as the 4-cell stage, and becomes spatially restricted by late blastocyst stage. Perturbation of BMP signaling in preimplantation mouse embryos, whether by treatment with a small molecule inhibitor, with Noggin protein, or by over-expression of a dominant-negative BMP receptor, indicates that BMPs regulate cell cleavage up to the morula stage. These results indicate that BMP signaling is active during mouse pre-implantation development and is required for cell cleavage in preimplantation mouse embryos.

Keywords

BMP; Smad1; Morula; Preimplantation; Cell cleavage; Cell division

Introduction

Mammalian pre-implantation development has been best studied in the mouse. After fertilization, mammalian embryos undergo four rounds of cell division to reach the morula stage, a solid ball of approximately 16 cells. Cells on the inside of the morula differ from those on the outside in several ways including genes expressed, cell shape, and cell adhesion characteristics (Ziomek et al., 1982; Zernicka-Goetz et al., 2009; Rossant and Tam, 2009). Subsequently, a combination of symmetric and asymmetric cell divisions and cavitation transforms the morula into a blastocyst (32-cell stage) (Smith and McLaren, 1977). The

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Appendix A. Supporting information
Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.10.001.

blastocyst hatches from the zona pellucida in preparation for implantation at around embryonic day (E 4.5).

Inner cell mass (ICM) and trophectoderm (TE) cell differentiation is promoted by several transcription factors (TFs) that are expressed dynamically during this developmental transition. *Pou5f1/Oct4*, *Nanog*, and *Sox2* specify ICM cells while *Tead4* and *Cdx2* specify TE cells (Nichols et al., 1998; Avilion et al., 2003; Chambers et al., 2003; Strumpf et al., 2005; Wang and Dey, 2006; Yagi et al., 2007; Nishioka et al., 2008). TE cells can be further characterized by their location relative to the ICM as polar and mural TE cells. Polar TE cells are defined as those that overlay the ICM while the mural TE cells overlay the blastocoel cavity, with division of polar TE contributing cells to the mural TE compartment (Copp, 1978; Gardner and Davies, 2002). The essential roles of TFs such as *Pou5f1/Oct4*, *Nanog*, *Sox2*, *Tead4* and *Cdx2* in specifying ICM and TE lineages have been extensively studied. In contrast, our understanding of how cell division is regulated during pre-implantation development is relatively limited (CIemerych and Sicinski, 2005).

Recent single cell RNA-seq expression profiling (Tang et al., 2010) indicates BMP signaling components, including BMP ligands, receptors, and Smads, are all expressed in early stages of mouse pre-implantation development (Fig. S1). This raises the possibility that BMP signaling may function during mouse pre-implantation development.

Mouse mutants deficient in various BMP ligands, intracellular transducers, and receptors have underscored the importance of BMP signaling during gastrulation; *e.g.* Mishina et al. (1995), Macías-Silva et al. (1998), Solloway and Robertson (1999), Yi Se et al. (2009), Arnold et al. (2006), but has thus far failed to disclose a role(s) for BMP signaling at pre-implantation stages, possibly because of redundancies in BMP signaling that ensure the robustness of embryonic developmental programs. Smad1 and 5 single homozygous null mice have similar phenotypes as the Smad1$^{+/-}$: Smad5$^{+/-}$ double heterozygous mutants presumably because they are functionally redundant (Arnold et al., 2006). An inability to generate Smad1 and 5 homozygous null mice precluded genetic analysis of a role for BMP signaling in pre-implantation mouse development, illustrating the challenge of investigating the function of BMP signaling pathway components in development. Mice deficient in BMP type I receptor (BMPr1a) or type II receptor (BMPrII), however, were smaller and had fewer cells (Beppu et al. 2000; Mishina et al. 1995), supporting a potential involvement of BMP signaling in cell proliferation. Coucouvanis and Martin (1999) used *in situ* hybridization to demonstrate that *Bmp4* RNA is present exclusively in the ICM cells of the blastocyst. They also used *in vitro* culture of embryoid bodies made from aggregated PSA1 embryonal carcinoma cells to demonstrate that inhibition of BMP via expression of a dominant negative *Bmpr-Ib* blocked both cavitation and expression of *Hnf4a*, a marker of visceral endoderm.

To investigate if BMP signaling functions in mouse preimplantation embryonic development, we measured the direct output of BMP signaling during this period using two different approaches. The first method examines the transcriptional response of “BMP-indicator” mouse embryos, while the second measured the status of Smad1/5/8 phosphorylation within preimplantation stage embryos. We mapped and quantitated the dynamic activities of BMP signaling through high-resolution immunofluorescence imaging combined with a 3D segmentation method. We find that BMP signaling activity can be detected in all blastomeres as early as the 4-cell stage (E2.0) and becomes spatially restricted by late blastocyst stage. Perturbation of BMP signaling in embryos, by treatment with a pharmacologic inhibitor, by Noggin protein, or by overexpression of a dominant-negative BMP receptor, each delayed cell cleavage during early cleavage stages. Based on these results, we propose that BMP signaling is required for normal cell cycle during development of the preimplantation mouse embryo.

Results

BMP signaling activity in preimplantation embryos revealed by BRE-gal expression and the phosphorylation state of Smad1/5/8

We investigated for evidence of BMP activity during mouse preimplantation development using mice transgenic for a BMP-dependent *lacZ* reporter gene (BRE-gal) (Javier et al., 2012). BMP-responsive elements (BRE) are *cis*-acting sequences that respond to canonical Smad-dependent BMP signaling in the frog *Xenopus* (von Bubnoff et al., 2005), *Drosophila* (Yao et al., 2006), zebrafish (Alexander et al., 2011), and mouse (Javier et al., 2012; Doan et al., 2012). A BRE element was adapted to generate a BMP-dependent reporter gene by placing seven copies of the BRE in tandem upstream of a *X. laevis Id3*-gene minimal promoter:: *nlsLacZ* reporter gene (Maretto et al., 2003). BRE-gal mice identified SMAD-dependent BMP activity in E5.5 to E13.5 post-implantation stage mouse embryos (Javier et al., 2012; Doan et al., 2012). We used BRE-gal mice to analyze BMP signaling in the pre-implantation mouse embryo from morula (~E2.5) to blastocyst (~E3.5) stage (Fig. 1A). Nuclear β-gal activity was observed in both ICM and TE of blastocysts, although the activity in ICM was in general stronger than that observed in TE. To provide independent evidence that BMP signaling is active in the blastocyst, immunofluorescence analysis was performed to identify the phosphorylated form of Smad1/5/8 (hereafter referred to as p-Smad1), produced by receptor-activated BMP signaling. Phospho-Smad1 was detected in all nuclei of the E3.5 stage embryo (Fig. 1B). The difference in cellular patterns of X-gal staining (enriched expression in ICM) and p-Smad1 immunostaining (uniform expression) may be due to reduced sensitivity of the BRE-gal reporter compared to anti-pSmad1 staining. Alternatively, although both ICM and TE cells receive BMP signaling, the transcriptional machinery mediating BMP signaling in these lineages may differ, with only a subset of this activity being detected by the BRE-gal reporter construct. Immunostaining using a pan-Smad1 antibody showed predominantly cytoplasmic localization of Smad1 (Fig. 1C) suggesting that the majority of Smad1 present in the preimplantation stage embryos is unphosphorylated. This suggests that the availability of Smad1 is not rate limiting in regulating BMP signaling activity in the preimplantation stage mouse embryo.

Specificity of the p-Smad1 antibody was verified using E14Tg2a mouse embryonic stem (mES) cells. Following stimulation with *Bmp4* the mES cells show p-Smad1 staining within the nucleus, which was inhibited in the presence of either Noggin or a small molecule BMP antagonist LDN193189 (4-(6-(4-(piperazin-1-yl)phenyl)pyrazolo[1,5-a]pyrimidin-3-yl)quinoline hydrochloride) (Fig. S2A; Vogt et al., 2011). X-gal staining of BRE-gal ES cells also shows that BRE-gal induction was dependent on BMP signaling (Fig. S2B).

Western blot analysis of total protein from treated mES cells also detected p-Smad1 upon stimulation with *Bmp4*, which was prevented in the presence of LDN193189 (Fig. S2C). LDN193189 is a highly selective antagonist of the intracellular kinase domain of the BMP receptor isotypes ALK2 (activin receptor-like kinase 2, also known as ACVR1), ALK3 (BMPR1A) and ALK6 (BMPR1B) (Yu et al., 2008; Cuny et al., 2008). We also examined the effect of LDN193189 on the transcriptional response of known BMP-target genes and found that the inhibitor effectively down-regulated expression of *Id1*, *Id2* and *Id3* (Hollnagel et al., 1999) (Fig. S2D). Taken together, our data support that p-Smad1 staining in E3.5 mouse blastocysts is specific and that LDN193189 is an effective BMP antagonist.

BMP signaling is detected as early as the four-cell stage of mouse development

We determined the earliest stage at which p-Smad1 staining is detectable in preimplantation stage mouse embryos. Phospho-Smad1 staining is below the limit of detection in the 2-cell stage embryo, but is detected at the 4-cell stage and persists through late blastocyst (~100-cell stage) (Fig. 1D and E). The onset of p-Smad1 staining precedes the appearance of X-gal staining in the BRE-gal embryos, the latter first being detected at around the 16-cell morula stage (Fig. 1A). The observed delay in X-gal staining (Fig. 1A) could be explained by a required threshold concentration of p-Smad-1 to activate the BRE-gal reporter gene or that the BRE-gal reporter is not responsive to all BMP signaling events. Based on these results, we conclude that BMP signaling is activated by the 4-cell stage, prior to known activation of transcription factors including *Cdx2*, *Nanog*, *Tead4* and *Pou5f1/Oct4* that are required to establish the transcriptional network for ICM and TE cell lineage selections (Nichols et al., 1998; Avilion et al., 2003; Chambers et al., 2003).

Confocal imaging (Fig. 1F) revealed a non-uniform, punctate staining of p-Smad1 predominantly within the nucleus, but absent from the nucleolus. Nucleoli were identified by absence of Hoechst staining within the nucleus (Martin et al., 2005). This punctate staining can be detected as early as 4-cell stage (~E2.0) and persists until the blastocyst stage (E3.5–4.5). Similar punctate p-Smad1-staining pattern has been observed in other tissues (Eom et al., 2011; Hogg et al., 2010), and may represent transcriptional regulatory complexes.

Increased p-Smad1 in the ICM

We investigated whether average cellular phospho-Smad1 levels changed during early pre-implantation development. To quantitate changes we developed a computer program that segments individual nuclei in three-dimensional confocal stacks (a sample output of an analysis is shown in Fig. 2A, and volume rendering of an embryo in Fig. 2B; see “Experimental procedures” section for more detail and accuracy measurements, Fig. S3A). Mean p-Smad1 levels at the four and eight cell stages were not significantly different (p = 0.16). Likewise, mean p-Smad1 levels remained constant at the¼16, 32, and 64-cell stages (Fig. 2C, S3B and C).

Next, we asked whether p-Smad1 levels differed in inner cells versus outer cells. To address this question we quantified p-Smad1 levels in manually annotated inner and outer cells using image segmentation (see the “Experimental procedures” section). No significant difference was observed in ~16-cell stage embryos (Fig. 2D left, $p = 0.2$, $n = 18$, Fig. S3C). In contrast, p-Smad1 levels were enriched in ICM cells at the ~64-cell stage blastocyst (Fig. 2D right, $p<0.001$, $n = 13$, Fig. S3C). We also compared p-Smad1 levels in manually annotated polar/mural TE cells, using the embryonic axis and ICM to distinguish the two cell types. At the 64-cell stage, polar TE cells had higher p-Smad1 levels than mural TE cells (Figs. 2E, $p<0.001$; see Fig. 2F for spatial distribution of p-Smad1 in a sample embryo). The observation that ICM cells displayed more intense p-Smad1 staining than TE cells (Fig. 2D, E and Fig. S3D) and that *Bmp4* was specifically expressed in the ICM cells (Fig. S1), combined with a study of the mitotic index of different regions of the TE (Copp, 1978), suggests secretion of *Bmp4* from the ICM may influence the mitotic index of TE cells with proximity to a higher local concentration of BMP correlating with higher mitotic index in polar TE *versus* mural TE.

BMP signaling in cell division

To investigate functions of BMP signaling during pre-implantation development, BMP signaling was inhibited using the small molecule BMP antagonist LDN193189. Culture of 8-cell stage embryos in 1 μM LDN193189 for 24 h blocked formation of a blastocoel cavity (Fig. 3A). We verified LDN193189 was blocking BMP signaling with p-Smad1 immunostaining, which was weaker in LDN193189-treated embryos (Fig. 3B, top panels). Similarly, BRE-gal reporter gene activity was also reduced in morula stage embryos treated with LDN193189 (Fig. 3B, bottom panels). We quantified p-Smad1 levels in control ($n = 4$, ~54-cells per embryo) and LDN193189-treated embryos ($n = 8$, ~30-cells embryo) using image segmentation per (see the “Experimental procedures” section). Mean p-Smad1 levels were reduced in LDN193189-treated embryos (Fig. 3C, $p<0.001$).

LDN193189-treated embryos had fewer cells compared to *in vitro*-cultured control embryos (Fig. 3D, $p<0.001$). Two criteria suggest the reduction in cell number after LDN193189-treatment is not due to increased cell death. The first is absence of DNA condensation and nuclear fragmentation, characteristics of apoptosis (White, 1996) in DNA stained embryos treated with 1 μM LDN193189. Second, apoptosis was not detected in embryos treated with 1 μM LDN193189 ($n = 16$) using a TUNEL assay, although at a higher concentration of LDN193189 (2.5 μM), apoptosis could be detected (Fig. S4). These results suggest that inhibition of BMP signaling by 1 μM LDN193189 interferes with normal blastocyst development by reducing mitotic index and not by increasing rate of apoptosis.

We next investigated whether pre-implantation embryos displayed stage-specific sensitivity to LDN193189-mediated inhibition of BMP signaling. Pre-implantation embryos were isolated at different stages (E2.5, E3.0, and E3.5), then cultured *in vitro* for 24 hours in the presence of 1 μM LDN193189 (Fig. 4A). Over 50% of 8-cell embryos (“early morula”) cultured in this manner failed to form normal blastocysts (Fig. 4Bb and Bb′), while late morula and blastocyst stage embryos treated with LDN193189 formed morphologically normal blastocysts (Fig. 4B and C). These results identify an important BMP signaling period in 8-cell early embryos and indicate that exposure to 1 μM LDN193189 does not cause death of the embryo. We further determined that LDN193189 treatment for as little as 12 h was sufficient to affect cell cleavage (Fig. S5A). Embryos treated with lower

LDN193189 concentrations (0.25 μM) displayed similar, albeit weaker, effects (Fig. S5B). We also cultured LDN193189-treated embryos beyond 24 h. While some embryos failed to develop further, the surviving embryos were able to form blastocysts at later stages, suggesting that BMP signaling inhibition causes developmental delay.

Overexpression of a dominant negative BMP receptor blocks cell cleavage

One concern of using chemical inhibitors is possible non-specific effects of the drug—in this case, inhibition of other ALK family receptors. To obtain independent evidence for a function of BMP signaling in early embryonic cell cleavage, we used a genetic approach. A DNA construct (CMV-DNBR-HA) encoding an epitope-tagged dominant negative type I BMP receptor 1a (BMPR1a/ALK3) was generated (Lim et al., 2004). The ability of this construct to affect BMP signaling was tested in mES cells. As with LDN193189 treatment, expression of CMV-DNBR-HA inhibited phosphorylation of Smad1 upon *Bmp4* stimulation, (Fig. S6A, lanes 3, 4). To examine the effects of expressing DNBR-HA in embryos, we microinjected CMVDNBR-HA into a single blastomere of 2-cell stage embryos and analyzed the effect at the 8-cell stage of development (Fig. 5A). The presence of DNBR-HA on the surface membrane of the descendants of injected blastomeres was confirmed by immunostaining (Fig. S6B). The cell number in CMV-DNBR-HA/CMV-β-gal co-injected and control CMV-β-gal injected embryos were quantified after 24 h of *in vitro* culture. Embryos expressing CMV-DNBR-HA/CMV-β-gal had an average of 6.2±0.2 cells, while CMV-β-gal injected embryos had 8.2±0.4 cells. Thus, DNBR-HA expressing embryos have fewer cells than control CMV-β-gal injected embryos (Fig. 5B and C; $p<0.001$), and reduced levels of p-Smad1 staining (Fig. S6C; $p<0.001$). Cell lineage analysis (β-gal expressing cells) also revealed that fewer cells were derived from CMV-DNBR-HA-expressing blas- tomeres (an average of 2.5±0.1 cells) compared to those contributed from the uninjected control blastomere (contralateral side, 3.7±0.2 cells). We used an additional independent method to inhibit BMP signaling in pre-implantation embryos, by removing the zona pellucida using acid Tyrode's followed by incubation of the 8-cell stage embryos with Noggin protein, a BMP antagonist. Noggin treatment also resulted in developmental delays (Fig. 6D). These results provide independent confirmation that inhibition of BMP signaling affects cell cleavage in preimplantation stage embryos.

Time lapse imaging of LDN193189-treated embryos

To better define the cell cleavage defects seen in mouse embryos after the LDN193189 treatment, we performed time-lapse imaging of CAG:H2B-GFP mouse embryos that express GFP tagged histone H2B in the presence and absence of LDN193189 (Kurotaki et al., 2007). Fig. 5E shows the images of CAG:H2B-GFP e1.5 embryos (2-cell stage) after 60 h of *in vitro* culturing with and without LDN193189. LDN193189-treated embryos (bottom panel) develop significantly slower than the untreated embryos (top panel). We quantified the average cell-cycle time of each cleavage, and found that 2nd, 3rd, 4th and 5th cleavages are all significantly delayed in LDN193189-treated embryos (Fig. 5F). Throughout this experiment, no evidence of apoptosis was detected. Hence, LDN193189-treatment appears to cause an increase in cell cycle time without causing apoptosis.

Inhibition of BMP signaling delays ICM and TE cell segregation

We hypothesized that if BMP signaling is required for the blastocyst formation, blockade of BMP signaling by LDN193189 might disrupt ICM and TE cell lineage specification in the embryo. Nanog and Cdx2 positive cells are present in the morula stage embryo, and by the early blastocyst stage they segregate to mark ICM and TE cells (Dietrich and Hiiragi, 2007). We examined whether inhibiting BMP signaling via LDN193189 alters Nanog and/or Cdx2 expression. Embryos at 8-cell stage were treated with 1 μM LDN193189, cultured *in vitro* for 24 h, and subjected to immunofluorescence staining for the transcription factors Nanog and Cdx2 followed by 3D segmentation (Fig. 6A). As noted previously, LDN193189 treated embryos showed delay in cell cleavage (Fig. 6B). Exposure to LDN193189 caused reduction of Cdx2 in outside cells, while Nanog expression was relatively unaffected (Fig. 6C and D). To determine whether the difference in Cdx2 expression could be a consequence of developmental delay, we compared the expression of Nanog and Cdx2 to that found in cell number matched embryos (~30-cell stage embryo, 12 h control). The relative expression levels of Nanog and Cdx2 were similar suggesting that the difference in gene expression between control and LDN193189-treated embryos is due to developmental delay.

Discussion

We have examined the presence of BMP signaling during development of the preimplantation stage mouse embryo and investigated its function during this process. The results indicate that BMP signaling is active as early as the 4-cell stage of development and is required to regulate rate of cell cleavage up to the morula stage.

Results of experiments using three independent methods provide consistent support for the requirement of BMP signaling during pre-implantation development. Inhibition of BMP signaling by LDN193189 using concentrations up to 1 μM interferes with cell cleavage, but does not cause cell death. Expression of a dominant negative BMPR1a/ALK3 in mosaic embryos, and exposure of whole embryos to the BMP-antagonist Noggin cause similar effects on rate of cell division. Based on these findings we postulate that inhibition of BMP signaling via ALK3 reduces cell number in the blastocyst by increasing cell cycle length. How might BMP signaling influence rate of cell division? BMP signaling can directly control expression of *Id* genes, which encode proteins that heterodimerize with basic helix-loop-helix (bHLH) proteins thereby inhibiting their function. Basic HLH proteins have been implicated in regulation of the cell cycle (von Bubnoff et al., 2005; Massari and Murre, 2000; Murre et al., 1989). *Id2* and *Id3* are differentially expressed in preimplantation stage embryos (Tang et al., 2010; Guo et al., 2010) and forced expression of *Id* genes can bypass the mES cells requirement for BMP for self-renewal (Ying et al., 2003). Hence, regulation of the cell cleavage by BMP signaling might be mediated via differential expression of *Id2* and *Id3*.

Interestingly, BMP signaling can also negatively regulate cell division in the early mouse embryo. Epiblast specific loss of ACVR1/ALK2 affects the ability of ventral cells in the node to enter G_0 (Komatsu et al., 2011). Deletion of ALK2 attenuated the level of stabilized $p27^{Kip1}$ cyclin-dependent kinase inhibitor normally required to inhibit nodal cell proliferation and formation of a primary cilium. Together with the results of the present study, this suggests BMP signaling can have opposite effects on cell cycle in the early mouse embryo depending on the specific BMP signaling pathway and cell type involved.

Signaling through BMP receptors can be transduced via Smad4-dependent canonical (Smad1/5/8) pathways and Smad4-independent non-canonical (BMP/TGF-β-activated kinase-1, TAK1) pathway (Yagi et al., 1995; Yamaguchi et al.,1999; Deynck and Zhang, 2003; Moustakas and Heldin, 2005). At present, the relative contribution of these pathways to cell division in preimplantation embryos is not fully understood. Mutation of *Smad4* in mice results in defective gastrulation (Takaku et al., 1998; Chu et al., 2004) although analysis of embryos at preimplantation stages was not reported in these studies. It would be of interest to investigate if *Smad4*-mutant pre-implantation embryos have fewer cells compared to control embryos. BMP signaling might also influence cell division via the TAK1 non-canonical signaling pathway. Smad1 activity can be inhibited by phosphorylation of its linker region by ERK, which results in exclusion of Smad1 from the nucleus (Kretzschmar et al., 1997) and its association with Smurf1 which ubiquitinylates Smad1 leading to its degradation (Fuentealba et al., 2007; Sapkota et al., 2007). TAK1 can maintain Smad1 activity indirectly by inhibiting ERK. The mechanism of inhibition involves p38-dependent activation of a PP2A complex that binds and dephosphorylates MEK and ERK (Westermarck et al., 2001). Interestingly, preimplantation mouse embryos express all four genetic forms of p38 (α,β,δ,γ) and pharmacologic inhibition of p38 α and β forms can reversibly halt mouse embryo development at the 8–16 cell stage but not before (Natale et al., 2004). Hence, BMP signaling, in part via TAK1, might be required for cell division of mouse embryos following compaction.

BRE-gal ES cells respond well to exogenously added BMP stimulation as indicated by strong β-gal activity (Fig. 1A). However, BRE-gal mES cells do not respond to endogenous levels of BMPs present in serum under *in vitro* culture condition. We postulate that this is because BRE-gal mES cells respond to moderate levels of BMP signaling and the amount of BMP signaling present in *in vitro* culture condition is insufficient to provide strong β-gal activity. Consistent with this notion, weak β-gal activity was observed when BRE-gal mES cells were incubated in X-gal substrate for a longer period of time. Additionally, in the monolayer of BRE-gal mES cells, we observed patches of cells showing stronger β-gal activity (Fig. S2B, serum control). These X-gal positive ES cells are likely to represent ES cells responding to high concentrations of local BMP signaling influenced by neighboring cells. In that light, it is interesting to note that X-gal staining of mouse morula stage embryos is significantly stronger than that of *in vitro* cultured BRE-gal ES cells, suggesting that the endogenous level of BMP activity present in mouse morula stage and blastocysts is relatively high.

The available expression data for BMP signaling components during mammalian development suggests that qualitatively different BMP signaling may occur across preimplantation stages. Both Tang et al. (2010) and Wicher et al. (personal communication) observed that the inner and outer cells of morula-stage mouse embryos display differential expression of BMP ligands and receptors. BMP6 and BMP7 are expressed early and their expression declines quickly after fertilization, while *Bmp4* expression becomes enriched in the ICM of developing blastocysts (Coucouvanis and Martin, 1999; Fig. S1) BMP receptor

1a (Bmpr1a/ALK3) and 1b (Bmpr1b/ALK6) are differentially expressed zygotically, whereas the type II receptor *Bmpr2* is expressed exclusively in outside cells (Wicher et al., personal communication). BMP ligands are also known to bind to other ALK family receptors to activate Smad1/5/8 signaling (Roelen et al., 1997; Maserbourg et al., 2005). Based on these expression data, we hypothesize that BMP signaling inputs for ICM and TE cells are mediated via utilization of different BMP ligands and receptors, and thus transcriptional machinery. This model is consistent with our observations indicating the presence of BMP signaling in both ICM and TE cells, but displaying differential transcriptional response toward BRE-reporter gene in ICM and TE cells (Fig. 1). The differential response of ICM and TE cells toward BMP signaling is likely to depend on the utilization of different transcription factors present in ICM and TE cells, which are expected to partner with Smad1/4 differentially.

These instances of qualitatively different BMP signaling during blastocyst formation may not be apparent by examination of p-Smad1 immunostaining, as p-Smad1 staining represents a general readout of overall canonical BMP signaling activity and this approach is unable to distinguish different pathways of BMP signaling. Specifically, different sets of targets can be modulated at different developmental time in different tissues, dependent on the availability of different Smad partners and receptors. It will be important to better define the spatio-temporal expression patterns of various BMP signaling components within the embryo in order to elucidate the roles these components play during preimplantation stage mouse development, a crucial step in the reconciliation and incorporation of BMP signaling pathway data into the current gene regulatory network addressing blastocyst formation in the mouse.

Experimental procedures

Detailed description of materials and methods are described in supplemental information

Embryo acquisition and in vitro culture—Preimplantation stage mouse embryos were collected at desired embryonic day by flushing uterine horns or oviducts with Dulbecco's Modified Eagle Medium (DMEM) with Hepes (10 mM, pH 7.3) media. Embryos were rinsed in flushing and holding media three times before culturing *in vitro* in micro-droplets containing KSOMaa (Life Technologies) and solvent control-DMSO (Sigma Aldrich), which were layered with light mineral oil (Sigma Aldrich) When necessary, LDN193189 (Stemgent) or Noggin (R&D Systems) were included in KSOMaa micro-droplets. Mouse embryos were staged according Gardner (1997).

ES cell culturing, cell transfection, and Western blot—Mouse E14Tg2a ES cells were maintained in GMEM medium (Sigma Aldrich) with 10% FBS (Hyclone) DNBR-HA and pCMV-β-gal constructs were transfected into ES cells using Lipoectamine2000 (Invitrogen). For Western blot analysis, cells were homogenized in RIPA buffer and subjected to gel electrophoresis. Primary antibodies (see Table S2) were anti-p-Smad1 (1:500, Millipore), anti-Smad (1:200, Santa Cruz Biotechnology), anti-HA (1:200, Santa Cruz biotechnology), or anti-Tubulin (1:10,000, Life Technologies).

Quantitative polymerase chain reaction (qPCR)—RNAs were isolated after treatment of E14Tg2a mES cells with hBMP4 (R&D Systems) or LDN193189 (Stemgent) using Trizol (Life Technologies). Reverse transcription was performed using MMLV Reverse Transcriptase according to manufacture's guideline. Quantitative PCR reactions were performed using Sybr-Green (Roche) and the primers listed in Supplemental Materials and methods.

X-gal and immunofluorescence staining—For X-gal staining, E14Tg2a cells were rinsed in pre-warmed PBS, fixed in 0.05% glutaraldehyde at room temperature and incubated in X-gal (10 μg/ml) at 37 °C. Preimplantation stage mouse embryos were fixed in 4% paraformaldehyde, washed, and incubated with X-gal overnight.

For immunostaining, E14Tg2a mES cells were fixed with 3.7% formaldehyde in 1X PBS, rinsed with 1X PBS, and incubated in permeabilization buffer (0.24% Triton-X100) for 15 min at room temperature. After pre-incubation with blocking solution, the cells were incubated with indicated primary antibodies, anti-p-Smad1/5/8 (1:100, Millipore) or anti-SMAD1/5/8 (1:200, Santa Cruz Biotechnology) overnight. Secondary antibodies were goat anti-rabbit-Alexa555 (1:200, Life Technologies) and donkey anti-mouse Cruz647 (1:200, Santa Cruz Biotechnology). Preimplantation stage mouse embryos were rinsed in Acid Tyrode's solution, (Sigma Aldrich), followed by a 30 min fixation with 3.7% formal-dehyde. Primary antibodies used (see Table S2) were anti-p-Smad1/5/8 (1:50 & 1:100, Millipore), anti-Smad1/5/8 (1:200, Santa Cruz Biotechnology), anti-Cdx2 (1:200, BioGenex), and anti-Nanog (1:200, Cosmo Bio USA) Additionally, E14Tg2a mES cells and embryos were stained with Hoechst (2 μg/ml, Sigma Aldrich) and Phalloidin-488 (1:40 dilution, Life Technologies) for nuclear and F-actin staining, respectively. Embryos were placed on a glass slide coated with a 1% agarose pad and compressed to a 3:1 aspect ratio. All confocal images were acquired using a 20 × 0.8 NA (mES cells) and 63 × 1.4 NA (embryos) objective, on an Axioobserver Z1 Zeiss 780 confocal microscope with Zen2009 software. Z-stack images were acquired at 0.3 μm intervals.

Live imaging—Live imaging of H2BeGFP embryos was performed as previously described (Kurotaki et al., 2007). A Leica microscope equipped with Hamamatsu 1K EM-CCD was used.

Fluorescence quantification via image segmentation—We generated segmentation masks for individual nuclei in fluorescence microscopy images of the early mouse embryo. First, we manually annotated cell centers based on DNA images. Cell labels (such as inside/outside/ICM/trophectoderm) were manually assigned at this stage. Second, we preprocessed the DNA image for segmentation by normalizing, blurring, and inverting the image. Third, we generated segmentation masks by running active contours initialized from the annotated cell centers (De Solorzano et al., 2001). Expansion of active contour boundaries was constrained by areas of low intensity in the original DNA image and by collisions with neighbors. A sample segmentation is given in Fig. 2A. Lastly, we quantified p-Smad1/Nanog/Cdx2 concentration by computing the average pixel intensity in individual nuclear segmentation masks.

Embryos in each experiment were imaged in the same session; this enabled direct comparison of fluorescence contents across embryos in the same experiment. In order to verify accuracy of our segmentation procedure, we compared fluorescence contents derived from our procedure *versus* manual segmentations (generated using the Fiji Segmentation Editor; Schindelin et al., 2012) and found close agreement (R^2 of 0.98–0.94, Fig. S3A). In order to minimize the impact of fluorescence attenuation along the z-axis of the image, we only used "top layer" nuclei in subsequent analysis (nuclei whose projection to the top of the stack was at least 10% free of overlap with nuclei closer to the top of the stack): fluorescence from "top layer" nuclei is minimally attenuated since there is a minimal amount of tissue to traverse. In addition, mitotic and polar body cells were annotated based on DNA morphology and excluded from subsequent analysis.

Above, we assay fluorescence levels via concentration (*i.e*., average pixel intensity). An alternative method of assaying fluorescence levels is to compute total content (*i.e*., summed pixel intensities). In 64-cell stage embryos, significant differences in p-Smad1 levels exist in ICM/TE cells when measured via total content. However, these differences are relatively small (7%) compared to results derived p-Smad1 concentrations (27%, Table S1). We speculate that nuclear p-Smad1 concentration may be a better readout of BMP signaling than total nuclear p-Smad1 content.

Data analysis

All p-values were computed using Student's t-test (R studio, Matlab). Images were visualized using ImageJ (NIH http://imagej.nih.gov/ij/).

Dominant negative BMP receptor 1a expression construct and DNA microinjections—Bmpr1aCA-pCIG pRosa26-DEST (gift from Edwin Monuki, Lim et al., 2004) was used to generate DNA encoding a dominant negative BMP receptor (DNBR-HA), which was sub-cloned into pCS2+HA. Subsequently, the DNBR-HA was subcloned into pCMV-β (MacGregor et al., 1991). The human cytomegalovirus (CMV) immediate early promoter (IEP), a splice acceptor–donor region, precedes the DNBR-HA sequence. Lastly, an oligo adapter with the P2A sequence was subcloned into pCS2-mCherry. The P2A-mCherry fragment was cloned into the pCMV-DNBR-HA vector to complete the construct that expresses both DNBR-HA and mCherry under transcriptional control of the CMV IEP. C57BL/ 6NTac 3-week old females were superovulated and embryos were harvested at the 2-cell stage (embryonic day e1.5) (Nagy et al., 2003). DNA fragments were gel purified, passed through a 0.22 μm filter and injected into a single blastomere of 2-cell stage embryos at a final concentration of 1.5 ng/μl total DNA. After microinjection, embryos were washed four times and transferred in KSOMaa media and incubated at 37 °C under hypoxic conditions (90% N_2, 5% CO_2, 5% O_2) in 25 μl microdrops of pre-equilibrated media in a Planer BT37 (Planer PLC, Sunbury-On-Thames, England) until the required developmental stage was reached. Images were collected using Olympus DP70.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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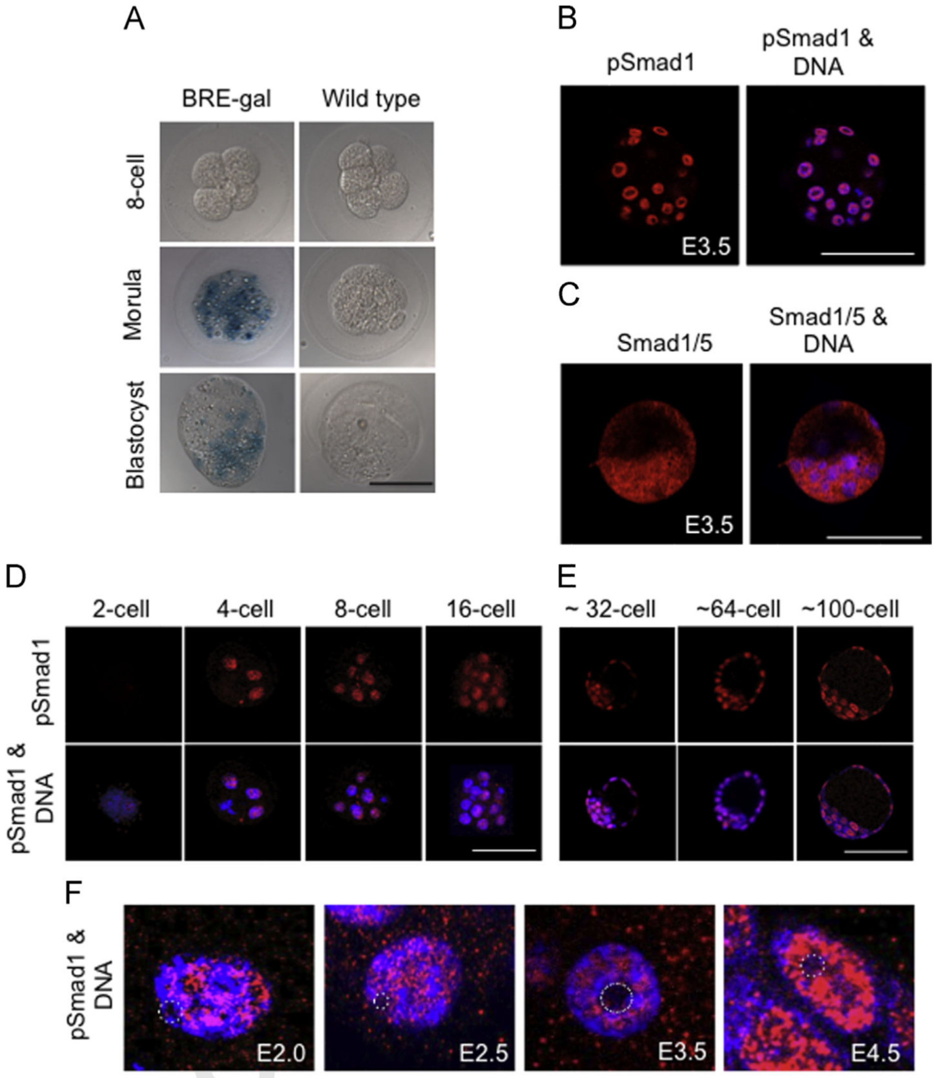

Fig. 1.

Bre-gal reporter activity and location of p-Smad1 in preimplantation mouse embryos. (A) Homozygous BRE-gal and CD1 e3.5 mouse embryos (8-cell, morula, and blastocyst stage) stained for β-gal activity using X-gal. X-gal staining is stronger in the ICM compared to the TE of mouse blastocysts. Non-transgenic (wild type) embryos do not show X-gal staining. (B and C) E3.5 mouse embryos immunostained with p-Smad1 and Smad1 antibodies, respectively. (D and E) Timeline of p-Smad1 activity in developing mouse embryos between E1.5 and E4.5 stages (2–100-cell stages). (F) Nuclei of e2.5-e4.5 mouse embryos stained for p-Smad1 and DNA, nucleoli lacking pSmad1 are indicated by white dotted circles. Scale bar = 100 μm.

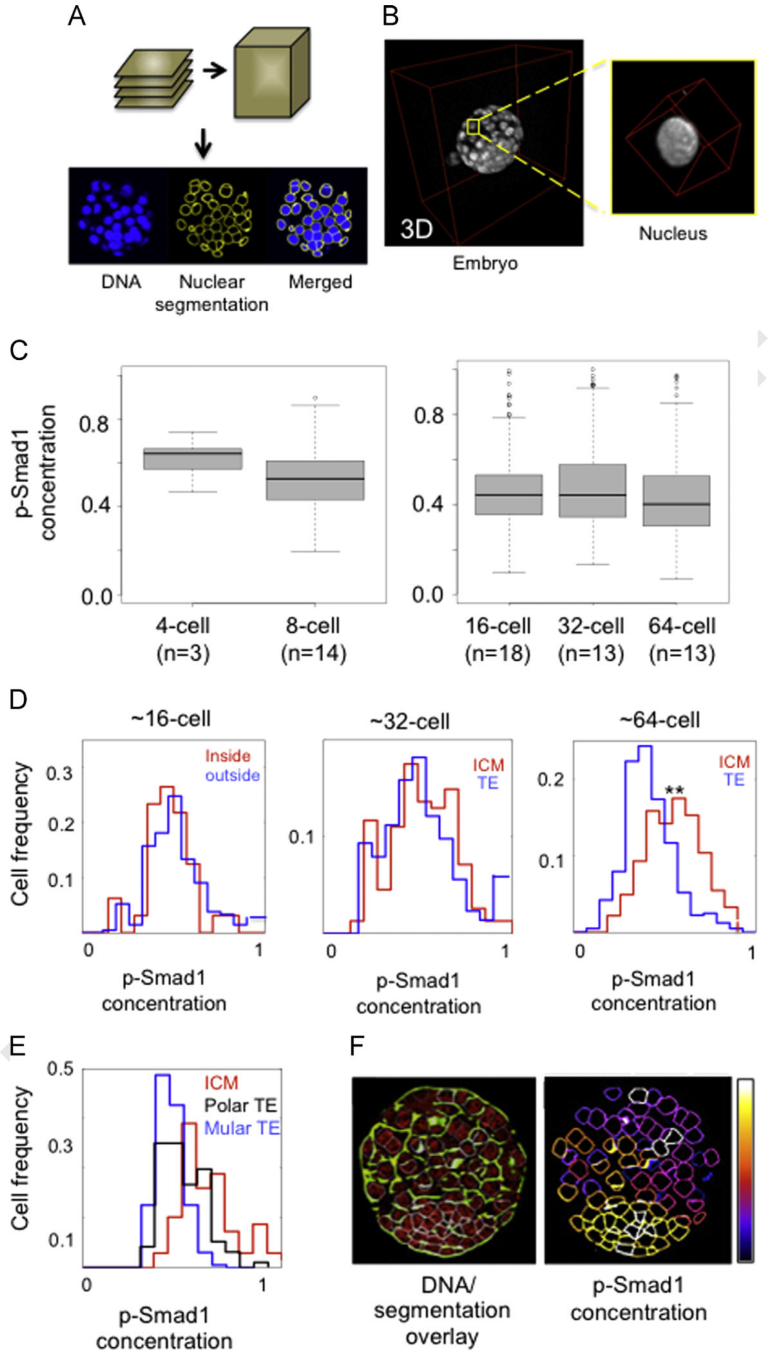

Fig. 2.

Distribution of p-Smad1 in preimplantation embryos. (A) Schematic of data acquisition. Z-stacks of each randomly oriented embryo were collected using 63 × objective at 0.3 μm intervals, and subjected to a 3D segmentation approach. DNA in blue (right), segmented nuclei in yellow (middle), and merged image (right). (B) A 3D image of an embryo after segmentation and a magnified nucleus. (C) Nuclear p-Smad1 concentrations in the blastomeres of pre-morula (4–8-cell) and later stage embryos (~16-, 32-, and 64-cell). (D) Distribution of nuclear p-Smad1 concentrations at 16-, 32- and 64-cell stages. p-

Smad1concentrations in inside and outside cells of 16-cell stage embryos are 0.43 and 0.46, respectively. p-Smad1 concentrations in ICM and TE cells of 32-cell stage blastocysts are 0.47 and 0.45, respectively. p-Smad1 concentrations of ICM cells of 64-cell stage embryos (0.49, $n = 314$ cells) are notably higher than those in TE cells (0.38, $n = 485$ cells, $p<0.001$). (E) p-Smad1 staining levels in ICM ($n = 104$ cells), Polar TE ($n = 142$ cells), and Mural TE ($n = 213$ cells) in ~100-cell stage embryos. (ICM *vs* Polar TE, $p<0.001$; ICM v Mural TE, $p<0.001$; Polar *vs* Mural TE, $p<0.001$). (F) Nuclear p-Smad1 concentration in individual nuclei; ICM is located at the bottom of the embryo shown. Florescence intensity differences were color-coded on segmentation outlines (from low to high: purple, orange, yellow and white). The numerical numbers from 0 to 1 indicate the relative fluorescence intensity range. p-values were derived using Student's t-test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

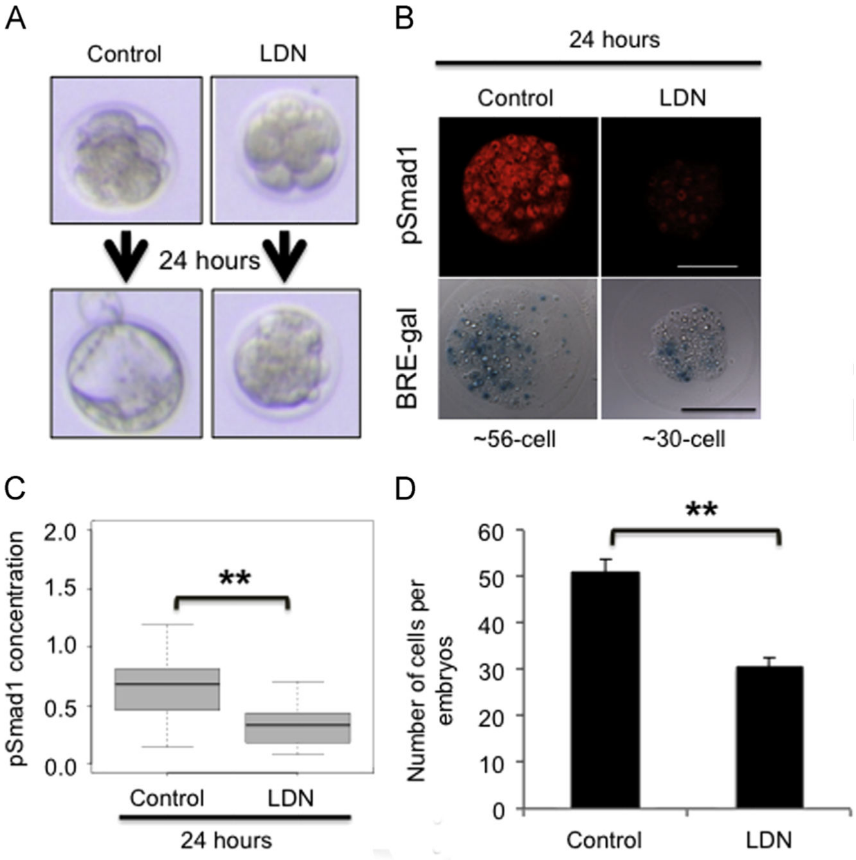

Fig. 3.

BMP signaling inhibition affects cell cleavage. (A) Images of embryos collected at E2.5 (pre-compaction, top panels) and incubated with and without LDN193189 for 24 h (bottom panels). (B) Decrease in p-Smad1 and X-gal staining in embryos cultured in LDN193189 for 24 h. Scale = 100 μm. (C) Quantification of p-Smad1 in control (*n* = 5, mean concentration of 0.66) and LDN193189-treated (*n* = 8, mean concentration of 0.40, *p*<0.001) embryos. The data is reported as nuclear concentration. (D) Decrease in cell numbers in embryos treated with LDN193189. The average numbers of cells are 51±2.5 cells for control (*n* = 33) and 30.6±2 cells at 1 μM LDN193189 (*n* = 38, *p*<0.001).

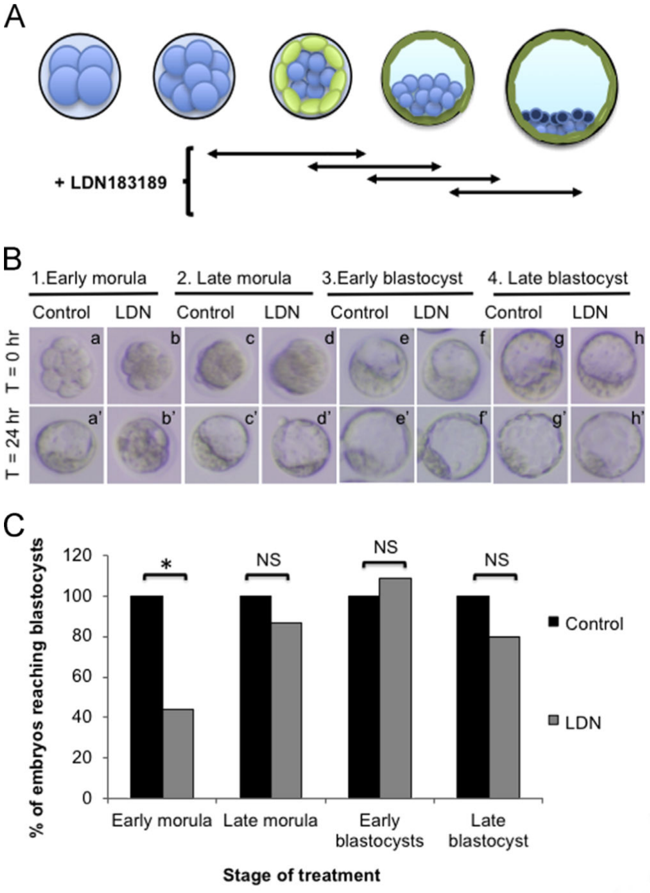

Fig. 4.

Temporal limits of LDN193189 sensitivity. Experimental schematic determining the BMP signaling sensitive period. (B) Embryos at early morula (uncompacted), late morula (compacted), early blastocyst (~32-cell) and late blastocyst (~60-cell) stages before (a–h) and 24 h after LDN193189 treatments (a′–h′). (C) Percentages of embryos reaching blastocysts stage (a and a′) *n* = 37, (b and b′) *n* = 52, (c and c′) *n* = 72, (d and d′) *n* = 60, (e and e′) *n* = 14, (f and f′) *n* = 13, (g and g′) *n* = 48, (h and h′) *n* = 46). *p<0.05 based on Student's *t* test.

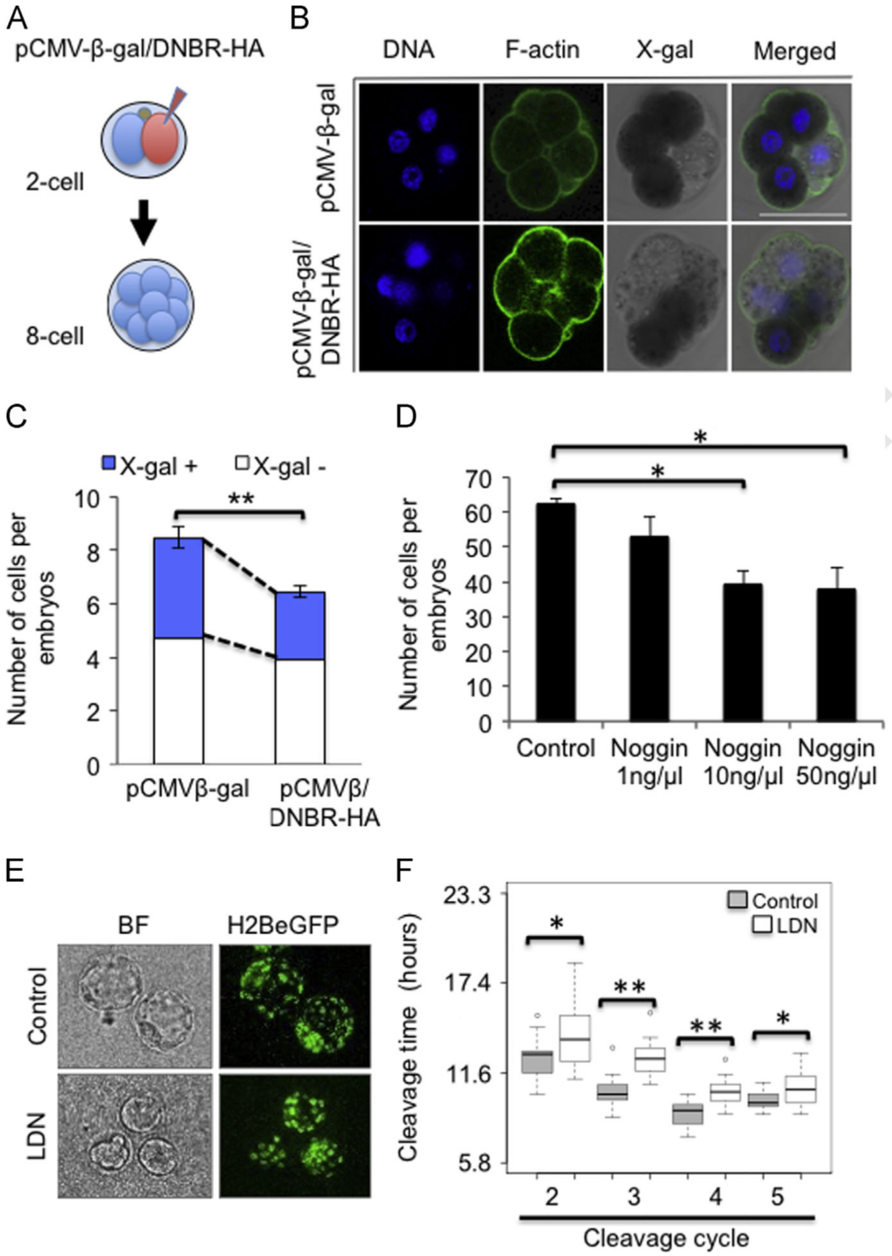

Fig. 5.

Dominant-negative BMP receptor overexpression affects cell cleavage. (A) Schematic diagram showing DNA microinjection experiment. (B) Effects of DNBR-HA expression on cell cleavage. CMV-β gal injected embryos with and without CMV-DNBR-HA were subjected to X-gal staining. Dark grey colored blastomeres in X-gal panels indicate the descendants of CMV-β-gal injected blastomeres. Scale bar = 100 μm. (C) Bar graph showing the effects of DNBR-HA on cell cleavage. Average total cell numbers per embryos after pCMVβ and pCMVβ/DNBR-HA injections are ± 0.4 cells and 6.45 ± 0.2 cells, respectively. The number of X-gal lineage traced blastomeres for pCMVβ and pCMVβ/DNBR-HA are 3.7 ± 0.2 cells and 2.5 ± 0.1 cells, respectively. For pCMVβ (*n* = 27) and for pCMVβ/DNBR-HA(*n* = 40). (D) Decrease in total cell numbers after Noggin treatment.

Total cell numbers after each Noggin treatment are 62.5 ± 1.5 cells (*n* = 3, control),53.1 ± 5.7 cells (*n* = 7,1ng/µl), 39.1 ± 4.1cells (*n* = 8, 10ng/µl), and37.8 ± 6.3 cells (*n* = 9, 50ng/µl). (E) Images of H2BeGPF with and without LDN193189. (F) LDN193189 treatment delays cell cleavage. Cell cleavage lengths were measured for control (*n* = 14) and LDN treated (*n* = 19) embryos at the 2nd, 3rd, 4th, and 5th, cleavage. *$p<0.05$, **$p<0.001$, BF, bright field. Data are averages of ±SEM.

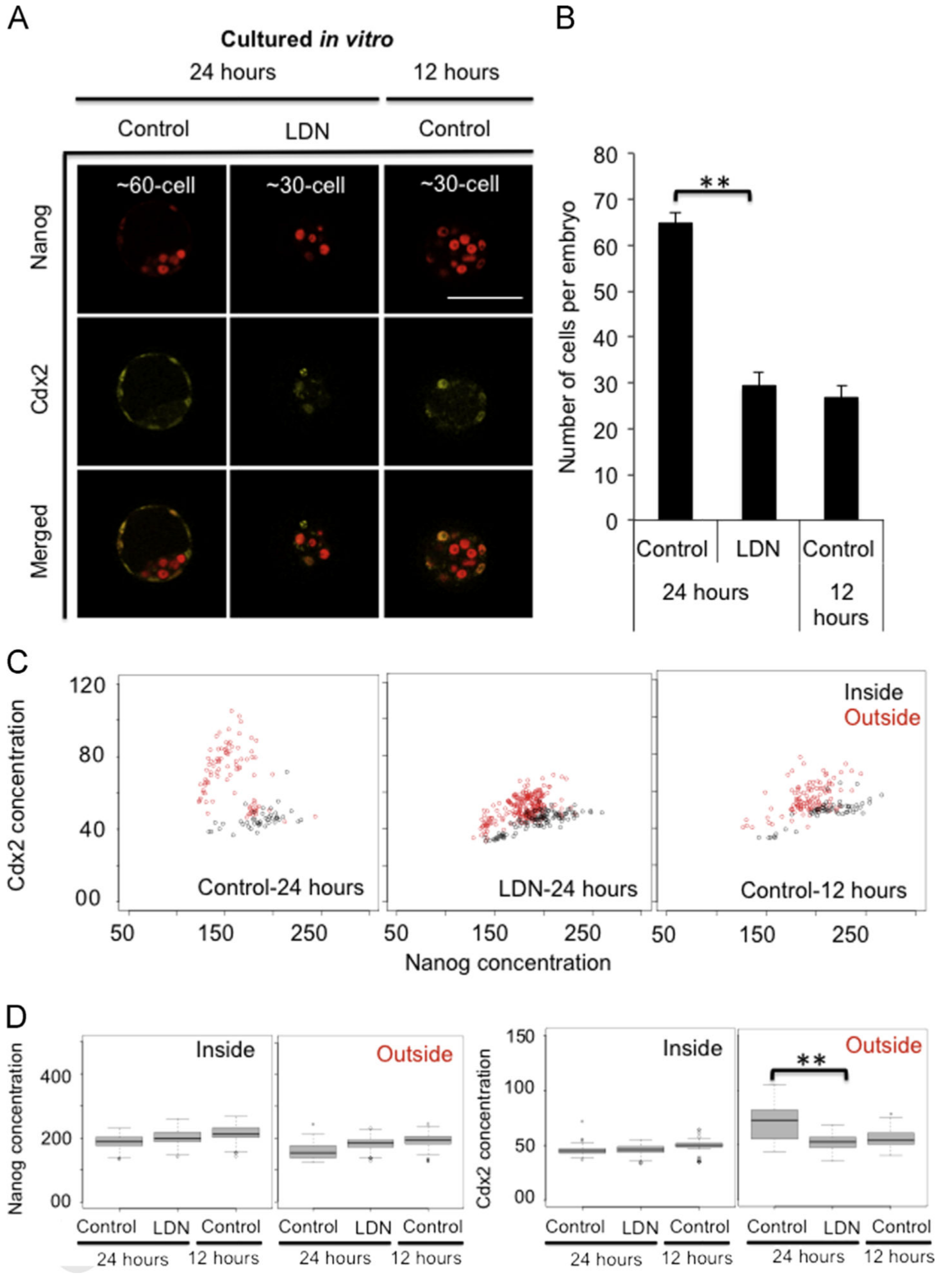

Fig. 6.

BMP signaling inhibition delays blastocyst development. (A) Embryos isolated at E2.5 were cultured *in vitro* with and without LDN193189 for 24 h and stained for Nanog and Cdx2 (left and middle). Cell number-matched control embryos cultured for 12 h (right). (B) The average total cell number of LDN193189 treated embryos (~30 cells per embryo, *n* = 12) is significantly reduced when compared to the embryos cultured for 24 h (~60 cells per embryo, *n* = 4), but is comparable to the 12 h cultured embryos (30 cells per embryo, *n* = 8). (C and D) Scatter plots and box- whisker plots of Nanog and Cdx2 concentrations in individual blastomeres (inside black and outside red) blastomeres. Blastomeres of LDN193189 treated embryos displayed a significant decrease in *Cdx2* concentration (control mean of 72.4 and LDN193189 mean of 52.8, $p<0.001$). Scale = 100 μm, SEM is reported. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)