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BMP signalling is required for extra-embryonic ectoderm development during pre-to-post-implantation transition of the mouse embryo

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ABSTRACT

At implantation, the mouse embryo undergoes a critical transformation which requires the precise spatiotemporal control of signalling pathways necessary for morphogenesis and developmental progression. The role played by such signalling pathways during this transition are largely unexplored, due to the inaccessibility of the embryo during the implantation when it becomes engulfed by uterine tissues. Genetic studies demonstrate that mutant embryos for BMPs die around gastrulation. Here we have aimed to dissect the role of BMPs during pre- to post-implantation transition by using a protocol permitting the development of the embryo beyond implantation stages *in vitro* and using stem cells to mimic post-implantation tissue organisation. By assessing both the canonical and non-canonical mechanisms of BMP, we show that the loss of canonical BMP activity compromises the extra-embryonic ectoderm development. Our analyses demonstrate that BMP signalling maintains stem cell populations within both embryonic/extra-embryonic tissues during pre- to post-implantation development. These results may provide insight into the role played by BMP signalling in controlling early embryogenesis.

KEY WORDS: BMP, Extraembryonic Ectoderm, Trophoblast Stem Cells, Embryonic Stem Cells

INTRODUCTION

On the fourth day after fertilization, the mouse blastocyst implants into the uterus and undergoes growth accompanying with a series of morphological changes leading to formation of an elongated egg cylinder structure. In this process, the initially amorphous epiblast (EPI), transforms into a polarized cup-shaped epithelium located at the distal region of the egg cylinder, whereas the polar trophectoderm (TE) develops into the extra-embryonic ectoderm (ExE) at the proximal region. Both of these tissues will become enveloped by another extra-embryonic tissue, the primitive endoderm (PE)-derived visceral endoderm (VE) (Bedzhov et al., 2014a; Rivera-Perez and Hadjantonakis, 2014; Rossant and Tam, 2009). During this transition, the embryo must maintain its stem cell populations, resident both in embryonic and extra-embryonic compartments in order to permit further development (Boroviak et al., 2014; Hemberger et al., 2020; Kunath et al., 2004). These stem cell populations are present only transiently in early embryos as they quickly differentiate into various somatic cells throughout development, but failure to maintain stemness in these early stages, and premature differentiation may result in loss of lineage specification or tissue growth, and compromise further development.

BMPs are members of the TGF-beta family of signalling components, which transduce signals by binding to complexes of type I and II serine/threonine kinase receptors (Heldin et al., 1997; Massague and Wotton, 2000). Ligand binding induces receptor phosphorylation which then signal via two independent downstream pathways: the canonical pathway through Smad1/5/9, and the non-canonical pathway through p38-MAPK (Miyazono et al., 2010). It has been shown that embryos mutant for BMP signalling are able to develop to the post-implantation stages, however they exhibit developmental abnormalities and fail to gastrulate, and die around E8.5 (Beppu et al., 2000; Chu et al., 2004; Di-Gregorio et al., 2007; Lawson et al., 1999; Sirard et al., 1998). Recently, we demonstrated that BMP signalling regulates the correct pre-implantation development of both extra-embryonic lineages, PE and TE, but not the embryonic lineage (Graham et al., 2014). However, it has not been possible to investigate the role of this pathway during implantation, when the blastocyst transforms into the egg cylinder, due to an embryo inaccessibility as it implants. Here, we utilise a system to culture mouse embryos through implantation stages *in vitro* (Bedzhov et al., 2014b; Morris et al., 2012) to investigate the specific role of BMPs during this pre- to -post-implantation transition. We further exploit mouse embryonic stem cells (ESCs) and trophoblast stem cells (TSCs) to recapitulate EPI and ExE tissue organisation in a 3D *in vitro* platform. Through pharmacologically mediated loss of function experiments, our results show that, importantly, canonical BMP mechanism, but not the non-canonical mechanism, regulates proper ExE development at implantation. Our data suggest that the compromised development of ExE tissue is due to the decreased proliferative capacity of TSC population under BMP inhibition. Although EPI continues correct tissue remodelling at peri-implantation stages, we found that BMP/Smad inhibition promotes accelerated cell death in EPI at early post-implantation onwards. ESCs in 3D culture, mimicking EPI development *in*

vitro, prematurely upregulate expression of differentiation markers in the absence of BMP/Smads, highlighting causes of improper maintenance of EPI tissue *in vivo*. Altogether we demonstrate that canonical BMP activities are required to ensure extra-embryonic tissue development and to maintain embryonic identity during pre- to post-implantation transition.

RESULTS

Inhibition of BMP signalling prevents normal development of extra-embryonic lineages

In order to determine whether BMP signalling is present and play a role in peri-implantation development, we cultured mouse embryos from the blastocyst stage for 48h until they reached the egg cylinder stage in the presence of three different inhibitors of the BMP pathway **(Fig. 1A-B, S1A-D)**. To block canonical (Smad-dependent) BMP pathway, we used both Noggin, a BMP ligand antagonist, and Dorsomorphin, a small molecule chemical inhibitor to selectively inhibit Smad1/5/9 signalling (Graham et al., 2014; Walsh et al., 2010; Yu et al., 2008). To selectively inhibit p38-MAPK signalling and block the non-canonical (Smad-independent) pathway, we used SB203580 (SB) (Anderson and Darshan, 2008; Sozen et al., 2015). When BMP activity was lost, embryos developed clear morphological abnormalities, in line with previous reports (Beppu et al., 2000; Chu et al., 2004; Di-Gregorio et al., 2007; Sirard et al., 1998; Tremblay et al., 2001). Particularly in Noggin and Dorsomorphin-treated groups, embryos displayed disrupted development: compared to control counterparts, embryos were noticeably smaller, contained fewer cells, and the percentage of embryos that developed to egg cylinder stage was significantly reduced **(Fig. 1C-E, Supplementary Movies 1-3)**. Our quantification of cell numbers in all lineages at the early post-implantation stage of development (48h of *in vitro* culture), showed that the loss of canonical BMP activity caused significant reductions in cell number across all lineages in a dose-dependent manner, with losses being most pronounced in ExE and VE lineages **(Fig. 1F, G)**. However, blocking non-canonical BMP activity through SB treatment incurred much milder reductions in cell numbers that were not significant **(Fig. 1H)**. After careful examination of developing embryos treated with Dorsomorphin, we observed a range of phenotypes as assessed by ExE tissue size: (i) decreased ExE (between 50-100µm in length along the proximal-distal axis; 45%), (ii) poorly defined ExE (less than 50µm in length; 35%), and (iii) a complete lack of ExE (0µm; 20%) **(Fig. 2A, B)**. Embryos in the third category consisted of only EPI and surrounding VE layer **(Fig. 2A, B)**.

Both TE and ExE contain TSCs which can be isolated from these tissues and grown *in vitro* (Tanaka et al., 1998; Uy et al., 2002). The proliferative potential of both of these tissues is dependent on this TSCs population and a failure to maintain these stem cells would be expected to affect ExE formation (Tanaka et al., 1998). We therefore next investigated whether the absence of BMP might affect stem cell organisation. After analysing the active BMP signalling in TSCs by immunoflourescence and qRT-PCR **(Fig. 2C, D),** we embedded TSCs in 3D Matrigel (to mimic basal membrane normally provided in the embryo and surrounds ExE compartment), in conditions required for TSC maintenance (Ohinata and Tsukiyama,

2014; Tanaka et al., 1998), and then added Noggin (50ng/mL), Dorsomorphin (0.1µM/mL); or SB (0.5µM/mL) for 72h **(Fig. 2E-G).** We found that, like BMP-inhibited embryos, organisation of TSC structures without BMP signalling was variable. Specifically, 40% of structures cavitated with decreased cell numbers, 30% did not cavitate and were poorly organised (30%), and 30% of structures contained dying cells (identified by pyknotic and fragmented cell nuclei) **(Fig. 2H, I)**. This range of phenotypes parallel those observed in the ExE compartment of post-implantation embryos following BMP inhibition **(Fig. 2A-B)**, indicating that TSC provides an *in vitro* model to mimic ExE phenotypes. Together, these results indicate an important involvement of canonical BMPs in pre- to post-implantation transition and specifically in the development of the extra-embryonic tissue.

Disrupted ExE tissue development after BMP suppression is a result of the decreased stem cell proliferation

Our findings of impaired development of ExE with the decreased number of cells, first directed us to investigate the expression of molecular markers responsible for ExE lineage identity in the absence of BMP signalling. We assessed transcription factors critical to ExE specification, and also required for maintenance of TSCs during the pre- to post-implantation transition including Eomes, Cdx2, Tfap2c and Elf5 (Latos and Hemberger, 2014; Latos et al., 2015; Lee et al., 2016). We did not observe a significant change in the expression of these transcription factors in the ExE lineage following the 48h of any inhibitor treatments used, compared to the controls **(Fig. S2A, B)**. 3D TSC structures demonstrated similar outcome when analysed for same markers **(Fig. S2C, D).** To investigate specifically, we transfected TSCs with lipofectamine/siRNA complexes and cultured the cells for 48h to knocked down *Smad5* in TSCs by siRNA and block intracellular BMP/Smad activity **(Fig. S2E, F)**. Upon phosphorylation by BMP receptors, R-Smads 1, 5, 8 form complexes with Smad4 and translocate to the nucleus. Abrogation of any one of the R-Smads functionally disrupts Smad-signalling **(Fig. S2E)** (Nishimura et al., 2003). After *Smad5* KD*,* although the expression levels of core TFs in TSCs were decreased, it was not significant **(Fig. S2G)**. These results suggested that BMPs may not directly control cell identity at implantation.

We next sought to investigate other possible underlying reasons leading to tissue lost at implantation. It is known that stem cells possess remarkable proliferative capacity. Dysregulation or loss of the proliferation ultimately leads to stem cell depletion which then can lead to distrusted tissue maintenance (Orford and Scadden, 2008). Promoted by our observation that BMP inhibition disrupts tissue development, we analysed proliferation in both ESCs and TSCs structures grown in Matrigel, as *in vitro* models mimicking EPI and ExE development, respectively. These 3D structures were assessed for the expression of Phospho-Histone-H3 (H3S10-P) protein, a marker of proliferation (Chadee et al., 1999; Strahl and Allis, 2000). No changes were observed in ESC structures **(Fig. 3A, B)**, but a significant reduction of H3S10-P expressed cells was detected in TSC structures following BMP inhibition **(Fig. 3C, D)**. When we analysed embryos developed *in vitro*, no difference was detected between control and inhibitor-treated embryos in terms of the average percentage

of cells positive for H3S10-P **(Fig. 3E, F)**. However, time course analysis of change in cell number in EPI and ExE lineages showed significant reduction of cell numbers in ExE lineage **(Fig. 3G)**. This suggests cell cycle length is proportionally longer in BMP inhibited embryos, which ultimately leads to less growth in the ExE **(Fig. 3G)**. Overall, this supports our previous observation of impaired growth in ExE lineage in embryos.

Absence of BMP leads to increased cell death in the EPI lineage at post-implantation

Since the decreased cell number in ExE tissue can also occur as a result of increased cell death, we analysed this possibility in embryos grown in the absence of BMP signalling. First, we let embryos to develop in the presence of 500ng/ml^{-1} of Noggin, 1µM/mL of Dorsomorphin or 10µM/mL SB from blastocyst to egg cylinder stage and examined the expression of cleaved-caspase-3, an apoptotic cell marker in each developing tissue **(Fig. 4A)**. After analysing three consecutive stages representing peri- and early post-implantation, no significant increase for the average percentage of cells expressing cleaved-caspase-3 was observed neither in VE nor ExE tissues at any time point **(Fig. 4B)**. This lends credence to the hypothesis that the absence of canonical BMP activity drives a prolonged cell cycle length, ultimately leading to impaired tissue growth **(Fig. 3G).**

Embryo development requires coordinated morphogenesis between embryonic and extra-embryonic lineages. When cleaved-caspase-3 expression was analysed in EPI lineage, we found no significant difference in early time points of peri-implantation development in the absence of canonical BMPs **(Fig. 4A, B).** Notably, the most significant increase in cell death was found in the EPI at the post-implantation time point (4.4-fold for Noggin, 10.6-fold for Dorsomorphin, 2.2-fold for SB, compared with control embryos) **(Fig. 4B)**. To verify these results, we filmed the development of control and Noggin-treated embryos under time-lapse microscope during pre- to post-implantation transition, in the presence of SYTOX, a fluorescent reporter of cell death, and visualized the dying cells in real-time (Bedzhov and Zernicka-Goetz, 2014; D'Sa-Eipper et al., 2001). We observed SYTOX-positive cells were mainly concentrated in the EPI tissue of the developing egg cylinder after Noggin treatment **(Fig. 4C)**. Moreover, ESCs embedded in 3D Matrigel, mimicking the EPI tissue morphogenesis, also showed increased cell death after dorsomorphin treatment **(Fig. 4D)**.

Overall, our analyses indicate divergent roles for canonical BMP signalling in ExE versus EPI development. While BMP signalling tunes the rate of cell-cycle in the ExE, its inhibition promotes cell death in the EPI, indicating a pro-survival role.

Loss of BMP/Smad signalling induces cell differentiation in 3D ESC rosettes

At the time of embryo implantation, EPI cells transit through distinct pluripotent states and become primed for differentiation. Naïve pluripotency is established in the non-polar EPI cells of the blastocyst (Nichols and Smith, 2009). Upon implantation, cells in the EPI lineage polarize and a central lumen forms (Bedzhov and Zernicka-Goetz, 2014). This morphological remodelling of the EPI at pre-to-post-implantation transition can be recapitulated in vitro

using cultured ESCs embedded in Matrigel, termed ESC rosettes (Bedzhov and Zernicka-Goetz, 2014; Shahbazi et al., 2017). Exploiting this *in vitro* model, we first asked whether BMP signalling plays a role in EPI morphogenesis. To do this, we analysed self-organization and lumenogenesis of ESC spheres grown in Matrigel for 48h with podocalyxin, a marker for polarisation and lumen formation **(Fig. 5A)**. After BMP inhibition, all ESC structures showed polarized organization with a central cavity, similar to control **(Fig. 5A)**. We next cultured blastocysts derived from CAG:GFP line that exhibits GFP in the membrane (Rhee et al., 2006) during pre- to post-implantation transition and filmed their development with confocal microscopy. As the blastocysts proceeded through post-implantation *in vitro*, EPI cells become polarised and displayed cavity formation upon Dorsomorphin treatment in spite of severe failure in ExE formation **(Fig. 5B)**. These results indicated that EPI morphological remodelling during pre- to post-implantation development is not affected by the BMP activity.

Despite unaffected EPI morphogenesis, in order to further explore whether the loss of BMP signalling influences fate dynamics and differentiation, we analysed the molecular signature in EPI-like ESC rosettes. Stem cell culture conditions in the presence of GSK3B and MEK inhibitors, and the leukemia inhibitory factor (called 2i-LIF), allow self-renewal and reduce intercellular heterogeneity to maintain ESCs in a naïve pluripotent state (Ying et al., 2008). Standard 2D-culture on gelatin-coated plates, in the absence of 2i-LIF has previously been used to induce differentiation in ESCs (Boroviak et al., 2014). Thus, we used gelatin group as a positive control to induce differentiation in ESCs, and to compare them with the EPI-like ESC rosettes grown in our 3D Matrigel protocol. We first analysed expression of *Id* genes, downstream targets of *Smads* and also known for maintaining stemness in ESCs (Li and Chen, 2013). We first confirmed that Id genes were downregulated in ESCs grown on gelatin as a result of induced differentiation **(Fig. 5C)**. Id genes were highly expressed in ESC rosettes grown in Matrigel and found to be decreased when BMP signalling was inhibited, as expected after loss of BMP activity **(Fig. 5C)**. Further assessment with marker genes associated with early differentiation of the EPI, including *Acsl4, Dll3, Fgf5* and *Otx2* (Ghimire et al., 2018), showed significant increase in ESC rosettes in the absence of BMP **(Fig. 5C)**. These results indicate that blocking the BMP/Smad activity results in activation of early differentiation genes in pluripotent cells. This observation is in line with previous studies demonstrating BMP signalling promotes DNA hypo-methylation in ESC culture (Gomes Fernandes et al., 2016). The present experiments also suggest that the premature differentiation in embryonic lineage may be associated with increased cell death following post-implantation stages.

DISCUSSION

It is well known that elimination of individual components of the BMP signalling pathway prevents development beyond implantation (Beppu et al., 2000; Chu et al., 2004; Sirard et al., 1998; Yang et al., 1998). These BMP mutant embryos show reduced size and tissue disorganisation at early post-implantation stages and eventually die around

gastrulation (Beppu et al., 2000; Chu et al., 2004; Di-Gregorio et al., 2007; Lawson et al., 1999; Sirard et al., 1998). However, the cause of this dysregulation has remained elusive, with the cell physiological events downstream of the disruption to BMP signalling being not fully investigated. There have been major experimental barriers to studying development across the peri-implantation period due to the small size of embryos at this stage and their inaccessibility during implantation. In recent years, these barriers have largely been lifted with the emergence of *in vitro* culture methods (Bedzhov et al., 2014b; Bedzhov and Zernicka-Goetz, 2014; Morris et al., 2012) and stem cell models (Beccari et al., 2018; Bedzhov and Zernicka-Goetz, 2014; Desbaillets et al., 2000; Harrison et al., 2017; Morgani et al., 2018; Sozen et al., 2018; van den Brink et al., 2020; Warmflash et al., 2014). Here we use these approaches to directly investigate the role of this critical signalling pathway during the blastocyst to egg cylinder transition utilizing complementary loss of function approaches. We find that in three BMP depletion strategies, blocking canonical BMP activity severely impaired the growth of ExE tissue. Our studies suggest that BMP signalling does not affect embryo remodelling at implantation, but is required for maintaining both embryonic and extra-embryonic tissue development soon after implantation **(Fig 5D)**.

Lineage-specific stem cells are maintained in small numbers by specialized niches (Rossant, 2007). Self-renewal of these stem cells is vital for tissue maintenance and growth, generating progressively specialized progeny that ultimately yield the fully differentiated cells of the adult. Blastocyst-derived lineage-specific stem cells mimic mouse post-implantation tissue and embryo organisation *in vitro* as shown previously by us and others (Beccari et al., 2018; Harrison et al., 2017; Sozen et al., 2018; ten Berge et al., 2008; van den Brink et al., 2020; van den Brink et al., 2014). In this study, we aimed specifically to investigate the consequences of BMP signalling on tissue organisation in their individual 3D stem cell-based platforms. To this end, we used ESCs and TSCs embedded in 3D Matrigel, which largely recapitulates the *in vivo* environment and provides a simplified platform to study the impacts of autocrine signalling on embryo tissues. To investigate how ExE cells become decreased in number following BMP inhibitor treatment, we examined the embryos and TSCs in 3D culture for their proliferation capacity. The TSC niche in the mouse is present within the polar TE before implantation, whereas after implantation the ExE fulfils this role. Correct proliferation, differentiation and apoptosis of this extra-embryonic lineage is fine-tuned in response to cellular signalling, regulating stem cell maintenance and thereby tissue organisation (Tanaka et al., 1998). Correct proliferation, differentiation and apoptosis of this extra-embryonic lineage is fine-tuned in response to cellular signalling, regulating stem cell maintenance and thereby tissue organisation (Tanaka et al., 1998; Uy et al., 2002). Following BMP inhibition, we found evidence for decreased proliferation in TSCs in 3D culture, as judged by the mitotic index measured by H3S10-P staining. This indicates the absence of ExE is a consequence of the lack of TSC maintenance. Although BMP-depleted embryos showed a similar proportion of H3S10-P-positive cells as controls, they had less ExE cells in total. Less ExE cells was not due to differences in cell death (Fig. 4), but likely due to differences in cell proliferation rate. Given these findings, we propose that the improper development of ExE

in the absence of BMP activity is due to the loss of proliferative potential in the TSC population. Further analyses of proliferation dynamics are required to fortify this proposal.

The ExE is not only essential for mammalian extra-embryonic placental formation, but also crucial for the survival of the embryo proper in utero. Previous reports stress a role for Bmp4 from the ExE in acting as a paracrine factor essential for EPI development after implantation, and for further patterning at gastrulation (Winnier et al., 1995; Yang et al., 1998). We therefore suggest a requirement for BMP signalling in the EPI possibly through providing the physiological machinery to regulate autocrine-paracrine signal exchange between embryonic and extraembryonic tissues which would be ultimately essential in maintaining the embryonic EPI. However, given the limitations of our study, by which BMP inhibition is applied to the entire conceptus, targeted elimination of signalling activity in ExE tissue and its effects to the EPI remain to be determined. Additionally, beyond survival effects, our results indicate a role for BMP in regulating the cellular differentiation, with a lack of signalling promoting early differentiation of the 3D ESC rosettes. This supports previous observations on BMP signalling through Smads modulates lineage priming in ESCs in 2D culture (Gomes Fernandes et al., 2016). This stage specific effect of BMPs on EPI development suggests that in earlier developmental stages, Bmp/Smad activity is dispensable for the maintenance and survival of the EPI lineage, but that it might regulate differentiation. As development progresses, ectopic EPI cell death follows, possibly heralded by decreased development of the extra-embryonic tissues.

The potential differences between Dorsomorphin, a small molecule, and Noggin, an extracellular ligand, were previously reported in different contexts, showing that small molecules permit more precise temporal control over BMP signalling, while Noggin shows slow effectiveness (Hao et al., 2008). Although we observed similar phenotype after both Noggin and Dorsomorphin treatments, the effect on the embryo growth appeared more pronounced upon Dorsomorphin treatment (Fig. 1C, G, H). This effect could reflect intrinsic differences between the small molecules and protein-based antagonists, and possibly results from the ability of the small molecule to rapidly penetrate the multiple cell layers of the embryo, while Noggin may not infiltrate cell layers as effectively. That said, a caveat to consider with pharmacologically mediated loss and gain of function experiments is the potential off-target effects (Strahle and Grabher, 2010). In addition, it was reported by some that Smad1/5/9 phosphorylation is absent in the ExE (Di-Gregorio et al., 2007; Yamamoto et al., 2009), while others support the notion that BMP signalling is active in extraembryonic tissues (Coucouvanis and Martin, 1999; Javier et al., 2012; Tremblay et al., 2001). Given that BMP receptors are expressed in the ExE (Mishina et al., 1995; Pijuan-Sala et al., 2019), a functional role of BMP signalling in this tissue before gastrulation is likely (Kishigami and Mishina, 2005). Nevertheless, we cannot rule out possible indirect effects of BMP signalling and/or inter-lineage crosstalk for the phenotypes observed in this study. Further studies using knock-out strategies for specific BMP components, or the development of small molecules which are more selective for BMP signalling will be essential to clarify this point.

Although the exact mechanism by which disrupted ExE formation is not fully determined, our findings provide new insights into pre-to-post implantation developmental dynamics and suggest a critical role for BMP signalling in regulating this process.

In conclusion, our results suggest multiple tissue-specific roles for BMP signalling during peri-implantation mouse embryo development. Our data indicated that BMP function within the ExE to maintain its proliferation via its TSCs niche; and in the EPI to mediate cell identity and survival. Overall, the observed defects in stem cell populations in both embryonic and extra-embryonic lineages result in abnormal tissue development at implantation/early post-implantation and may explain the consequent developmental failure at later developmental stages as described previously. Thus, combining analyses of *in vitro* cultured embryos and stem-cell models, has allowed us to uncover these early phenotypes, which may have been masked by the influence of maternal factors in the oviduct and uterus in *in vivo* studies.

FIGURE LEGENDS

Fig. 1. Blocking BMP activity in embryos at pre- to post-implantation transition (A) Protocol for peri-implantation culture and development in mouse. Time scale above: development *in vivo*; below: equivalent developmental timings *in vitro*. **(B)** A representative image of E4.75 blastocyst stage embryo at 0h of IVC, and *in vitro* developed egg cylinder at 48h of IVC. Asterisks mark EPI. **(C)** Morphological appearances of *in vitro* cultured embryos in each condition. Oct4 for EPI; Gata4 for VE. **(D)** Egg cylinder phenotypes 48h after Noggin treatments. Nog 300, 300ng/ml^{-1}; Nog 500, 500ng/ml^{-1}; Nog 800, 800ng/ml^{-1} **(E)** Developmental frequency for blastocyst developed to egg cylinder in 48h of IVC culture. Nog 300, 300ng/ml^{-1}; Nog 500, 500ng/ml^{-1}; Nog 800, 800ng/ml^{-1}; Dorso 0.5, 0.5µM/mL**;** Dorso 1, 1µM/mL; SB 5, 5µM/mL; SB 10, 10µM/mL**. (F-H)** Relative number of cells in each lineage compared with controls (n=30) in embryos treated with **(F)** Noggin (300ng/ml^{-1}, n=25; 500ng/ml^{-1}, n=32; 800ng/ml^{-1}, n=22), **(G)** Dorsomorphin (0.5µM/mL, n=29; 1µM/mL, n=36) **(H)** SB203580 (5µM/mL, n=34; 10µM/mL, n=36), respectively. **P*<0.05, ***P*<0.01, ****P*=0.001 (Student's t-test). All error bars=SEM. All scale bars=20µm.

Fig. 2. Impaired ExE development a result of the lack of TSCs maintenance. (A) Phenotypes of egg cylinders 48h after Dorsomorphin treatments (i) decreased ExE region (top), (ii) poorly defined ExE region (middle), (iii) no ExE region at all (bottom). White dashed lines mark outline the ExE compartment where the measurement was performed. **(B)** Percentages of each phenotype of ExE observed *in vitro* cultured egg cylinders 48h after Dorsomorphin treatment (0.5µM/mL). At least 20 embryos analysed per group. **(C)** TSCs were cultured on 2D-monolayer-culture conditions under serum-free (defined) or serum-containing conditions. Scale bars=20µm. Quantification on the right shows P-Smad1/5/9 immunofluorescence intensity in TSCs. P-Smad1/5/9 expression in TSCs found increased in Serum-containing condition and upon BMP4 treatment. P-Smad1/5/9 intensity was

normalised to the DNA-channel (DAPI) and a mean average was taken. ***P=0.001 (ANOVA test), n=35 per group. Error Bars= SEM. (D) qRT-PCR analysis of the expression of BMP signalling target genes (Id1, Id2, Id3) in TSCs grown in different conditions. **P<0.01, ***P=0.001 (Student's t-test) (3 separate experiments). Error bars=SEM. **(E)** Scheme of protocol to 3D-embeded TSCs in Matrigel. **(F)** An example of polarised TSCs structure 48h after control and dorsomorphin (0.1µM/mL) conditions. **(G)** Bmpr1 expression in polarised TSCs structure **(H)** Phenotypes of TSC structures in 3D 72h after Dorsomorphin treatments (0.1µM/mL; n=50): (i) cavitated structure with decreased cell number (left) (ii) non-cavitated and poorly organised structures (middle), and (iii) cells undergoing cell death (right). **(I)** Percentages of each phenotype of TSC structures observed 72h after Dorsomorphin treatment in Matrigel (top) and number of cells per structure (bottom). *P<0.05, **P<0.01, ***P=0.001 (Student's t-test). All error bars=SEM. All scale bars=20µm.

Fig. 3. Proliferation assessment of 3D ESC/TSC structures and *in vitro* cultured embryos. **(A, B)** Quantification showing the number of cells positive for H3S10-P expression in ESC structures and **(C, D)** TSC structures. At least 20 structures analysed for per group. ***P*<0.01 (Student's t-test). **(E)** Expression of H3S10-P in embryos developed *in vitro*. **(F)** Quantification showing the number of cells positive for H3S10-P expression in each lineage in control (n=9), Noggin (500ng/ml^{-1}, n=10), dorsomorphin (1µM/mL, n=10), SB203580 (5µM/mL, n=9) treatment for 48h. Error Bars= SEM. Scale bars=20µm. **(G)** Time-course quantification of cell number in EPI (Oct4) and ExE (Tfap2c) lineages in the developing embryo. Graphs show relative number of cells in each lineage after 24, 36 and 48h IVC compared with controls in embryos treated with **(F)** Noggin (500ng/ml^{-1}), **(G)** Dorsomorphin (1µM/mL) **(H)** SB203580 (5µM/mL), respectively. N=6 per group. **P*<0.05, ***P*<0.01, ****P*=0.001 (Student's t-test). Error bars=SEM.

Fig. 4. Cell death in BMP-inhibited embryos. (A) Cleaved-caspase-3 expression at 24h, 36h and 48h time-points during pre-to-postimplantation development in the presence of Noggin (500ng/ml^{-1}), Dorsomorphin (1µM/mL) or SB203580 (5µM/mL). **(B)** Quantifications showing the percentage of cells positive for cleaved-caspase-3 expression in each lineage at 24 (top), 36 (middle), 48h (bottom) in control and inhibitor treatments (at least 6 embryos analysed per group) **P*<0.05, ****P*=0.001 (ANOVA followed by Tukey test). Error bars=SEM. **(C)** Still images of time-lapse recording of an embryo forming egg-cylinder *in vitro*. Dying cells are marked by SYTOX, a red cell death reporter (control n=11; Dorsomorphin n=13). White dashed-line marks outline of the embryo, yellow dashed-line marks the site of the emerging proamniotic cavity. Scale bars=20µm. **(D)** Cleaved-caspase-3 expression in 3D ESC rosettes in control and dorsomorphin treated group. Quantification shows cleaved caspase-3-positive apoptotic cells in the ESC rosettes (n=28 per group). *****P*=0.0001 (Student's t-test). All error bars=SEM. All scale bars=20µm.

Fig. 5. BMP inhibition does not affect EPI morphological remodelling but triggers cell differentiation in ESC rosettes. (A) Formation of polarized rosettes in control versus BMP-

inhibitor treated ESCs cultured in 3D Matrigel. The rate of self-organization in ESCs was examined at 24, 36, 48h. Scale bars=20µm. **(B)** Still images of time-lapse recording of *in vitro* cultured CAG-GFP* embryo. Note that a single cavity emerges from the center of the EPI rosette, marked by yellow dashed line. Scale bars=20µm. *CAG promoter-driven transgenes become silenced in extraembryonic lineages in this particular line (Rhee et al. 2006; Griswold et al. 2011, Abe and Fujimora, 2013, Bedzhov and Zernicka-Goetz, 2014). **(C-D)** qRT-PCR analysis of *Id* genes and differentiation-related genes (*Acsl4, Dll3, Fgf5, Otx2*) in ESCs rosettes. *P<0.05, **P<0.01, ***P=0.001 (ANOVA followed by Tukey test) (5 experiment per group). Error Bars= SEM. **(E)** Illustration shows roles of BMP signalling in pre-to-post-implantation development.

MATERIALS AND METHODS

Embryo culture through peri-implantation

Embryos were cultured as described in Bedzhov et al, 2014 (Bedzhov et al., 2014b). Blastocysts were recovered from the mother at 4.5 days *post coitum* by uterine flushing with M2 medium. Recovered blastocysts then had their mural trophectoderm manually dissected away, before blastocysts were plated in ibiTreat microscopy plastic µ-plates (Ibidi) and cultured in IVC1 medium (Advanced DMEM/F12 supplemented with 20% heat-inactivated FBS, 2 mM l-glutamine, penicillin-streptomycin (25 µg/ml), 1× ITS-X (10 mg/l insulin, 5.5 mg/l transferrin, 0.0067 mg/l sodium selenite and 2 mg/l ethanolamine), 8 nM β-estradiol, 200 ng/ml progesterone and 25 µM N-acetyl-l-cysteine). After 24h in culture, the medium was changed to IVC2 medium (Advanced DMEM/F12 supplemented with 30% KSR, 2 mM l-glutamine, penicillin-streptomycin (25 µg/ml), 1× ITS-X (10 mg/l insulin, 5.5 mg/l transferrin, 0.0067 mg/l sodium selenite and 2 mg/l ethanolamine), 8 nM β-estradiol, 200 ng/ml progesterone and 25 µM N-acetyl-l-cysteine).

Post-implantation embryo recovery and culture

4- to 6-week-old F1 mice from the C57Bl6/CBA crosses were naturally mated and sacrificed at midday after 5 days *post-coitum*. The uterus was recovered and embryos were manually dissected from deciduae in M2 medium using fine forceps. Following the recovery embryos cultured in IVC2 medium for 24h.

Stem cell culture

ESCs were cultured at 37˚C and 5% CO2 on gelatinized tissue-culture grade plates and passaged once confluent. Cells were cultured in 'ES medium' (DMEM with 15% FBS, 2mM L-glutamine, 0.1mM 2-ME, 0.1mM NEAA, 1mM sodium pyruvate, and 1% penicillin-streptomycin) supplemented with PD0325901 (1uM), CHIR99021 (3uM) (2i) and leukaemia inhibitory factor (0.1mM, LIF)). TSCs were cultured at 37˚C and 5% CO2, in TS medium (RPMI 1640 (Sigma) with 20% FBS, 2mM L-glutamine, 0.1mM 2-ME, 1mM sodium pyruvate, and 1% penicillin-streptomycin), plus FGF4 (Peprotech) and heparin (Sigma)) in the presence of

inactivated DR4 MEFs. For the purpose of immunoflourescence staining on 2D-monolayer-cell-culture and qRT-PCR experiments, TSCs grown in MEF-conditioned TS medium in order to avoid additional signal/mRNA from MEF cells. Cells were passaged when wells became 80% confluent.

Cell Lines used in the study

All experiments were performed using E14 or 129 mouse ES cells, wild-type TS cells , and TS_EGFP cells (Tanaka et al., 1998). Wild type TS cells derived in M.Zernicka-Goetz's lab from CD1 mouse with following the protocol by (Tanaka, 2006).

'3D embedded' culture in Matrigel

ES or TS cell colonies were dissociated to single cells by incubation with 0.05% trypsin-EDTA at 37˚C. Cells were pelleted by centrifugation for 5 min/1,000 rpm, washed with PBS, and re-pelleted. The pellet was re-suspended in Matrigel (BD, 356230). The cell suspension was plated on ibiTreat microscopy plastic µ-plates (Ibidi) and incubated at 37˚C until the Matrigel solidified. The plate was then filled with pre-warmed N2B27 medium for ES cells; TS medium for TS cells. Cells were cultured at 37˚C and 5% CO2.

qRT–PCR for analysis

Total RNA was extracted from cells using Trizol reagent as per the manufacturer's instructions (Ambion). cDNA synthesis was performed with 1 µg of total RNA according to the manufacturer's instructions (Applied Biosystems). The amounts of mRNA were measured using SYBR Green PCR Master Mix (Ambion). Relative levels of transcript expression were assessed by the ΔΔCt method, with *Gapdh* as an endogenous control. For qRT-PCR primers used, see Supplementary Data Table 2.

siRNA Transfection

siRNA was transfected using LipofectAMINE RNAiMAX (Invitrogen) according to the manufacturer's instructions. The siRNA sequences are as follows: siSmad5 5'-ACGTCATACATTTACATTTAA-3'. Transfected TSCs were cultured for 36h at 37˚C and 5% CO2, in MEF-conditioned TS medium. For the confirmation of knockdown, cells were harvested 36h after siRNA transfections. Total RNA extraction and cDNA synthesis were performed as explained above. Relative levels of transcript expression were assessed by the ΔΔCt method, with *Gapdh* as an endogenous control.

Immunofluorescence

Cells/embryos were fixed with 4% paraformaldehyde for 20 mins at room-temperature, then washed in PBS. Permeabilization was performed with 0.3% Triton-X-100, 0.1% Glycin in PBS for 15 minutes at room-temperature. Primary antibody incubation was performed overnight at 4˚C. The following day, cells were washed, then incubated overnight in secondary antibody at 4˚C. DAPI in PBS (5mg/ml) was added prior to confocal imaging. For antibodies and dilutions used, see Supplementary Data Table 1.

Time-Lapse Imaging

Confocal time-lapse imaging during in vitro culture was performed using spinning-disc microscope system (Intelligent Imaging Innovations). The embryos were imaged every 15 or 30 min in 100 mm image stacks of 8 µm z-planes. Analysis of cell death in the developing egg-cylinder was carried out using SYTOX Red nucleic acid stain (Life technologies) according to the manufacturer's instructions. Images were processed using Slidebook 5.0 (Intelligent Imaging Innovations)

Confocal Microscopy Imaging, Processing and Analysis

All images were acquired using a Leica SP5 or SP8 confocal microscope, and all analyses were carried out using open-source image analysis software 'Fiji'.

Statistics

Statistical tests were performed on GraphPad Prism 7.0 software for Windows. Data were checked for normal distribution and equal variances before each parametric statistical test was performed. Error bars represent standard error of the mean in all cases, unless otherwise specified. Figure legends indicate the number of independent experiments performed in each analysis.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: B.S., M.Z-G.; Methodology & Investigation: B.S. Writing: B.S, M.Z-G.; Supervision: N.D., M.Z-G. Funding: B.S., N.D., M.Z.-G.

References

Anderson, G.J., Darshan, D., 2008. Small-molecule dissection of BMP signaling. Nat Chem Biol 4, 15-16.

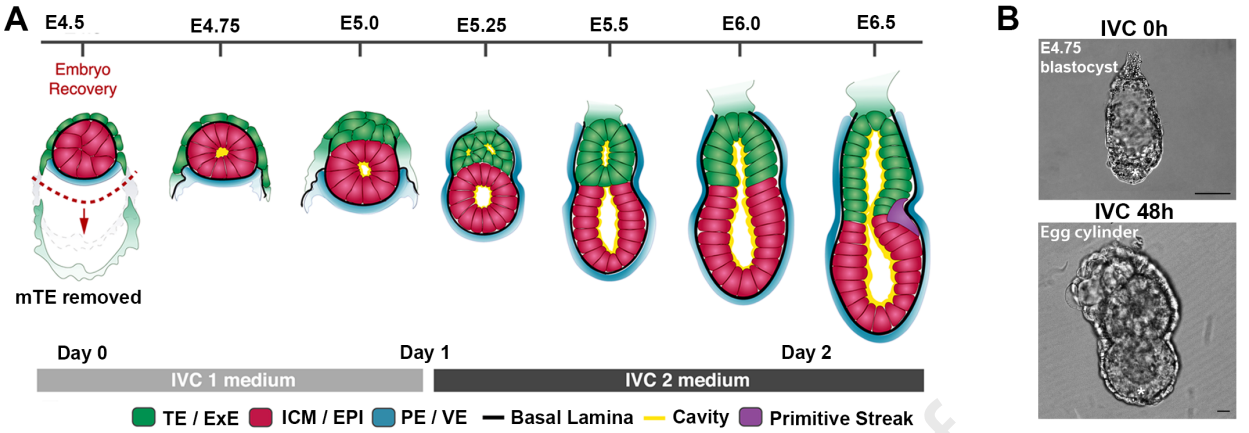
Beccari, L., Moris, N., Girgin, M., Turner, D.A., Baillie-Johnson, P., Cossy, A.C., Lutolf, M.P., Duboule, D., Arias, A.M., 2018. Multi-axial self-organization properties of mouse embryonic stem cells into gastruloids. Nature 562, 272-276.
Bedzhov, I., Graham, S.J., Leung, C.Y., Zernicka-Goetz, M., 2014a. Developmental plasticity, cell fate specification and morphogenesis in the early mouse embryo. Philos Trans R Soc Lond B Biol Sci 369.
Bedzhov, I., Leung, C.Y., Bialecka, M., Zernicka-Goetz, M., 2014b. In vitro culture of mouse blastocysts beyond the implantation stages. Nat Protoc 9, 2732-2739.
Bedzhov, I., Zernicka-Goetz, M., 2014. Self-organizing properties of mouse pluripotent cells initiate morphogenesis upon implantation. Cell 156, 1032-1044.
Beppu, H., Kawabata, M., Hamamoto, T., Chytil, A., Minowa, O., Noda, T., Miyazono, K., 2000. BMP type II receptor is required for gastrulation and early development of mouse embryos. Dev Biol 221, 249-258.
Boroviak, T., Loos, R., Bertone, P., Smith, A., Nichols, J., 2014. The ability of inner-cell-mass cells to self-renew as embryonic stem cells is acquired following epiblast specification. Nat Cell Biol 16, 516-528.
Chadee, D.N., Hendzel, M.J., Tylipski, C.P., Allis, C.D., Bazett-Jones, D.P., Wright, J.A., Davie, J.R., 1999. Increased Ser-10 phosphorylation of histone H3 in mitogen-stimulated and oncogene-transformed mouse fibroblasts. The Journal of biological chemistry 274, 24914-24920.
Chu, G.C., Dunn, N.R., Anderson, D.C., Oxburgh, L., Robertson, E.J., 2004. Differential requirements for Smad4 in TGFbeta-dependent patterning of the early mouse embryo. Development 131, 3501-3512.
Coucouvanis, E., Martin, G.R., 1999. BMP signaling plays a role in visceral endoderm differentiation and cavitation in the early mouse embryo. Development 126, 535-546.
D'Sa-Eipper, C., Leonard, J.R., Putcha, G., Zheng, T.S., Flavell, R.A., Rakic, P., Kuida, K., Roth, K.A., 2001. DNA damage-induced neural precursor cell apoptosis requires p53 and caspase 9 but neither Bax nor caspase 3. Development 128, 137-146.
Desbaillets, I., Ziegler, U., Groscurth, P., Gassmann, M., 2000. Embryoid bodies: an in vitro model of mouse embryogenesis. Exp Physiol 85, 645-651.
Di-Gregorio, A., Sancho, M., Stuckey, D.W., Crompton, L.A., Godwin, J., Mishina, Y., Rodriguez, T.A., 2007. BMP signalling inhibits premature neural differentiation in the mouse embryo. Development 134, 3359-3369.
Ghimire, S., Van der Jeught, M., Neupane, J., Roost, M.S., Anckaert, J., Popovic, M., Van Nieuwerburgh, F., Mestdagh, P., Vandesompele, J., Deforce, D., Menten, B., Chuva de Sousa Lopes, S., De Sutter, P., Heindryckx, B., 2018. Comparative analysis of naive, primed and ground state pluripotency in mouse embryonic stem cells originating from the same genetic background. Sci Rep 8, 5884.
Gomes Fernandes, M., Dries, R., Roost, M.S., Semrau, S., de Melo Bernardo, A., Davis, R.P., Ramakrishnan, R., Szuhai, K., Maas, E., Umans, L., Abon Escalona, V., Salvatori, D., Deforce, D., Van Criekinge, W., Huylebroeck, D., Mummery, C., Zwijsen, A., de Sousa Lopes, S.M., 2016. BMP-SMAD Signaling Regulates Lineage Priming, but Is Dispensable for Self-Renewal in Mouse Embryonic Stem Cells. Stem Cell Reports 6, 85-94.
Graham, S.J., Wicher, K.B., Jedrusik, A., Guo, G., Herath, W., Robson, P., Zernicka-Goetz, M., 2014. BMP signalling regulates the pre-implantation development of extra-embryonic cell lineages in the mouse embryo. Nat Commun 5, 5667.
Hao, J., Daleo, M.A., Murphy, C.K., Yu, P.B., Ho, J.N., Hu, J., Peterson, R.T., Hatzopoulos, A.K., Hong, C.C., 2008. Dorsomorphin, a selective small molecule inhibitor of BMP signaling, promotes cardiomyogenesis in embryonic stem cells. PLoS One 3, e2904.
Harrison, S.E., Sozen, B., Christodoulou, N., Kyprianou, C., Zernicka-Goetz, M., 2017. Assembly of embryonic and extraembryonic stem cells to mimic embryogenesis in vitro. Science 356.
Heldin, C.H., Miyazono, K., ten Dijke, P., 1997. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature 390, 465-471.

Hemberger, M., Hanna, C.W., Dean, W., 2020. Mechanisms of early placental development in mouse and humans. Nat Rev Genet 21, 27-43.
Javier, A.L., Doan, L.T., Luong, M., Reyes de Mochel, N.S., Sun, A., Monuki, E.S., Cho, K.W., 2012. Bmp indicator mice reveal dynamic regulation of transcriptional response. PLoS One 7, e42566.
Kishigami, S., Mishina, Y., 2005. BMP signaling and early embryonic patterning. Cytokine Growth Factor Rev 16, 265-278.
Kunath, T., Strumpf, D., Rossant, J., 2004. Early trophoblast determination and stem cell maintenance in the mouse--a review. Placenta 25 Suppl A, S32-38.
Latos, P.A., Hemberger, M., 2014. Review: the transcriptional and signalling networks of mouse trophoblast stem cells. Placenta 35 Suppl, S81-85.
Latos, P.A., Sienerth, A.R., Murray, A., Senner, C.E., Muto, M., Ikawa, M., Oxley, D., Burge, S., Cox, B.J., Hemberger, M., 2015. Elf5-centered transcription factor hub controls trophoblast stem cell self-renewal and differentiation through stoichiometry-sensitive shifts in target gene networks. Genes Dev 29, 2435-2448.
Lawson, K.A., Dunn, N.R., Roelen, B.A., Zeinstra, L.M., Davis, A.M., Wright, C.V., Korving, J.P., Hogan, B.L., 1999. Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes & development 13, 424-436.
Lee, C.Q., Gardner, L., Turco, M., Zhao, N., Murray, M.J., Coleman, N., Rossant, J., Hemberger, M., Moffett, A., 2016. What Is Trophoblast? A Combination of Criteria Define Human First-Trimester Trophoblast. Stem cell reports 6, 257-272.
Li, Z., Chen, Y.G., 2013. Functions of BMP signaling in embryonic stem cell fate determination. Experimental cell research 319, 113-119.
Massague, J., Wotton, D., 2000. Transcriptional control by the TGF-beta/Smad signaling system. The EMBO journal 19, 1745-1754.
Mishina, Y., Suzuki, A., Ueno, N., Behringer, R.R., 1995. Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. Genes Dev 9, 3027-3037.
Miyazono, K., Kamiya, Y., Morikawa, M., 2010. Bone morphogenetic protein receptors and signal transduction. Journal of biochemistry 147, 35-51.
Morgani, S.M., Metzger, J.J., Nichols, J., Siggia, E.D., Hadjantonakis, A.K., 2018. Micropattern differentiation of mouse pluripotent stem cells recapitulates embryo regionalized cell fate patterning. Elife 7.
Morris, S.A., Grewal, S., Barrios, F., Patankar, S.N., Strauss, B., Buttery, L., Alexander, M., Shakesheff, K.M., Zernicka-Goetz, M., 2012. Dynamics of anterior-posterior axis formation in the developing mouse embryo. Nat Commun 3, 673.
Nichols, J., Smith, A., 2009. Naive and primed pluripotent states. Cell Stem Cell 4, 487-492.
Nishimura, R., Hata, K., Ikeda, F., Matsubara, T., Yamashita, K., Ichida, F., Yoneda, T., 2003. The role of Smads in BMP signaling. Frontiers in bioscience : a journal and virtual library 8, s275-284.
Ohinata, Y., Tsukiyama, T., 2014. Establishment of trophoblast stem cells under defined culture conditions in mice. PloS one 9, e107308.
Orford, K.W., Scadden, D.T., 2008. Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. Nature reviews. Genetics 9, 115-128.
Pijuan-Sala, B., Griffiths, J.A., Guibentif, C., Hiscock, T.W., Jawaid, W., Calero-Nieto, F.J., Mulas, C., Ibarra-Soria, X., Tyser, R.C.V., Ho, D.L.L., Reik, W., Srinivas, S., Simons, B.D., Nichols, J., Marioni, J.C., Gottgens, B., 2019. A single-cell molecular map of mouse gastrulation and early organogenesis. Nature 566, 490-495.
Rhee, J.M., Pirity, M.K., Lackan, C.S., Long, J.Z., Kondoh, G., Takeda, J., Hadjantonakis, A.K., 2006. In vivo imaging and differential localization of lipid-modified GFP-variant fusions in embryonic stem cells and mice. Genesis 44, 202-218.
Rivera-Perez, J.A., Hadjantonakis, A.K., 2014. The Dynamics of Morphogenesis in the Early Mouse Embryo. Cold Spring Harb Perspect Biol 7.

Rossant, J., 2007. Stem cells and lineage development in the mammalian blastocyst. Reprod Fertil Dev 19, 111-118.
Rossant, J., Tam, P.P., 2009. Blastocyst lineage formation, early embryonic asymmetries and axis patterning in the mouse. Development 136, 701-713.
Shahbazi, M.N., Scialdone, A., Skorupska, N., Weberling, A., Recher, G., Zhu, M., Jedrusik, A., Devito, L.G., Noli, L., Macaulay, I.C., Buecker, C., Khalaf, Y., Ilic, D., Voet, T., Marioni, J.C., Zernicka-Goetz, M., 2017. Pluripotent state transitions coordinate morphogenesis in mouse and human embryos. Nature 552, 239-243.
Sirard, C., de la Pompa, J.L., Elia, A., Itie, A., Mirtsos, C., Cheung, A., Hahn, S., Wakeham, A., Schwartz, L., Kern, S.E., Rossant, J., Mak, T.W., 1998. The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. Genes Dev 12, 107-119.
Sozen, B., Amadei, G., Cox, A., Wang, R., Na, E., Czukiewska, S., Chappell, L., Voet, T., Michel, G., Jing, N., Glover, D.M., Zernicka-Goetz, M., 2018. Self-assembly of embryonic and two extra-embryonic stem cell types into gastrulating embryo-like structures. Nat Cell Biol 20, 979-989.
Sozen, B., Ozturk, S., Yaba, A., Demir, N., 2015. The p38 MAPK signalling pathway is required for glucose metabolism, lineage specification and embryo survival during mouse preimplantation development. Mech Dev 138 Pt 3, 375-398.
Strahl, B.D., Allis, C.D., 2000. The language of covalent histone modifications. Nature 403, 41-45.
Strahle, U., Grabher, C., 2010. The zebrafish embryo as a model for assessing off-target drug effects. Dis Model Mech 3, 689-692.
Tanaka, S., 2006. Derivation and culture of mouse trophoblast stem cells in vitro. Methods Mol Biol 329, 35-44.
Tanaka, S., Kunath, T., Hadjantonakis, A.K., Nagy, A., Rossant, J., 1998. Promotion of trophoblast stem cell proliferation by FGF4. Science 282, 2072-2075.
ten Berge, D., Koole, W., Fuerer, C., Fish, M., Eroglu, E., Nusse, R., 2008. Wnt signaling mediates self-organization and axis formation in embryoid bodies. Cell Stem Cell 3, 508-518.
Tremblay, K.D., Dunn, N.R., Robertson, E.J., 2001. Mouse embryos lacking Smad1 signals display defects in extra-embryonic tissues and germ cell formation. Development 128, 3609-3621.
Uy, G.D., Downs, K.M., Gardner, R.L., 2002. Inhibition of trophoblast stem cell potential in chorionic ectoderm coincides with occlusion of the ectoplacental cavity in the mouse. Development 129, 3913-3924.
van den Brink, S.C., Alemany, A., van Batenburg, V., Moris, N., Blotenburg, M., Vivie, J., Baillie-Johnson, P., Nichols, J., Sonnen, K.F., Martinez Arias, A., van Oudenaarden, A., 2020. Single-cell and spatial transcriptomics reveal somitogenesis in gastruloids. Nature 582, 405-409.
van den Brink, S.C., Baillie-Johnson, P., Balayo, T., Hadjantonakis, A.K., Nowotschin, S., Turner, D.A., Martinez Arias, A., 2014. Symmetry breaking, germ layer specification and axial organisation in aggregates of mouse embryonic stem cells. Development 141, 4231-4242.
Walsh, D.W., Godson, C., Brazil, D.P., Martin, F., 2010. Extracellular BMP-antagonist regulation in development and disease: tied up in knots. Trends Cell Biol 20, 244-256.
Warmflash, A., Sorre, B., Etoc, F., Siggia, E.D., Brivanlou, A.H., 2014. A method to recapitulate early embryonic spatial patterning in human embryonic stem cells. Nat Methods 11, 847-854.
Winnier, G., Blessing, M., Labosky, P.A., Hogan, B.L., 1995. Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes Dev 9, 2105-2116.
Yamamoto, M., Beppu, H., Takaoka, K., Meno, C., Li, E., Miyazono, K., Hamada, H., 2009. Antagonism between Smad1 and Smad2 signaling determines the site of distal visceral endoderm formation in the mouse embryo. J Cell Biol 184, 323-334.
Yang, X., Li, C., Xu, X., Deng, C., 1998. The tumor suppressor SMAD4/DPC4 is essential for epiblast proliferation and mesoderm induction in mice. Proc Natl Acad Sci U S A 95, 3667-3672.
Ying, Q.L., Wray, J., Nichols, J., Batlle-Morera, L., Doble, B., Woodgett, J., Cohen, P., Smith, A., 2008. The ground state of embryonic stem cell self-renewal. Nature 453, 519-523.

Yu, P.B., Hong, C.C., Sachidanandan, C., Babitt, J.L., Deng, D.Y., Hoyng, S.A., Lin, H.Y., Bloch, K.D., Peterson, R.T., 2008. Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol 4, 33-41.

Fig. 1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Day 2
IVC 1 medium
IVC 2 medium
TE / ExE
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Cavity
Primitive Streak

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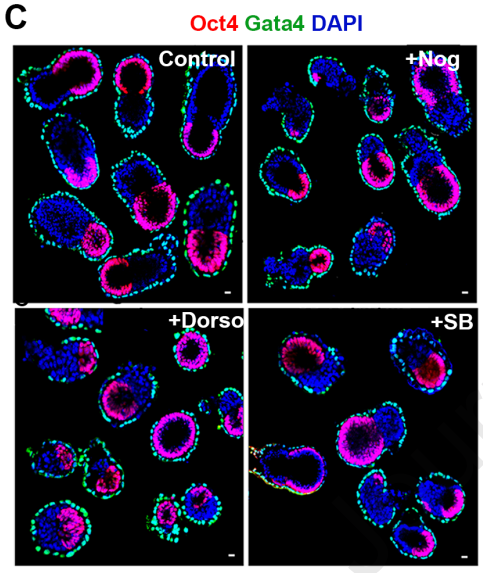
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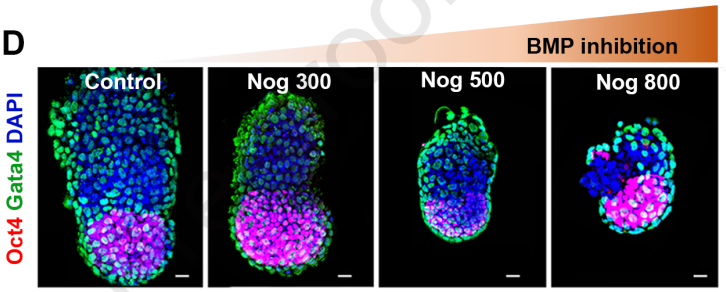
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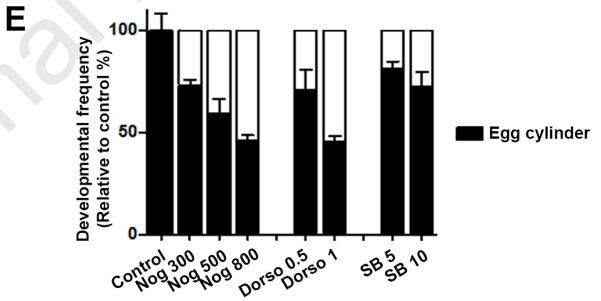
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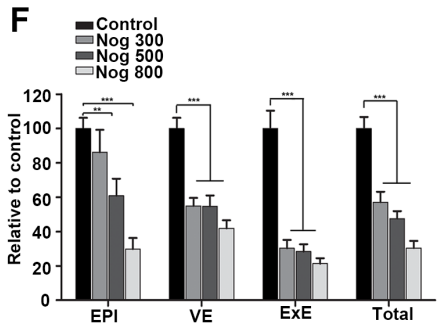
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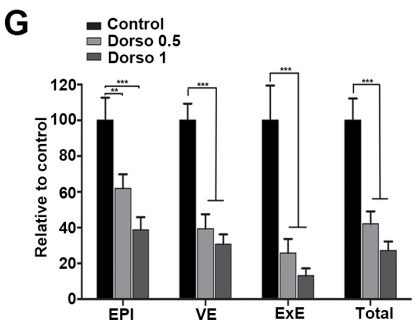
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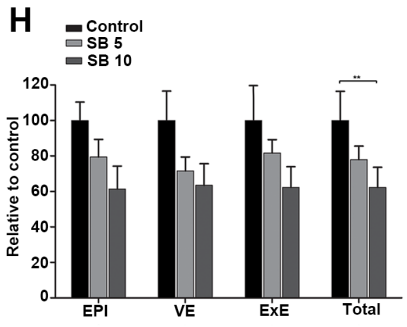
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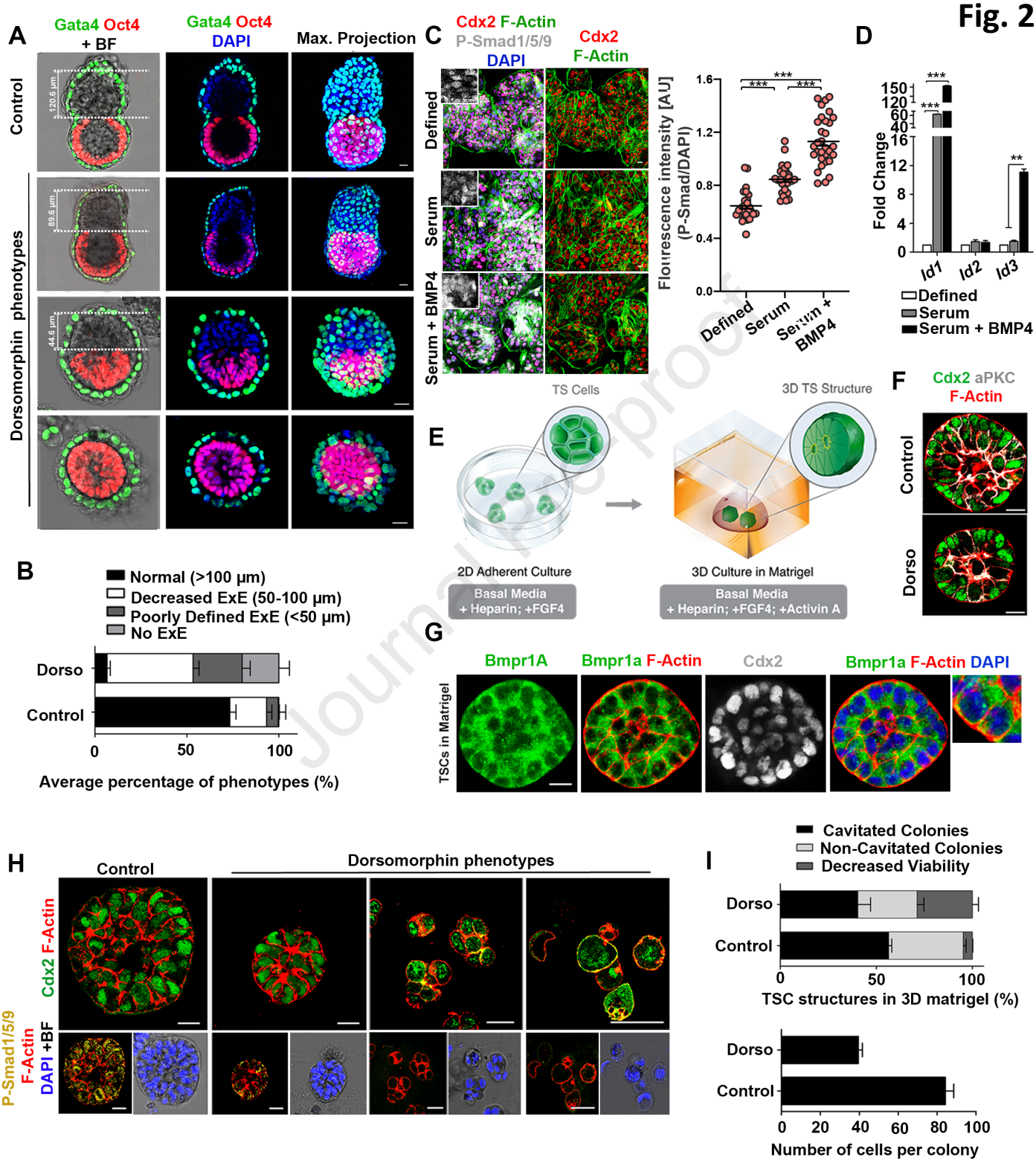
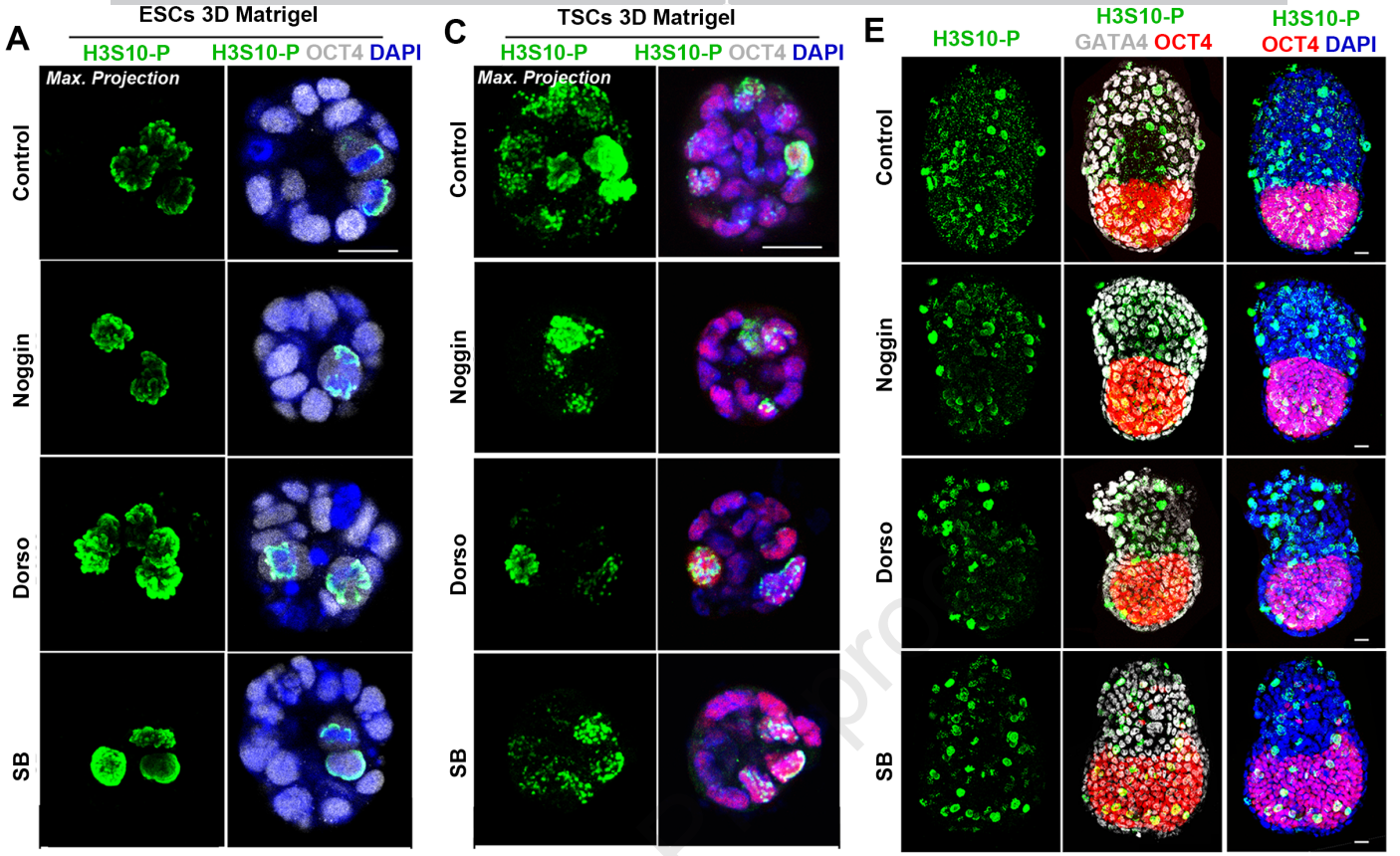
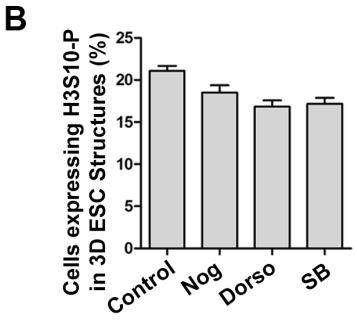
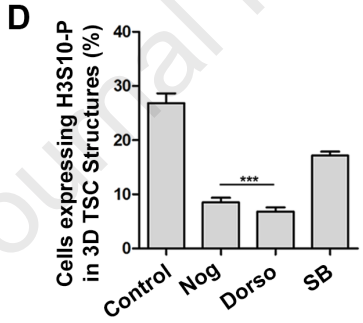
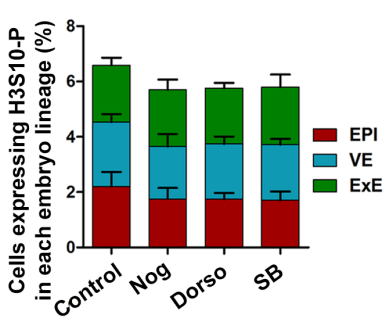
Fig. 2

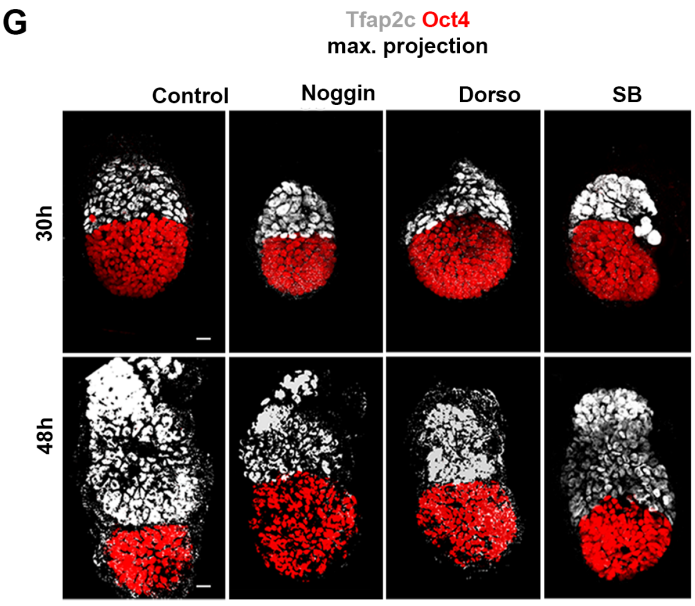


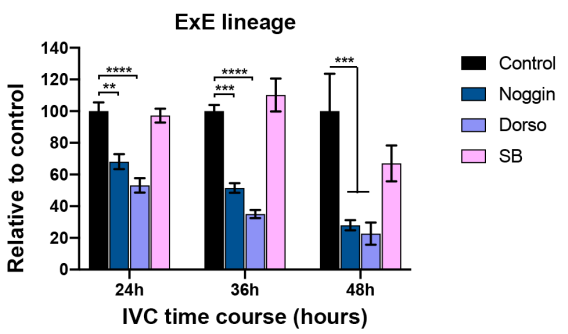












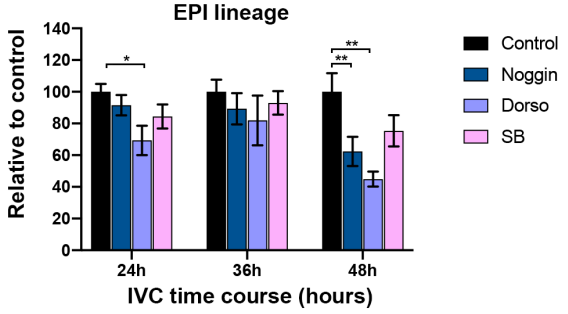


Fig. 4

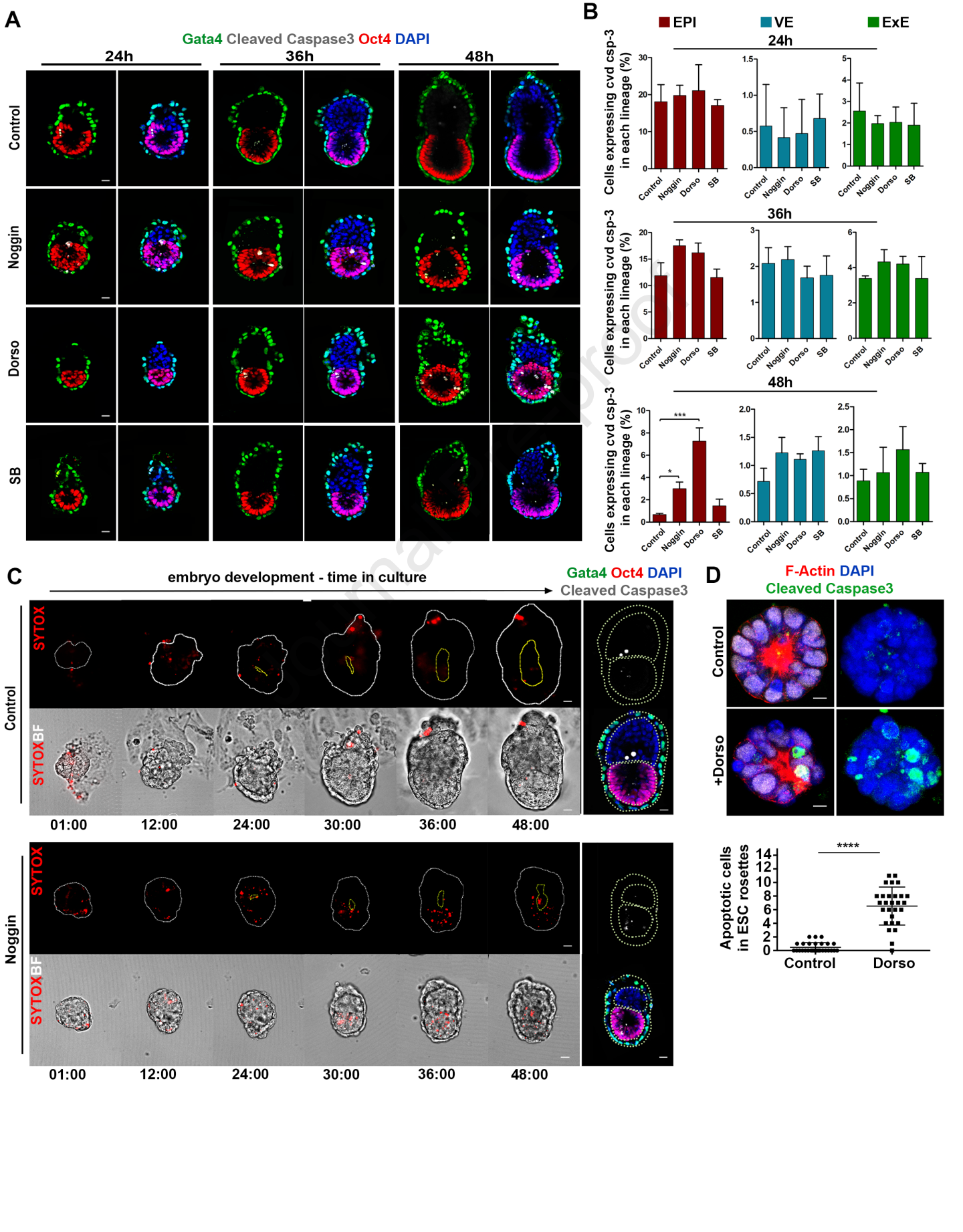

A
Gata4 Cleaved Caspase3 Oct4 DAPI
24h
36h
48h
Control
Noggin
Dorso
SB
B
EPI
VE
ExE
24h
36h
48h
Cells expressing cvd csp-3 in each lineage (%)
Control
Noggin
Dorso
SB
C
embryo development - time in culture
Gata4 Oct4 DAPI
Cleaved Caspase3
SYTOX
SYTOXBF
Control
Noggin
01:00
12:00
24:00
30:00
36:00
48:00
D
F-Actin DAPI
Cleaved Caspase3
Control
+Dorso
Apoptotic cells in ESC rosettes
****
Control
Dorso

Fig. 5

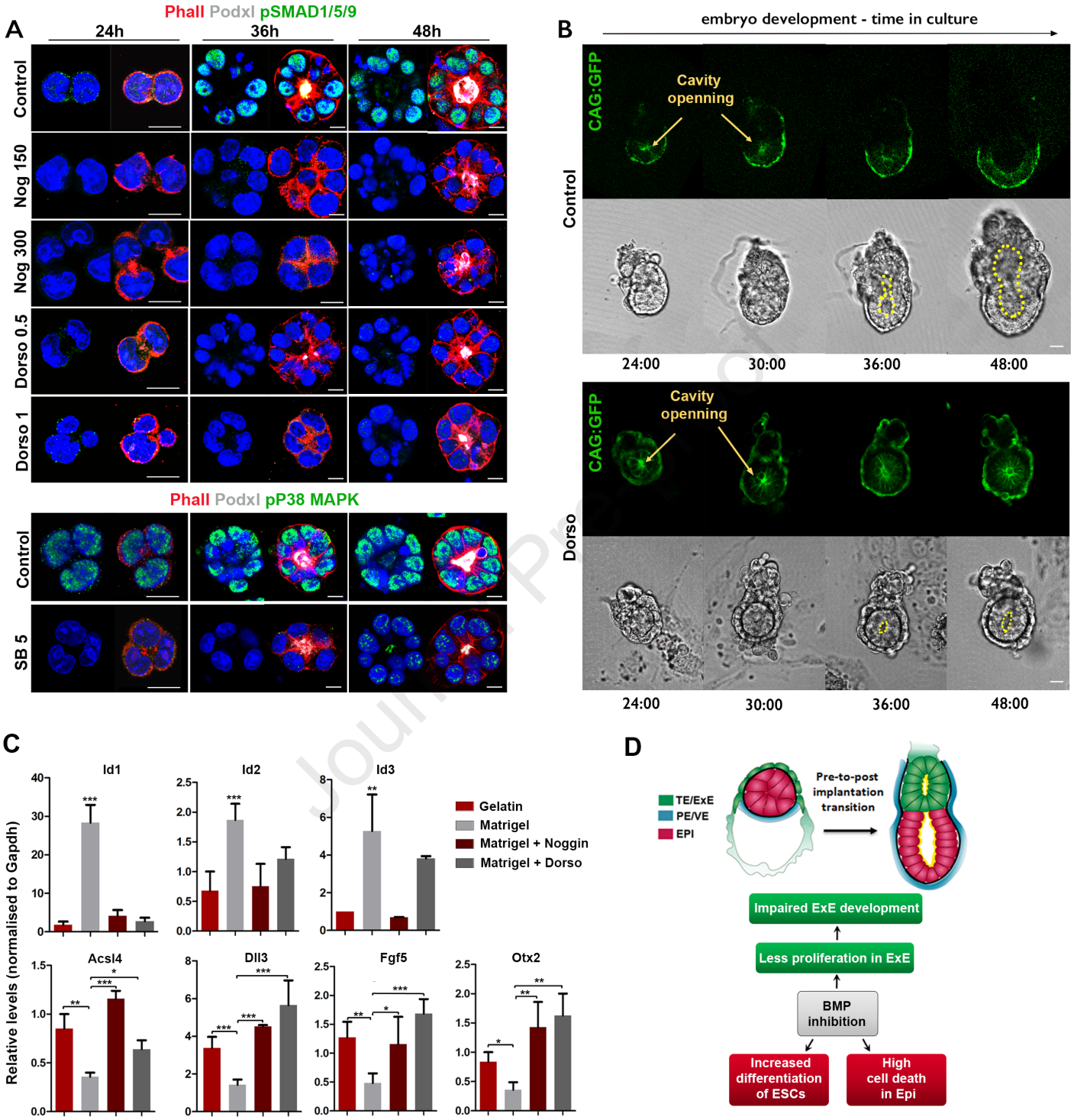
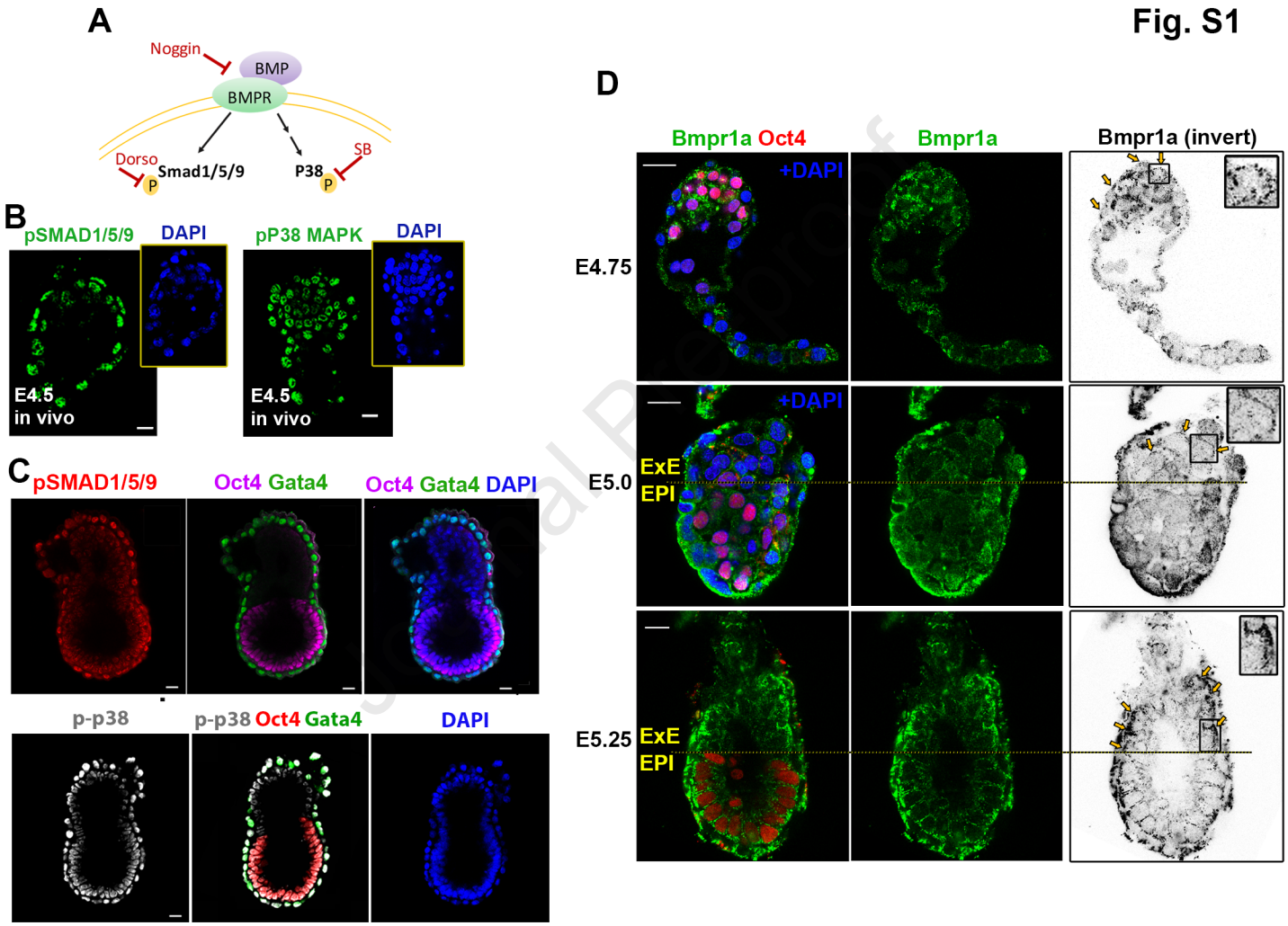
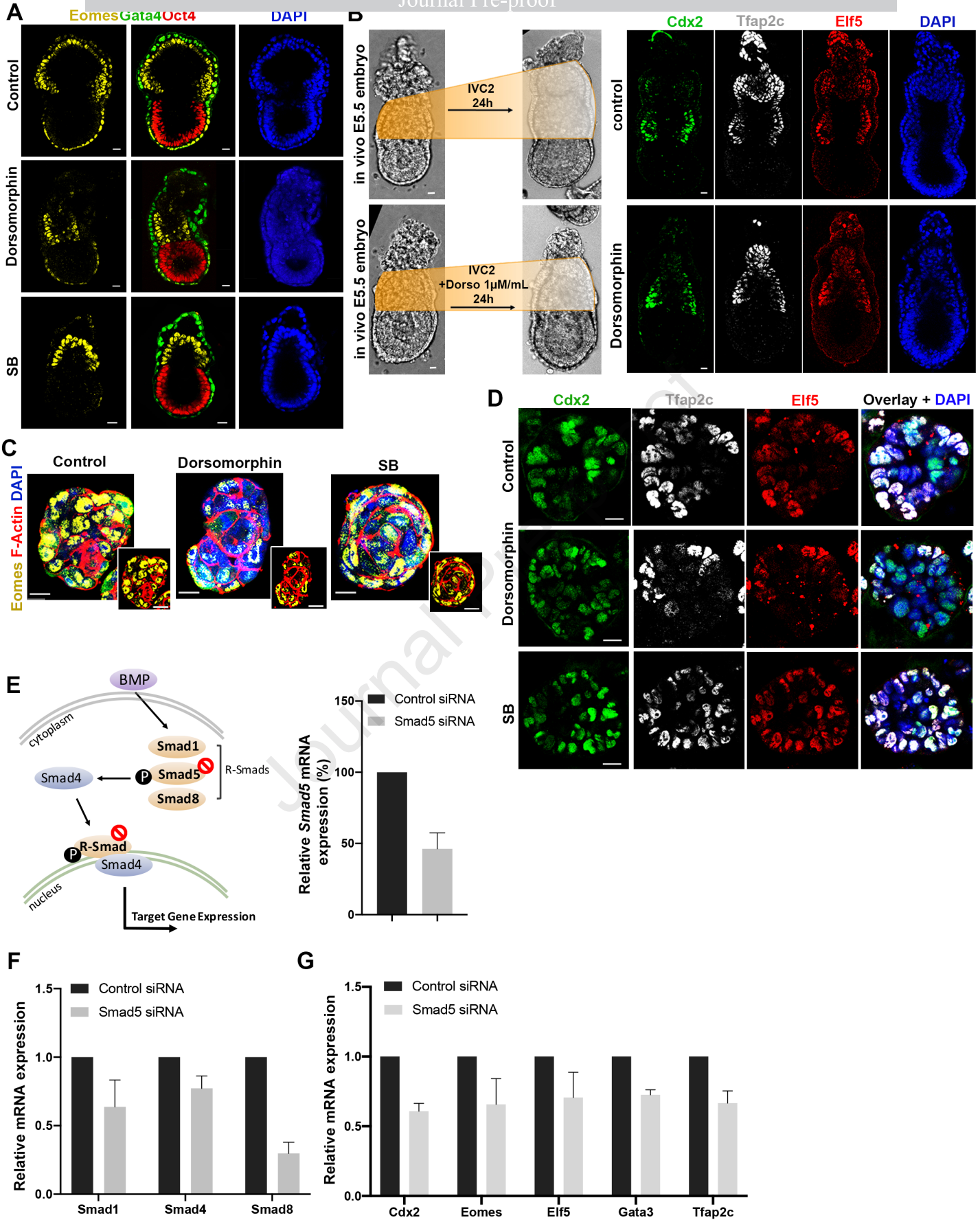

A
Phall Podxl pSMAD1/5/9
24h
36h
48h
Control
Nog 150
Nog 300
Dorso 0.5
Dorso 1
Phall Podxl pP38 MAPK
Control
SB 5
B
embryo development - time in culture
CAG:GFP
Cavity openning
Control
24:00
30:00
36:00
48:00
CAG:GFP
Cavity openning
Dorso
24:00
30:00
36:00
48:00
C
Id1
Id2
Id3
Acsl4
Dll3
Fgf5
Otx2
Relative levels (normalised to Gapdh)
Gelatin
Matrigel
Matrigel + Noggin
Matrigel + Dorso
D
TE/ExE
PE/VE
EPI
Pre-to-post implantation transition
Impaired ExE development
Less proliferation in ExE
BMP inhibition
Increased differentiation of ESCs
High cell death in Epi

Fig. S1


A
Noggin
BMP
BMPR
Dorso
Smad1/5/9
P
P38
SB
P
B
pSMAD1/5/9
DAPI
E4.5
in vivo
pP38 MAPK
DAPI
E4.5
in vivo
C
pSMAD1/5/9
Oct4 Gata4
Oct4 Gata4 DAPI
p-p38
p-p38 Oct4 Gata4
DAPI
D
Bmpr1a Oct4
+DAPI
Bmpr1a
Bmpr1a (invert)
E4.75
E5.0
ExE
EPI
E5.25
ExE
EPI



Supplementary table 1- Antibodies used in this study

Antibody (species)	Vendor	Number	Dilution
Oct 3/4 (mouse)	Santa cruz	sc-5279	1:200
Tbr2/Eomes (rabbit)	Abcam	ab23345	1:400
aPKC (rabbit)	Santa cruz	sc-17781	1:200
Podocalyxin (rat)	R&D systems	MAB1556	1:400
Cdx2 (mouse)	Launch diagnostics	MU392-UC	1:200
GFP (rat)	Nacalai biochemicals	04404-84	1:2000
Tfap2c (rabbit)	Santa cruz	sc-8977	1:200
Elf5 (goat)	Santa cruz	Sc-9645	1:200
H3S10-P (rabbit)	Cell signalling technologies	9701	1:400
BmpR1a (rabbit)	Biorbyt	orb420681	1:200
Phospho-p38 MAPK (rabbit)	Cell signalling technologies	9211	1:200
Phospho-SMAD 1/5/9 (rabbit)	Cell signalling technologies	13820P	1:100
Gata4 (Goat)	Santa cruz	sc-1237	1:200
Cleaved caspase-3 (rabbit)	Cell signalling technologies	#9664	1:200
F-actin (Phalloidin 488)	Life Technologies (Thermofisher scientific)	A12379	1:1000
Alexa 488 (Donkey anti-rat)	Life Technologies (Thermofisher scientific)	A21208	1:500
Alexa 568 (Donkey anti-mouse)	Life Technologies (Thermofisher scientific)	A10037	1:500
Alexa 647 (Donkey anti-rabbit)	Life Technologies (Thermofisher scientific)	A31573	1:500
Alexa 647 (Donkey anti-goat)	Life Technologies (Thermofisher scientific)	A21447	1:500

Supplementary table 2- qPCR primers used in this study

Gene	Forward (5' to 3')	Reverse (5' to 3')
Cdx2	AGTGAGCTGGCTGCCACACT	GCTGCTGCTGCTTCTTCTTGA
Eomes	TCGCTGTGACGGCCTACCAA	AGGGGAATCCGTGGGAGATGGA
Elf5	ATTCGCTCGCAAGGTTACTCC	GGATGCCACAGTTCTCTTCAGG
Id1	CCTAGCTGTTCGCTGAAGGC	CTCCGACAGACCAAGTACCAC
Id2	TCCGGTGAGGTCCGTTAGG	CAGACTCATCGGGTCGTCC
Id3	CTGTCGGAACGTAGCCTGG	GTGGTTCATGTCGTCCAAGAG
GAPDH	CGTATTGGGCGCCTGGTCAC	ATGATGACCCTTTTGGCTCC
Tfap2c	TGCCCACGTCACTCTCCTCA	TCCGTCCCCCAAGATGTGGT
Gata3	GGGTTCGGATGTAAGTCGAG	CCACAGTGGGGTAGAGGTTG
Ascl4	CCTGAGGGGCTTGAAATTC	GTTGGTCTACTTGGAGGAACG
Dll3	GCTGGTGTCTTCGAGCTACAA	TGCTCCGTATAGACCGGGAC
Fgf5	AACTCCATGCAAGTGCCAAAT	CGGACGCATAGGTATTATAGCTG
Otx2	TATCTAAAGCAACCGCCTTACG	GCCCTAGTAAATGTCGTCCTCTC

- Embryo remodelling during pre- to post-implantation transition occurs normally in the absence of BMP.
- BMP is required for maintaining both embryonic and extra-embryonic tissue development soon after implantation.
- Loss of canonical BMP activity compromises extra-embryonic ectoderm development via decreasing the proliferative potential of TSCs.
- The lack of BMP signalling promotes early differentiation of 3D ESC rosettes and increases cell death in EPI.