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Role of autocrine bone morphogenetic protein signaling in trophoblast stem cells[†]

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Abstract

The Bone Morphogenetic Protein (BMP) pathway is involved in numerous developmental processes, including cell growth, apoptosis, and differentiation. In mouse embryogenesis, BMP signaling is a well-known morphogen for both mesoderm induction and germ cell development. Recent evidence points to a potential role in development of the extraembryonic compartment, including trophectoderm-derived tissues. In this study, we investigated the effect of BMP signaling in both mouse and human trophoblast stem cells (TSC) in vitro, evaluating the expression and activation of the BMP signaling response machinery, and the effect of BMP signaling manipulation during TSC maintenance and differentiation. Both mouse trophoblast stem cells (mTSC) and human trophoblast stem cells (hTSC) expressed various BMP ligands and the receptors BMPR1A and BMPR2, necessary for BMP response, and displayed maximal active BMP signaling when undifferentiated. We also observed a conserved modulatory role of BMP signaling during trophoblast differentiation, whereby maintenance of active BMP signaling blunted differentiation of TSC in both species. Conversely, the effect of BMP signaling on the undifferentiated state of TSC appeared to be species-specific, with SMAD-independent signaling important in maintenance of mTSC, and a more subtle role for both SMAD-dependent and -independent BMP signaling in hTSC. Altogether, these data establish an autocrine role for the BMP pathway in the trophoblast compartment. As specification and correct differentiation of the extraembryonic compartment are fundamental for implantation and early placental development, insights on the role of the BMP signaling in early development might prove useful in the setting of in vitro fertilization as well as targeting trophoblast-associated placental dysfunction.

Summary sentence
Autocrine Bone Morphogenetic Protein (BMP) signaling regulates the development of the extraembryonic compartment by modulating trophoblast stem cell maintenance and blunting differentiation.

Keywords: BMP signaling, trophoblast stem cells, extraembryonic ectoderm, extravillous trophoblast, syncytiotrophoblast, spongiotrophoblast, labyrinthine trophoblast, trophoblast giant cells

Introduction

The placenta is an organ of fetal origin that connects the fetus to the mother, provides nutrients and gas exchange, produces hormones for fetal growth, and negotiates with the maternal immune system to maintain the pregnancy through term. Abnormal placental development and function can result in multiple complications, affecting both the baby, such as preterm birth and intrauterine growth restriction, and the mother, like preeclampsia and placenta accreta [1, 2]. Normal and abnormal development of the placenta involve poorly understood processes, but progress has been made through establishment of in vitro models, including trophoblast stem cells (TSC), that recapitulate in vivo trophoblast during placental development [3, 4].

Mouse trophoblast stem cells (mTSC) can be derived from preimplantation blastocyst and peri-implantation embryos up to E8.0 [3, 5]. They are thought to represent a trophoblast population in the polar trophectoderm (TE) of the embryo and extraembryonic ectoderm (ExE)/chorion of the early postimplantation mouse placenta, and have been largely used to study mechanisms of trophoblast differentiation and function [6]. Human trophoblast stem cells (hTSC) have only recently been derived from both blastocyst-stage embryos and first trimester placental tissues [4]. Although the exact in vivo population represented by hTSC remains unknown, these cells have been derived from the ITGA6^{+} cytotrophoblast (CTB) layer of the placenta, and have been shown to be bipotential, with the ability to differentiate into both multinucleated human chorionic gonadotropin (hCG)-secreting syncytiotrophoblast (STB) and invasive HLA-G[+] extravillous trophoblast (EVT). Both mTSC and hTSC are powerful tools to dissect the role of different signaling pathways in early trophoblast differentiation in vitro, either through genetic or environmental manipulation.

The Bone Morphogenetic Protein (BMP) pathway is a branch of the Transforming Growth Factor Beta (TGF-β)

signaling pathway, comprised of at least 15 classic BMPs, and is involved in numerous developmental processes, including cell growth, apoptosis, and differentiation [7]. The BMP signal is transduced by a tetramer complex, formed by combination of two type I receptor subunits (either IA or IB) and two type II receptor subunits, each of which contain a cytoplasmic serine–threonine kinase domain. In the canonical SMAD-dependent pathway, ligand binding to the receptor complex initiates the recruitment and phosphorylation of SMAD1/5/9 proteins, which subsequently bind SMAD4 and translocate into the nucleus, where they control the expression of downstream targets. Noncanonical (SMAD-independent) pathways involve activation of MAPK, TAK-p38, PI3-kinase, CDC42, and LIMK [8]. This means that activation of BMP signaling has a complex downstream effect, including cross talk with multiple other pathways. Small molecule inhibitors have been used to specifically target both the canonical and noncanonical BMP signaling pathways in order to identify the role of different branches in various cell types and organ systems, both in vivo and in vitro [9].

In mouse, BMP signaling is a well-known morphogen for both mesoderm induction and germ cell development in postimplantation embryos [10, 11]. However, increasing evidence suggests a role during earlier stages of development. In preimplantation mouse blastocysts, BMP signaling components are expressed asymmetrically in the cells that will become the TE and the inner cell mass, with active BMP signaling observed in the former [12]. Treatment of mouse preimplantation embryos with BMP inhibitors or a dominant negative form of *Bmpr2* caused defects in extraembryonic lineages [12], whereas BMP4-treated mouse embryos showed a delay in blastocyst formation [13]. Similarly, in human, single-cell transcriptomic analysis of blastocyst-stage embryos showed differential expression of the branches of the TGF*β* pathways between cells of the inner cell mass and the TE, with BMP signaling components expressed preferentially in the TE compartment [14]. In early postimplantation mouse embryos, BMP4 is known predominantly as a growth factor secreted by trophoblast cells of the ExE/chorion, with a fundamental paracrine effect on the embryo, necessary for initiation of gastrulation [15, 16]. However, phosphorylated SMAD1/5/9 has also been localized to the ExE, and knockout of the *Bmpr1a* gene leads to peri-implantation lethality, associated with absence of both mesoderm and ExE development, as noted by lack of *Eomes* expression [17]. Much less is known about the role of BMP signaling in human postimplantation development in vivo for both technical and ethical reasons. In vitro, BMP4 is known to drive differentiation of human embryonic stem cells into different lineages depending on culture conditions, with mesoderm and trophoblast differentiation initiated in WNT-dependent and independent manner, respectively [18, 19]. These findings, as well as the presence of an active BMP machinery in the trophoblast progenitor cells residing in the ExE/chorion, suggest a potential autocrine role for BMP signaling on trophoblast cells.

In this study, we set out to investigate the effect of BMP signaling in both mouse and human TSC in vitro, evaluating first the expression and activation of BMP signaling machinery, and subsequently the effect of BMP signaling manipulation and exogenous BMP4 treatement during TSC maintenance and differentiation, respectively.

Material and methods

The data underlying this article are available in the article and in its online supplementary material.

Bmp4-HA cloning

For the generation of the *Bmp4*-overexpressing plasmid, the *Bmp4* mRNA was cloned by PCR from the pRIAS-mBMP4 plasmid (gift from Cliff Tabin, plasmid #14001; Addgene #578) with the addition of an HA-tag and the restriction enzyme sites for BamHI and XhoI for sense insertion. The amplified construct was inserted into the pcDNA 3.1+ plasmid for constitutive expression. Correct in-frame insertion was verified by sequencing. mTSCs were transfected with Lipofectamine-2000 (Invitrogen) according to manufacturer's instructions. In brief, mTSC were grown to 60% confluence in 6-well plates and then incubated with the Lipofectamine/DNA complexes overnight including a cotransfection with GFP. The next day cells were washed with phosphate-buffered saline (PBS) and new medium was added. After 24 h, selection of transfected cells was started with puromycin up to a final concentration of 5ug/ml. Two independent clones were expanded and characterized.

Mouse TSC culture and differentiation

Mouse work was conducted under IACUC-approved protocol S09063. mTSC were derived from E3.5 blastocysts of Sv129 mice as previously described [20]. mTSC were cultured at 37°C and 5% CO_2 in RPMI 1640 culture medium (Corning) supplemented with 20% fetal bovine serum (FBS, Omega Scientific), 1 mM sodium pyruvate (Invitrogen), 55 nM *β*-mercaptoethanol (Invitrogen), 25 ng/ml fibroblast growth factor 4 (FGF4; Sigma), and 1 *μ*g/ml heparin (Sigma), with 70% of medium being conditioned for 72 h on inactivated mouse embryonic fibroblasts (MEFs). For differentiation, mTSC were grown in basic mTSC medium without FGF4, heparin, and MEF-conditioned medium for 7 days. For differentiation in the presence of exogenous BMP4, 100 ng/ml BMP4 was added to the normal differentiation conditions for 5 days. For differentiation in low-serum conditions, cells were serum-starved for 5 h and differentiation was conducted in media supplemented with 2.5% FBS (low serum) and 100 ng/ml BMP4 (R&D Systems) for 5 days.

Human placental explants, isolation of primary CTB, and human TSC derivation

All placental tissues were collected under a protocol approved by the Human Research Protections Program Committee of the University of California San Diego Institutional Review Board; all patients gave informed consent for collection and use of these tissues. Villous explants were dissected from first trimester placental tissues and cultured in RPMI media supplemented with 10% FBS and pen/strep/gentamicin for 24 h with or without 100 ng/ml of exogenous BMP4. Primary CTB, isolated from first trimester placentae ($n = 3$), were purified using sequential trypsin digestion and Percoll gradient purification as previously described [21]. Before RNA extraction for qPCR analysis, CTB purity was verified by flow cytometry for EGFR expression [19]. For hTSC derivation, following Percoll gradient purification, the trophoblast fraction was MACS-purified with a PE-conjugated anti-ITGA6 antibody (Biolegend #313612; cell line 1049) or an APC-conjugated anti-EGFR antibody (Biolegend #352906; cell line 1048).

Cells were then plated on 6-well plates that were coated with 5 μg/ml of collagen IV for at least 1 h and cultured in the original Okae media [4]. Once cells lines were established, they were switched to a modified complete media, with addition of 100 ng/ml FGF2, 20 ng/ml Noggin, and 50 ng/ml HGF for maintenance [22]. Cells were passaged using TrypLE as previously described [4]. The two hTSC lines used in this study (1048 and 1049) were previously characterized [23].

EVT and STB differentiation of hTSC

EVT differentiation was performed following a modified Okae protocol by plating 75 000 cells/well onto 6-well plate precoated with 20 μg/ml fibronectin in the Okae EVT differentiation media [4, 23]. On day 3, EVT medium was changed to remove NRG1 and to increase Matrigel concentration to 0.5% until day 5. For STB differentiation, the Okae protocol was slightly modified [4]. Briefly, 100 000 cells/well were plated onto a 6-well plate coated with 2.5 μg/ml of collagen IV into TSC complete media. The next day, media was changed into STB differentiation media containing forskolin. Media was changed at day 3 and experiment was collected at day 6. Exogenous BMP4 was added during differentiation at 100 ng/ml.

BMP signaling inhibitor treatment

For the experiments with small molecule inhibitors, both mTSC and hTSC cells were plated and cultured for 4 days in the respective growth media. The small molecule inhibitors (or same volume:volume ratio of DMSO) were added at the following final concentrations: LDN-193189 at 1-5uM (LDN, STEMCELL Technologies), DMH1 at 4 μM and 8 μM DMH1 (Sigma–Aldrich), and 5Z-7-Oxozeaenol at 100 nM and 250 nM (OXO, Sigma).

Flow cytometry

Cells were stained with a PE-conjugated HLA-G antibody (ExBio MEMG/9), an APC-conjugated EGFR antibody (Biolegend #352906), or relative isotype control antibodies for 1 h in FACS buffer (10% FBS and 0.03% Sodium Azide in PBS) as previously described [19]. Positive cells were quantified at a Canto flow cytometer (BD Biosciences) at the UCSD Human Embryonic Stem Cell Core. Experiments were performed at least in triplicate. Data presented are from one representative experiment.

Cell proliferation assay

Cell proliferation was measured using the Click-iT™ Edu Alexa flour™ 488 flow cytometer assay kit (ThermoFisher Scientific, #C10425,) following manufacturer's instructions. Briefly, mTSCs overexpressing *Bmp4* and hTSC were differentiated as described above scaled-down to a 12-well plate. At the end of the differentiation protocol, cells were incubated for 2 h with 5-ethynyl-2′-deoxyuridine (EdU). DAPI was then added as a nuclear stain and cells were analyzed by flow cytometry. Data were expressed as percentage of cells in S-phase. Experiments were measured in triplicates and data were shown as mean ± SD.

Immunofluorescence

First trimester villous explants and 1048 hTSC were fixed in 4% PFA/PBS after 24 h of treatment with 100 ng/ml of BMP4. They were stained with rabbit anti-P-SMAD1/5/9 antibody (Cell Signaling Technology #13820) overnight, followed by secondary antirabbit 488-conjugated (Thermo Fisher Scientific #A11008) or 594-conjugated (Thermo Fisher Scientific #A11005) Alexa Fluor IgG antibody incubation for 1 h. Nuclei were stained by DAPI during washes and pictures were taken on a Leica microscope.

PCR and RT-qPCR

RNA was collected and purified using the Nucleospin kit according to manufacture protocol. Five hundred nanograms of RNA was reverse-transcribed into cDNA and either used undiluted for PCR (Go Taq™ Master Mix, Promega) or diluted 1:10 for qPCR with SYBR Green. Regular PCR products were run on a 2% agarose gel. qPCR data were analyzed according to the $\Delta\Delta Ct$ method using 18S as house-keeping gene and normalization performed compared to d0 (differentiation experiments) or DMSO vector control (inhibitor treatment experiments). Graphs show relative FC ($2^{-\Delta\Delta Ct}$) with SEM as error bars. Statistical analysis was performed using GraphPad (Prism). See Supplementary Table 1 for primer sequences.

Western blot

Cells were lysed in modified RIPA buffer containing 50 mM Tris/HCl, 150 mM NaCl, 01% (v/v) Nonidet P-40, and 0.1% (w/v) SDS supplemented with fresh protease and phosphatase inhibitors (Roche Applied Science) according to the manufacturer's protocol. The protein content was determined by BCA protein assay (Thermo Scientific). A total of 30 μg of total protein was loaded onto a 12% denaturing polyacrylamide gel for separation and then transferred to PVDF membranes by electrophoresis. Membranes were blocked with 5% nonfat dried milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (Sigma) for 1 h and then incubated overnight with primary antibodies (Supplementary Table 2). After incubation for 1 h with secondary HRP-conjugated IgG antibodies (Supplementary Table 2), signal was captured on film. Densitometry quantification was performed using ImageJ and data were normalized to β-actin.

Statistical analysis

All experiments were performed at least in triplicate. Data were analyzed by using an unpaired *t*-test with Welch correction or one-way ANOVA, as required, using GraphPad Prism and statistical significance was set at $P < 0.05$.

Results

Active BMP machinery in mTSC

We started by investigating the presence of the BMP response machinery in mTSC. mTSC expressed the BMP receptor subunits *Bmpr1a* and *Bmpr2*, but not *Bmpr1b*, both in the undifferentiated state (D0) and after differentiation for 7 days upon removal of growth factors and conditioned media (Figure 1A and B). mTSC also expressed various BMP ligands (Supplementary Figure S1A), including *Bmp2* and *Bmp4*, and the BMP transducing proteins, *Smad1* and *Smad5* (Supplementary Figure S1B). Active canonical BMP signaling was present in undifferentiated mTSC, as demonstrated by phosphorylation of SMAD1/5/9 (Figure 1C). Following differentiation, both total SMAD1 and phosphorylated SMAD1/5/9 were decreased (Figure 1C). These data point to the presence of active BMP signaling in undifferentiated
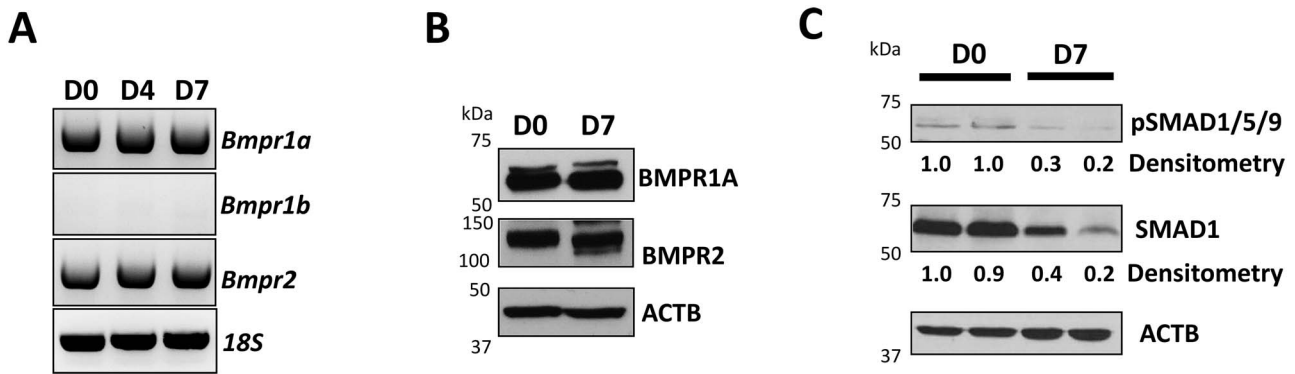
Figure 1. BMP signaling is active in undifferentiated mTSC. (A) RT-PCR of BMP receptor subunits during mTSC differentiation, (18S = loading control). (B) Western blot of BMP receptors in undifferentiated (D0) and differentiated (D7) mTSC. (C) Phosphorylated SMAD1/5/9 and total SMAD1 protein expression in undifferentiated (D0) and differentiated (D7) mTSC by western blot (ACTB = β-actin = loading control). Densitometry data normalized to β-actin and expressed as ratio of D0.

mTSC, and suggest active autocrine signaling in the stem cell state, which rapidly decreases following differentiation.

Role of BMP signaling in undifferentiated mTSC

To study the role of BMP signaling in mTSC in the undifferentiated state, we treated mTSCs with different small molecule inhibitors, targeting both SMAD-dependent and independent branches of the signaling pathway. We initially treated mTSC with the small molecule LDN-193189, a commonly used canonical BMP-specific inhibitor that blocks the kinase activity of both BMPR1 subunits at concentrations between 2μM and 10 μM [9, 24, 25]. Dose–response experiments in mTSC showed cellular toxicity with complete cell death at concentrations of 4 μM and above (Supplementary Figure S2A), but no effects on mTSC morphology or marker expression at lower concentrations (Supplementary Figure S2B and C). We next tried the small molecule DMH1 [26, 27], a dorsomorphin analog highly selective for the BMP pathway, which inhibits phosphorylation of SMAD1/5/9, and 5Z-7-Oxozeaenol (OXO), a SMAD-independent BMP inhibitor, which targets various kinases, including TAK1 [12, 28, 29]. Based on previous publications [12, 26–28] and following a dose–response assay to evaluate cell toxicity, we identified the optimal concentrations for use (8 μM for DMH1 and 250 nM for OXO) (data not shown). Treatment with DMH1 for 96 h showed little-to-no morphological changes (Figure 2A) and only a slight down-regulation of one of the markers of the undifferentiated TSC state (*Epcam*) (Figure 2B), without any associated up-regulation of markers of differentiation (Figure 2C). Treatment with OXO, on the other hand, caused rapid morphological changes, with flattening of TSC colonies (Figure 2A), and was associated with down-regulation of all TSC-associated markers tested (*Cdx2, Epcam,* and *Esrrb*) (Figure 2B), as well as up-regulation of early markers of both labyrinthine trophoblast (the SynT-I marker, *Syna*) and junctional zone/spongiotrophoblast (*Tpbpa* and *Blimp-1*) (Figure 2C). Since OXO is known to be a potent inhibitor of TAK1, we next tested a specific TAK1 inhibitor (TAKinhib) on undifferentiated mTSC, but found no effects on either morphology or markers of differentiation (data not shown). These data suggest a possible role for SMAD-independent autocrine BMP signaling in mTSC maintenance.

Role of BMP signaling during mTSC differentiation

To investigate the role of BMP signaling in mTSC differentiation, we added exogenous BMP4 during differentiation, induced by withdrawal of FGF4 and conditioned media. Although defined serum-free culture conditions have been established for mTSC [30], differentiation in serum-free conditions causes high levels of cell death [30]. Our mTSC differentiation medium includes 20% serum, which is known to contain BMP signaling bioactive factors. To observe the effect of exogenous BMP4 during differentiation, undifferentiated mTSC were serum-starved for 5 h, then differentiated in low serum (2.5% FBS) in the presence of exogenous BMP4 (100 ng/ml). Maintenance of active BMP signaling during BMP4 supplementation in low-serum differentiation was confirmed by maintenance of phosphorylated SMAD1/5/9 (Figure 3A). Exogenous BMP4 treatment did not prevent down-regulation of the stem cell marker, *Cdx2*, during differentiation in low serum (Figure 3B). Although culture in low serum decreased expression of some differentiation markers, like *Prl3d1* (encoding placental lactogen 1) and *Prl3b1* (encoding placental lactogen 2), addition of exogenous BMP4 further decreased expression of *Tpbpa* (a marker of spongiotrophoblast) and *Prl3d1* (a marker of trophoblast giant cells/TGC) (Figure 3B). We observed similar decreases in expression of *Tpbpa, Prl3d1,* and *Prl3b1* when we performed mTSC differentiation with exogenous BMP4 in normal (20%) serum conditions (Supplementary Figure S3), suggesting reduced BMP signaling is required for proper mTSC differentiation.

To validate the results with exogenous BMP4, we over-expressed BMP4 in mTSC using a constitutively-expressed HA-tagged *Bmp4* plasmid construct (Figure 3C). mTSC clones overexpressing HA-tagged BMP4 showed maintenance of phospho-SMAD1/5/9 and total SMAD1 during differentiation (Figure 3D). Compared to wild-type (WT) mTSC, two independent mTSC clones expressing HA-tagged BMP4 (O.E1.1 and O.E2.2) showed blunted differentiation, with low expression of multiple differentiation markers, including *Tpbpa, Prl3d1, Syna, Blimp1* (Figure 3E), and *Prl3b1* (not shown). However, again, down-regulation of the stem cell marker *Cdx2* was not affected (Figure 3E). Cells overexpressing BMP4 also showed higher proliferation than WT cells after 5 days of differentiation, consistent
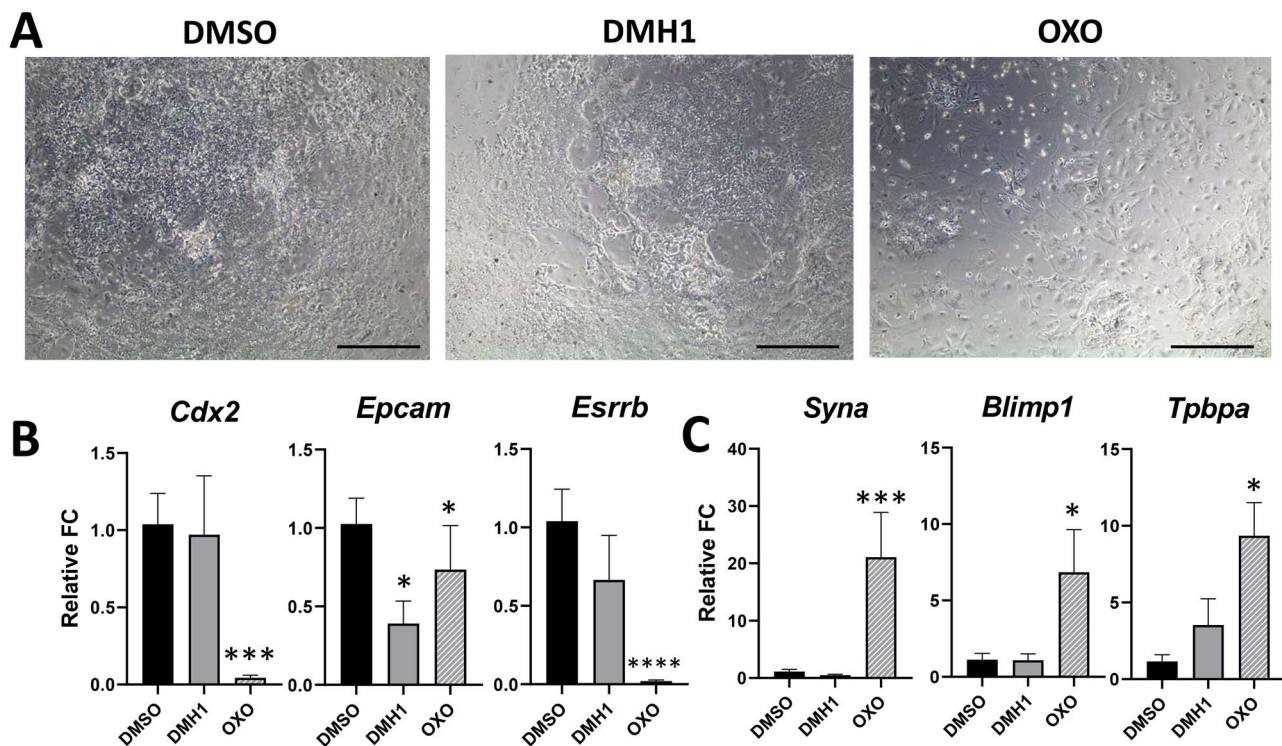
Figure 2. BMP inhibition causes differentiation in a Smad-independent manner. (A) Bright field pictures of mTSC cultured in growth media and treated for 4 days with DMSO (control), DMH1, and 5Z-7-Oxozeaenol (OXO). Treatment with OXO showed large areas of flattening differentiating cells. Scale bar 100 μm. (B) qPCR for mTSC markers *Cdx2*, *Epcam*, and *Esrrb* in mTSC cultured in growth media and treated for 4 days with DMSO or BMP inhibitors. (C) qPCR for mTSC differentiation markers *Syna*, *Blimp1*, and *Tpbpa* in cells cultured in growth media and treated for 4 days with DMSO or BMP inhibitors. $*P \leq 0.05$, $***P \leq 0.001$, $****P \leq 0.0001$ compared to DMSO.

with delayed differentiation (Figure 3F). These data show that continuous BMP signaling blunts or delays mTSC differentiation, suggesting that a decrease in BMP signaling (following FGF4/conditioned media withdrawal) is necessary for proper mTSC differentiation.

Active BMP machinery in primary CTB and hTSC

We next turned our attention to human trophoblast, and started by evaluating the expression of the BMP signaling machinery in first trimester CTB, where the trophoblast progenitor population is thought to reside. First trimester CTB showed expression of the ligand *BMP4*, as well as the receptor subunits *BMPR1A* and *BMPR2*, but not *BMPR1B* (Figure 4A). To assess the pathway activation state, we stained first trimester placental explants for P-SMAD1/5/9. Explants showed low basal level of active BMP signaling in regular media (Supplementary Figure S4). Addition of exogenous BMP4 caused increased P-SMAD1/5/9 staining, specifically in CTB and the proximal cell column compartment, with low or no response in distal cell column and STB layers (Supplementary Figure S4). These data suggest a potential role for BMP signaling in the progenitor compartment of first trimester placenta, where hTSC are thought to reside. Therefore, we continued by investigating the BMP signaling machinery, using the recently-established in vitro hTSC model.

We recently derived and characterized two hTSC lines from first trimester placental tissues: #1049 (derived from a male placenta; Figure 4B), and #1048 (derived from a female placenta; data presented in supplementary figures) [23]. We differentiated both hTSC lines into EVT and STB

(Supplementary Figures S5A and D, S6A and G), and confirmed down-regulation of the trophoblast progenitor marker *TP63* and up-regulation of the respective differentiation markers (Supplementary Figures S5B and E, and S6B and H), by qPCR. STB differentiation was further confirmed by secretion of the hormone hCG in the media with an ELISA-based assay (Supplementary Figures S5C and S6C), while cell surface expression of HLA-G protein by flow cytometry further confirmed EVT differentiation (Supplementary Figures S5F and S6I).

We evaluated BMP signaling machinery in both hTSC cell lines, and found that, similar to first trimester CTB, undifferentiated hTSC (day 0/D0) expressed the BMP receptor subunits *BMPR1A* and *BMPR2*, with little-to-no *BMPR1B* (Figure 4C,D,F and Supplementary Figure S6D,E,J,K). These cells also expressed a panel of BMP ligands, including *BMP2* and *BMP4*, and the BMP transducing protein *SMAD1* and *SMAD5* (Figure S7A and B). They responded to exogenous BMP4 treatment with correct nuclear localization of P-SMAD1/5/9 (Supplementary Figure S7C). Upon differentiation into STB, protein levels of BMP receptors decreased in both cell lines as shown by western blot (Figure 4D and Supplementary Figure S6E), although this decrease was not always reflected at the mRNA level (Figure 4C and Supplementary Figure S3D). During EVT differentiation, BMP receptor levels remained stable at both the mRNA and protein levels (Figure 4C,F and Supplementary Figure S6J–K). Undifferentiated cells showed basal levels of P-SMAD1/5/9, which decreased during both STB and EVT differentiation, while levels of total SMAD1 decreased or remained stable (Figure 4E,G and Supplementary Figure S6F,L). These data
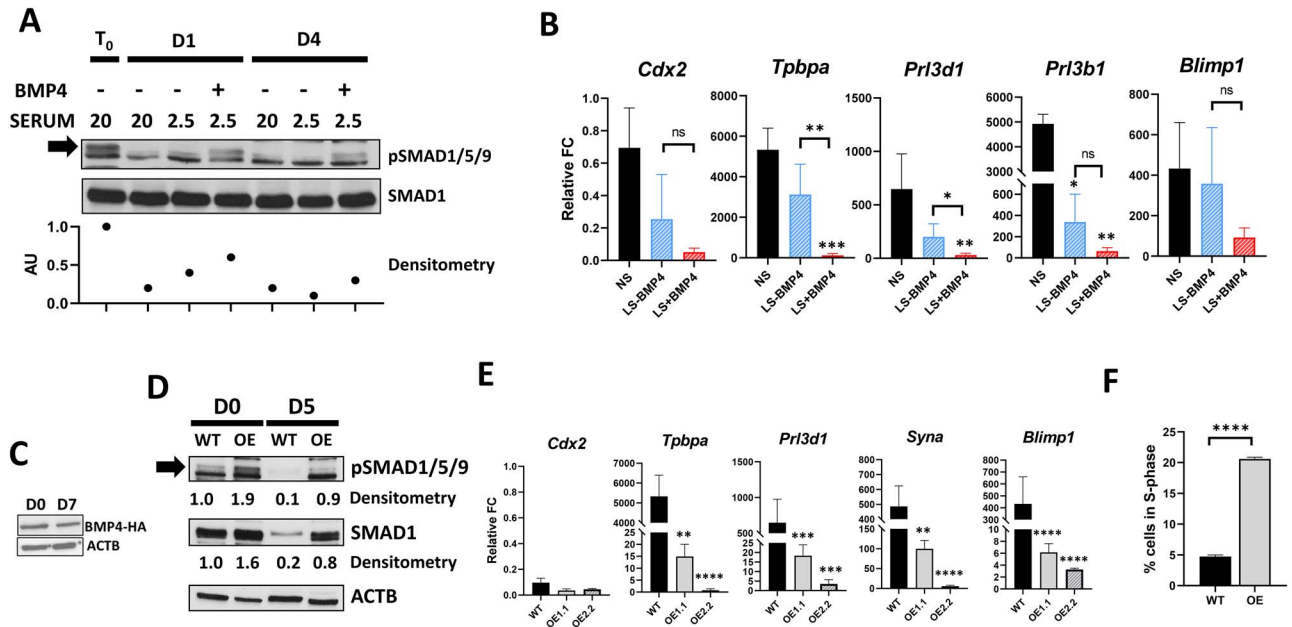
Figure 3. BMP4 delays mouse TSC differentiation. (A) Phosphorylated SMAD1/5/9 and total SMAD1 protein expression during mTSC differentiation in normal serum (20%) and low serum (2.5%) with addition of exogenous BMP4 by western blot. Densitometry normalized to total SMAD1 and expressed as ratio of T0. (B) qPCR for undifferentiated mTSC marker, *Cdx2*, and differentiation markers at d5 of differentiation in normal serum (NS), and low serum (LS) with and without addition of exogenous BMP4. Relative fold change calculated compared to D0. Asterisk directly above column indicates statistical analysis compared to NS. Asterisk above bracket indicates statistical significance between LS-BMP4 and LS + BMP4. (C) Western blot of HA-tagged BMP4 in *Bmp4* OE cell line at D0 and D7 of differentiation. (ACTB = β-actin = loading control). (D) Western blot of phosphorylated SMAD1/5/9, and total SMAD1 in wild-type (WT) mTSC compared to a clone constitutively overexpressing BMP4 (OE) at D0 and D5 of differentiation. Densitometry data normalized to β-actin and expressed as ratio of WT D0. (E) qPCR for differentiation markers expressed in wild-type (WT) and two independent mTSC clones constitutively overexpressing BMP4 during differentiation (OE1.1 and OE2.2) after 5 days of differentiation. Relative fold change calculated compared to D0. (F) Cell proliferation assay of WT and BMP4 OE mTSC at d5 of differentiation. Statistical analysis compared to WT D5. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$. Arrows indicate specific bands on western blots.
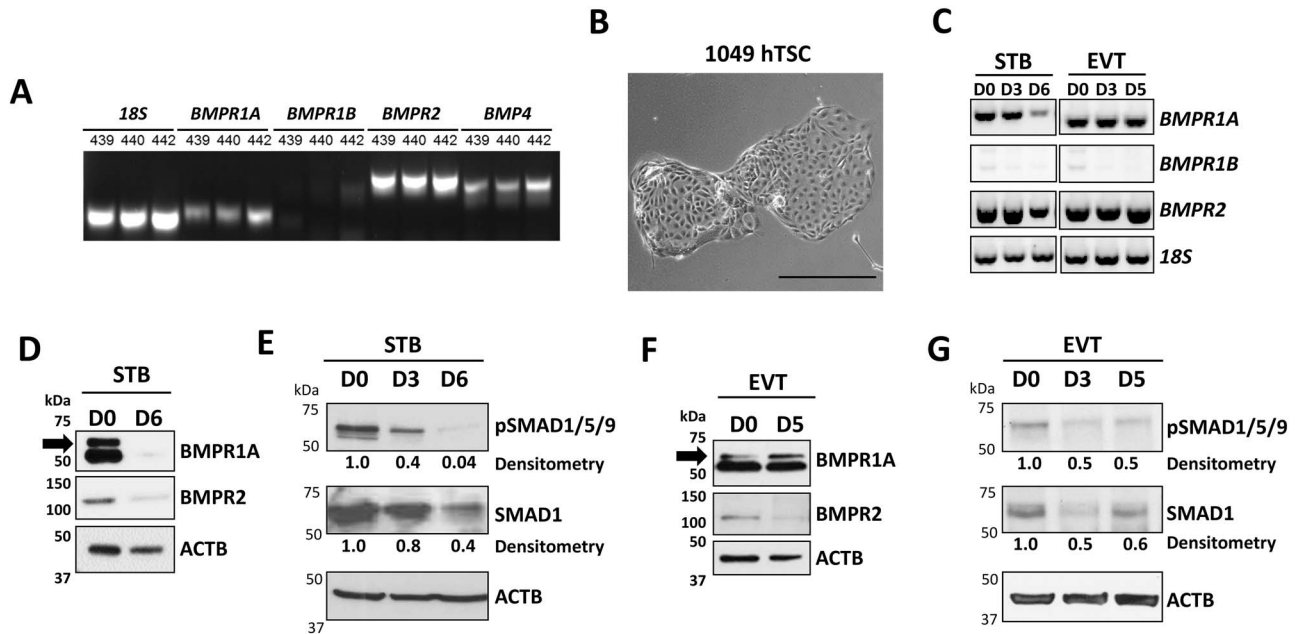

Figure 4. BMP signaling is active in human progenitor cells. (A) RT-PCR of BMP receptor subunits and *BMP4* in first trimester primary CTB preps ($n = 3$), (18S = loading control). (B) Bright field picture of novel hTSCs derived from first trimester placental tissues. Scale bar 250 μm. (C) RT-PCR of BMP receptor subunits during hTSC differentiation into STB and EVT. (D) Western blot of BMP receptors in undifferentiated (D0) and STB differentiated hTSC (D6). (E) Phosphorylated SMAD1/5/9 and total SMAD1 protein expression during differentiation of hTSC into STB by western blot. (F) Western blot of BMP receptors in undifferentiated (D0) and EVT differentiated hTSC (D5). (G) Phosphorylated SMAD1/5/9 and total SMAD1 protein expression during differentiation of hTSC into EVT by western blot. (ACTB = β-actin = loading control). Arrows indicate specific bands on western blots. Densitometry data normalized to β-actin and expressed as ratio of D0.
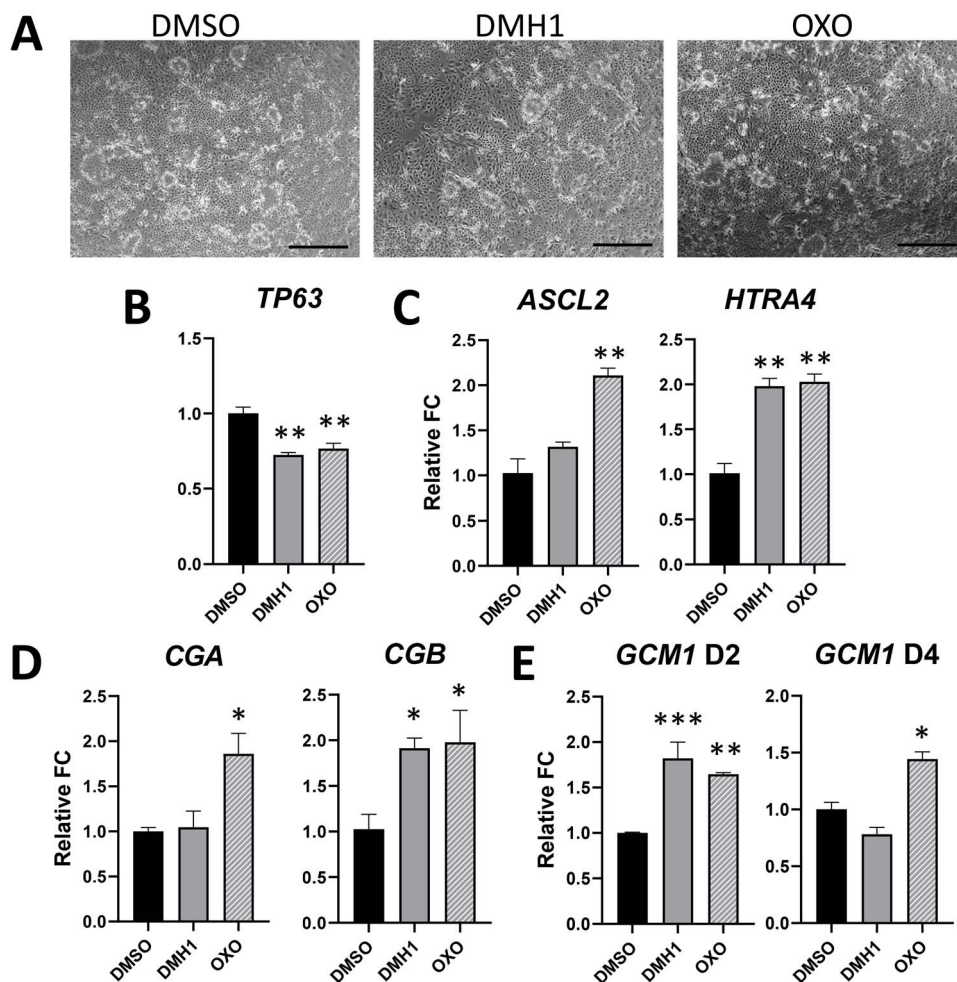
Figure 5. BMP inhibition causes differentiation of hTSC. (A) Bright field pictures of hTSC cultured in growth media and treated for 4 days with DMSO (control), DMH1, and 5Z-7-Oxozeaenol (OXO). Scale bar 100 μm. (B) qPCR for hTSC marker *TP63* in cells cultured in growth media and treated for 4 days with DMSO or BMP inhibitors. (C) qPCR for hTSC differentiation markers *ASCL2* and *HTRA4* at day 4 of treatment with of DMSO/BMP inhibitors. (D) qPCR for *CGA* and *CGB* genes at day 4 of treatment with DMSO/BMP inhibitor treatment. (E) qPCR for *GCM1* at day 2 and day 4 of DMSO/BMP inhibitor treatment. $*P \leq 0.05, **P \leq 0.01, ***P \leq 0.001$ compared to DMSO.

suggest that, similar to mTSC, BMP signaling in hTSC is active in the undifferentiated state and decreases with differentiation, pointing to a potential role for this pathway in regulation of the hTSC stem cell state.

Role of BMP signaling in undifferentiated hTSC

To investigate the role of the BMP signaling pathway in hTSC, we treated the cells with the small molecule inhibitors DMH1 and 5Z-7-Oxozeaenol (OXO) while cultured in regular growth media. Based on previous publications [12, 26–28] and following a dose–response assay, we identified optimal concentrations to be 4 μM for DMH1 and 100 nM for OXO (data not shown). Although 4 days of treatment with the small molecule inhibitors did not significantly affect hTSC morphology (Figure 5A), we observed a slight, but significant, down-regulation of undifferentiated hTSC marker *TP63* (Figure 5B). We also observed an up-regulation of EVT markers *ASCL2* (OXO only) and *HTRA4* (both OXO and DMH1) by qPCR (Figure 5C). STB markers were also up-regulated: *CGA* (OXO only) and *CGB* (both OXO ad DMH1) (Figure 5D). *GCM1*, a marker of both STB and EVT differentiation, was up-regulated at day 2 with both inhibitors and remained high at day 4 with OXO (Figure 5E). Although the effects were more

subtle than observed with OXO-treated mTSC, the results with DMH1 and OXO treatment of hTSC suggest a possible contribution from both SMAD-dependent and -independent pathways in maintenance of the undifferentiated hTSC state.

Role of BMP signaling during hTSC differentiation

To investigate the role of BMP signaling during hTSC differentiation, we first applied exogenous BMP4 during STB differentiation [4] (Supplementary Figure S8A). We observed dramatic cell detachment and death around D4 (Supplementary Figure S8B), with no consistent changes in expression of STB-associated markers in either hTSC line (Supplementary Figure S8C and D). Although this suggests a role for dampened BMP signaling in STB viability, the onset of cell death early in this differentiation precluded a more thorough evaluation of the effect of BMP signaling during STB differentiation.

Subsequently, we assessed the effect of exogenous BMP4 in EVT differentiation of hTSC. Similar to the observations in mTSC, addition of exogenous BMP4 (Figure 6A and B and Supplementary Figure S9A) significantly inhibited EVT differentiation. Although the TSC marker, *TP63*, was correctly downregulated (Figure 6C and Supplementary Figure S9B),
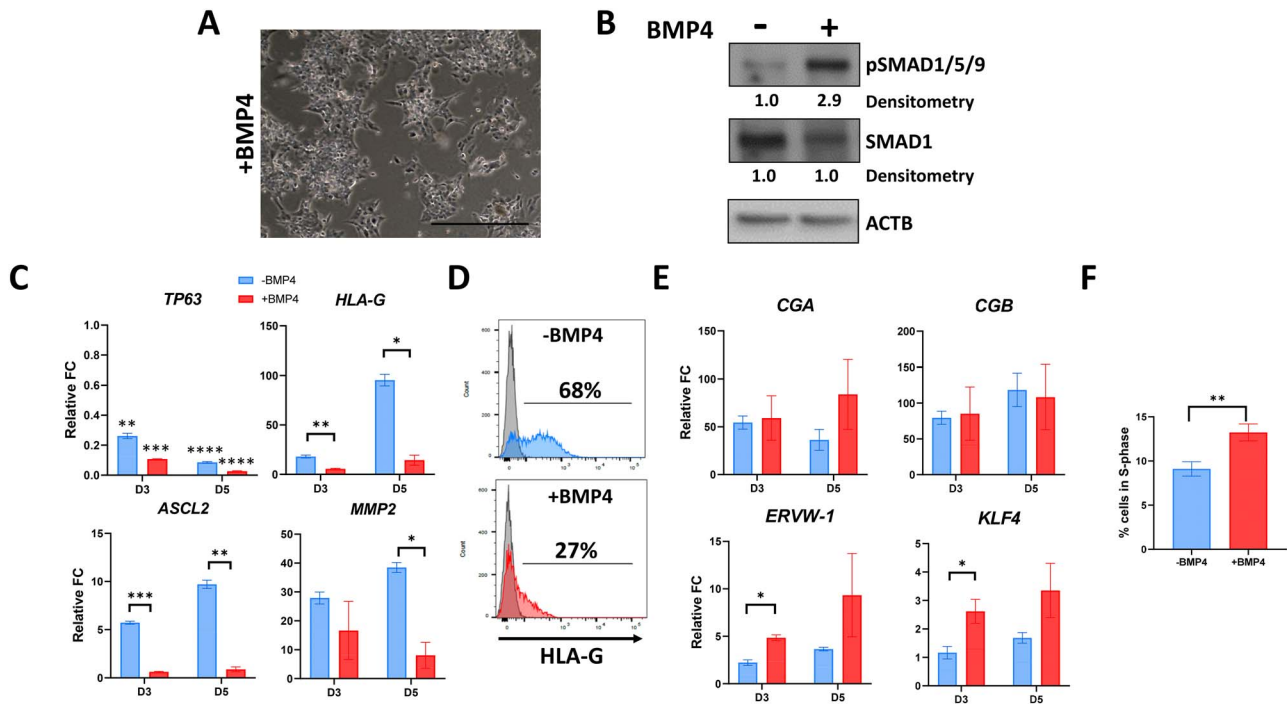
Figure 6. Addition of BMP4 delays human TSC differentiation into EVT. (A) Bright field pictures of hTSC differentiated for 5 days following EVT protocol in the presence of exogenous BMP4. Scale bar 100 μm. (B) Phosphorylated SMAD1/5/9 and total SMAD1 protein expression during hTSC differentiation into EVT in the presence of exogenous BMP4. Densitometry data normalized to β-actin and expressed as ratio of D0. (C) qPCR for hTSC marker *TP63* and EVT markers *ASCL2*, *HLA-G*, and *MMP2* of hTSC differentiated into EVT with exogenous BMP4. (D) Flow cytometry analysis of cell surface HLA-G in hTSC differentiated into EVT with (red) and without (blue) exogenous BMP4. Isotype control in gray, HLA-G staining in blue or red. (E) qPCR for STB markers *CGA*, *CGB*, *ERVW-1* (encoding for Syncytin-1), and *KLF4* of hTSC differentiated into EVT with exogenous BMP4. (F) Percentage of cells in S-phase after 5 days of EVT differentiation with and without exogenous BMP4. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

we observed significantly lower expression of EVT markers, *ASCL2*, *HLA-G*, and *MMP2*, by qPCR (Figure 6C and Supplementary Figure S9C), as well as a decrease in cell surface expression of HLA-G by flow cytometry (27% with BMP4, compared to 68% without) (Figure 6E and Supplementary Figure S9D). The blunted EVT differentiation was not accompanied by a significant increase in STB differentiation, although we observed small but significant increase in some STB markers in a cell line-specific manner: *ERVW-1* (also known as syncytin 1) and *KLF4* at day 3 in 1049 hTSC (Figure 6E), and *CGA* but not *CGB* at day 5 in 1048 hTSC (Supplementary Figure S9E). In addition, cell proliferation was higher in differentiating cells treated with BMP4, consistent with delayed differentiation (Figure 6F). These data suggest a potential role for dampened BMP signaling in EVT differentiation.

Discussion

In vivo, the role of BMP4, originating in the trophoblast compartment, in the initiation of gastrulation is well-characterized [10, 11]. However, little is known about BMP signaling within the extraembryonic compartment itself. In this study, we used the in vitro TSC model systems to dissect the role of BMP signaling in the maintenance and differentiation of this compartment in both mouse and human. Both mTSC and hTSC expressed all the necessary components of the BMP machinery, including multiple BMP ligands, and displayed maximal active BMP signaling in the undifferentiated state, which decreased with differentiation (Figure 7A). We also observed a conserved modulatory role of BMP signaling during trophoblast differentiation, whereby addition

of BMP4 blunted differentiation of TSC in both species. In the mouse, Di-Gregorio et al. have shown the presence of phospho-SMAD1/5/9 in the proximal ExE/chorion portion of the embryo, adjacent to the epiblast, but not in the distal extraembryonic region (ectoplacental cone) closer to the uterine implantation site, indicating that BMP signaling decreases as trophoblast differentiate within this compartment (Figure 7B) [17]. This suggests that a gradual decrease in BMP signaling may be necessary for differentiation within this compartment in vivo, which matches our in vitro data with mouse TSC.

Two previous studies have investigated the role of BMP signaling in the peri-implantation mouse embryo [12, 31]. Specifically, they have shown that treatment of mouse embryos with small molecule inhibitors, targeting both canonical and noncanonical BMP signaling, impaired development of the extraembryonic compartment. Furthermore, data from 3D Matrigel-embedded mTSC models suggest a role for the canonical BMP pathway in cell proliferation [31]. In our study, the effect of BMP signaling on the undifferentiated state of TSC appeared to be more complex and species-specific compared to the role observed during differentiation. In the mouse, although we observed higher phospho-SMAD1/5/9 levels in the undifferentiated cells, the stronger effect elicited by the Smad-independent inhibitor 5Z-7-Oxozeaenol (OXO) suggests a tight connection with other MAPKKK pathways. Previous studies have shown that at the concentrations used in this study, OXO is a potent and selective inhibitor of TAK1 [28, 32, 33] but, at higher concentrations, it can affect other kinases [28, 29]. Since treatment with an alternative TAK1 inhibitor did not reproduce the results observed with OXO, further studies are required to identify the exact mechanism
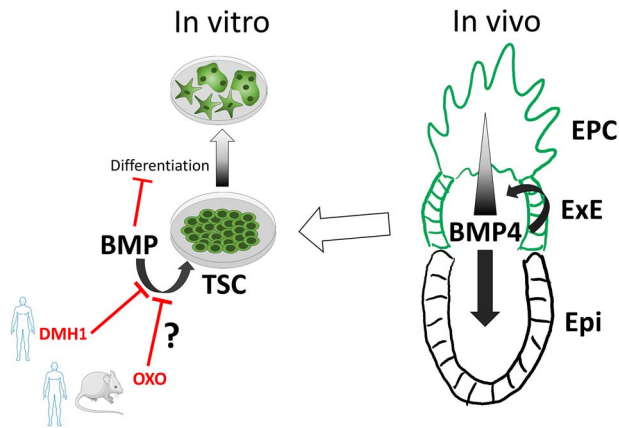
Figure 7

Figure 7. Autocrine role of BMP signaling pathway in vitro and in vivo. Autocrine BMP signaling modulates TSC maintenance in vitro in a species-specific manner, whereby in mouse the highest effects were observed upon noncanonical SMAD-independent signaling inhibition (OXO), whereas in human both the canonical (DMH1) and noncanonical (OXO) branches were involved. Decrease BMP signaling is necessary for TSC differentiation in vitro as addition of exogenous BMP4 blunted trophoblast differentiation in both mouse and human TSC. These data suggest that autocrine BMP signaling contributes to the maintenance of a trophoblast progenitor population in the postimplantation extraembryonic ectoderm (ExE) in vivo. Decrease BMP signaling is necessary for correct differentiation toward the ectoplacental cone (EPC) while paracrine BMP signaling into the embryo initiates gastrulation and differentiation into progenitor germ cells of the epiblast (Epi).

through which this small molecule affects undifferentiated TSC, including whether this effect is indeed downstream of BMP receptors.

In undifferentiated hTSC, the effect of the BMP inhibitors seemed to be more subtle, though perhaps mediated by both SMAD-dependent and independent branches of the BMP pathway. The differences between the effects of these inhibitors on undifferentiated TSC in these two species are perhaps not surprising, given the known differences between the culture conditions required for TSC derivation and maintenance. These differences, in turn, may be correlated to the species-specific characteristics of the trophoblast layer, labyrinthine in the mouse and stratified epithelial in human. Although we observed a conserved role for BMP signaling in blunting TSC differentiation in this study, studies in equine models suggest a role for BMP4 signaling in promoting binucleate trophoblast differentiation in the chorionic girdle [34]. Similar to mouse and human, bovine peri-implantation concepti express *BMP2* and −*4* as well as BMP receptors in the trophoblast cells, but addition of exogenous BMP4 showed conflicting results, with decreased trophoblast proliferation and reduced expression of mRNA for *IFN-tau* [35]. Further studies into the various roles of BMP signaling in maintenance and differentiation of trophoblast in different placental mammals could reveal further details about the mechanism(s) of divergent evolution of this transient organ.

One limitation of the mTSC model is the mixed (nonlineage-directed) differentiation upon FGF4/conditioned media withdrawal, resulting in a mixture of labyrinthine and junctional zone trophoblast cell types in vitro, precluding the evaluation of the effects of continuous BMP signaling on lineage-specific differentiation. The hTSC system, on the other hand, provided a platform for this distinction,

showing that BMP signaling mainly interfered with EVT differentiation, while during STB differentiation it might affect cell survival. The STB differentiation protocol is driven by the syncytialization capacity of forskolin, a cAMP pathway activator that induces expression of fusogenic genes as well as hCG. Forskolin is known to induce a rapid and potent ERK1/2 and p38MAPK phosphorylation in BEWO cells [36]. BMP4 is also known to activate the same pathways in a Smad-independent manner in other cell types, including smooth muscle cells [37, 38]. Although the focus of this study was on the conserved role of decreasing BMP signaling needed for proper differentiation of mTSC, as well as EVT differentiation of hTSC, questions remain regarding the role of BMP signaling in cell proliferation and survival in the context of STB differentiation. Given the role of EVT in uterine wall invasion and vascular remodeling, processes which are key to early placental development [39, 40], the study of BMP signaling in this context may provide insight into placenta-based pregnancy complications, including preeclampsia, fetal growth restriction, and placenta accreta spectrum. Of course, the in vitro nature of these models is also a limitation, necessitating confirmatory studies using animal models and human tissues. Studies using trophoblast-specific knockout of BMP receptors will be important in mice; however, investigating the role of this signaling pathway in the peri-implantation human embryo will be more difficult. One possibility would be the use of a new 3D stem cell-based embryo ("STEMbryo") model, which can recapitulate certain steps in early embryonic development, using a mix of embryonic and TSC [41, 42]. Use of such a model system could not only avoid the ethical issues surrounding use of human embryos, but, given that these cells can be more easily and separately manipulated, this model would provide a robust platform to investigate the role and directionality of different signaling pathways within and between the embryonic and extraembryonic compartments in the peri-implantation period.

In summary, this study has revealed a potential role for autocrine BMP signaling in TSC of both mouse and human. Further studies are required to evaluate the role of specific ligands and receptors in this key pathway in differentiation of trophoblast and development of the placenta in vivo. Since abnormalities of trophoblast differentiation are associated with poor implantation or placental dysfunction later in pregnancy, our results have implications for understanding possible causes of miscarriage and/or preeclampsia.

Supplementary material

Supplementary material is available at *BIOLRE* online.

Acknowledgment

Conflict of interest

The authors have no conflict of interest.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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