UNIVERSITY OF CALIFORNIA SAN DIEGO

BMP Signaling in Mouse Trophoblast Stem Cell Maintenance and Differentiation

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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Professor Alon Goren
Professor Deborah Yelon

2019

The Thesis of Jennie Au is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2019

EPIGRAPH

Be of good cheer. Do not think of today's failures,
but of all the success that may come tomorrow.
You have set yourself a difficult task,
but you will succeed if you persevere;
and you will find a joy in overcoming obstacles.

Helen Keller

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LIST OF ABBREVIATIONS

TSC	Trophoblast Stem Cell
BMP	Bone Morphogenetic Protein
Bmpr	Bone Morphogenetic Protein Receptor
Syna	Syncytin A
Tpbpa	Trophoblast Specific Protein Alpha
Pl1	Placental Lactogen 1
Pl2	Placental Lactogen 2
Cdx2	Caudal Type Homeobox 2
Esrrb	Estrogen Related Receptor Beta
Blimp1	PR Domain Containing 1 (Prdm1)
Gcm1	Glial Cells Missing Homolog 1

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This thesis in full is unpublished material that will be prepared for submission for publication of the material. Au, Jennie; Wakeland, Anna; Soncin, Francesca; Parast, Mana. The thesis author was the primary investigator and author of this material.

ABSTRACT OF THE THESIS

BMP Signaling in Mouse Trophoblast Stem Cell Maintenance and Differentiation

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Jennie Au

Master of Science in Biology

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Professor Mana Parast, Chair
Professor Heidi Cook-Anderson, Co-Chair

Previous studies have shown the fatal effects of BMP treatment on early mouse blastocysts, but never in isolated trophoblast stem cells. The question being addressed in this research investigates the role of BMP signaling in mouse trophoblast stem cell maintenance and differentiation. TSC are evaluated through the addition and removal of BMP signaling via exogenous Bmp4, overexpression of Bmp4, knockdown of Bmpr2,

and small molecule inhibitors. The addition of exogenous Bmp4 inhibited some differentiation markers, while the overexpression of Bmp4 inhibited all the differentiation markers tested. However, there was insufficient knockdown of Bmpr2 using inducible shRNA, leading to the use of small molecule inhibitors to remove BMP signaling. The use of LDN193189, DMH1, 5Z-7-Oxozeaenol, and Takinib in TSC cultures resulted in the largest effect morphologically and in differentiation markers with 5Z-7-Oxozeaenol, targeting the non-canonical BMP signaling pathway. Although TAK1 was determined not to be the target of 5Z-7-Oxozeaenol, suggesting other targets such as NF-κB or JNK/p38. Overall, the findings suggest that BMP signaling maintains TSC in their undifferentiated state and inhibits differentiation into their respective lineages. Importantly, this study may provide more insight on how BMP signaling affects early mouse placental development and is thus relevant to placental complications such as pre-term birth, preeclampsia, and placenta accreta.

CHAPTER 1: INTRODUCTION

The placenta is an organ of fetal origin that develops in the uterus of eutherian mammals during pregnancy. It connects the fetus to the mother and provides nutrients and gas exchange via the umbilical cord. It also protects the fetus from the maternal immune system, as well as providing necessary hormones for fetal growth. The placenta is a temporary organ, which is delivered after the baby during labor. Abnormal placental development and functions can result in multiple complications, affecting both the baby, such as pre-term birth and intra-uterine growth restriction, and the mother, like preeclampsia and placenta accreta. Normal and abnormal development of the placenta are poorly understood processes and further studies are necessary to improve pregnancy outcomes.

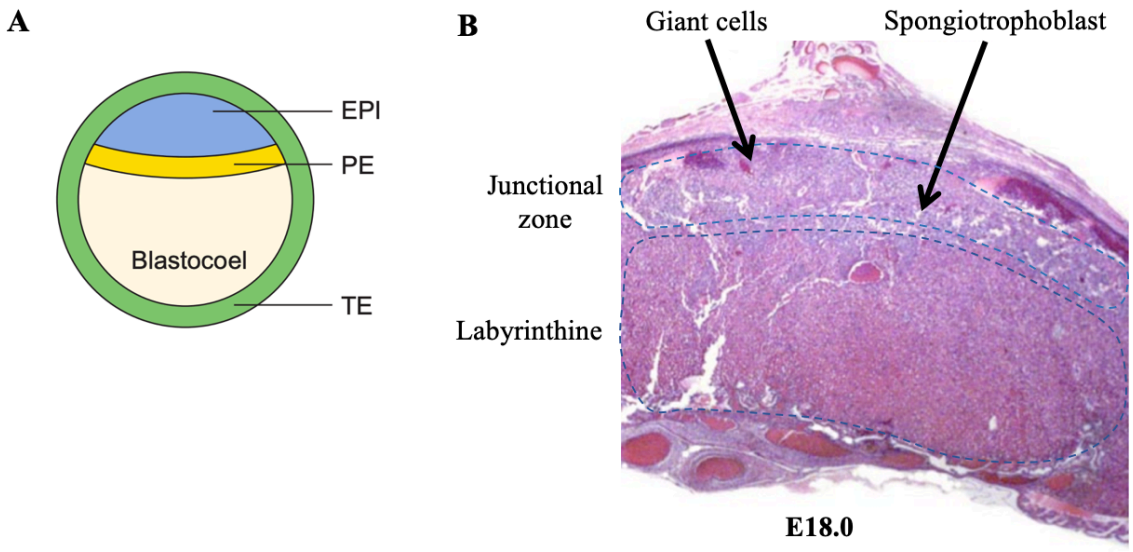


Figure 1: Development of blastocyst and mouse placenta. (A) The blastocyst is comprised of the epiblast (EPI), primitive endoderm (PE), and trophectoderm (TE) in both humans and mouse. Reprinted from Rossant, 2015. (B) The mouse placenta at E18.0 is comprised of a labyrinthine layer and junctional zone. Figure 1B courtesy of K. Benirschke.

The early stages of placental development are similar between eutherian mammals, including human and mouse. After fertilization and a few rounds of cell division, the blastocyst forms, which is composed of an outer cell layer, the trophectoderm, and the inner cell mass (ICM) (Figure 1A). The ICM further develops into the embryo proper, while the trophectoderm gives rise to the epithelial cells (trophoblast) of the placenta, which originate from a small population of trophoblast stem cells (TSC). In the mouse, such TSC can be isolated from both pre- and early post-implantation blastocysts and maintained *in vitro* under specific culture conditions. It is known that the ICM secretes fibroblast growth factor 4 (FGF4) and Nodal, which signal to the polar trophectoderm, where the TSC reside, and help to maintain this niche [10]. Similarly, TSC can be maintained *in vitro* with provision of FGF4 and Activin A/Nodal in the culture media. Upon the removal of these two signals, TSC differentiate into various trophoblast cell types that make up the distinctive layers of the placenta. In the mouse, we distinguish three main trophoblast cell types: the multinucleated syncytiotrophoblast (STB) in the labyrinthine layer, and the trophoblast giant cells (TGC) and spongiotrophoblast in the junctional zone (Figure 1B). Expression of specific markers is used to identify each trophoblast cell type in the mouse, including *Cdx2*, *Esrrb*, Gcm1, *Syna*, *Tpbpa*, *Pl1*, *Pl2, and Blimp1*. *Cdx2* and *Esrrb* are markers of undifferentiated TSC, while the other four markers identify various lineages of differentiated cells. *Gcm1* is a marker of progenitor of STB, *Syna* a marker of STB, *Tpbpa* and *Blimp1* markers of spongiotrophoblast, and *Pl1* and *Pl2* markers of TGC.

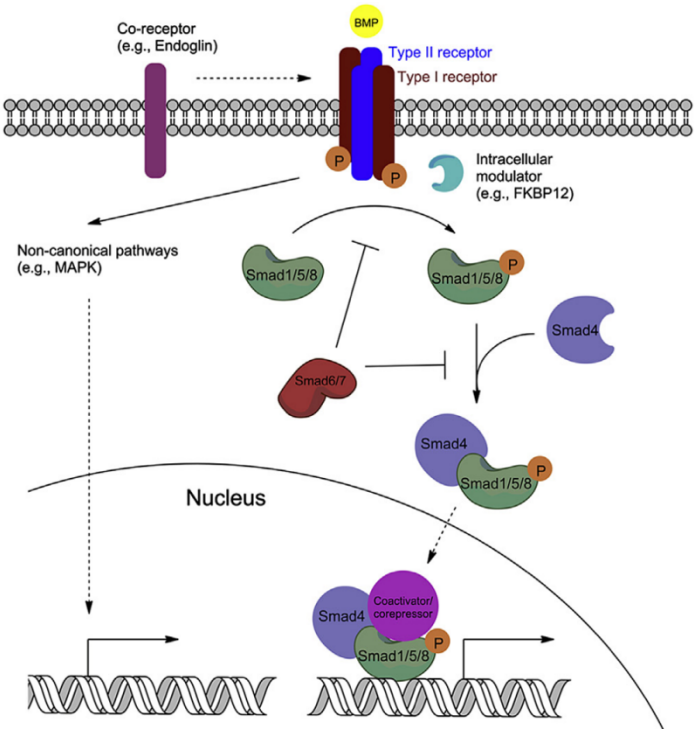


Figure 2: BMP signal transduction pathway. BMPs signal via the canonical, Smad-dependent pathway or various non-canonical pathways. Reprinted from Wang, 2014.

A key developmental signaling pathway is the bone morphogenetic protein (BMP) signaling pathway, which is involved in cell growth, apoptosis, and differentiation. There are two modes for BMP signaling: the canonical and non-canonical pathways (Figure 2). Canonical BMP signaling is mediated by Smad proteins, whereas non-canonical BMP signaling is mediated by other lesser known mechanisms, such as MAPK, TAK-p38, PI3-kinase, Cdc42, and LIMK [2]. The signal transduction machinery is composed of two receptors, type I and II, with type I including two subunits, alpha and beta. The receptors form a heterotetrametric complex comprised of two dimers each of type I and type II. The constitutively active type II receptor transphosphorylates the type I receptor. The phosphorylation of the BMP receptors allows for the recruitment and

phosphorylation of Smad1/5/8, which then form a complex with Smad4 and translocate into the nucleus, where, in conjunction with other proteins, they regulate the expression of specific downstream gene targets [14].

The role of BMP signaling has been studied in numerous *in vitro* and *in vivo* systems. For example, Bmpr1a and Bmpr1b have different expression patterns for the specification and differentiation of osteoblasts and adipocytes *in vitro* [1]. This study showed that the BMP signaling pathway is crucial in several cell types for proper cell proliferation and differentiation. Several *in vivo* studies demonstrate the lethality of BMP receptor mutations. For example, a Bmpr1a mutation causes peri-implantation lethality in mice [9] and Bmpr2 deficient mice exhibit embryo lethality prior to gastrulation [7]. These studies suggest that the BMP machinery is required for proper early embryo development.

Moreover, the treatment of mouse blastocysts with BMP inhibitors or dominant negative Bmpr2 caused defects in extra-embryonic lineages [3]. At the same time, Bmp4-treated mouse embryos showed a delay in blastocyst formation [5]. These studies suggest that BMP signaling might play a role in the establishment, maintenance, and/or early differentiation of the mouse trophectoderm in the blastocyst.

In this study, our aim is to evaluate the role of BMP signaling in mouse trophoblast stem cells, with respect to both maintenance and differentiation. To this end, we begin with investigating the presence of the BMP machinery and signaling both during TSC maintenance and differentiation. Because BMP signaling decreased during TSC differentiation, we further investigate the role of BMP signaling in these systems by

manipulating the activation of the signals both during TSC maintenance and differentiation using multiple approaches. Maintenance of the BMP signal during differentiation was obtained both by addition of exogenous Bmp4 in culture and by establishment of Bmp4-overexpressing mouse TSC lines. Inhibition of BMP signaling during TSC maintenance was achieved by inducible shRNA-mediated knockdown of Bmpr2 and by the use of small molecule inhibitors. Some inhibitors include LDN193189, dorsomorphin homolog 1 (DMH1), 5Z-7-Oxozeaenol, and Takinib. Both analogs of BMP inhibitor dorsomorphin, LDN193189 and DMH1 are BMP receptor type I inhibitors; however, DMH1 has higher selectivity. 5Z-7-Oxozeaenol is a non-canonical BMP signaling inhibitor with several targets such as IL-1-induced activation of NF-κB, JNK/p38, and TAK1. Takinib is a highly selective TAK1 inhibitor. Our results show that BMP signaling is required for the maintenance of TSC in their undifferentiated state, and that loss of BMP signaling is necessary for optimal trophoblast differentiation.

CHAPTER 2: METHODS

Generation of Overexpressed and Knockdown Plasmids

For the generation of the Bmp4-overexpressing plasmid, the BMP4 gene 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. Correct in-frame insertion was verified by sequencing.

For the generation of Bmpr2 shRNA constructs, the BLOCK-iT™ RNAi Designer tool from Invitrogen was used. Five sequences were picked, three targeting the open reading frame (ORF) and two targeting the 3' untranslated region (UTR, the tool could not find any suitable 5' UTR sequences). The sequence CTCGAG was used as the short hairpin loop. For a comprehensive list of the target sequences, please see Table 1. shRNA/Scramble oligos were aligned at the bench and cloned into the inducible Tet-pLKO-neo plasmid (gift from Dmitri Wiederschain plasmid #21916; Addgene). This plasmid includes a Tet-On system for the tetracycline-mediated induction of the insert expression (Figure 3).

Table 1: Target shRNA sequence. Five sequences were generated to target varying regions of the Bmpr2 sequence and a Scramble sequence for a negative control.

	Target Sequence
ORF1	5' – GCTGCTGTAGTACAGATTTAT – 3'
ORF2	5' – GGAGCAGTATATAAAGGTTCC – 3'
ORF3	5' – GCTTGTGATGGAGTATTATCC – 3'
UTR4	5' – GCAGCAAGGACTTGCTTTAAA – 3'
UTR5	5' – GCAGACAAATTGTTACCATGT – 3'
Scramble	5' – CCTAAGGTTAAGTCGCCCTCG – 3'

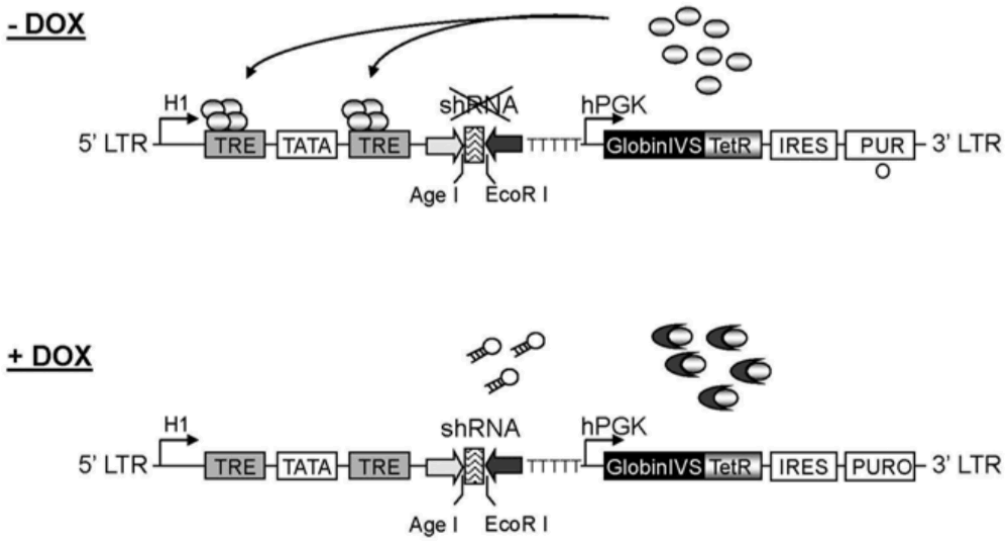


Figure 3: Schematic of pLKO-Tet-On and its mechanism of action. The vector contains the necessary components for the inducible expression of shRNA in target cells. In the absence of tetracycline/doxycycline, shRNA expression is suppressed by constitutively-expressed TetR protein. Upon the addition of tetracycline or doxycycline to the growth media, shRNA expression is triggered resulting in target gene knockdown. Reprinted from Wiederschain, 2009.

Mouse Cell Culture

Mouse TSC were derived from E3.5 blastocysts from wild-type Black 6 and Sv 129 mice as previously described [12]; one cell line from each background was used for all experiments. TSC were cultured at 37ºC and 5% CO_2 in RPMI 1640 culture medium (Corning) supplemented with 20% fetal bovine serum (FBS, Omega Scientific), 1mM sodium pyruvate (Invitrogen), 55 nM β-mercaptoethanol (Invitrogen), 25 ng/ml fibroblast growth factor 4 (FGF4; Sigma), and 1 µg/ml heparin (Sigma) with 70% of medium being conditioned for 72 hours on inactivated mouse embryonic fibroblasts. For differentiation conditions, TSC medium was used as described above without supplementation with FGF4, heparin, or conditioned medium. For low serum conditions, cells were serum

starved for 2 hours and the experiment was conducted in media supplemented with only 2.5% FBS with the addition of 100 ng/ml BMP4 (R&D Systems).

TSC were transfected with Lipofectamine-2000 (Invitrogen) according to manufacturer's instructions with slight modifications. In brief, TSC were grown to 60% confluence in 6-well plates and then incubated with the Lipofectamine/DNA complexes overnight; all cells were co-transfected with a CMV-GFP lentiviral vector to identify transfected cells. The next day cells were washed with PBS and new medium was added. After 24 hours, cells were treated with the appropriate antibiotics to allow selection of transfected cells. For the generation of the Bmpr2 shRNA stable cell lines, GFP positive colonies were selected in culture. Tetracycline hydrochloride (Sigma-Aldrich) was used at 1 μg/ml to induce shRNA expression and gene knockdown.

For the experiments with small molecule inhibitors, TSC were plated and cultured in growth media. The following inhibitors were used: LDN193189 (STEMCELL Technologies), 5Z-7-Oxozeaenol (Sigma-Aldrich), DMH1 (Sigma-Aldrich), and Takinib (Sigma-Aldrich). All inhibitors were dissolved in dimethyl sulfoxide (DMSO). The DMSO dilution equivalent to the highest concentration of inhibitor was used as a vehicle control. Concentrations of inhibitors were based on the literature and IC_{50} value of each target, please see Table 2.

Table 2: List of small molecule inhibitors. Small molecules tested in this study, along with their targets and IC_{50} values.

Name	Targets
LDN193189	• Inhibitor of BMP type I receptors ALK2 (IC_{50} = 5 nM), ALK3 (IC_{50} = 30 nM)
DMH1	• Selective inhibitor of BMP type I receptors ALK2 (IC_{50} = 107.9 nM)
5Z-7-Oxozeaenol	• Potent inhibitor of the MAPKKK TAK1 (IC_{50} = 8 nM) • Inhibits IL-1-induced activation of NF-κB (IC_{50} = 83 nM) and JNK/p38.
Takinib	• Selective inhibitor of TAK1/MAP3K7 (IC_{50} = 9.5 nM)

RNA preparations, cDNA Synthesis, and RT and Quantitative Real-Time PCR

Total RNA from TSC cultures was isolated using the NucleoSpin ® RNA isolation kit (Macherey-Nagel). 500 ng of RNA was reverse transcribed using the PrimeScript™ RT Reagent Kit (Takara) in a 10µl reaction. The cDNA was diluted 10 fold in nuclease-free water and 4 µl of the diluted cDNA, along with 12.5 µM of each primer and POWER SYBR Green PCR master mix (Applied Biosystems) were used for the PCR reaction. Quantitative real-time PCR (qRT-PCR) reactions were performed in triplicate for each sample in a StepOnePlus™ Real-Time PCR System (Applied Biosystems) with a one-step program: 95°C, 10 min; 95°C, 10 s, 60°C, 1 min, for 40 cycles. For the analysis of the small molecule inhibitors, the cDNA was diluted to 3 ng/µl in nuclease-free water and 1 µl of the diluted cDNA, along with 12.5 µM of each primer and SYBR® Premix Ex Taq™ (Takara) were used for the PCR reaction. qRT-PCR reactions were performed in triplicate for each sample in a QuantStudio 5 Real-Time

PCR System (Applied Biosystems) with a one-step program: 95°C, 30 s; 95°C, 5 s, 60°C, 34 s, for 40 cycles. A melting curve analysis was arranged for determination of PCR product specificity. For each reaction, a cycle threshold (Ct) value was recorded. The mean Ct value of triplicate reactions was used to determine the relative gene expression normalized against 18S, using the comparative Ct (ΔΔCt) method [8]. For a comprehensive list of primers used in this study please see Table 2. For the RT-PCR, the samples were run for 35 cycles and visualized on a 2% agarose gel with 1:1000 of GelRed Nucleic Acid Stain (41003, Biotium).

Table 3: List of primer sequences. Sequence of primers used for qPCR.

F, forward; R, reverse.

Gene name	Primer Sequence
Blimp1	F, 5' – GCAAAGAGGTTATTGGCGTGG – 3' R, 5' – TTCACGTAGCGCATCCAGTT – 3'
Bmpr2	F, 5' – AGCACAGAGGCCCATTCTC – 3' R, 5' – CTTGTGTTGACTCACCTATCTGT – 3'
Cdx2	F, 5' – CCAGCTCTTTGCCTCTCTGT – 3' R, 5' – TGCCTCTGGCTCCTGTAGTT – 3'
Esrrb	F, 5' – GGGAGCTTGTGTTCCTCATC – 3' R, 5' – CTACCAGGCGAGAGTGTTCC – 3'
Gcm1	F, 5' – AACACCAACAACCACAACTCC – 3' R, 5' – CAGCTTTTCCTCTGCTGCTT – 3'
Pl1	F, 5' – TGGTGTCAAGCCTACTCCTTT – 3' R, 5' – CAGGGGAAGTGTTCTGTCTGT – 3'
Pl2	F, 5' – CCAACGTGTGATTGTGGTGT– 3' R, 5' – TCTTCCGATGTTGTCTGGTG – 3'
Syna	F, 5' – CCCTTGTTCCTCTGCCTACTC – 3' R, 5' – TCATGGGTGTCTCTGTCCAA – 3'
Tpbpa	F, 5' – CGGAAGGCTCCAACATAGAA – 3' R, 5' – TCAAATTCAGGGTCATCAACAA – 3'
m18s	F, 5' – CGCGGTTCTATTTTGTTGGT – 3' R, 5' – AACCTCCGACTTTCGTTCTTG – 3'

Western Blot

Protein extracts were prepared with 1% Triton X-100, 0.5% SDS, in 1X TBS supplemented with HALT protease inhibitor (Roche, Mannheim, Germany) and EDTA according to the manufacturer's protocol. The protein content was determined with the Pierce BCA protein assay kit (Thermo Scientific). A total of 20 µg of total protein was separated on a 10% denaturing polyacrylamide gel and electrophoretically transferred to PVDF membranes. Membranes were blocked with either 5% non-fat dried milk or 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% (v/v) Tween-20 (Hoefer), followed by incubation with the primary antibody. Primary antibodies included rabbit-anti-Phospho-Smad1/5/8 (9511, Cell Signaling), rabbit-anti-Smad1 (6944, Cell Signaling), rabbit-anti-Bmp4 (PA5-19683, Invitrogen), rabbit-anti-Bmpr1a (ab38560, Abcam), mouse-anti-Bmpr1b (ab78417, Abcam), rabbit-anti-Bmpr2 (ab96826, Abcam), mouse-anti-TetR (TET02, Boca Scientific), and mouse anti-beta-Actin (A5441, Sigma). Upon HRP-conjugated secondary antibody incubation (Cell Signaling), bound complexes were detected using Pierce™ ECL Western Blotting Substrate (ThermoFisher).

CHAPTER 3: RESULTS

CHAPTER 3.1: PRESENCE OF BMP MACHINERY IN TSC

The first set of questions was whether TSC express components of the BMP machinery, both in their undifferentiated state and during differentiation. We observed continuous expression of Bmpr1a and Bmpr2 RNA, with little to no expression of Bmpr1b, by RT-PCR (Figure 4A). Bmp4 RNA was expressed at day 0 and down-regulated during differentiation (Figure 4A). At the protein level, we observed consistent expression of BMPR1A, BMPR2, and BMP4 during in both undifferentiated TSC (Figure 4B). We observed decreased levels of pSMAD1/5/8 and total SMAD1, indicating decreased BMP signaling in TSC during differentiation (Figure 4C). Therefore, we confirmed that, not only are BMP machinery components expressed in TSC, but there is an overall decrease in BMP signaling with TSC differentiation. This prompted us to further investigate the role of BMP signaling in TSC.

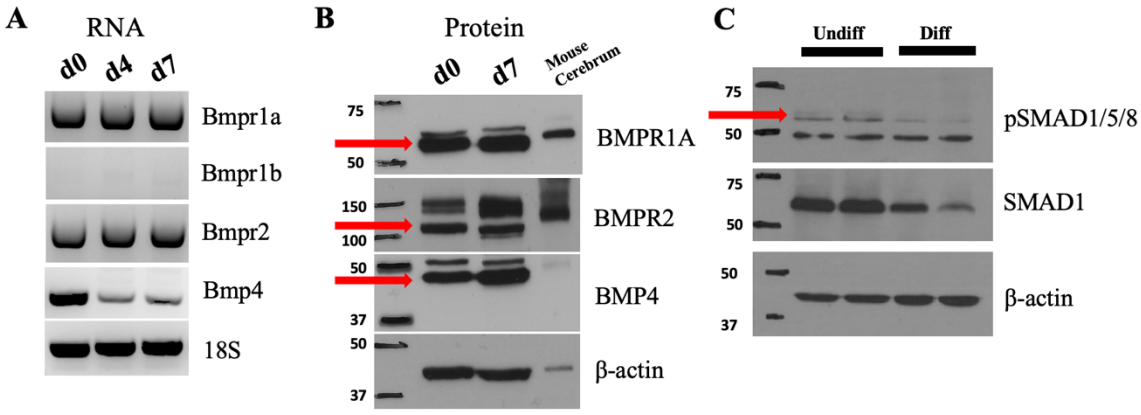


Figure 4: BMP Machinery in TSC. (A) RT-PCR of BMP receptors and Bmp4 during TSC differentiation. (B) Western blot of BMP receptors and BMP4 during TSC differentiation. (C) Phosphorylated SMAD1/5/8 and SMAD1 protein expression in undifferentiated and differentiated TSC.

CHAPTER 3.2: ADDITION OF BMP4 ON TSC

To examine the role of BMP signaling in TSC differentiation, we used two methods to supplement Bmp4 into the cells: first, through the addition of exogenous Bmp4 into the culture and second, through the generation of Bmp4-overexpressing TSC lines.

The addition of exogenous Bmp4 in low serum condition maintained pSMAD1/5/8 levels during TSC differentiation (Figure 5A). In these conditions, we observed a reduction in the expression levels of the differentiation markers *Syna*, *Tpbpa*, and *Pl2* (Figure 5B). To independently verify that the effect on the differentiation markers were due to the addition of exogenous Bmp4, we generated TSC constitutively expressing an HA-tagged Bmp4. pSMAD1/5/8, SMAD1, and BMP4 levels were maintained during differentiation in clone 3.1, while they decreased in the wild-type cells, as previously observed (Figure 5C). With the overexpression of Bmp4, all tested differentiation markers *Syna*, *Tpbpa*, *Pl1*, and *Pl2* were reduced significantly in multiple TSC clones (Figure 5D). Together, both sets of experiments suggest that maintenance of BMP signaling blunts TSC differentiation. Next, we looked into the effect of interfering with BMP signaling during TSC maintenance.

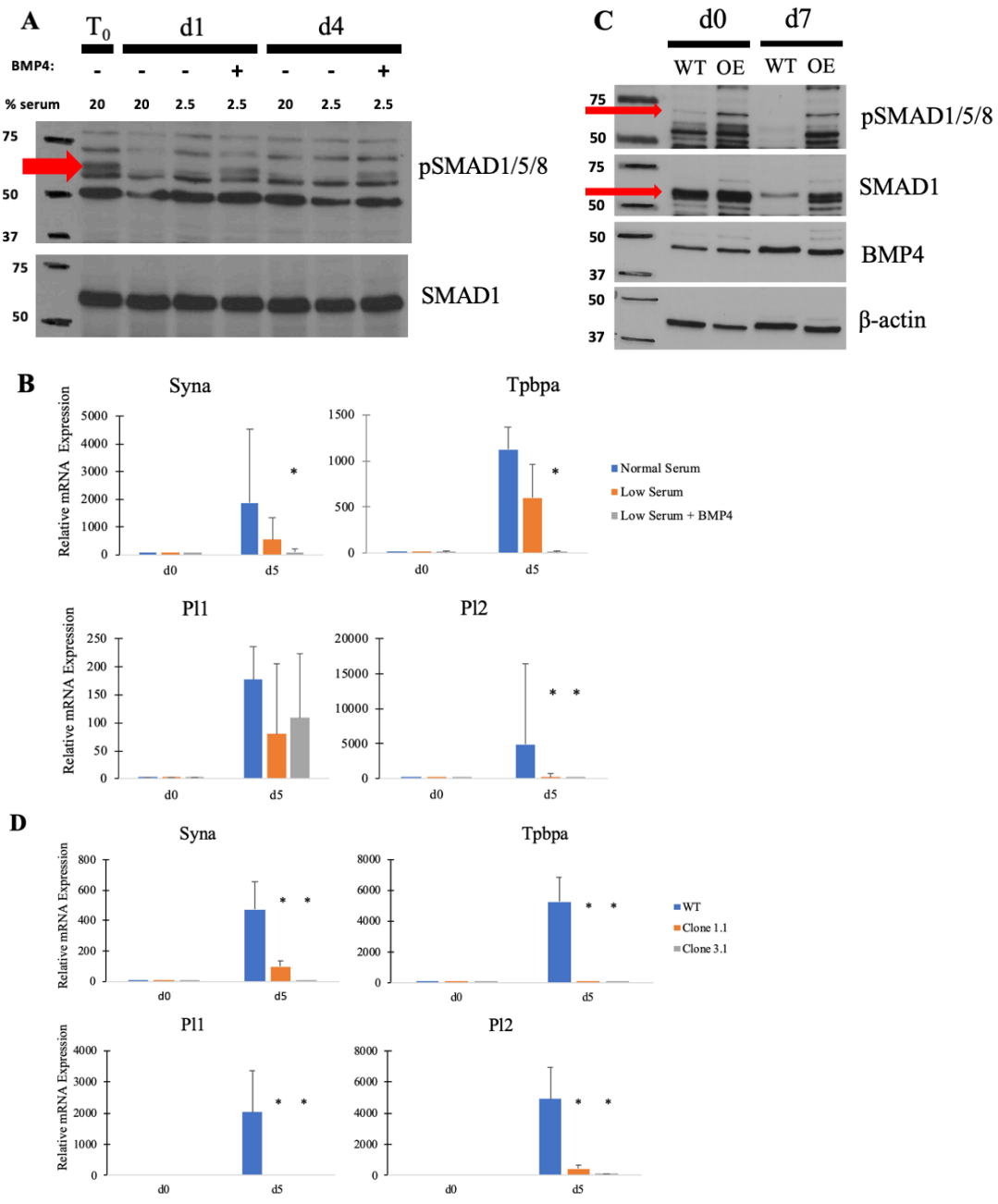


Figure 5: Addition of Bmp4 blunts TSC differentiation. (A) Western blot of BMP signaling with the addition of exogenous BMP4. (B) qPCR of differentiation markers with the addition of exogenous Bmp4. (C) Western blot of wild-type and overexpressed BMP4 clone 3.1 during differentiation. (D) qPCR of differentiation markers in Bmp4-overexpressing clone 1.1 and 3.1. (*Syna*, Syncytin A; *Tpbpa*, Trophoblast Specific Protein Alpha; *Pl1*, Placental Lactogen 1; *Pl2*, Placental Lactogen 2). * $P < 0.05$ (Student's t-test)

CHAPTER 3.3: REMOVAL OF BMP SIGNALING IN TSC

To examine the role of BMP signaling in TSC maintenance, we again used two methods: an inducible Bmpr2-shRNA system and small molecule inhibitors.

Lentiviral Bmpr2-specific shRNA constructs were introduced into TSC as described in Chapter 2 (Methods). Co-transfection with GFP plasmid suggested low transfection efficiency of TSC (Figure 6A). However, overtime G418 sulfate-resistant cells were established. Based on cell morphology, addition of tetracycline in growth media showed increased cell differentiation compared to vehicle (DMSO) control and no tetracycline conditions (Figure 6B). However, qPCR showed insufficient knockdown of Bmpr2, with each of the shRNA constructs tested, and no differences in the level of trophoblast stem cell markers *Cdx2* and *Esrrb* between the three conditions (Figure 6C). We verified that the insufficient knockdown was not due to low expression of TetR, as presence/absence of tetracycline in growth media did not change TetR expression levels (Figure 6D). Because of the insufficient knockdown of Bmpr2 using shRNA, we switched to using chemical inhibitors of BMP signaling.

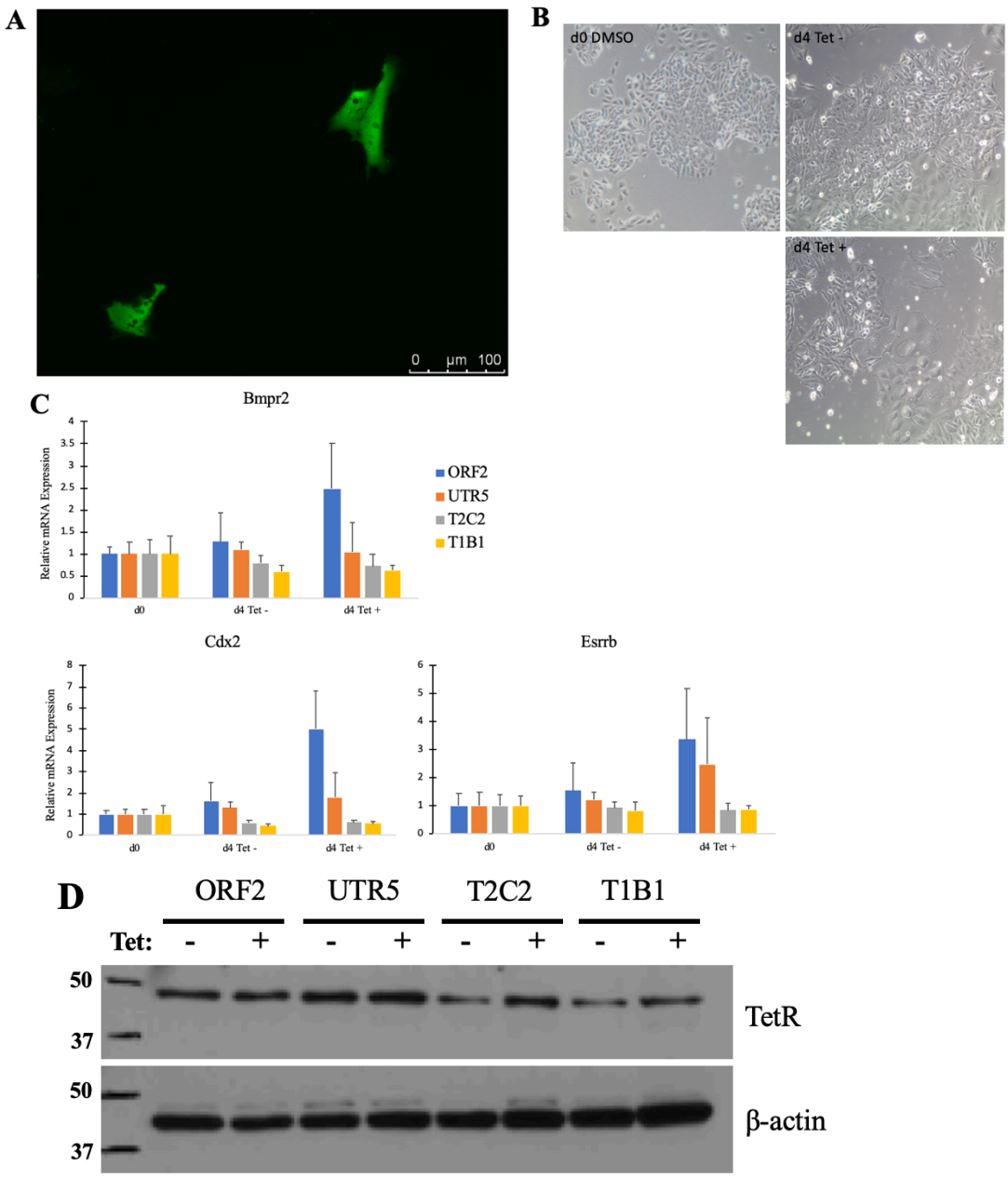
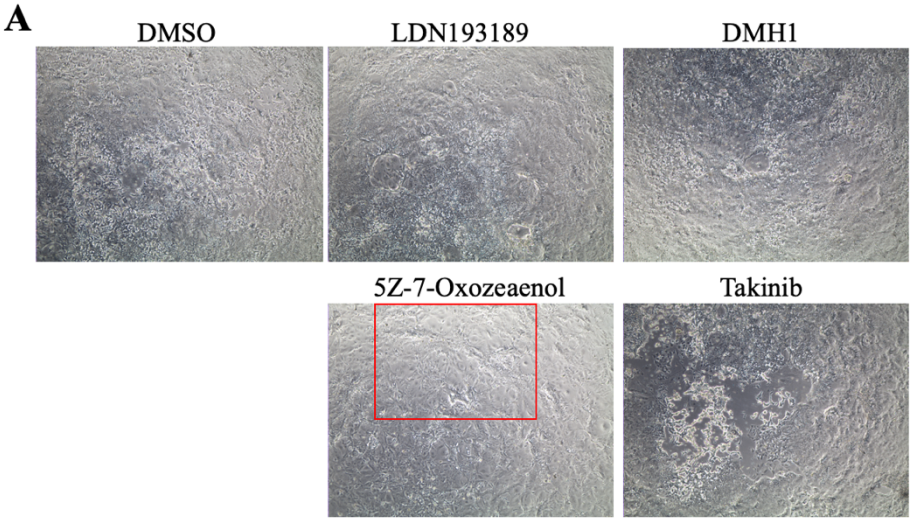
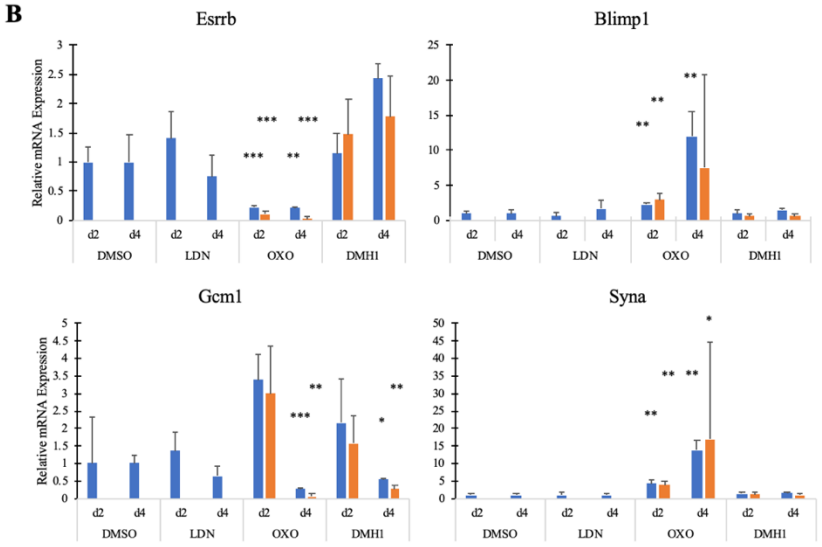


Figure 6: Insufficient Bmpr2 knockdown via shRNA. (A) GFP-positive TSC confirm appropriate transfection with shRNA. (B) TSC with and without the addition of tetracycline in growth media. (C) qPCR of stem cell markers in TSC transfected with one of four different inducible Bmpr2-specific shRNA constructs. (D) Western blot confirming TetR expression in TSC transfected with one of four different inducible Bmpr2-specific shRNA constructs. (*Bmpr2*, Bone Morphogenetic Protein Receptor 2; *Cdx2*, Caudal Type Homeobox 2; *Esrrb*, Estrogen Related Receptor Beta).

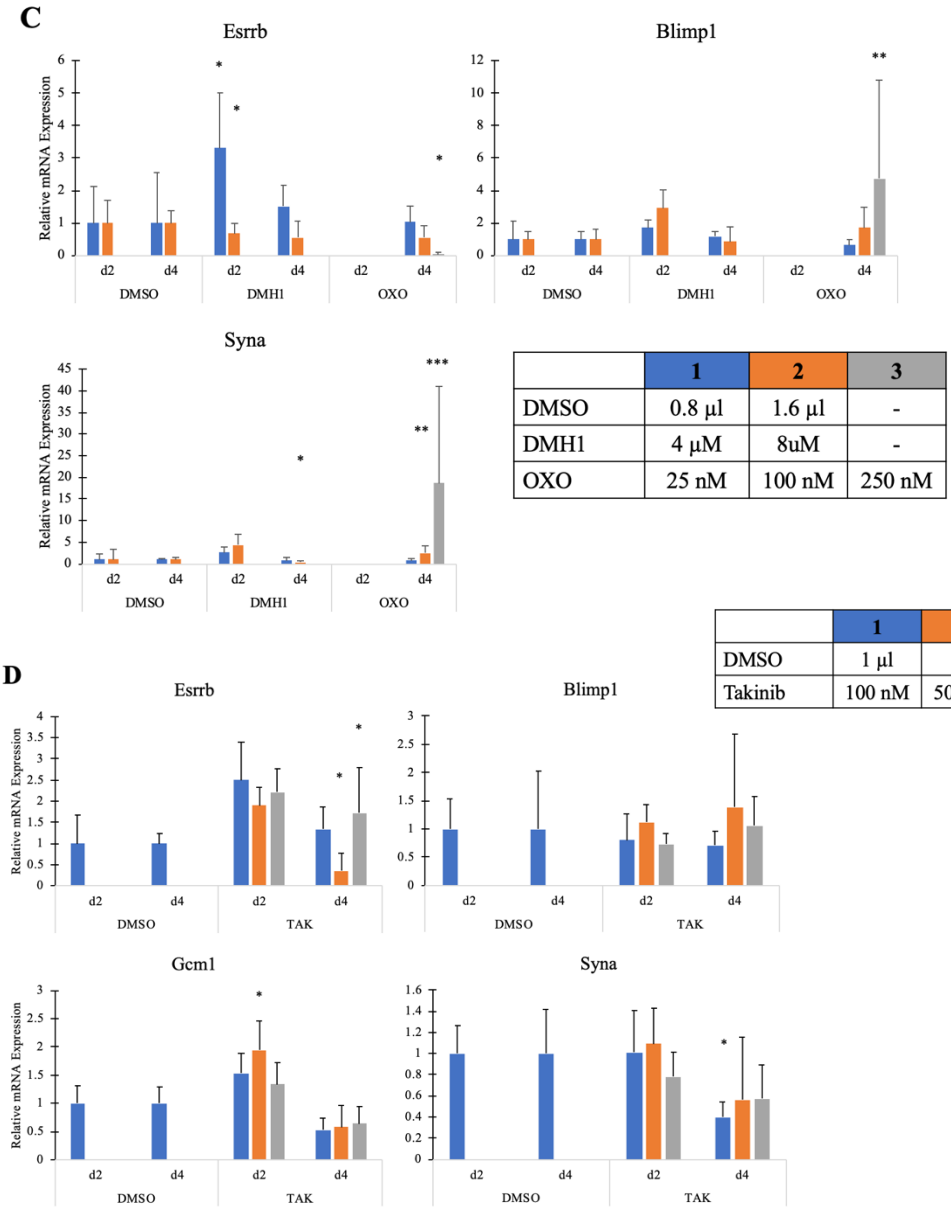
Small molecule inhibitors (concentrations listed in Figure 7) were introduced in TSC cultures in growth media based on previous literature and IC_{50} values. Addition of LDN193189, DMH1, and Takinib did not induce any differences in cell morphology compared to vehicle (DMSO) control, while 5Z-7-Oxozeaenol did (Figure 7A). LDN193189 was toxic at the higher concentration (4 μM). qPCR showed that LDN193189 (2 μM) and DMH1 did not significantly alter any markers. In comparison to the other inhibitors, 5Z-7-Oxozeaenol decreased the TSC marker *Esrrb,* and increased differentiation markers *Blimp1* and *Syna* (Figure 7B). We subsequently tried higher concentrations of DMH1, since no effects or toxicity were noted at lower concentrations; simultaneously, we also tried lower concentrations of 5Z-7-Oxozeaenol in order to identify the most likely target of this inhibitor, with one such target, TAK1, having an IC_{50} of 8 nM. Neither higher DMH1 concentrations, nor lower concentrations of 5Z-7-Oxozeaenol, showed any effects on marker expression by qPCR (Figure 7C). The lowest effective concentration of 5Z-7-Oxozeaenol continued to be 250 nM, leading to a decrease in *Esrrb* with a significant increase in *Blimp1* and *Syna* (Figure 7C). While this made TAK1 an unlikely target of 5Z-7-Oxozeaenol, to further exclude this as the exact target, we decided to try Takinib, a specific inhibitor of TAK1. qPCR, however, showed no effects on all TSC markers tested (Figure 7D). At the end, only 5Z-7-Oxozeaenol, at 250 nM or above, caused significant changes in morphology and meaningful changes in trophoblast stem cell/differentiation markers.





	1	2
DMSO	4 µl	-
LDN	2 µM	4 µM
OXO	250 nM	500 nM
DMH1	0.5 µM	5 µM

Figure 7: 5Z-7-Oxozeaenol has the largest effect on TSC maintenance. (A) TSC with the addition of the vehicle DMSO control and respective small molecule inhibitors in growth media at day 4. Only treatment with 5Z-7-Oxozeaenol shows large areas of flattening cells. (B) qPCR of stem cell (Esrrb) and differentiation (*Blimp1, Gcm1, Syna*) markers in TSC with the addition of the vehicle DMSO control, LDN, Oxozeaenol, and DMH1. (*Esrrb*, Estrogen Related Receptor Beta; *Blimp1*, PR Domain Containing 1 (*Prdm1*); *Gcm1*, Glial Cells Missing Homolog 1; *Syna*, Syncytin A). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's t-test)



	1	2	3
DMSO	0.8 µl	1.6 µl	-
DMH1	4 µM	8uM	-
OXO	25 nM	100 nM	250 nM

	1	2	3
DMSO	1 µl	-	-
Takinib	100 nM	500 nM	1000 nM

Figure 7 (Cont'd): (C) qPCR of stem cell and differentiation markers in TSC with the addition of the vehicle DMSO control, higher concentration of DMH1, and lower concentration of Oxozeaenol. (D) qPCR of stem cell and differentiation markers in TSC with the addition of the vehicle DMSO control and Takinib. (*Esrrb*, Estrogen Related Receptor Beta; *Blimp1*, PR Domain Containing 1 (*Prdm1*); *Gcm1*, Glial Cells Missing Homolog 1; *Syna*, Syncytin A). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's t-test)

CHAPTER 4: DISCUSSION

In vivo, the role of BMP signaling, originating from trophoblast, in the initiation of gastrulation and mesoderm induction in mice is well known [6]. Previous studies have shown that BMP signaling inhibition impairs trophectoderm development in mouse embryos [3], suggesting a potential autocrine role in trophoblast. The confirmation of the BMP machinery in TSC justify the use of this *in vitro* system to further investigate the role of BMP signaling in trophoblast cells. We observed phosphorylation of SMAD1/5/8 in undifferentiated TSC. Upon differentiation, both levels of pSMAD1/5/8 and total SMAD1 as well as Bmp4 RNA level decreased leading us to hypothesize that 1) BMP signaling is required for maintenance of TSC; and 2) that its down-regulation is needed for proper differentiation of TSC.

Bmp4 treatment has been shown to delay blastocyst formation, inhibiting further development of the embryo [6]. We found that addition of exogenous Bmp4 during differentiation maintained phosphorylation of SMAD1/5/8 as well as levels of total SMAD1 well into differentiation. This led to a reduction in differentiation markers such as *Syna* and *Tpbpa*, markers of syncytiotrophoblast and spongiotrophoblast lineages, respectively, suggesting that Bmp4 inhibits the differentiation of TSC. To confirm these results, Bmp4-overexpressing TSC lines were established. During differentiation of these cells, pSMAD1/5/8 was maintained during differentiation, while in the wild-type TSC, pSMAD1/5/8 was appropriately diminished during differentiation, confirming that the overexpression of Bmp4 is maintaining BMP signaling well into differentiation. In two Bmp4-overexpressing TSC clones, all differentiation markers were significantly reduced,

further indicating that differentiation of the TSC to spongiotrophoblast, giant cells, and syncytiotrophoblast are all inhibited in the presence of continuous Bmp4 signaling.

With the blunting of differentiation upon continuous BMP signaling, we next investigated the effects of inhibiting BMP signaling during TSC maintenance. In previous studies, the use of dominant negative Bmpr2 and Smad4 caused defects in the extra-embryonic lineages of mouse embryos via both the canonical and non-canonical BMP pathways [3]. We first used an inducible lentiviral shRNA to knockdown Bmpr2 in TSC. Presence of GFP expression confirmed successful transfection of TSC, albeit with low efficiency. Induction of shRNA expression, however, did not show changes, in either Bmpr2 knockdown, or in TSC marker expression. Western blot confirmed that this was not due to lack of TetR expression (necessary for tetracycline induction). This led us to the conclusion that most likely, all shRNA constructs used in this study were unable to sufficiently target this gene for proper knockdown. In the future, designing additional sequences to target different regions within the ORF and 3' UTR, and including the 5' UTR region, may improve knockdown.

Next, we turned to the use of small molecule inhibitors to block BMP signaling in undifferentiated TSC. In previous studies, the use of Noggin, 5Z-7-Oxozeanol, and Dorsomorphin had decreased the number of trophoblast cells in the mouse embryo [3]. Previous experiment by other members of the Parast lab had shown some effects on TSC with the use of dorsomorphin; however, DMH1 has been shown to be a more selective inhibitor for Bmpr1 [4]. Therefore, I used LDN193189, DMH1, as well as 5Z-7-Oxozeaenol to determine the initial effects of these small molecule inhibitors. Neither

morphology, nor stem cell/ differentiation markers, were altered with the addition of LDN193189 and DMH1. LDN193189 showed toxicity at 4 μM, and thus could not be further evaluated as a BMP inhibitor. Several higher concentrations of DMH1 were tried but again showed little changes. However, there were significant effects noted in morphology and marker expression with the addition of 5Z-7-Oxozeaenol, indicating loss of stemness and enhanced differentiation of TSC. To further evaluate the specificity of 5Z-7-Oxozeaenol, lower concentrations of 5Z-7-Oxozeaenol were tried. Effects of 5Z-7-Oxozeaenol were only seen starting at a concentration of 250 nM, and not at the lower concentrations of 25 and 100 nM, suggesting that TAK1 was unlikely to be the target (with an $IC_{50} = 8$ nM). Nevertheless, to completely exclude TAK1 as the target of 5Z-7-Oxozeaenol, Takinib, a selective TAK1 inhibitor [13], was tried at varying concentrations, without any effects noted on TSC morphology or marker expression. In the end, only 5Z-7-Oxozeaenol showed an effect on TSC, inducing differentiation in the presence of growth media. Since, TAK1 was eliminated as a target of 5Z-7-Oxozeaenol in TSC, I hypothesize that another non-canonical BMP signaling molecule may be the target; possibilities include IL-1-induced activation of NF-κB or JNK/p38.

In this study, our aim was to evaluate the role of BMP signaling in mouse trophoblast stem cells, with respect to both maintenance and differentiation. We confirmed the presence of BMP machinery in TSC, with decreased BMP signaling in differentiated compared to undifferentiated TSC. This led to the hypothesis that 1) BMP signaling is required for maintenance of TSC; and 2) that its down-regulation is needed for proper differentiation of TSC. With the addition of Bmp4 via exogenous

supplementation in culture and overexpression, we observed blunting of TSC differentiation. We next investigated the effects of inhibiting BMP signaling during TSC maintenance. However, we were unsuccessful at establishment of genetic knockdown, leading us to switch to using small molecule inhibitors. Among multiple inhibitors tested, only 5Z-7-Oxozeaenol, a small molecule which targets non-canonical BMP signaling, showed a significant effect morphologically and by qPCR of trophoblast stem cell and differentiation markers. With TAK1 eliminated as the target of 5Z-7-Oxozeaenol, I hypothesize that other non-canonical BMP signaling, such as NF-κB or JNK/p38, may be involved in TSC maintenance. Overall, the results indicate that BMP signaling is required for the maintenance of TSC in their undifferentiated state, and that loss of BMP signaling is necessary for optimal trophoblast differentiation. Notably, this study provides more insight on how BMP signaling affects early mouse placental development and is thus relevant to understanding complications in early pregnancy, such as miscarriage.

This thesis in full is unpublished material that will be prepared for submission for publication of the material. Au, Jennie; Wakeland, Anna; Soncin, Francesca; Parast, Mana. The thesis author was the primary investigator and author of this material.

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