ROLE OF SMAD4 IN MORPHOLOGICAL AND MIGRATORY PROPERTIES OF MOUSE TROPHOBLAST STEM CELLS

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By

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Abbreviations Used

β-cat – β-catenin
AB-9 – Acid blue-9
BMP – Bone morphogenetic protein
Cdx2 – Caudal type homeobox -2
CM – Conditioned Medium
Co-Smad – Common mediator Smad
E-cad – E-cadherin
ECM – Extracellular Matrix
EMT – Epithelial-Mesenchymal transition
FGF – Fibroblast growth factor
ICM – Inner cell mass
I-Smad – Inhibitory Smad
KO - Knockout
MAPK – Mitogen activated protein kinase
MEF – Mouse embryonic fibroblast
MET – Mesenchymal-Epithelial transition
MT-1 – Mutant-1
MT-2 – Mutant-2
N-cad – N-cadherin

R-Smad – Receptor regulated Smad

RT- Room Temperature

TGF-β – Transforming growth factor β

TS cells – Trophoblast Stem cells

WT – Wild type

ZO-1 – Zona occludens -1
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Introduction

Mouse embryonic development and trophoblast stem cells

The development of a mouse embryo begins as a fertilized zygote that undergoes a series of cleavages leading to the formation of the blastocyst (Figure 1), which then forms the trophectoderm and the inner cell mass (ICM), the first two lineages segregated during mammalian development. In culture conditions, the inner cell mass form the embryonic stem cells and the trophectoderm form the trophoblast stem (TS) cells. The ICM forms the precursors to the future embryo proper, called the epiblast, whereas the trophectoderm gives rise to the extra-embryonic lineage of the embryo. The trophectoderm is a layer of epithelial cells and the TS cells form a colony of monolayered flat epithelial cells in vitro. The TS cells are important for implantation and formation of the fetal component of the placenta (Figure 2). The placenta is a complex organ that is required for exchange of nutrients and gases between the mother and fetus (Rossant and Cross, 2001). The TS cells can be derived from pre-implantation and post-implantation embryos in vitro (Rielland et al., 2008; Tanaka et al., 1998). The TS cells in vivo receive their signals from the epiblast; hence under in vitro culture conditions they are cultured with mouse embryonic fibroblasts and supplemented with FGF-4 and Heparin to proliferate and maintain the stem cell characteristics (Tanaka et al., 1998). In vivo the
FGF-4 is induced by Nodal, a TGF-β related signaling molecule, to prevent differentiation and maintain the stem cell characteristic (Guzman-Ayala et al., 2004). FGF-4 mediated maintenance of stem cell characteristic requires MEKK activation, a downstream signaling molecule of the mitogen activated protein kinase (MAPK) pathway (Abell et al., 2009). The trophectoderm is divided into the polar trophectoderm that lines the ICM and the mural trophectoderm that lines the blastocoel cavity. The polar trophectoderm forms the extra-embryonic ectoderm, which consists of the ectoplacental cone (that differentiates into the secondary trophoblast giant cells and the spongiotrophoblast layer) and labyrinth layer (Cross, 2000, 2005). The labyrinth layer is the site of component exchange between the mother and fetus and is formed by the chorion-allantois fusion. The chorion is derived from extra-embryonic ectoderm and allantois from the extra-embryonic mesoderm. The spongiotrophoblast layer is the middle layer in between the trophoblast giant cell layer and the labyrinth layer. It is required for eroding the maternal endothelial cells and secreting anti-angiogenic factors to prevent growth of maternal vasculature in the fetal placenta (Cross, 2000, 2005; Hughes et al., 2004). The spongiotrophoblast cells are surrounded by maternal blood. The mural trophectoderm undergoes the first wave of differentiation and forms the primary giant cells important for implantation by mediating the attachment and invasion of blastocysts into the uterine epithelium (Hemberger et al., 2004; Hu and Cross, 2010). The invasive nature of the trophoblast giant cells facilitates the maternal-fetal connection. The trophoblast giant cells are also required for different paracrine and endocrine signaling occurring at the maternal-fetal junction that is critical to maintain a successful
pregnancy (Hu and Cross, 2010). TS cells differentiate into trophoblast giant cells in the absence of FGF-4 and Nodal signaling (Cross, 2005; Hughes et al., 2004). Any defects in formation of the placenta leads to embryonic lethality (Rossant and Cross, 2001).

Figure 1: Representation of a blastocyst at E4.5

Figure 2: Fetal components of the placenta
**TGF-β signaling**

TGF-β-related signaling plays important roles in development and pathological conditions by regulating various processes including cell proliferation, differentiation, apoptosis, migration, and cell fate. The TGF-β superfamily ligands include the TGF-βs, Nodal/Activins, and BMP ligands. Signaling through TGF-β superfamily members occurs through two pathways: the canonical pathway and the non-canonical pathway. The canonical pathway involves Smads downstream of the receptors (Figure 3). The signaling cascade is initiated by ligand binding to the transmembrane TGF-β type II receptor (serine-threonine kinases) that upon activation heterodimerizes, phosphorylates and activates another transmembrane receptor, the TGF-β type I receptor. The TGF-β type I receptor then activates the downstream signaling through Smads. There are at least three types of Smads involved in the downstream signaling pathway that regulate gene expression. All Smads are transcription factors, including the receptor regulated Smads – Smad 1/2/3/5/8, the common mediator Smad – Smad4 and the inhibitory Smads – Smad 6/7. The Smad 1, 5, 8 are downstream of the BMP signaling pathway whereas the Smad 2 & 3 are downstream of the TGF-β, Nodal/Activin signaling. Smad4 is the central mediator for many of these pathways. Activation of TGF-β type I receptor leads to activation of receptor regulated Smads by phosphorylation, which binds the co-Smad Smad4. This complex then enters the nucleus and regulates transcription of gene expression positively or negatively (Ross and Hill, 2008).
Figure 3: TGF-β signaling pathway

Signaling through TGF-β type I and type II receptors activate R-Smads which complex with Smad4 and enter the nucleus to regulate TGF-β mediated gene transcription. R-Smad (receptor regulated Smads) – Smad1/2/3/5/8, T.F – other transcription factors.

Smad4 and development

TGF-β signaling has an important role in development. Knockout studies of many of the ligands, receptors, or the downstream effectors of TGF-β signaling, like the Smads, have severe defects in development (Goumans and Mummery, 2000). Smad4 knockout
embryos display the most defects compared to other Smad knockouts, and die around E7.5 (Goumans and Mummery, 2000). They show growth retardation and several embryonic and extra-embryonic lineage defects. Smad4 KO embryos do not gastrulate and hence do not form the mesoderm. They do not form distinct embryonic and extra-embryonic boundary (Chu et al., 2004; Sirard et al., 1998; Yang et al., 1998). The growth retardation of Smad4 KO embryos are shown to be not due to apoptosis but due to reduced cell proliferation (Sirard et al., 1998; Yang et al., 1998). Smad4 KO embryos have small to no extra-embryonic region (Yang et al., 1998). This shows the significance of Smad4 requirement in development of both embryonic and extra-embryonic region of the mouse embryo. There are many studies being conducted to study the requirements of Smad4 in the epiblast but its role in the extra-embryonic region is unclear.

**EMT in development and in TS cells**

In addition to signaling via secreted molecules, cells also communicate and interact with their environment via a variety of junctional complexes formed at the sites of intercellular adhesion. These junctional complexes connect a cell to its neighbors and also the cytoskeleton or the extracellular matrix (ECM). The junctional protein expression and its connection with the cytoskeleton depend on the cell type and also its migratory property. Anchoring (eg. Adherens junction), communicating (eg. Gap junction), and occluding junctions (eg. Tight junction) are the three main types of junctions found between cells especially the epithelial cells. Adherens junctions are belt-like structures necessary for the stability of epithelial tissues. E-cadherin is a calcium
dependent transmembrane junctional protein that forms homophilic interaction with other cadherin molecules expressed by neighboring cells. The intracellular domains of E-cadherin interact with β-Catenin, which is part of the cytoplasmic complex that links the adherens junctions to the actin cytoskeleton. Tight junction proteins line body surfaces and organs and are involved in preventing leakage of substances between the cells or between the basolateral and apical domains. They are impermeable to macromolecules but permeability to small molecules depends on the cell type. The formation of a tight junction requires the formation of adherens junction first. Blocking the formation of adherens junctions blocks the formation of tight junctions. During migration, epithelial cells lose their epithelial characteristics, undergo cytoskeletal reorganization and acquire a mesenchymal phenotype. This change in phenotype and character of epithelial cells is called as Epithelial-Mesenchymal transition (EMT). During EMT, the epithelial cells first lose their tight junction proteins followed by loss of adherens junction proteins (e.g. Loss of Zona occludens-1 occurs before the loss of E-cadherin). Gap junctions connect the cytoplasm of neighboring cells for transport of molecules (Alberts B, 2002).

EMT is a process that occurs during development, pathological conditions such as cancer metastasis, and also during wound healing (Kalluri, 2009). This process is reversible in many cases and is called as Mesenchymal to epithelial transition (MET). The term “transition” highlights the reversible plasticity of both processes. EMT and MET are processes that occur in development during implantation, gastrulation, and organogenesis, where extensive cellular migration and sometimes, invasion occur. During EMT the epithelial cells lose their epithelial characteristic by undergoing several
biochemical and physiological changes to obtain mesenchymal phenotype and characteristics (Figure 4). These changes increase the migratory and invasive properties of the cells by activation of several transcription factors, re-organization and secretion of cytoskeletal proteins and inducing production of ECM degrading enzymes (Kalluri and Weinberg, 2009). There are several prototypical genes that are turned on/off or show changes in localization during EMT. For example, the protein levels of E-cadherin and ZO-1 (Zona occludens-1) are typically reduced whereas other genes such as N-cadherin, Snail, Slug, Zeb1, Twist, etc. (Zeisberg and Neilson, 2009) show increased expression. Some cytoplasmic proteins such as β-catenin localize to the nucleus during EMT (Kim et al., 2002; Satterwhite and Neufeld, 2004; Zeisberg and Neilson, 2009). The primary EMT in mouse occurs during implantation in the trophoblast giant cells. The trophectoderm cells undergo morphological and behavioral transitions resembling EMT to form trophoblast giant cells (Sutherland, 2003) that invade into the maternal uterine epithelium to establish the maternal-fetal connection (Adamson et al., 2002; Hemberger et al., 2004). This invasiveness also partly resembles EMT (Aplin, 1996; Hohn and Denker). The next main developmental event that involves EMT occurs during gastrulation. Any defects in gastrulation lead to failure in development of the embryo as a whole (Kalluri, 2009). In the absence of Smad4, embryos do not gastrulate (Chu et al., 2004; Sirard et al., 1998; Yang et al., 1998).
During EMT, *E-cadherin* (adherens junction protein) is down regulated following down regulation of *ZO-1* (tight junction protein) and re-localization of *β-catenin* from cytoplasm to nucleus occurs.
**Objectives**

Based on the phenotypes of *Smad4* null embryos, we hypothesize that *Smad4* is required for the proper development of the extra-embryonic lineages, especially the one associated with the extra-embryonic ectoderm. Many aspects of the trophectoderm lineage development can be recapitulated in TS cells. Therefore, this thesis focuses on examining the properties of *Smad4* null TS cells that were derived in our lab. The following hypotheses were proposed and tested:

1. *Smad4* is required for maintaining the epithelial morphology of the TS cells. The rationale for this hypothesis comes from the abnormal shape of *Smad4* mutant TS cells. Transmission through dye microscopy was done to quantitate the difference in the thickness of the wild type and *Smad4* KO TS cells. Antibody staining was carried out to assess the difference in epithelial architecture of the wild type and *Smad4* KO TS cells.

2. *Smad4* is involved in regulating the migratory behavior and epithelial junctions of the TS cells. To test this hypothesis wound healing and trans-well assays were performed to study the migratory and invasive behavior. Immunofluorescent staining was done to understand the role of *Smad4* in the maintenance of epithelial architecture.

3. *Smad4* is required for regulating the proliferation and differentiation of TS cells. BrdU incorporation assays and phospho-H3 staining were done to assess the role of
Smad4 in TS cells proliferation. Growing TS cells in the absence of stem cell renewal factors assessed the differences in their differentiation rates.

Figure 5: Schematic representation of the prediction of role of Smad4 in TS cells
Materials and Methods

Transmission through dye microscopy

Materials

Acid Blue-9 (AB-9) (50mg/mL solution) was obtained from Dr. Model and diluted to 5mg/mL using TS Base medium (20%FBS, 1% 10mM 2-mercaptoethanol, 1% 200mM L-glutamine, 1% 100mM sodium pyruvate and 1X PenStrep), 70% Conditioned Medium (CM) (30%TS Base + 70% of medium collected from 200µg Mitomycin C treated CD1p3 embryonic fibroblasts) with FGF-2 (Peprotech, 25ng/mL) and Heparin (Sigma, 1µg/ml), Wild type and Smad4 knockout TS cells, Phase contrast microscope, Image analysis software (Image J).

Method

Wild type and Smad4 KO TS cells were cultured on separate 35mm dishes in 70% Conditioned Medium with FGF-2 and heparin for two days at 37°C and 5% CO2. On the second day, the medium was aspirated and 9x22 cover slip was placed on the cells and sealed onto the dish using vacuum grease leaving enough space above the cells for them to maintain their normal morphology. Just enough AB-9 was then added from one side of the cover slip using a P20 pipette. Excess AB-9 will lead to change in concentration of
the dye. The cells were then viewed under an Olympus IX-70 inverted microscope and images were acquired. The sample was illuminated by a quartz-halogen lamp through a NA - 0.5 condenser, a 630/10 band-pass filter installed on the condenser wheel. The images were collected with an UPlanApo x10/0.4 objective as 16-bit images using the software Slidebook. The acquired images were analyzed with Image J. The images were converted to 32bit images and the background was subtracted. Taking an image of a blank cover slip on a dish with AB-9 only and no cells on it gave the background value. The image was then converted into logarithmic scale and divided by .110(α value) (Figure 7 - a, c, e) (Gregg et al., 2010). The thickness of about 3-5 cells in a colony was measured using Image J and processed in Excel.

Trans-well assays

Materials

Trans-well inserts covered with 8μm pore filters (GREINER ThinCert Tissue Culture Inserts) at the bottom, 24 well plate, Matrigel(10 mg/mL), 70% Conditioned Medium (CM) (30%TS Base + 70% of medium collected from 200μg Mitomycin C treated 10cm CD1p3 Feeder plates) with FGF-2 and Heparin, 4% Formaldehyde, 1X PBS (phosphate buffer saline), Hematoxylin stain 2- Gill method, Q-tips, Slide, Cover slip (24 X 50mm), Histochoice mounting media, Wild-type and Smad4 KO TS cells, Leica dissection scope, Spot Advanced software.
Methods

For Invasion assay (Hohn and Denker, 2002), the transwell inserts were coated with 100 µl matrigel (BD Biosciences, 1:20 dilution) in 70% CM with FGF-2 and Heparin overnight at room temperature whereas non-coated transwell inserts were used for mobility assay. The transwells with matrigel were rehydrated with 100 µl medium at 37°C for a minimum of two hours before use. The transwell inserts were placed into the wells of a 24-well plate and 200µl of medium containing about 20x10^3 cells were added to the top of the inserts, whereas about 400µl medium alone was added to the wells. Media at the bottom of the inserts were changed every day and the filters were collected on Day1, Day3, and Day5 and fixed with 4% formaldehyde for 5mins, rinsed twice with 1X PBS 3mins each time. Then the inserts were stained with 200µl Hematoxylin stain overnight at room temperature. The inserts were then rinsed with distilled water until they were clean. The cells on the top of the inserts were scraped off using Q-tips. The filters were then peeled off from the insert and mounted onto a slide with the bottom-side of the filter facing up using Histochoice mounting medium and the images were captured using a Leica Dissection scope and Spot advanced software at magnification of 2X.

Wound-healing Assay

Materials

A P20 pipette tip, phase contrast microscope, Stage incubator connected to CO₂ and H₂O supply, Image analysis software (Image J), 70% Conditioned Medium (CM) (30%TS Base + 70% of medium collected from 200 µg Mitomycin C treated 10cm
CD1p3 Feeder plates) with FGF-2 and Heparin, OPTI-MEM with 0.02% FBS, FGF-2 and Heparin, wild-type and Smad4 KO TS cells on a 35mm culture dish.

**Method**

The cells were grown in a 0.1% gelatin coated 35mm tissue culture dish until they reached confluency in 70% Conditioned Medium. Medium was changed everyday. Once the cells reached confluency, they were starved with OPTI-MEM with 0.02% FBS, FGF-2 and Heparin for 5 hours and then a scratch was made with a P20 pipette tip and a time-lapse experiment was performed in the stage incubator on an IX-70 inverted microscope. Phase images were acquired using a CPlano 10X/0.3 objective every 15 minutes. Image J was used to track and measure the distance of migration of the cells. Microsoft Excel was used to generate a graph.

**Immunofluorescent Staining of junctional proteins and trophoblast lineage marker**

**Materials**

4% PFA (paraformaldehyde), 0.5% Triton-X100, 1X PBS (phosphate buffer saline), DAPI with Vecta Shield, Topro-3 (1mm Invitrogen T3605), rat anti-E-cadherin (Invitrogen 131900), rabbit anti-ZO-1 (Invitrogen 402300), rabbit anti-β-catenin (CST 9587), rabbit anti-N-cadherin (Epitomics 2019-1), mouse anti-Cdx-2 (Invitrogen MU392A-UC), donkey anti-rabbit AF488 (Invitrogen A21206), Rabbit anti-rat AF488 (Invitrogen A21210), donkey anti-mouse AF488 (Invitrogen A21202), cells on cover slip (22x22 mm), slide (25x75x1 mm)
**Methods**

*Smad4* KO TS cells were grown on 0.1% gelatin coated cover slip with feeders. The TS cells were grown to 60% confluency and the cover slips were then fixed with 4% PFA (paraformaldehyde) for 20mins at RT. The cover slip was then permeabilized with 0.5% Triton-X100 for 10mins at RT and washed with 1X PBS three times for 5mins each time. After the wash, the cover slips were incubated with primary antibody (1:100) diluted in PBS at 4°C overnight and washed 3 times with 100 µl 1X PBS for 5 min each. The cover slips were then incubated in secondary antibody (1:500) diluted in PBS dilution for 1 hr at RT in the dark. The cover slips were then washed 3 times with 1X PBS and incubated with Topro3 (Invitrogen, 1:500) for 15 minutes at RT in the dark. Finally, the cover slips were mounted with 4 µl of Slow Fade glycerol (~0.1 µg/ml DAPI) on microslides. Edges of the cover slips were sealed with clear nail polish and images were obtained using epifluorescence microscopy.

**Cell proliferation and Differentiation**

**Materials**

4%PFA (paraformaldehyde), 0.5% Triton-X100, 1X PBS (phosphate buffer saline), DAPI with Vecta Shield, Topro-3, rabbit anti-phospho-Histone3 (Upstate 06-570), G3G4-mouse anti-BrdU (DSHB), donkey anti-mouse AF488 (Invitrogen A21202), donkey anti rabbit Cy3 (Jackson IR 711-165-152), cells on cover slip (22x22 mm), 2M HCl, 1X TBE, BrdU, phospho-H3
Methods

Cell proliferation

*Smad4* KO TS cells were grown on 0.1% gelatin coated cover slip with feeders. The TS cells were grown to 60% confluency and treated with BrdU for 2 hours. Cover slips were then fixed with 4% PFA (paraformaldehyde) for 20 mins at RT. The cover slip was then permeabilized with 0.5% Triton-X100 for 10 mins at RT, incubated in 2M HCl for 30 mins at 37°C (wet chamber), washed with TBE and 1X PBS twice for 5 mins each time with each reagent. After the wash, the cover slips were incubated in anti-BrdU (1:100) and anti-phospho H3 (1:1000) antibody diluted in PBS in 4°C overnight and washed 3 times with 100 µl 1X PBS for 5 min each. The cover slips were incubated in secondary antibody (1:500) diluted in PBS for 1 hr at RT in the dark. The cover slips were then washed 3 times with 1X PBS and incubated with Topro3 (1:500) for 15 minutes at RT in the dark. Finally cover slips were mounted with 4 µl of Slow Fade glycerol (~0.1 µg/ml DAPI) on microslides. Edges of the cover slips were sealed with clear nail polish and images were obtained using epifluorescence.

Cell Differentiation

20x10^3 cells of *Smad4* KO and wild-type TS cells were plated onto 4-wells in stem cell culture medium and then deprived from self-renewing factors the next day. The medium was changed everyday. The number of differentiating cells for each day was quantified by counting the number of cells with perinuclei. The number of differentiated
cells in the medium with self-renewing factors from the corresponding day of
differentiation was considered as the base level of differentiation.
Results

Altered Morphology in Smad4 KO TS cells

The TS cells were immunostained with Cdx2, a transcription factor expressed in the trophoderm cells of the blastocyst (Beck et al., 1995) and cultured TS cells (Yang et al., 1998) whose expression is downregulated during differentiation. Our blastocyst-derived wild type and mutant cells lines express Cdx2 (Figure 6-b,d,f), consistent with their trophoderm lineage. The lineage identity of these cells was confirmed with expression analysis of additional lineage-specific markers (data not shown).

The wild-type TS cells form a flat epithelial colony whereas in the absence of Smad4 the TS cells lose this flat epithelial morphology (Figure 6-a,c,e). The Smad4 KO TS cell lines show two kinds of phenotypic behavior which is described as MT-1 and MT-2. Due to obvious difference in morphology between the wild-type and Smad4 KO TS cells, the difference in thickness was quantitated between these cells using transmission through dye microscopy (Gregg et al., 2010).
Figure 6: Morphology of Wild-type and Smad4 KO TS cells

In the absence of Smad4, TS cells lose their typical flat and epithelial morphology but maintain their lineage identity by expression of Cdx-2.

(a,c,e) – Phase images of wild-type and Smad4 KO TS cells; (b,d,f) Immunofluorescent staining of Cdx2 in wild-type and Smad4 KO TS cells

The wild-type TS cells have an average thickness of 5 ± 1.9 µm whereas the Smad4 KO TS cell line of MT-1 phenotype is 18 ± 3.5 µm thick and the MT-2 phenotype is 10 ± 3 µm thick on average from a n of 5 colonies. Transmission through dye microscopy measurement demonstrates that the Smad4 KO TS cells are at least two times taller than the wild-type cells in a colony (Figure 7-b, d, f). The results are statistically significant at 99% confidence interval (CI).
Figure 7: Measurement of thickness of TS cells using Transmission through dye microscopy

TS cells are thicker in the absence of Smad4 along the sections shown in red in a,c,e. (a,c,e) – Wild-type and Smad4 KO colonies under Transmitted light using 633nm filter (b,d,f) – Excel profiles of 5-6 wild-type and Smad4 KO TS cells generated using Microsoft Excel.

Increased migration and invasion in Smad4 KO TS cells

Unlike wild type TS cells that form flat colonies with distinct intercellular boundaries Smad4 null TS cells (MT-1) are thicker and tend to remain closer to each other in a colony. Under normal culture conditions MT-1 mutant cells frequently acquire a new morphology that appears fibroblastic and mesenchymal like (MT-2). The MT-2 cells show signs of migration as single cells out of the colony. The studies included in this thesis focus on the MT-2 Smad4 mutant TS cells.
In order to assess the differences in migration between the wild-type and mutant TS cells, transwell *in vitro* migration and invasion assays were performed. The transwell migration assay and the transwell invasion assay indicate that the *Smad4* KO TS cells are significantly more motile and invasive by Day 5 when compared to the wild-type TS cells (Figure 8 and Figure 9).

![Figure 8: Transwell Migration assay](image)

**Figure 8: Transwell Migration assay**

**TS cells are more motile in the absence of *Smad4* by day 5.**

Wild-type (upper panel) and *Smad4* KO(lower panel) TS cell migration to the bottom side of the filter at Days 1, 3, and 5.
Figure 9: Transwell invasion assay

TS cells are more invasive in the absence of Smad4 by day 5.
Wild-type (upper panel) and Smad4 KO (lower panel) TS cell invasion to the bottom of the filter on Days 1, 3, and 5.

Increased migration and Loss of cell-cell contact in Smad4 KO TS cells

The results of the trans-well assays indicate that in the absence of Smad4 the TS cells are more mobile and invasive. In order to observe the migratory behavior of the Smad4 KO TS cells in comparison with the wild-type TS cells the well-established scratch assay (Liang et al., 2007) was performed. The TS cells were serum starved for 5 hours prior to video imaging.
The wild type TS cells migrate in a coordinated manner as a sheet of cells whereas the mutant TS cells migrate as individual cells in an uncoordinated way, frequently detaching themselves from their neighbours, a behavior indicative of loss of epithelial architecture (Figure 10). The analysis of the distance of migration of the TS cells using Image J revealed that the mutants migrate longer distances compared to the wild-type TS cells at every two hour time point (Figure 11a). The Smad4 KO TS cells show migration in different directions compared to unidirectional movement in the wild-type TS cells (Figure 11b). The numbers in the image indicate the endpoint and the direction of the track of migration (Figure 11b).

Figure 10: Wound Healing assay
In the absence of Smad4 TS cells migrate individually and show loss of epithelial architecture and coordinated movement.
Wild-type(upper panel) and Smad4 KO(lower panel) TS cells at time T=0,7 and 15 hours after the creation of the wound
Figure 11a: Distance of migration of TS cells

TS cell shows increased migration in the absence of Smad4 at every timepoint observed.

Figure 11b: TS cell migration track

Smad4 KO (MT-2) TS cells migrate in multiple directions and longer distances compared to wild-type TS cells, indicating loss of coordinated movement.

The numbers indicate the endpoint of the track of migration, arrows indicate the direction of migration.
Loss of cell-cell junctional proteins and change in localization of EMT associated proteins

The wound-healing assay indicates the loss of epithelial architecture in the Smad4 KO TS cells when they attempt to fill in the wound. Immunofluorescent staining of the tight junction ZO-1 and the adherens junction protein E-cadherin (Figure 12) shows disrupted pattern of these junctional proteins in the Smad4 KO TS cells compared to wild-type TS cells. The cells appearing to be moving out of the colony had decreased E-cadherin patterning. The single cells present outside the colony have no membrane ZO-1 but ZO-1 appears to be present in the nucleus in Smad4 KO TS cells (Figure 12). Nuclear ZO-1 is also observed in MEFs (data not shown). The change in expression levels of epithelial junctional proteins suggests that the TS cells in the absence of Smad4 fail to form an intact epithelium, the first sign of cells undergoing EMT. To confirm this observation, immunostaining of EMT associated junctional proteins (N-cadherin and β-catenin) was performed. Upregulation of N-cadherin and nuclear localization of β-catenin is usually identified in cells undergoing EMT. Changes in localization of these proteins were observed in Smad4 KO TS cells as expected. N-cadherin showed cytoplasmic localization whereas β-catenin showed nuclear expression in the single cells near the colony in the Smad4 KO TS cells compared to membrane localization of these proteins in the wild-type TS cells (Figure 13). The Smad4 KO TS cells show disruption in patterning of N-cadherin and β-catenin in the colony (Figure 13). All these results are consistent with the hypothesis that Smad4 null TS cells have a propensity to undergo EMT.
Figure 12: Immunofluorescent staining of adherens junction protein $E$-cadherin and tight junction protein $ZO$-1

In the absence of $Smad4$, the epithelial architecture of TS cells is lost either by down regulation of $E$-cadherin (upper panel) and $ZO$-1 (lower panel) in single cells in the membrane or disrupted membrane staining of these proteins in colonies.

(a,b) Wild-type TS cell colony (c-f) Mutant TS cell colony (a-d) Immunofluorescent staining (e,f) phase contrast images

(The red arrow indicates single cells and the grey arrow shows cells with loose cell-cell contacts at the edge of the colony)
Figure 13: Immunofluorescent staining of adherens junction protein β-catenin and tight junction protein N-cadherin

In the absence of Smad4, proteins involved in EMT shows disrupted membrane β-catenin (upper panel) and N-cadherin (lower panel) staining in colonies; cytoplasmic localization of N-cadherin in single cells and cells at the edge of the colony; nuclear localization of β-catenin in single cells and cytoplasmic localization of β-catenin in cells at the edge of the colony.

(a,b) Wild-type TS cell colony (c-f) Mutant TS cell colony (a,c) Topro-3 and β-catenin (b,d) Topro-3 and N-cadherin (e,f) β-catenin and N-cadherin (blue arrow indicates single cells and grey arrow shows cells at the edge of the colony)
Cell proliferation and Differentiation

Control of cell proliferation and differentiation are important processes during development. Hyper-proliferation leads to formation of tumor and hypo-proliferation leads to growth retardation and defects in development (Yang et al., 1998). Similarly disruption in timing of differentiation affects proper establishment of maternal-fetal connection (Hu and Cross, 2010). Cells in S-phase were stained with anti-BrdU antibody after a 2-hour treatment with BrdU and cells in M-phase were labeled with phosphoH3. The percentage of cells that are in and that underwent S-phase and the cells in M-phase were calculated and plotted as shown (Figure 14). There is no significant difference in proliferation rates between wild-type and Smad4 KO TS cells.

Figure 14: Cell proliferation
Loss of Smad4 does not affect TS cell proliferation.
But *Smad4* KO TS cells cultured in differentiation medium differentiate at a higher rate compared to wild-type TS cells (Figure 15).

**Figure 15: Differentiation Rate**
In the absence of *Smad4* TS cells differentiate faster in differentiation medium compared to wild-type TS cells.
Discussion

Role of Smad4 in proliferation and differentiation

Mouse TS cells represent an excellent model system to study the role of different signaling pathways in trophectoderm lineage development and ultimately, placenta morphogenesis (Senner and Hemberger, 2010). Signaling through members of the TGF-β superfamily has a very significant role in development. The Nodal/Activin signaling is known to be important for the development of both the maternal and fetal component of the placenta. Misregulation of Nodal signaling in either of these tissues results in a defective placenta and failure of pregnancy (Park and Dufort, 2011a; Park and Dufort, 2011b). Nodal is shown to limit giant cell differentiation, expand the spongiotrophoblast layer, and allow multipotent stem cells to adopt a labyrinth cell fate (Ma et al., 2001). TS cells grown in differentiation medium with Activin do not differentiate terminally into trophoblast giant cells, instead they show intermediate differentiation to a labyrinth cell fate (Natale et al., 2009). This shows the significance of TGF-β superfamily member mediated signaling in maintaining the multipotency of TS cells in addition to FGF signaling.

Trophoblast giant cell differentiation from the mural trophectoderm is the first sign of differentiation seen during embryogenesis. Trophoblast giant cell differentiation
is required for implantation and subsequent placentation. Implantation occurs by the invasion of the maternal uterine by trophoblast giant cells. The trophoblast giant cells show endoreduplication, a process involving chromosomal DNA replication without undergoing mitosis or cytokinesis resulting in an increased ploidy level. During endoreduplication cells exit from cell cycle and enter into endocycle in which replication occurs but not mitosis (Hu and Cross, 2010). It is also shown that exit from cell cycle is required for trophoblast giant cell differentiation to occur (Soloveva and Linzer, 2004). Cell cycle arrest occurs by down regulation of c-myc, G1/S cyclins, and up regulation of Cyclin dependent kinase (CDK) inhibitors like p15, p16^{INK4a}, p19^{Arf}, p57 and p21 (Donovan and Slingerland, 2000). Trophoblast differentiation by deprivation of FGF signaling occurs through p57 mediated inhibition of CDK1 (Ullah et al., 2008). In INK4a/Arf^{Δ2, 3} cells, addition of TGF-β increased cell proliferation and reduced differentiation whereas blocking the antibodies for TGF-β and Activin reduced proliferation and increased differentiation in the presence of FGF-4 (Erlebacher et al., 2004). Hence, TGF-β/Activin signaling is required to maintain the multipotency of TS cells and inhibition of this pathway leads to differentiation. Consistent with the literature, knocking down Smad4, a downstream molecule of the TGF-β superfamily ligand dependent signaling pathway, increases differentiation. This indicates that increased differentiation of TS cells in the absence of FGF-4 occurs in a Smad4 dependent manner. DNA cell cycle analysis using flow cytometry of wild type and Smad4 KO TS cells deprived from FGF-4 will provide us information on role of Smad4 in endoreduplication during differentiation. Looking at the role of Smad4 in endoreduplication will help us
identify the defects in trophoblast giant differentiation and subsequent effects of implantation and placental morphogenesis. In the presence of FGF-4, the presence or absence of Smad4 in TS cells does not appear to have any effect on differentiation.

Most of the TGF-β ligands and downstream molecules are known to act as tumor suppressors and loss of such proteins are observed in many types of cancer (Donovan and Slingerland, 2000; Ross and Hill, 2008). In the breast carcinoma line MDA-MB-468 lacking Smad4; epithelial growth inhibition is restored by addition of Smad4 through p21 expression induced by TGF-β/activin pathway (Pardali et al., 2005). TGF-β induced CDK inhibitor p15 leads to the down regulation of c-myc, a transcription factor that promotes cell proliferation (Seoane et al., 2001). These results support a role for Smad4 in inhibiting growth and proliferation in many types of cells. But knocking down Smad4 shows reduced proliferation in both the embryonic and extra-embryonic regions of E6.5 embryos (Yang et al., 1998) which could be due to loss of Smad4 dependent TGF-β signaling as shown by Dr. Glimcher’s lab (Erlebacher et al., 2004). But under in vitro culture conditions, Smad4 KO TS cells do not show significant difference in proliferation rate compared to the wild-type TS cells. The differences in proliferation between the Smad4 KO extra-embryonic region in vivo and TS cells in vitro might be due to unavailability of signals from the epiblast. FGF-4 is shown to act upstream of MAPK pathway to maintain trophoblast stem cell proliferation. Disruption in MAPK pathway inhibits FGF-4 induced Jun kinase and p38 pathway and induces differentiation in TS cells (Abell et al., 2009; Hatano et al., 2003; Simmons and Cross, 2005). Gene expression profiling of proteins downstream of FGF-MAPK signaling and proteins involved in cell
cycle at different time points will give us information on effects of Smad4 on proliferation, differentiation and stem cell maintenance. This is would aid in identifying targets to control for proliferation and differentiation defects of the trophectoderm lineage.

**Role of Smad4 in EMT**

Smad4 is shown to be required for maintaining a proper epithelial morphology in numerous cancer cell line-based studies. Loss of Smad4 in colorectal cancer relates to reduction in expression of E-cadherin, an adherens junction protein and addition of a Smad4 re-expressing clone restored E-cadherin expression (Muller et al., 2002). TGF-β treated Smad4-deficient pancreatic carcinoma BxPC-3 cells display increased motility and reduced E-cadherin expression (Vogelmann et al., 2005). Consistent with the known roles of Smad4 in maintenance of epithelial architecture, we have observed greatly reduced E-cadherin expression in motile cells at the edge of Smad4-null TS cell colonies and complete lack of E-cadherin expression in detached single cells. We then decided to examine the membrane pattern of ZO-1, a tight junction protein and an epithelial marker that is also down regulated during EMT (Kalluri and Weinberg, 2009; Xu et al., 2009). It is also indicated from unpublished data that ZO-1 is down regulated in lung fibroblasts during TGF-β induced EMT (Thomas et al., 2007). ZO-1 is a member of the membrane associated guanylate kinase (MAGUK) like proteins. Madin-Darby canine kidney (MDCK) and Cercopithecus aethiops monkey kidney (CV-1) cells show nuclear expression of α+ isoform of ZO-1 in subconfluent cell cultures (Gottardi et al., 1996). Nuclear association of these relates to the extent and maturity of cell-cell contact (Bauer
et al., 2010; Gottardi et al., 1996) and is expressed in blastocysts prior to formation of nascent blastocoele. The mislocalization ZO-1 in Smad4-null TS cells strongly suggests that Smad4 is also required to maintain stable cell-cell contact and epithelium formation in cells of trophectoderm lineage. Loss of E-cadherin expression leads to cytoplasmic and sometimes nuclear re-localization of membrane-associated β-catenin. β-catenin, another adherens junction protein associated with E-cadherin in epithelial cells, is a downstream molecule in the Wnt signaling pathway. Smad4 loss is associated with increased Wnt/β-catenin signaling in mammary epithelial cells and tooth morphogenesis (Li J, 2011). In colon carcinoma cells restoration of Smad4 suppresses the Wnt/β-catenin signaling induced migration capacity (Li et al., 2003). In Smad4 negative breast cancer cell line, Smad2 is shown to enhance WNT signaling through β-catenin and TCF (Tian et al., 2009) indicating the suppressive role for Smad4 in regulating WNT signaling. Another mechanism by which TGF-β related signaling regulates EMT is via up-regulation of N-cadherin, another adherens junction protein (Hirota et al., 2008). Increased expression of N-cadherin is considered a hallmark of EMT (Kalluri and Weinberg, 2009; Micalizzi et al., 2010; Tumbarello et al., 2005; Xu et al., 2009). In NMuMG, normal and malignant oral epithelium N-cadherin is up regulated during TGF-β induced EMT. (Diamond et al., 2008; Zeisberg and Neilson, 2009) In NMuMG cells this TGF-β induced expression of N-cadherin is dependent on Smad4 (Deckers et al., 2006). Our results show cytoplasmic localization of N-cadherin in Smad4 KO TS cells. In Smad4 null TS cells EMT is independent of TGF-β signaling. Immuno-blotting or real time analysis of the expression level of N-cadherin will provide us quantitative
information on its up regulation or down regulation. All of these indicate a suppressive role for Smad4 in EMT, consistent with our results that show decreased E-cadherin, ZO-1 expression on the membrane and relocalization of N-cadherin and β-catenin in Smad4 null TS cells.

Migration plays a significant role in EMT. Role of Smad4 in migration and its persistence has been studied widely in keratinocytes and tumor cell lines of epithelial origin. Smad4 has been implicated in either promoting (Deckers et al., 2006) or inhibiting (Hesling et al., 2011) cellular migration, depending on the system. It is clear from our studies of Smad4-deficient TS cells that Smad4 plays a role in inhibiting cellular migration. In keratinocytes, deletion of Smad4 leads to single cell, uncoordinated movement (Imamichi et al., 2005) similar to Smad4 KO TS cells, which show uncoordinated and multi-directional migration from our in vitro scratch assay (Figure 11b).

One potential target of Smad4-dependent signaling in cell migration can be members of the Rho GTPases family such as Cdc42, Rac and RhoA, all well-known regulators of cell migration. Studying the interaction between the Rho GTPases and Smad4 dependent pathways will help us understand the role of Smad4 in inhibiting migration in wild-type TS cells under normal culture conditions. The Rho GTPases are involved in the remodeling of the cytoskeleton and in regulating migration. It is also involved in inducing mesenchymal expression through a serum response factor, which is shown to be involved in cytoskeletal re-organization and regulation of focal adhesion proteins like focal adhesion kinase (FAK), β-integrin, talin, vinculin expression in ES
cells thereby regulating cellular migration (Hill et al., 1995; Schratt et al., 2002; Tumbarello and Turner, 2007). Paxillin and Hic-5 are two focal adhesion proteins involved in regulating the migration of cells (Owens et al., 2010). Paxillin links actin and extracellular matrix (ECM) together that is usually found in the cytoplasm. TGF-β1 induces cell shape changes by paxillin α phosphorylation during EMT. Paxillin δ suppresses the integrin signaling to paxillin α that reduces its phosphorylation and thus inhibits migration in NmuMG and CHO.K1 cells, a characteristic found in epithelial cells (Thomas et al., 2007; Tumbarello and Turner, 2007). Hic-5, another member of the paxillin family induced during TGF-β mediated EMT is involved in cell shape changes (Tumbarello et al., 2005). It is shown that paxillin δ and Hic-5 have a reciprocal expression in TGF-β induced EMT in NMuMG cells (Thomas et al., 1999; Tumbarello et al., 2005; Tumbarello and Turner, 2007) indicating role of Hic-5 in mesenchymal phenotype. It is shown that a breast cancer line and a fibrosarcoma line have the plasticity to switch between two distinct migratory modes, the mesenchymal mode and amoeboid mode. And the switch is related to changes in morphological, migratory, and invasive behavior of cells. Increased levels of Hic-5 and Rac1-GTP is associated with the mesenchymal mode and the increase in levels of paxillin and RhoA-GTP is associated with the amoeboid mode of behavior. Cdc42 is common for both modes of behavior. Hic-5 was up regulated in a realtime based super array performed in our Smad4 KO TS cells showing the amoeboid phenotype. It would be interesting to see in Smad4 null TS cells, if addition of Smad4 into culture medium could restore the epithelial morphology and restricted migration of wild-type TS cells. Immunofluorescent staining experiments
of the cytoskeletal elements would give us information on the patterning defects in the absence of Smad4. Microarray and real time based assays will let us identify the downstream targets of Smad4 in TS cells. Analyzing the behavior of these proteins by over expressing them in wild-type TS cells and observing their migratory behavior will help us identify the role of Smad4 in regulating these proteins and thereby regulating migration.

During EMT, in addition to changes in migratory properties, cells also acquire invasive characteristics. As a tumor suppressor, loss of Smad4 is frequently associated with acquisition of invasive properties of tumor cells. Prostrate cancer cells lacking both Smad4 and PTEN, another tumor suppressor, are much more invasive than cells lacking PTEN alone, demonstrating the inhibitory effect of Smad4 on cellular invasion (Deakin and Turner, 2011). Restoration of Smad4 in PK-1, a pancreatic adenocarcinoma cell line lacking Smad4 show reduction in invasiveness indicating an inhibiting role for Smad4 in invasion (Ding et al., 2011). Our TS cells in the absence of Smad4 show increased invasiveness assuring its role in inhibiting the invasiveness of the TS cells. Further analysis of the expression of proteins involved in invasion that are known to be targets of TGF-β will let us identify the targets of Smad4 in regulating invasive capacity of the TS cells. Hypo or hyper invasion of the maternal uterine does not establish a proper maternal-fetal connection and so it is required that the invasive capacity of the TS cells be controlled. As Smad4 KO TS cells show much more invasion compared to wild-type TS cells it is important to identify the downstream targets of Smad4 and its role in regulating them.
Role of Smad4 in cancer:

*Smad4* is a well-known tumor suppressor, and loss of *Smad4* is associated with pancreatic, gastrointestinal, skin and a wide range of other cancers (Duda et al., 2003; Hoot et al., 2008). Most cells during carcinogenesis acquire properties like increased proliferation, angiogenesis, migration and invasiveness. They also show anchorage independent growth. But our results do not show defects of proliferation in TS cells lacking *Smad4*. Testing anchorage independent growth of *Smad4* null TS cells by culturing them in soft agar, subsequent injection of these cells into adult mice, and observing for tumor growth will provide us information on the tumorigenic properties of the *Smad4* in TS cells.

Though most of *TGF-β* signaling occurs in a Smad-dependent manner, *TGF-β* is also known to induce EMT in cooperation with other signaling pathways, such as the Ras pathway, ERK-MAPK pathway, PI3K pathway (Bakin et al., 2000), Rho A pathway. (Ahmed and Nawshad, 2007; Clements et al., 2005) These pathways are independent of *Smad4* at least in some breast, colon and pancreatic carcinoma cell lines (Ahmed and Nawshad, 2007; Ozdamar et al., 2005). Identifying the targets of *Smad4* in regulating cell migration will aid targeting certain downstream effectors for drug discovery during loss of *Smad4* in developmental and pathological conditions.

Conclusion

Studies on the role of TGF-β superfamily mediated signaling and its downstream signaling in development and pathological conditions have been the focus for many years
now. But the role of Smad4, one of the downstream TGF-β signaling molecules, in placental development is still unclear. Smad4 KO TS cells were derived in our lab to understand the role of Smad4 in TS cells. TS cells were derived from the trophectoderm lineage that forms the fetal component of the placenta in vivō. From our experimental studies, Smad4 is shown to play a role in maintaining a proper epithelial morphology and architecture in TS cells. Smad4 KO TS cells show increased thickness and form colonies with abnormal cell junctions. Lack of Smad4-mediated signaling leads to increased motility and EMT-like behavior. Smad4 KO TS cells are more motile, invasive, display uncoordinated movement, and show changes in localization of proteins associated with EMT. Smad4 deletion in TS cells also leads to increased differentiation although proliferation is not affected. Our studies thus indicate that Smad4 plays a significant role in regulating EMT in TS cells. Insights into the role of Smad4 in the trophectoderm lineage will lead us to a better understanding of the biology of the placenta.
References


