2009 Vision Grant: Interim Progress Report

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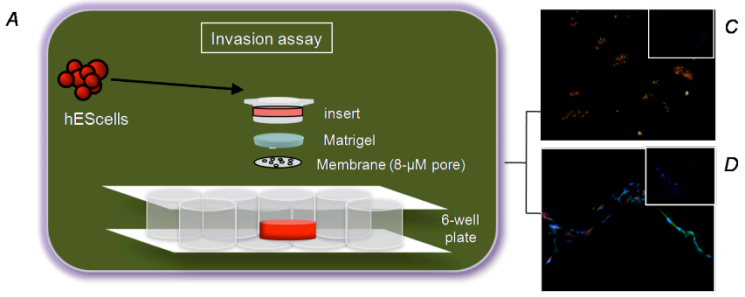
I sincerely thank Preeclampsia Foundation for awarding "Vision Grant" for supporting my research proposal "Extra Villous Trophoblast Models Developed from Human Embryonic Stem Cells". The research project was aimed at developing a novel cell culture model to derive invasive extravillous trophoblast cells (EVT) utilizing human embryonic stem cells and bone morphogenetic protein 4 (BMP-hESC). Defective EVT invasion into maternal uterus and blood vessels is one of the root causes of preeclampsia (PE). The model system outlined in this proposal offers considerable advantages over the existing cell based models to conduct research on preeclampsia. It offers an unabated supply of EVT cells that mitigates the requirement for continual supply of first trimester human placentas, which are not always readily available. We anticipate that the cell model also affords a means of studying the effects of O2 and other environmental inputs that perturb EVT development and invasion, generate a comprehensive list of intrinsic factors that might predispose to PE, and finally validate the role of candidate genes in the etiology of PE. The proposal consisted of two aims. The progress made so far regarding both the aims, and future approaches are outlined briefly below:

Aim-1: Isolate and characterize the invasive populations from BMP4 treated hESC.

This goal of this aim was to a) characterize the HLAG positive (+) invasive population and b) obtain the transcriptional profile of the resulting putative EVT-cells and compare them to their in vivo counterparts.

As for Aim-1a, BMP-hESC model was thoroughly characterized. We noticed that some regions of the BMP-hESC stained brightly for HLAG, the canonical marker of EVT lineage. Other genes, such as *SLC3A2* and *PPARγ* thought to be involved in EVT emergence were also up-regulated in BMP-hESC by d4. Additionally, strong evidence for EVT presence has been obtained from "invasion" assays (Fig. 1A & B). In this experiment, undifferentiated H1 (WA01) and H9 (WA09) hESC cultured under 20% and 4% O_2 were dissociated by collagenase and suspended in mouse embryonic fibroblast (MEF)-conditioned hESC medium. Equal



Source of Variation	% of total variation	P value
Interaction	1.61	0.3881
treatment	68.63	0.0003
oxygen	14.34	0.026

Source of Variation	% of total variation	P value
Interaction	0.29	0.7362
treatment	64.84	0.0008
oxygen	15.61	0.0343

Fig. 1. BMP4-hESC invasion chamber assay. A) Schematic depiction of invasion assay. hESC are plated onto the insert which has a layer of Matrigel coated on a 8-μM porous membrane. The insert with the cells is placed in a single well of a 6-well plate and cultured in MEF conditioned hESC medium lacking FGF2 with 10ng/ml BMP4. (B) Results from 3-different invasion assays with both H1 & H9 hESC. Error bars represent SEM from 3 experiments. Values are expressed as percentage of invaded cells in BMP4 Vs FGF2. The blue bar represents invaded cells in 20% O_2 and those in red represent 4% O_2. The invaded cells stain positive for (C) CDX2 (green) and (D) MSX2 (Green) as well as for KRT7 (Red) (C&D). The staining controls are shown in the insets.

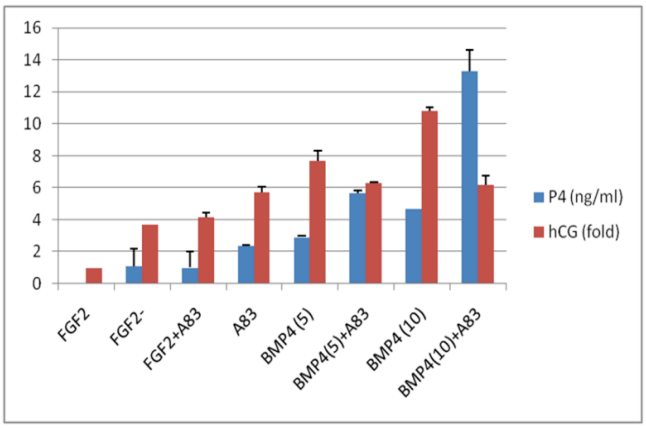


Fig. 2. The response of H1 cells to the ALK2/3/6 inhibitor A83-01 (1 µM) alone and in presence of FGF2 and BMP4 under high O_2 conditions. Colonies were maintained on hESC medium plus FGF2 (FGF2; left), which provides the control. Some hCG is produced in controls under these conditions because under high O_2 there is a tendency for some spontaneous differentiation to occur. In absence of FGF2 (2nd lane), differentiation occurs more rapidly and areas of the colonies show visible differentiation (not shown). A83 promotes production of hCG and P4, and especially P4 relative to hCG when BMP4 (5 ng/ml and 10 ng/ml) is also present.

numbers of hESC from both O_2 conditions were transferred onto Matrigel coated inserts (BD Bioscience) suspended in 6-well plates. Cultures were supplemented with either BMP4 (10 ng/ml) to drive TR formation or FGF2 (8 ng/ml) without BMP4 to maintain pluripotency. After d 6, when differentiation was well advanced, cells that had migrated to the underside of the membrane were counted (Fig. 1B). Cultures exposed to BMP4 under 20% O_2 had significantly more invaded cells than those cultured in FGF2. Low O_2 reduced but did not eliminate cell migration. The invaded cells stained for CDX2, HLA-G, KRT-7, MSX2, (Fig. 1C & 1D) & ID2 (not shown), suggesting that they were EVT. Accordingly, it should be possible to manipulate, enrich, and study EVT cells during their early commitment to the lineage.

We additionally hypothesized that A83-01(A83), a more potent and specific inhibitor of ALK4/5/7 than SB431542, would drive differentiation of hESC into trophoblast even more effectively than SB431542 as was reported recently. As expected, at the recommended concentration of 1 µM, A83 under 20 % O_2 quickly converted colonies of H1 to a phenotype quite similar to that produced in response to BMP4 at 10 ng/ml and promoted hCG and progesterone (P4) production (Fig.2). Both the inhibitor and BMP4 alone and in combination increased expression of *HLAG*. They also up-regulated *KRT7*, *CD9*, and syncytin (*ERVWE1*) (Fig. 3). A83 with BMP4 appeared to enhance P4 production, but was less effective than BMP4 alone in promoting hCG secretion. These experiments need to be confirmed and replicated in another line. However, there is the suggestion that manipulating the relative concentrations of A83, BMP4, and FGF2 might allow us to bias differentiation towards different sub-lineages and allow enrichment of EVT rather than ST. We are in the process of determining conditions most optimal for EVT differentiation. Additionally, collagen matrices (I & IV) which are relatively tougher compared to Matrigel have also being employed to obtain a homogeneous HLAG positive EVT population (Fig.4).

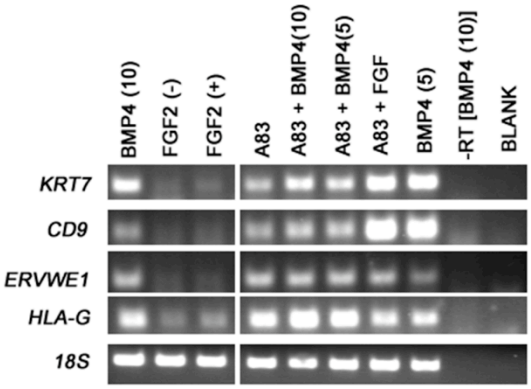


Fig. 3. TR gene expression in hESC determined by RT-PCR in response to 6-day treatment with A83 and BMP4 supplementation. H1 hESC cultured on Matrigel substratum in MEF conditioned hESC medium without FGF2 (standard medium) under 20% O2 conditions were supplemented with either A83 (1µM A83-01) or BMP4 (5 ng/ml and 10 ng/ml) alone or in combination. Cultures in standard medium with no FGF [FGF2 (-)], and those with 8 ng/ml FGF2 [FGF2 (+)], both lacking A83 and BMP4, serve as controls for the experiment. Additionally, A83 with FGF2 was also investigated. The samples were probed for lineage restricted TR markers such as *ERVWE1* (Syncytin) for ST, and *CD9* & *HLAG* for EVT, as well as the general TR marker *KRT7*. 18S is the loading control. Note that A83 by itself or in combination with either BMP4 or FGF2 strongly up-regulated *HLAG* all TR markers.

For Aim-1b, in order to perform comparative microarray analysis of HLAG + primary EVT cells and BMP-hESC, primary EVT cells have been enriched by magnetic bead assisted cell sorting (MACS) using anti-HLAG antibody from 3 separate human placental samples. Additionally, cytotrophoblast population was also sorted using TROP1 antibody. Secondly, HLAG+ cells have been sorted from whole BMP-hESC colonies from two experiments. Following the third sorting experiment, RNA from all the samples will be collected, quality tested and submitted for microarray analysis.

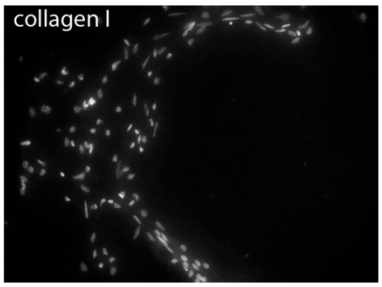


Fig. 4. Invasion of BMP-hESC through type I collagen. Here, H1 hESC cultured on a collagen matrix in invasion chambers were exposed to BMP4 in absence of FGF2 for 6-days. Cells that passed through the 3 μm pores were stained by DAPI. Notice the elongated shape of the nuclei and the column-like associations of invaded cells.

Aim-2: Delineate mechanisms underpinning the emergence of EVT cell by manipulated expression of *HIF-1-alpha (HIF-1α)*

Our hypothesis was that, persistent HIF-1α under normoxia is a principal etiology of some forms of PE. To test the hypothesis, we are in the process of generating stably transfected hESC lines carrying a reverse tetracycline transactivator gene (FUW-M2rtTA; Addgene Plasmid:20342) and a tet-inducible HIF-1α triple mutant (TM) (residues P402/ N803 mutated to alanine and P564 to glycine) resistant to degradation and inactivation. The HIF-1A TM was already generated and sequence verified. Drug resistant feeder lines were also acquired. The HIF1A-TM is in the process of being cloned upstream of IRES site in IRES-GFP/ PuroR (Addgene: Plasmid 14430). hESC stably transduced for TM can then be selected by puromycin selection and the expression of the TM gene monitored by GFP expression and Western blotting following addition of DOX to the medium. These experiments are technically straightforward, and are envisaged to be completed within the next 3 months of the project duration.