Vol.2, No.4, 163-175 (2012)
http://dx.doi.org/10.4236/scd.2012.24021

Stem Cell Discovery

Bone morphogenetic protein-4 affects both trophoblast and non-trophoblast lineage-associated gene expression in human embryonic stem cells

Margaret L. Shirley[1,2*], Alison Venable[1*], Raj R. Rao[3], Nolan L. Boyd[4], Steven L. Stice[1,5,6], David Puett[1#], Prema Narayan[7#]

[1]Department of Biochemistry and Molecular Biology, University of Georgia, Athens, USA;
[#]Corresponding Author: puett@bmb.uga.edu
[2]Department of Psychiatry, University of California, San Francisco, USA
[3]Department of Chemical and Life Science Engineering, School of Engineering, Virginia Commonwealth University, Richmond, USA
[4]Cardiovascular Innovation Institute, University of Louisville, Louisville, USA
[5]Regenerative Bioscience Center, University of Georgia, Athens, USA
[6]Department of Animal and Dairy Sciences, University of Georgia, Athens, USA
[7]Department of Physiology, Southern Illinois University School of Medicine, Carbondale, USA;
[#]Corresponding Author: pnarayan@siumed.edu

Received 5 May 2012; revised 4 June 2012; accepted 1 July 2012

ABSTRACT

Human embryonic stem cells (hESC) can be induced to differentiate to trophoblast by bone morphogenetic proteins (*BMP*s) and by aggregation to form embryoid bodies (EB), but there are many differences and controversies regarding the nature of the differentiated cells. Our goals herein were to determine if BG02 cells form trophoblast-like cells (a) in the presence of *BMP*4-plus-basic fibroblast growth factor (*FGF*-2) and (b) upon EB formation, and (c) whether the *BMP*4 antagonist noggin elicits direct effects on gene expression and hormone production in the cells. Transcriptome profiling of hESC incubated with *BMP*4/*FGF*-2 showed a down-regulation of pluripotency-associated genes, an up-regulation of trophoblast-associated genes, and either a down-regulation or no change in gene expression for many markers of the three embryonic germ layers. Yet, there was up-regulation of several genes associated with mesoderm, ectoderm, and endoderm, strongly suggesting that differentiation to trophoblast-like cells under the conditions used does not yield a homogeneous cell type. Several genes, heretofore unreported, were identified that are altered in hESC in response to *BMP*4-mediated differentiation. The production of human chorionic gonadotropin (hCG), progesterone, and estradiol in the differentiated cells confirmed that trophoblast-like cells were obtained. Gene expression by EB was characterized by an up-regulation of a number of genes associated with trophoblast, ectoderm, endoderm, and mesoderm, and the production of hCG and progesterone confirmed that trophoblast-like cells were formed. These results suggest that, in the presence of *FGF*-2, BG02 cells respond to *BMP*4 to yield trophoblast-like cells, which are also obtained upon EB formation. Thus, *BMP*4-mediated differentiation of hESC represents a viable cell system for studying early developmental events post-implantation; however, up-regulation of non-trophoblast genes suggests a somewhat diverse response to *BMP*4/*FGF*-2. Noggin altered the transcription of a limited number of genes but, not surprisingly, did not lead to secretion of hormones.

Keywords: Human Embryonic Stem Cells; Trophoblasts; Bone Morphogenetic Protein-4; Embryoid Bodies; Noggin

1. INTRODUCTION

In the human blastocyst, the first step of differentiation from the morula, composed of totipotent cells, yields the inner cell mass and trophoblast. The former differentiates into the hypoblast, leading to the extraembryonic endoderm and the epiblast, that differentiates to give the amniotic ectoderm and the primitive ectoderm. The primitive ectoderm, in turn, differentiates into the embryonic

[*]These authors contributed equally to this work.

ectoderm and the primitive streak, the latter giving rise to extraembryonic mesoderm, primitive mesoderm, and embryonic endoderm. The trophectoderm is composed of epithelial cells and differentiates into several lineages. The trophoblast precursor cells begin rapid proliferation into cytotrophoblasts, with some fusing to form multinucleate syncytiotrophoblasts, a terminally differentiated trophoblast cell; in addition, villous and extravillous cytotrophoblasts are formed [1-3]. One of the hallmarks of trophoblast differentiation is the production of the heterodimeric glycoprotein hormone, human chorionic gonadotropin (hCG), that is secreted throughout gestation, being essential for progesterone production by the corpus luteum in the first trimester of human pregnancy [4,5]. There is a paucity of adequate cell models for studying early trophoblast development, and a reliable system will greatly facilitate progress in the area of human reproduction.

Recent studies have shown that human embryonic stem cells (hESCs) can undergo differentiation into trophoblast-like cells spontaneously from colonies [6], growth of the cells beyond confluence [7], or embryoid bodies (EBs) [3,8-12]. Similarly, trophoblast-like cells can be obtained from hESCs via induced differentiation from the bone morphogenetic proteins (*BMP*s) 2, 4, and 7 [13-24]. Fortunately, timely reviews are available [25-28]. Others have reported that RNAi-mediated knockdown of Oct4 in hESCs can also lead to trophoblast-like cells [29,30]. The *BMP*s, members of the transforming growth factor-b (TGFb) superfamily, function to regulate many aspects of development which act by binding to cell surface serine/threonine kinase receptors that, in turn, phosphorylate particular Smads, thus enabling them to enter the nucleus and act as transcriptional regulators [31-34].

These cellular models for trophectoderm differentiation begin to fill a long-awaited need for new systems to study early development, particularly in view of the major differences between human and mouse trophoblast [35-37]. Yet, the characteristics of the human trophoblast cells obtained by *BMP*-mediated differentiation vary, sometimes quite significantly, depending upon the cell type, culture conditions (including the presence or absence of growth factors, particularly *FGF*2), and mode of differentiation [10,18,38]. Moreover, in a number of instances there are considerable differences and controversies regarding the product(s) of differentiation obtained with *BMP*. For example, *BMP*4-mediated differentiation was found to yield little evidence of trophoblast-like cells under standard culture conditions [38]. Another study found that 14 genes were highly up-regulated as determined by microarrays, in addition to many others [21], while another report did not identify six of these particular genes [17]. Also, differences in cell morphology and gene markers have been noted in *BMP*-treated hESCs [18,21]. Explanations for many of these controversial reports were recently provided by two groups. Yu *et al.* [22] reported that b*FGF* (*FGF*-2), which acts via the MEK-ERK pathway, redirects *BMP*4-mediated differentiation from trophectoderm to mesendoderm as judged by the expression of brachyury. The activated MEK-ERK pathway sustains NANOG expression leading to a b*FGF*-independent induction of mesendoderm by *BMP*4. Another recent paper concluded that in the cooperative presence of *FGF*2, *BMP*4 (that acts through brachyury and *CDX*2) leads to the induction of mesoderm, not trophoblast [39].

The present study was undertaken with the goals of: 1) extending the previous reports that were based on a variety of cell lines, different culture conditions, and at times conflicting results, and 2) to critically examine the trophoblast and non-trophoblast *BMP*-regulated genes, particularly since a number of the non-trophoblast *BMP*-regulated genes have been noted in the earlier studies. We chose to use the hESC line, BG02 (karyotype 46, XY), that has not been thoroughly studied in hESC differentiation to trophoblast. This cell line was established from a 6-day embryo with an embryo grade of 3CC [7]. Herein, *BMP*4, in the presence of *FGF*2, was incubated with adherent colonies of BG02 cells to initiate trophoblast differentiation. Identical studies were also done in the presence of the *BMP*4 antagonist noggin, since it has been shown that noggin alters hESC gene expression and morphology, perhaps leading to differentiation toward early neuroectoderm [18]. In addition, trophoblast differentiation was initiated by formation of EBs. Particular emphasis was placed on quantitative profiling of a variety of genes via qRT-PCR, and measurements were made to determine hCG and steroid hormone production by the cells and EBs. Our results have many similarities with the findings of others, including for example the down-regulation of pluripotency genes, the up-regulation of trophoblast genes, and placental hormone production, but there are also notable differences, particularly with the up-regulation of certain genes associated with the three primary germ layers.

2. MATERIALS AND METHODS

2.1. Maintenance of Undifferentiated Cells

In order to ensure pluripotency, the BG02 cells, obtained with proper authorization from Bresagen, Athens, GA, were manually passaged every 2 - 3 days onto mitotically-inactivated mouse embryonic feeder (MEF) layers derived from E13.5 mouse fetuses. The MEF layer was removed from the hESC colony, which was then gently dispersed with the colony pieces being transferred to another 10 cm MEF-containing plate and treated with

hESC culture medium: 77% Dulbecco's Modified Eagle Medium (DMEM/F12; Gibco, Carlsbad, CA) supplemented with 15% fetal bovine serum (Hyclone, Logan, UT); 5% knockout serum replacement, 1% non-essential amino acids, 1% penicillin/streptomycin, and 1 mM L-glutamine (all from Gibco); 0.1 mM β-mercaptoethanol and 4 ng/mL basic fibroblast growth factor (*FGF*2; Sigma, St. Louis, MO); and 10 ng/mL leukemia inhibitory factor (LIF; Chemicon, Temecula, CA). Cells were passaged every 3 - 4 days. Two days after passage the medium was aspirated and replaced daily.

2.2. *BMP*4-Mediated Differentiation

The cells were passaged by gentle enzymatic digestion using cell dissociation buffer (Gibco) into 10 cm Matrigel-coated dishes (BD Bioscience, Boca Raton, FL). The BG02 cells were cultured in 50% DMEM/F12 medium that was conditioned by MEF layers [21] and then supplemented with 4 ng/mL *FGF*2. Experimental groups included incubation with 100 ng/mL *BMP*-4 (Quest Diagnostics, Lyndberg, NJ) and with 250 ng/mL noggin (Quest Diagnostics), with untreated hESC serving as controls. The medium was collected each day for analysis of secreted hormones. On day 7 the cells were harvested and quick-frozen for RNA extraction

2.3. Formation of Embryoid Bodies

Colonies were sliced into small pieces, then removed gently from the MEF layer where they were allowed to aggregate randomly in suspension on agarose plates. EBs were grown on agarose dishes in 12 mL of the hESC medium described above. Each culture began with ~50 EBs and ended with ~12 EBs of varying sizes. The loss of EB was attributed to aggregation of the individual units and/or to atresia/necrosis. On alternate days the culture plates were swirled to aggregate the EB with 6 mL of medium being removed and frozen. The same volume of fresh medium was added with the EBs then dispersed to prevent clumping.

2.4. Hormone Assays

Media collected from cell cultures and EBs were analyzed for secretion of three placental hormones namely hCG (the assay recognizes both heterodimer and hCGβ), progesterone, and estradiol using immunofluorescence-based assays. The hormones were measured with an Immulite 1000 with a tri-level internal control in human serum, Con6, being used to standardize all kits (Diagnostic Product Corporation, Los Angeles, CA).

2.5. qRT-PCR

Total RNA was isolated from BG02 (day 0, *i.e.* control, and incubated for 7 days) and from EBs at days 5, 22, and 50 of culture. hESCs were resuspended in 1 mL Trizol (Invitrogen, Carlsbad, CA) and triturated until homogenized. The integrity of isolated RNA isolated from the homogenates (Trizol, Molecular Research Corporation, Albany, NY) was verified and quantified using a RNA 600 Nano Assay (Agilent Technologies, Foster City, CA) and the Agilent 2100 Bioanalyzer. The cDNA Archive Kit (Applied Biosystems, Inc., Foster City, CA) was used to reverse transcribe 5 µg total RNA with the MultiScribe Reverse Transcriptase. Initially, reactions were incubated at 25˚C for 10 min and subsequently at 37˚C for 120 min. Quantitative PCR (Taqman) assays were selected for the transcripts to be evaluated from Assays-On-Demand (Applied Biosystems, Inc.) and incorporated into 384-well Micro-Fluidic Cards. The cDNA samples and 50 µl of GeneAmp Fast PCR master mix (2×) (Applied Biosystems, Inc.) were loaded in duplicate into respective channels on each microfluidic card and briefly centrifuged.

qRT-PCR and relative quantification were performed with the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Inc.), with the expression levels of all genes analyzed given relative to 18S rRNA expression. The results for differential expression between the treated and control samples were expressed as means and data with a C_t value greater than 35 were not analyzed. The qRT-PCR data on gene expression of *BMP*4-treated and noggin-treated hESCs on day 7 of culture were analyzed as ddC_t relative to day 0, *i.e.* undifferentiated hESCs. Data were collected for EBs at days 5, 22, and 50 of culture, in all cases using 18S rRNA as the internal reference gene. The values of ddC_t on days 22 and 50 are expressed relative to day 5. In the equations below, std = internal gene standard (18S rRNA), goi = gene of interest under experimental conditions, and con = gene of interest under control conditions. It is assumed that the amplification efficiency is identical under all conditions to give the normalized fold-change of the mRNA of interest in treated cells (experimental) relative to that of control cells [40,41].

$$\left(dC_t\right)_{goi} = \left(C_t\right)_{goi} - \left(C_t\right)_{std}, \left(dC_t\right)_{con} = \left(C_t\right)_{con} - \left(C_t\right)_{std}$$

$$ddC_t = \left(dC_t\right)_{goi} - \left(dC_t\right)_{con}, \text{Fold-change} = 2^{-ddc_t}$$

As defined, ddC_t is negative if the gene of interest under experimental conditions, *i.e.* BG02 cells plus *BMP*4 or plus noggin on day 7, is expressed at a higher level than the same gene under control conditions, *i.e.* untreated cells at day 0.

2.6. Data Analysis

Most of the PCR experiments were performed in trip-

licate, although in a few cases n was 2, 4, 5, or 6. Hormone measurements were done in triplicate, and the results are given as mean ± SEM. Data exceeding a fold-change of 2.0 (up-regulated) or –2.0 (down-regulated) were analyzed using a one-way analysis of variance (ANOVA) with the GraphPad Prism software. Results are given for changes with $P < 0.05$ and $P < 0.10$. Although the mean fold-change was large in some cases, significance was at times not reached, often due to n = 2 or to one value in three being particularly different from the others. Outliers were identified using the Grubbs' test (on-line GraphPad software). From over 400 hormone measurements, only about 1% was deemed outliers, and from over 700 qRT-PCR runs, only six and seven outliers were identified in the cell and EB data, respectively.

3. RESULTS

3.1. Effects of *BMP*4 and Noggin on Gene Expression and Hormone Production in hESC

Gene expression: The selection of 177 genes to investigate via qRT-PCR was based on several criteria. For example, it was important to include many that were established markers of pluripotency, trophoblast, ectoderm, endoderm, and mesoderm. In addition to these standard marker genes, others were also screened. Included in this list were genes encoding certain steroidogenic enzymes even if not specific for trophoblast and placenta, e.g. cytochrome P450 side-chain cleavage enzyme (*CYP*11*A*1) and aromatase (*CYP*19*A*1). Other genes encoding proteins for extracellular matrix and various aspects of cell function were monitored.

A heatmap, based on mean values of 2^{-ddc_t} and depicting gene expression of 146 genes in hESCs incubated with either *BMP*4 or with noggin for 7 days, both relative to control cells, is shown in **Figure 1**. **Table I** provides more information on many of the genes shown in **Figure 1** that are altered by 2-fold or more in response to *BMP*4. It can be seen that a number of genes are up-regulated by *BMP*4, a smaller number down-regulated, and a much larger number exhibit no major change in expression.

*BMP*4 leads to down-regulation of the pluripotency-associated genes: *DNMT*3*B*, *EBAF*, *FGF*2, *FGF*4, *FGFR*4, *FOXH*1, *GABRB*3, *GBX*2, *LDB*2, *POU5F*1, and *SOX*2, and an up-regulation of genes associated with differentiation to trophoblast or involved in implantation and/or placenta function: *CGB*, *CYP*11*A*1, *CYP*19*A*1, *ENPEP*, *EPAS*1, *GATA*2, *GATA*3, *HEY*1, *INHA*, *KRT*7, *MMP*9, *MSX*2, *PGF*, and *WNT5A*, genes that have also been identified by others (cf. [17,21] and references therein). Moreover, a comparison of *BMP*4-mediated hESC differentiation with human trophectoderm [42] identified

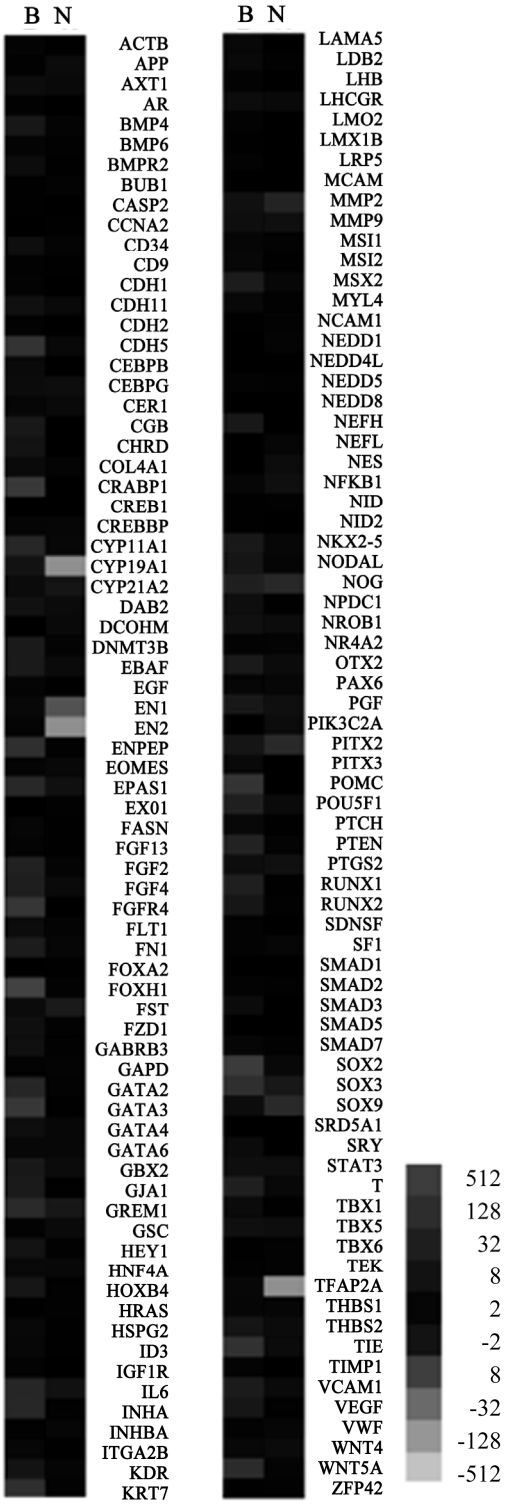


Figure 1. Heatmap showing the alterations in gene expression in hESC after seven days of incubation with either *BMP*4 (B) or noggin (N) relative to control. The data reflect qRT-PCR results given as mean values of 2^{-ddc_t}. The gray bars denote sufficiently low expression that could not be accurately determined, *i.e.* C_t greater than 35.

Table 1. *BMP*4-mediated changes in gene expression of hESCs[a].

Gene	Fold-change	Description
Trophoblast		
CGB	44.9[b]	Chorionic gonadotropin-b
*CYP*11*A*1	51.5[c]	Cytochrome P450 family 11, subfamily A
*CYP*19*A*1	8.9[b]	Cytochrome P450 family 19, subfamily A
ENPEP	244.6[b]	Glutamyl aminopeptidase
*EPAS*1	185.6[d]	Endothelial PAS domain protein 1
*GATA*2	200.2[d]	GATA binding protein 2
*GATA*3	336.2[d]	GATA binding protein 3
*HEY*1	18.5[b]	Hairy enhancement of split related with YRPW motif
INHA	89.9[c]	Inhibin a
*KRT*7	242.2[c]	Keratin 7
*MMP*9	8.6[c]	Matrix metalloproteinase 9
*MSX*2	174.1[d]	Msh homeobox 2
PGF	26.5[d]	Placental growth factor
*WNT*5*A*	101.8[d]	Wingless-type MMTV integration site family 5A
Pluripotency		
*DNMT*3*B*	−3.7[b]	DNA (cytosine-5)-methlytransferase 3 b
EBAF	−4.9[b]	Endometrial bleeding associated factor
*FGF*2	−5.3[b]	Fibroblast growth factor 2
*FGF*4	−4.5[b]	Fibroblast growth factor 4
*FGFR*4	−6.2[d]	Fibroblast growth factor receptor 4
*FOXH*1	−9.1[d]	Forkhead box protein H1
*GBX*2	−3.5[b]	Homeobox protein GBX2
*POU5F*1	−4.8[b]	POU class 5 homoebox 1
*SOX*2	−26.8[d]	SRY (sex determining region Y)-box 2
Mesoderm		
*BMP*4	24.1[d]	Bone morphogenetic protein 4
*BMPR*2	4.4[d]	Bone morphogenetic protein receptor, type II
*CDH*5	150.2[b]	Cadherin 5, type 2
*CDH*11	7.1[d]	Cadherin 1, type 2
CHRD	9.1[d]	Chordin
*GATA*4	13.7[b]	GATA binding protein 4
*GJA*1	–3.4[d]	Connexin 43, gap junction a3
KDR	–3.4[b]	Kinase insert domain receptor
*NKX*2-5	15.5[b]	Homeobox protein Nkx 2.5
*PITX*2	12.4[d]	Paired-like homeodomain transcription factor 2
*RUNX*1	60.9[d]	Runt-related transcription factor 1
*RUNX*2	14.3[b]	Runt-related transcription factor 2
*SOX*9	5.8[c]	SRY (sex determining region Y)-box 9
T	38.1[d]	Brachyury
*TBX*5	7.6[c]	T box transcription factor
TIE	−11.8[b]	Receptor tyrosine kinase
VEGF	13.2[b]	Vascular endothelial growth factor
Ectoderm		
*EN*1	2.7[b]	Engrailed homeobox 1
*FN*1	68.6[c]	Fibronectin 1
*HOXB*4	24.5[c]	Homeobox protein B4
Ectoderm		
*MMP*2	10.8[d]	Matrix metalloproteinase 2
*MSI*2	2.6[b]	Musashi homolog 2
*MYL*4	5.3[b]	Myosin light chain 4
NOG	44.4[c]	Noggin
*SOX*3	−5.2[d]	SRY (sex determining region Y)-box 3
Endoderm		
*GATA*6	7.3[b]	GATA binding protein 6
*HNF*4*A*	3.5[b]	Hepatocyte nuclear factor 4 a
NODAL	−2.0[b]	Nodal
Others		
*AKT*1	4.3[d]	Protein kinase B, PKB b
CEBPB	4.3[c]	CCAAT/enhancer binding protein b
CEBPG	3.9[c]	CCAAT/enhancer binding protein g
*COL*4*A*1	3.4[c]	Collagen, type IV, a 1
*CRABP*1	−11.8[c]	Cellular retinoic acid binding protein
*DAB*2	10.5[c]	Disabled homolog 2
FST	4.7[b]	Follistatin
*FZD*1	6.3[b]	Frizzled homolog 1
GREM1	−4.8[b]	Gremlin 1
*ID*3	2.4[b]	Inhibitor of DNA binding 3dominant negative HLH protein
*IL*6	80.1[b]	Interleukin 6
*LAMA*5	2.7[b]	Laminin, a 5
NEFH	−5.3[b]	Neurofilament heavy polypeptide
*NROB*1	6.4[c]	DAX-1 (nuclear receptor subfamily 0, group B, member 1)
*OTX*2	−13.1[b]	Orthodenticle homeobox 2
POMC	−8.4[b]	Proopiomelenocortin
PTCH	2.6[b]	Protein patched homolog 1
*PTGS*2	5.2[c]	Prostaglandin enteroperoxide synthase
PTEN	−3.4[b]	Phosphatase and tensin homolog
*SMAD*3	3.8[b]	Smad 3 (Mad homolog 3)
*VCAM*1	30.5[b]	Vascular cell adhesion protein 1
*WNT*4	3.2[c]	Wingless type 4

[a]Genes altered in expression 2-fold or more (see **Figure 1**); [b]$P > 0.10$; [c]$P \leq 0.10$; [d]$P \leq 0$.

many common genes, including ones we also found to be up-regulated: *FST*, *IL*6, and *VCAM*1.

Overall, *BMP*4 resulted either in no increase, and often even a down-regulation, of many genes associated with ectoderm (e.g. *EN*2, *EOMES*, *EXO*1, *FGF*13, *MSI*1, *NEFH*, *NES*, *NR*4*A*2, *PAX*6, *PITX*3, and *SOX*3), endoderm (e.g. *AFP*, *CER*1, and *NODAL*), and mesoderm (e.g. *CD*34, *GJA*1, *GSC*, *KDR*, *LMO*2, *SDNSF*, *TBX*1, and *TIE*). There are, however, important exceptions. Several canonical mesodermal gene markers were up-regulated, *BMP*4, *BMPR*2, *CDH*5, *CDH*11, *CHRD*, *GATA*4, *NKX*2-5, *PITX*2, *RUNX*1, *RUNX*2, *SOX*9, *T*, *TBX*5, and *VEGF*, as were the ectodermal markers, *EN*1, *FN*1, *HOXB*4, *MMP*2, *MSI*2, *MYL*4, and *NOG*, and the endodermal markers, *GATA*6 and *HNF*4*A*. Our transcriptome profiling also revealed the up-regulation of additional genes, including *AKT*1, *CEBPB*, *CEBPG*, *COL*4*A*1, *DAB*2, *FST*, *FZD*1, *IL*6, *LAMA*5, *NROB*1, *PTCH*, *PTGS*2, *SMAD*3, *VCAM*1, and *WNT*4, and the down-regulation of *CRABP*1, *GREM*1, *LHCGR*, *NEFH*, *OTX*2, *POMC*, *PTEN*, and *THBS*2. The regulation of *CEBPB*, *RUNX*2, *SOX*9, and *VEGF* by *BMP*4 has not, to the best of our knowledge, been reported by others.

The results in **Figure 1** demonstrate that noggin alone altered the transcription of several of the 177 genes surveyed. Genes that were up-regulated include *AFP*, *AKT*1 *CDH*5, *CEBPG*, *CER*1, *EBAF*, *EOMES*, *FGF*4, *GBX*2, *GSC*, *HNF*4*A*, *IL*6, *LHCGR*, *OTX*2, *POU*5*F*1, *SOX*2, *T*, *TBX*5, *THBS*2, and *VCAM*1. In addition, noggin down-regulated a number of genes, *CYP*19*A*1, *EN*1, *EN*2, *EPAS*1, *FST*, *GREM*1, *MMP*2, *MMP*9, *NFKB*1, *NOG*, *NROB*1, *PGF*, *PITX*2, *PTGS*2, *SOX*3, *SOX*9, and *TFAP*2*A*. It is worthy of note that the transcription of three of these down-regulated genes, *CYP*19*A*1, *EN*2, and *TFAP2A*, fall below the level of detection following treatment with noggin, *i.e.* C_t greater than 35.

Genes highlighted above that were altered similarly by both *BMP*4 and noggin, albeit in some cases to a greater or lesser degree, include, up-regulation: *AKT*1, *CDH*5, *CEBPG*, *IL*6, *TBX*5, *VCAM*1; and down-regulation: *GREM*1 and *SOX*3. These results may imply non-specific action of *BMP*4 and/or direct effects of noggin.

Of the genes surveyed, 31 were not expressed sufficiently to be accurately determined and hence could not be included in **Figure 1**. These genes are listed in the Supplement (**Figure S1**).

Phase-contrast microscopic images of the hESCs are given in the Supplement (**Figure S1**) showing control cells and cells incubated with *BMP*4 and with noggin. Consistent with the transcriptome profiling results, heterogeneity is apparent, but the *BMP*4-treated cells give more the appearance of syncytiotrophoblast while the noggin-treated cells are quite distinct.

Hormone production: The media concentrations of hCG, progesterone, and estradiol were measured daily, up to seven days, for cells incubated with *BMP*4 and with noggin (**Figure 2**). Following incubation with *BMP*4, the three hormones increased on days 6 and 7 relative to control cells. As expected, noggin itself had no effect on hormone production. The concentrations reached on day 7 with *BMP*4 are about 15, 10, and 0.6 ng/mL for hCG, progesterone, and estradiol, respectively.

3.2. Gene Expression and Hormone Production in EB

Gene expression: Expression of 74 genes by EBs is presented as a heatmap (derived from mean values of 2^{-ddc_t}) in **Figure 3** at 22 and 50 days, each relative to day 5. Some of the more prominently expressed genes up-regulated at day 22 and/or day 50 include *AFP*, *CGB*, *CHRD*, *DAB*2, *HSPG*2, *INHBA*, *PGF*, *PTGS*2, *RUNX*1, *TIMP*1, and *VEGF*, only a few of which are trophoblast-associated. Most of the genes shown in **Figure 3** are, however, either down-regulated or exhibit no appreciable change over time. Of the genes surveyed, an arbitrary selection of the 15 most highly expressed at day 5, as assessed by dC_t values, are (in decreasing order):

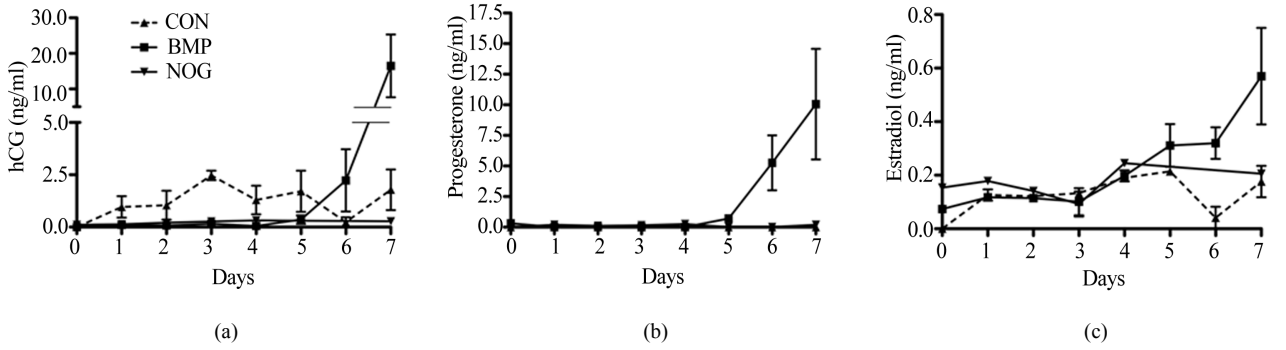


Figure 2. Medium concentrations of hCG (a), progesterone (b), and estradiol (c) of hESC. The cells were incubated in medium alone (CON), in medium containing *BMP*4 (*BMP*), or in medium with noggin (NOG).

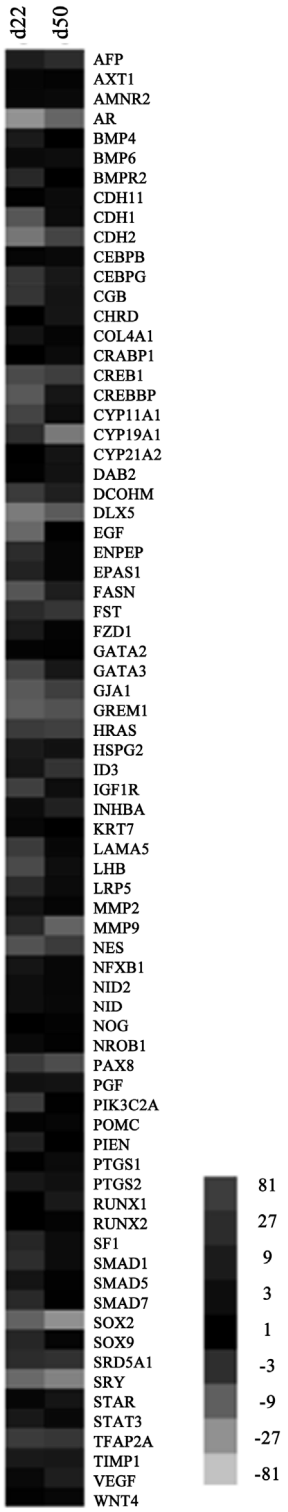


Figure 3. Heatmap showing changes in gene expression of EB at 22 and 50 days, relative to day 5 (mean values of 2^{-ddc_t}). The most prominently altered genes concomitant with time in culture are discussed in the text.

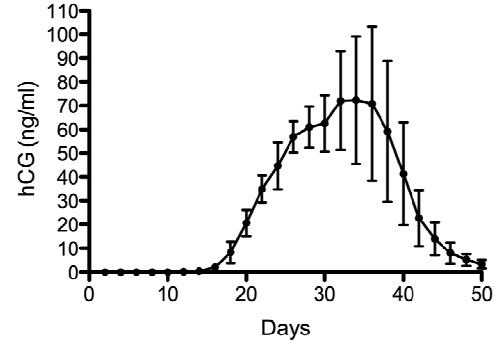
*GJA*1, *CREBBP*, *CDH*2, *ID*3, *CDH*1, *DLX*5, *SOX*2, *CREB*1, *HRAS*, *AR*, *AFP*, *CDH*11, *NES*, *GATA*3, and *GREM1* (data not shown). Supplement **Figure 2** (S. **Figure 2**) shows an H & E stained section of an EB at day 50, and the heterogeneous nature is quite apparent.

Hormone production: **Figure 4(a)** shows the media concentrations of hCG by EBs up to 50 days incubation. Considerable variability was noted in the hormone concentrations, attributed to the different sizes and numbers of EBs in each agarose dish. Moreover, viability may be decreasing after extended culture. hCG begins increasing on about day 18 and reaches a maximum on days 32 - 36 days. Under the conditions of the assay, where 50% of the EB medium is replaced every two days to ensure EB viability, the concentrations measured reflect new synthesis to a large extent, and, to a lesser degree, accumulation. Assuming that hCG is stable in the medium, it is possible to correct the concentrations for the total accumulated values at each two days of measurement. Doing so shows that hCG is continually synthesized between days 20 - 40 and then reaches a plateau of about 660 ng/mL between days 40 - 50. Media concentrations of progesterone follow a similar pattern to that of hCG **Figure 4(b)** and, when concentrations were corrected as described above, reach a plateau of about 1 ng/mL between days 40 - 50. The concentrations of estradiol were also measured (data not shown) and, when corrected as per hCG and progesterone, a maximal concentration of only about 0.04 ± 0.01 ng/mL was achieved. In view of the dynamic nature of EB size and number, quantification of hormone data is precluded.

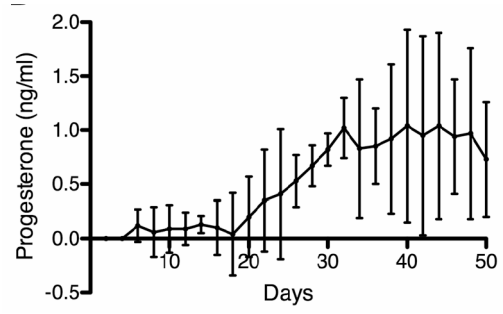
4. DISCUSSION

4.1. BG02 Cells

This study has shown that, in the presence of *FGF*2, *BMP*4 leads to differentiation of the hESC line, BG02, to trophoblast-like cells, as has been reported by several groups using different cell lines, e.g. H1, H7, H9, H14, HES-2, HES-3 and various culture conditions (cf. [17,19, 21,27]). We have also identified *BMP*4-mediated and specific up-regulation of the genes *AKT*1, *CEBPB*, *RUNX*2, *SOX*9, and *VEGF*. Of these, Xu *et al.* [21] reported only minimal and non-significant changes in *AKT*1 and *CEBPB*. These genes most likely reflect *BMP*4 signaling and are not specific to trophoblast, although *VEGF* is expected to become important in placental formation and development. *AKT*1 encodes an isoform of serine/threonine kinase B (PKB*α*) involved in cell survival, proliferation, metabolism, and angiogenesis. Its regulation by *BMP*4 and noggin may imply that the *BMP*4-regulated component may be via a non- canonical pathway. The intronless gene *CEBPB* encodes the bZIP transcriptional factor, CCAAT/enhancer binding pro-



(a)



(b)

Figure 4. Medium concentration of hCG (a) and progesterone (b) production by EB cultured up to 50 days. The variability at each day is attributable to the dynamic nature of EB, in particular the changing size, number, and possibly atresia and necrosis. As discussed in the text, the conditions are such that new hormone synthesis and secretion is the primary contributor with accumulated prior secretion contributing some as well. A correction for the total accumulated hCG and progesterone, assuming complete stability in the medium, shows that the majority of synthesis occurs between days 20 - 40, after which plateaus of about 660 ng/mL and 1 ng/mL, respectively, are reached. The concentrations of estradiol were also measured and found to be quite low, about 0.04 ± 0.01 ng/mL (data not shown).

tein-b, that forms homodimers or heterodimers with other members of the CEBP family, a, d, and g. *RUNX*2 is regulated by the *BMP* pathway and also encodes a transcriptional factor involved in osteogenesis. Another transcriptional factor, (sex determining region Y)-box 9, is encoded by *SOX*9 and participates in chondrogenesis. Vascular endothelial growth factor, a member of the cystine-knot growth factor family and encoded by *VEGF*, stimulates vasculogenesis and angiogenesis and is expected to function in placental development. The results reported herein demonstrate an effect of noggin on the expression of a number of genes. In some cases *BMP*4 and noggin alter gene expression similarly, suggesting either that the regulation by *BMP*4 may not be via a canonical signaling pathway or that *BMP*4 and noggin may alter transcription similarly. At this time it is not possible to relate the observed changes accompanying treatment with noggin with the early signs of neural differentiation noted by Pera *et al.* [18].

Xu *et al.* [21] first reported that, in the presence of *FGF*2, *BMP*2, *BMP*4, and *BMP*7 promoted differentiation of H1, H7, H9, and H14 cells, cultured in mouse embryonic fibroblast conditioned medium, into trophoblast cells. Later, others found that the induction of trophoblast by *BMP*4 required an inhibition of the Activin/Nodal signaling [43]. The seminal study by Xu *et al.* [21] was followed by other reports confirming *BMP*-mediated hESC differentiation to trophoblast-like cells. Das *et al.* [44], for example, also showed that *BMP*4 directed H1 and H9 cells to trophoblast, while *FGF*2 slowed this differentiation, with oxygen accelerating it and promoting formation of syncytiotrophoblast. Interestingly, it was found that *FGF*2, acting to maintain NANOG levels via the MEK-ERK pathway, is capable of switching *BMP*4-mediated differentiation of hESC to mesendoderm as documented by the expression of brachyury and other primitive streak markers [22]. A similar conclusion was reached by Bernardo *et al.* [39] who showed that differentiation of hESCs by *BMP*4 in the presence of *FGF*2 led to formation of mesoderm and inhibition of endoderm. They also reported that this differentiation was via the ERK pathway and mediated by brachyury and *CDX*2.

In a recent comprehensive study of *BMP*-mediated differentiation of hESC to trophoblast, Marchand *et al.* [17] performed microarray analysis using the Affymetrix Human Gene version 1.0 ST array, along with qRT-PCR on selected genes. H7 and H9 cells were incubated with *BMP*4 for various times, 0, 2, 4, 6, 8, and 10 days, following the removal of *FGF*2. They found that after 2 days *POU5F*1 and *NANOG* were dramatically down-regulated while trophoblast markers were up-regulated. Many new genes were identified and suggested to be involved in trophoblast formation, and pathway analysis provided considerable insight into the myriad signaling systems operative in the differentiation of hESCs to trophoblast. This study was augmented by another report [42] in which the transcriptome of trophectoderm cells obtained from 13 human blastocysts were compared with those of *BMP*4-mediated differentiation of hESCs [17]. Their results documented that *BMP*4-induced differentiation of hESCs offers a good model for studying trophoblasts and contributed significantly to a better delineation of the associated transcriptome.

Our results with *BMP*4 are, by and large, in agreement with the findings from the two major combined microarray and PCR investigations on *BMP*-induced differentiation of hESCs [17,21]. This is somewhat surprising since we and Xu *et al.* [21] maintained *FGF*2 along with

*BMP*4, while Marchand *et al.* [17] removed *FGF*2 from the medium when *BMP*4 was added. A summary of many of the genes found to be altered in the present study compared to other reports is given in the Supplement (S.2). A major difference between our results and those of Marchand *et al.* [17] is in the expression of the mesodermal markers, *BMP*4 and *T*. We observed increased expression of these two genes, while they reported a decrease or no change, attributable to their removal of *FGF*2 during *BMP*-mediated differentiation. Of interest, they found increased expression of *KDR*, while we noted a minimal decrease, albeit not significant. Increased expression of *MMP*9 was found herein, consistent with the findings of Xu *et al.* [21] and Schultz *et al.* [19], but Marchand *et al.* [17] reported down-regulation. With the BG02 cells, we found reduced expression of the pluripotent marker, *FOXH*1, whereas others did not [17,21]. These discrepancies may reflect cell-specific differences, culture differences, e.g. ±*FGF*2, or other factors.

While it has been convincingly documented that members of the *BMP* family lead to differentiation of hESC to trophoblast, there is also considerable evidence that experimental conditions have a profound effect on the type of differentiation obtained. For example, an earlier report on *BMP*4-mediated differentiation of BG02 cells identified the formation and outgrowth of an immature vascular system when the cells are grown in a 3D Matrigel substrate in an endothelial cell growth medium [13]. Further, Pera *et al.* [18] found that, in response to *BMP*2, *BMP*4, or *BMP*2/7, HES-2 and HES-3 hESC differentiated to extra-embryonic endoderm, with only a few percent of the cells having the appearance of the trophoblast precursors described by Xu *et al.* [21]. They also found that the *BMP* antagonist noggin blocks the differentiation to extra-embryonic endoderm and directs differentiation into neural precursors, differentiation that may be mimicked by secretion of gremlin by mouse embryo fibroblast feeder layers. Our results with noggin reflect some type(s) of differentiation, but there is no clear indication for preference of one major pathway. As judged by increased expression of *T*, *MIXL*1, and *WNT*3, others have found that short-term treatment of H1, H7, and H9 hESC with *BMP*4 resulted in the induction of mesoderm progenitor cells that can differentiate into hematopoietic and cardiac lineages [24]. Working with H7 cells, it was reported that *BMP*4 treatment failed to yield trophectoderm using mouse embryonic fibroblasts as a feeder layer; this, however, was overcome by using feeder-free cells on Geltrex-coated plates in StemPro [38]. Lastly, West *et al.* [45] have demonstrated that, in the presence of *BMP*4, the KIT ligand enhances differentiation to germ-like cells. Hence, additional work is needed to clarify the many experimental parameters associated with *BMP*-induced differentiation of hESC.

4.2. EB Derived from BG02 Cells

EB formation by these cells also yielded some degree of differentiation to trophoblast as evidenced by the up-regulation of *CGB* and the production of hCG and progesterone. The most highly expressed genes in EB on day 5 are: *GJA*1, *CREBBP*, *CDH*2, *ID*3, *CDH*1, *DLX*5, *SOX*2, *CREB*1, *HRAS*, *AR*, and *AFP*. Most of these were surveyed in the studies on cells receiving *BMP*4, and none were up-regulated. The results suggest that trophoblast-like cells are not forming to any significant extent by day 5, findings consistent with the absence of hCG and progesterone production until days 18 - 20. These data are consistent with those by Gerami-Naini *et al.* [8] on H1 cell-derived EB growing in Matrigel. They could not detect measureable hormone until about day 20 of culture, and, depending upon the conditions used, maximal production of hCG was reached on days 35 - 40, followed by a decline. In contrast to the results with the Matrigel-embedded EB, they found that in suspension culture EBs were producing hCG, progesterone, and estradiol by 48 h.

In our studies, a comparison of gene expression by EB on days 22 and 50, relative to day 5, with that of hESC receiving *BMP*4 for 7 days, provides additional evidence that differentiation to trophoblast is occurring by day 22, as evidenced by the up-regulation of *CGB*, *PGF*, *PTGS*2, *RUNX*1, and *VEGF*. These results are consistent with the hormone secretion data for hCG and progesterone. In addition to the above genes, there is also up-regulation of *AFP*, *BMP*6, *CHD*11, *CHRD*, DAB2, *HSPG*2, *INHBA*, *PTGS*2, and *TIMP*1. Not surprisingly, the EBs are apparently more heterogeneous in terms of constituent cell types than the *BMP*4-treated hESCs. For example, the early appearance (day 5) of *GJA*1, *CREBBP*, *CDH*2, *ID*3, *CDH*1, *DLX*5, *SOX*2, *CREB*1, *HRAS*, *AR*, *AFP*, *CDH*11, *NES*, *GATA*3, and *GREM*1, followed by the later up-regulation of *AFP*, *BMP*6, *CHD*11, *CHRD*, *DAB2*, *HSPG*2, *INHBA*, *PTGS*2, and *TIMP*1, indicates formation of trophoblast, endoderm, ectoderm, and mesoderm. Consistent with this finding was the observation some years ago that markers for the three embryonic germ layers were expressed in EBs prepared from H9 cells [46].

5. CONCLUSION

Overall, the results presented herein strongly support other reports concluding that, under certain conditions, *BMP*4 directs differentiation of hESCs to trophoblast-like cells. This conclusion notwithstanding, it is clear that experimental conditions, particularly the inclusion or exclusion of *FGF*2 during BMB-mediated differentiation, have a profound effect on the type of differentiation

achieved, and, moreover, based on our transcriptome profiling, it is highly likely that other cell types may be forming in response to *BMP*4. On the other hand, it may emerge that some of the other genes we found to have been up-regulated by *BMP*4 function in differentiation to trophectoderm and then to cytotrophoblasts (villous and extravillous) and syncytiotrophoblasts, or be involved in placental formation and function. The heterogeneity of the differentiated cells needs to be carefully established, but the *BMP*-mediated differentiation of hESCs to trophoblast, particularly in the absence of *FGF*2, certainly provides an attractive *in vitro* system for studying early differentiation events and gives a more homogeneous system than that of embryoid bodies. Lastly, our observation that the *BMP*4 antagonist noggin alters gene transcription of a subset of genes investigated may correlate with the morphological changes reported by others; however, more studies are required to map the transcriptional changes to neural differentiation.

6. ACKNOWLEDGEMENTS

We thank Mr. Roger Nilsen of the University of Georgia Functional Genomics Resource Facility for his expert assistance with the RNA analysis. This study was supported by NIH: R01DK033973 (D.P.), R01HD044119 (P.N.), and 5F32HL083741 (N.L.B.).

REFERENCES

[1] Rama, S. and Rao, A.J. (2003) Regulation of growth and function of the human placenta. *Molecular and Cellular Biochemistry*, **253**, 263-268. doi:10.1023/A:1026076219126

[2] Sullivan, M.H. (2004) Endocrine cell lines from the placenta. *Molecular and Cellular Endocrinology*, **228**, 103-119. doi:10.1016/j.mce.2003.03.001

[3] Udayashankar, R., Baker, D., Tuckerman, E., Laird, S., Li, T.C. and Moore, H.D. (2011) Characterization of invasive trophoblasts generated from human embryonic stem cells. *Human Reproduction*, **26**, 398-406. doi:10.1093/humrep/deq350

[4] Jameson, J.L. and Hollenberg, A.N. (1993) Regulation of chorionic gonadotropin gene expression. *Endocrine Reviews*, **14**, 203-221.

[5] Mesiano, S. (2009) The endocrinology of human pregnancy and fetoplacental neuroendocrine development. In: Strauss, J.F. and Barbieri, R.L., Eds., *Yen and Jaffe's Reproductive Endocrinology*. 6th Edition, Physiology, Pathophysiology and Clinical Management, Philadelphia, 249-281.

[6] Thomson, J.A., Itskovitz-Eldor. J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S. and Jones, J.M. (1998) Embryonic stem cell lines derived from human blastocysts. *Science*, **282**, 1145-1147. doi:10.1126/science.282.5391.1145

[7] Mitalipova, M., Calhoun, J., Shin, S., Wininger, D., Schulz, T., Noggle, S., Venable, A., Lyons, I., Robins, A. and Stice, S. (2003) Human embryonic stem cell lines derived from discarded embryos. *Stem Cells*, **21**, 521-526. doi:10.1634/stemcells.21-5-521

[8] Gerami-Naini, B., Dovzhenko, O.V., Durning, M., Wegner, F.H., Thomson, J.A. and Golos, T.G. (2004) Trophoblast differentiation in embryoid bodies derived from human embryonic stem cells. *Endocrinology*, **145**, 1517-1524. doi:10.1210/en.2003-1241

[9] Giakoumopoulos, M., Siegfried, L.M., Dambaeva, S.V., Garthwaite, M.A., Glennon, M.C. and Golos, T.G. (2010) Placental-derived mesenchyme influences chorionic gonadotropin and progesterone secretion of human embryonic stem cell-derived trophoblasts. *Reproductive Science*, **17**, 798-808. doi:10.1177/1933719110371853

[10] Golos, T.G., Pollastrini, L.M. and Gerami-Naini, B. (2006) Human embryonic stem cells as a model for trophoblast differentiation. *Semin Reproductive Medicine*, **24**, 314-321. doi:10.1055/s-2006-952154

[11] Harun, R., Ruban, L., Matin, M., Draper, J., Jenkins, N.M., Liew, G.C., Andrews, P.W., Li, T.C., Laird, S.M. and Moore, H.D. (2006) Cytotrophoblast stem cell lines derived from human embryonic stem cells and their capacity to mimic invasive implantation events. *Human Reproduction*, **21**, 1349-1358. doi:10.1093/humrep/del017

[12] Peiffer, I., Belhomme, D., Barbet, R., Haydont, V., Zhou, Y.P., Fortunel, N.O., Li, M., Hatzfeld, A., Fabiani, J.N. and Hatzfeld, J.A. (2007) Simultaneous differentiation of endothelial and trophoblastic cells derived from human embryonic stem cells. *Stem Cells and Development*, **16**, 393-402. doi:10.1089/scd.2006.0013

[13] Boyd, N.L., Dhara, S.K., Rekaya, R., Godbey, E.A., Hasneen, K., Rao, R.R., West, F.D., Gerwe, B.A. and Stice, S.L. (2007) *BMP*4 promotes formation of primitive vascular networks in human embryonic stem cell-derived embryoid bodies. *Experimental Biology and Medicine*, **232**, 833-843.

[14] Chen, G., Ye, Z., Yu, X., Zou, J., Mali, P., Brodsky, R.A. and Cheng, L. (2008) Trophoblast differentiation defect in human embryonic stem cells lacking PIG-A and GPI-anchored cell-surface proteins. *Cell Stem Cell*, **2**, 345-355. doi:10.1016/j.stem.2008.02.004

[15] Kee, K., Gonsalves, J.M., Clark, A.T. and Pera, R.A. (2006) Bone morphogenetic proteins induce germ cell differentiation from human embryonic stem cells. *Stem Cells and Development*, **15**, 831-837. doi:10.1089/scd.2006.15.831

[16] Liu, Y.P., Dovzhenko, O.V., Garthwaite, M.A., Dambaeva, S.V., Durning, M., Pollastrini, L.M. and Golos, T.G. (2004) Maintenance of pluripotency in human embryonic stem cells stably over-expressing enhanced green fluorescent protein. *Stem Cells and Development*, **13**, 636-645. doi:10.1089/scd.2004.13.636

[17] Marchand, M., Horcajadas, J.A., Esteban, F.J., McElroy, S.L., Fisher, S.J. and Giudice, L.C. (2011) Transcriptomic signature of trophoblast differentiation in a human embryonic stem cell model. *Biology of Reproduction*, **84**, 1258-1271. doi:10.1095/biolreprod.110.086413

[18] Pera, M.F., Andrade, J., Houssami, S., Reubinoff, B.,

Trounson, A., Stanley, E.G., Ward-van Oostwaard, D. and Mummery, C. (2004) Regulation of human embryonic stem cell differentiation by *BMP*-2 and its antagonist noggin. *Journal of Cell Science*, **117**, 1269-1280. doi:10.1242/jcs.00970

[19] Schulz, L.C., Ezashi, T., Das, P., Westfall, S.D., Livingston, K.A. and Roberts, R.M. (2008) Human embryonic stem cells as models for trophoblast differentiation. *Placenta*, **29**, S10-S16.

[20] Xu R.H. (2006) In vitro induction of trophoblast from human embryonic stem cells. *Methods in Molecular Medicine*, **121**, 189-202.

[21] Xu, R.H., Chen, X., Li, D.S., Li, R., Addicks, G.C., Glennon, C., Zwaka, T.P. and Thomson, J.A. (2002) *BMP*4 initiates human embryonic stem cell differentiation to trophoblast. *Nature Biotechnology*, **20**, 1261-1264. doi:10.1038/nbt761

[22] Yu, P., Pan, G., Yu, J. and Thomson, J.A. (2011) *FGF*2 sustains NANOG and switches the outcome of *BMP*4-induced human embryonic stem cell differentiation. *Cell Stem Cell*, **8**, 326-334. doi:10.1016/j.stem.2011.01.001

[23] Yu, X., Zou, J., Ye, Z., Hammond, H., Chen, G., Tokunaga, A., Mali, P., Li, Y.M., Civin, C., Gaiano, N. and Cheng, L. (2008) Notch signaling activation in human embryonic stem cells is required for embryonic, but not trophoblastic, lineage commitment. *Cell Stem Cell*, **2**, 461-471. doi:10.1016/j.stem.2008.03.001

[24] Zhang, P., Li, J., Tan, Z., Wang, C., Liu, T., Chen, L., Yong, J., Jiang, W., Sun, X., Du, L., Ding, M. and Deng, H. (2008) Short-term *BMP*-4 treatment initiates mesoderm induction in human embryonic stem cells. *Blood*, **111**, 1933-1941. doi:10.1182/blood-2007-02-074120

[25] Douglas, G.C., Vande-Voort, C.A., Kumar, P., Chang, T.C. and Golos, T.G. (2009) Trophoblast stem cells: Models for investigating trophectoderm differentiation and placental development. *Endocrine Reviews*, **30**, 228-240. doi:10.1210/er.2009-0001

[26] Golos, T.G., Giakoumopoulos, M. and Garthwaite, M.A. (2010) Embryonic stem cells as models of trophoblast differentiation: Progress, opportunities, and limitations. *Reproduction*, **140**, 3-9. doi:10.1530/REP-09-0544

[27] Pera M.F. and Trounson A.O. (2004) Human embryonic stem cells: Prospects for development. *Development*, **131**, 5515-5525. doi:10.1242/dev.01451

[28] Roberts, R.M., Ezashi, T. and Das, P. (2004) Trophoblast gene expression: Transcription factors in the specification of early trophoblast. *Reproductive Biology and Endocrinology*, **2**, 47. doi:10.1186/1477-7827-2-47

[29] Hay, D.C., Sutherland, L., Clark, J. and Burdon, T. (2004) Oct-4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells. *Stem Cells*, **22**, 225-235. doi:10.1634/stemcells.22-2-225

[30] Matin, M.M., Walsh, J.R., Gokhale, P.J., Draper, J.S., Bahrami, A.R., Morton, I., Moore, H.D. and Andrews, P.W. (2004) Specific knockdown of Oct4 and beta 2-microglobulin expression by RNA interference in human embryonic stem cells and embryonic carcinoma cells. *Stem Cells*, **22**, 659-668. doi:10.1634/stemcells.22-5-659

[31] Bragdon, B., Moseychuk, O., Saldanha, S., King, D., Julian, J. and Nohe, A. (2011) Bone morphogenetic proteins: A critical review. *Cell Signal*, **23**, 609-620. doi:10.1016/j.cellsig.2010.10.003

[32] Rider, C.C. and Mulloy, B. (2010) Bone morphogenetic protein and growth differentiation factor cytokine families and their protein antagonists. *The Biochemical Journal*, **429**, 1-12. doi:10.1042/BJ20100305

[33] Walsh, D.W., Godson, C., Brazil, D.P. and Martin, F. (2010) Extracellular *BMP*-antagonist regulation in development and disease: Tied up in knots. *Trends in Cell Biology*, **20**, 244-256. doi:10.1016/j.tcb.2010.01.008

[34] Zeng, S., Chen, J. and Shen, H. (2010) Controlling of bone morphogenetic protein signaling. *Cell Signal*, **22**, 888-893. doi:10.1016/j.cellsig.2009.12.007

[35] Malassine, A., Frendo, J.L. and Evain-Brion, D. (2003) A comparison of placental development and endocrine functions between the human and mouse model. *Human Reproduction*, **9**, 531-539. doi:10.1093/humupd/dmg043

[36] Rossant, J. (2001) Stem cells from the mammalian blastocyst. *Stem Cells*, **19**, 477-482. doi:10.1634/stemcells.19-6-477

[37] Smith, A.G. (2001) Embryo-derived stem cells of mice and men. *Annual Reviews of Cell and Developmental Biology*, **17**, 435-462. doi:10.1146/annurev.cellbio.17.1.435

[38] Erb, T.M., Schneider, C., Mucko, S.E., Sanfilippo, J.S., Lowry, N.C., Desai, M.N., Mangoubi, R.S., Leuba, S.H. and Sammak, P.J. (2011) Paracrine and epigenetic control of trophectoderm differentiation from human embryonic stem cells: the role of bone morphogenic protein 4 and histone deacetylases. *Stem Cells Development*, **20**, 1601-1614. doi:10.1089/scd.2010.0281

[39] Bernardo, A.S., Faial, T., Gardner, L., Niakan, K.K., Ortmann, D., Senner, C.E., Callery, E.M., Trotter, M.W., Hemberger, M., Smith, J.C., Bardwell, L., Moffett, A. and Pedersen, R.A. (2011) BRACHYURY and *CDX*2 mediate *BMP*-induced differentiation of human and mouse pluripotent stem cells into embryonic and extra embryonic lineages. *Cell Stem Cell*, **9**, 144-155. doi:10.1016/j.stem.2011.06.015

[40] Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods*, **25**, 402-408.

[41] Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, **29**, e45. doi:10.1093/nar/29.9.e45

[42] Aghajanova, L., Shen, S., Rojas, A.M., Fisher, S.J., Irwin, J.C. and Giudice, L.C. (2012) Comparative transcriptome analysis of human trophectoderm and embryonic stem cell-derived trophoblasts reveal key participants in early implantation. *Biology and Reproduction*, **86**, 1-21. doi:10.1095/biolreprod.111.092775

[43] Wu, Z., Zhang, W., Chen, G., Cheng, L., Liao, J., Jia, N., Gao, Y., Dai, H., Yuan, J. and Xiao, L. (2008) Combinatorial signals of activin/nodal and bone morphogenic protein regulate the early lineage segregation of human embryonic stem cells. *The Journal of Biological Chemistry*,

283, 24991-25002. doi:10.1074/jbc.M803893200

[44] Das, P., Ezashi, T., Schulz, L.C., Westfall, S.D., Livingston, K.A. and Roberts, R.M. (2007) Effects of *fgf* 2 and oxygen in the *bmp*4-driven differentiation of trophoblast from human embryonic stem cells. *Stem Cell Research*, **1**, 61-74. doi:10.1016/j.scr.2007.09.004

[45] West, F.D., Roche-Rios, M.I., Abraham, S., Rao, R.R., Natrajan, M.S., Bacanamwo, M. and Stice, S.L. (2010) KIT ligand and bone morphogenetic protein signaling enhances human embryonic stem cell to germ-like cell differentiation. *Human Reproduction*, **25**, 168-178. doi:10.1093/humrep/dep338

[46] Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., Soreq, H. and Benvenisty, N. (2000) Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Molecular Medicine*, **6**, 88-95.

1. Supplement

1.1. S1. The Following Genes Were Not Expressed Sufficiently to Be Measured in Control and *BMP*4-Mediated Differentiation of hESCs

AMH, *AMHR*2, *BAPX*1, *BMP*1, *BMPR*1*B*, *DLX*5, *ESR*1, *ESR*2, *FGF*5, *FIGF*, *FSHB*, *FSHR*, *GJA*5, *GJB*3, *IGF*1, *INHBB*, *IPF*1, *LAMR*1, *LY*6*G*6*D*, *NKX*2-2, *PAX*8, *PECAM*1, *PGR*, *PP*13, *PROML*1, *PTGS*1, *PTPRC*, *STAR*, *TAL*1, *TITF*1, *TSHB*, and *WT*1.

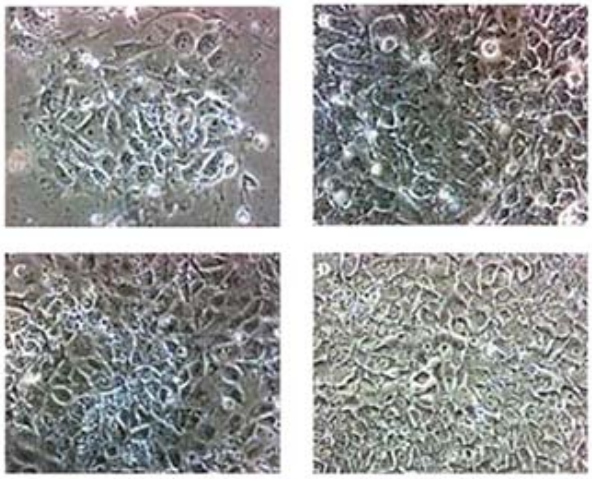


Figure S1. Phase-contrast microscopy of hESCs before and after incubation with *BMP*4 or noggin. (A) Cells at day 0 in media; (B) Cells at day 7 in media; (C) Cells at day 7 in media-plus-100 ng/mL *BMP*4; (D) Cells at day 7 in media-plus-250 ng/mL noggin. Cells incubated with *BMP*4 and with noggin exhibit distinct morphological changes.

1.2. S2. A Comparison of Our Results with *BMP*4-Induced Differentiation of hESCs and the Results of Others Gives the Following Similarities

We found increased expression of *CDH*11, *CGB*, *ENPEP*, *EPAS*1, *FN*1, *GATA*2, *GATA*3, *HEY*1, *KRT*7, *MSX*2, *PGF*, *PITX*2, and *WNT*5*A*, as well as decreased expression of *DNMT*3*B* and *POU*5*F*1, in agreement with Xu *et al.* [21] and Marchand *et al.* [17]. Consistent with the data of Xu *et al.* [21], we found increased expression of *CDH5*, *DAB2, MMP*9, and *WNT*4, along with decreased expression of *SOX*3. Our results and those of Marchand *et al.* [17] show increased expression of *BMPR*2, *COL*4*A*1, *CYP*11*A*1, and *CYP*19*A*1, decreased expression of *CRABP*1, *FGF*2, *GREM*1, *OTX*2, and *SOX*2, and either no changes or minimal changes in a number of other genes, including *AFP*, *GSC*, *NES*, *PAX*6, and others. Schultz *et al.* [19] also found increased expression in *CGB*, *GATA*2, *GATA*3, *KRT*7, and *MSX*2, and decreased expression of *POU*5*F*1 and *SOX*2.

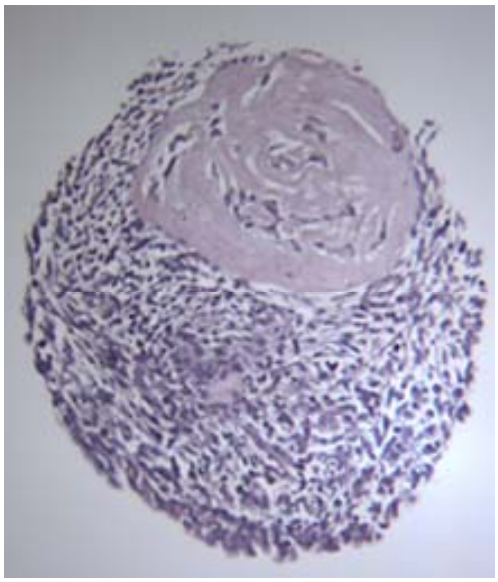


Figure S2. Following formation of embryoid bodies from hESCs and incubation for 50 days (d50), an embryoid body was fixed in formalin, embedded in paraffin, sectioned (5 microns), and stained with H&E.

