CHARACTERIZATION OF TROPHOBLAST-LIKE CELLS DERIVED FROM HUMAN EMBRYONIC STEM CELLS

by

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(Under the Direction of J. David Puett)

ABSTRACT

There are two *in vitro* models for forming trophoblast-like cells from human embryonic stem cells (hESCs): (i) in an adherent culture treated with bone morphogenic protein (BMP) 4, and (ii) cultured as embryoid bodies (EBs) and allowed to spontaneously differentiate. The hypothesis being tested is that in these two culture systems trophoblast-like cells are formed and they biosynthesize and secrete placental hormones. The goal of this project was to test this hypothesis by hormone analysis, gene expression analysis, and immunohistochemistry of the differentiated hESCs. Both culture conditions resulted in production of hCG, 17β-estradiol, and progesterone and up-regulation of trophoblast-related genes (CGB, KRT7, MMP9, GATA2, and GATA3). Side-chain cleavage enzyme (CYP11A1) and aromatase (CYP19A1), two enzymes necessary for steroid biosynthesis, were up-regulated in both culture conditions. In the study with BMP4, trophoblast formation was to the exclusion of all other cell types as germline markers and pluripotency markers were down regulated. The EBs stained positive for CYP11A1 and 3-beta-hydroxysteroid dehydrogenase in immunohistochemistry. Thus, trophoblast-like

differentiation can be achieved with HESCs by addition of BMP 4 or by specified culture conditions leading to EB formation, and these cells are capable of steroidogenesis.

INDEX WORDS: Human Embryonic Stem Cells (hESCs), Bone Morphogenic Protein 4 (BMP4), Embryoid Bodies (EBs), Trophoblast, Side-chain Cleavage Enzyme (CYP11A1), Aromatase (CYP19A1)

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Introduction/Literature Review

I. Introduction

Human embryonic stem (hESCs) cells can be induced to differentiate into trophoblast-like cells in response to bone morphogenic protein 4 (BMP4) treatment of adherent cultures plated on matrigel or as a result of spontaneous differentiation as embryoid bodies (EBs) [1,2]. These results were surprising as they accentuated the fundamental differences between hESCs and their murine counterparts. No growth factor guided trophoblast differentiation has yet been described in the murine field, nor has spontaneous differentiation into trophoblast under any culture conditions been observed. In contrast, BMPs are widely considered to differentiate murine embryonic stem cells (mESCs) into mesoderm when cultured as EBs or to aid in the maintenance of pluripotency when stem cells are in adherent cultures [3,4]. *In vivo*, BMPs are implicated in multiple developmental processes of the mouse including formation of Rathke's pouch in the pituitary, formation and migration of primordial germ cells, and the normal luteal cycle of adult mice [5,6]. BMP2 and BMP4 have since been described as driving hESCs down an extraembryonic endoderm pathway [7,8]. The goals of this research are to confirm the previous work with hESCs in both culture conditions (both adherent and EB forming) to verify and extend the description of the trophoblast-like cells produced, focusing primarily on their endocrine capabilities.

II. Literature Review

The first cell fate decision of a totipotent blastocyst occurs at E3.5 in a mouse when the trophoblast and inner cell mass (ICM) distinguish themselves. Forty to fifty trophoblast precursor cells encircle the ICM and later the blastocoel. Cells from the ICM can be extracted and cultured *in vitro* indefinitely and are termed embryonic stem cells [9,10]. These cell lines can differentiate into all three germ layers of the embryo including endoderm, ectoderm, and mesoderm, as well as some extraembryonic tissues, extraembryonic endoderm and extraembryonic mesoderm. The extraembryonic tissues, derived from the primitive endoderm and mesoderm, migrate to the trophoblast tissues and form the placenta (Fig. 1.1). In the murine system, the trophoblast precursor cells can also be extracted and cultured *in vitro* [11]. The cells differentiate into cytotrophoblasts which are mitotically active, mononucleated cells that fuse and become nondividing multinucleated syncytiotrophoblast. These trophoblast stem cells differentiate into trophoblast giant cells as indicated by placental lactogen 1 and MASH2 expression and increased DNA content by flow cytometry. The undifferentiated cells express mEOMES, CDX2, FGFR2, HAND1, and ERRβ all of which are indicative of murine trophoblast[11]. Trophoblast stem cells can form all the tissues of the trophoblast lineage, but they cannot form the placenta as this requires tissues derived from the ICM.

mESC lines were derived by Evans and Kaufman in 1981 [10]. The cells are characterized by the expression of multiple pluripotency markers including Oct3/4, a strictly controlled transcription factor. Oct3/4 contains a DNA-binding POU domain and two, one N- terminal and one C-terminal, trans-activation domains. In mice, Oct3/4 is regularly characterized as a differentiation lock into the fate of the ICM at the expense of trophoblast tissues as it can act to repress CDX2 and HAND1 within the ES cell [12,13]. This statement is substantiated by two

studies. In 1998, Nichols et al. [14] constructed a homozygous null mutant of Oct3/4 and showed that the mutant could not form an ICM. Instead, murine embryonic stem cell cultures derived from this line of genetically altered mice differentiated into trophoblast giant cells as they expressed murine trophoblast markers, SOX2, H19, and MASH2 [14]. In 2000, Niwa et al. [15] constructed an inducible Oct3/4 system, utilizing it to prove that the level of Oct3/4 protein in the cell can lead it down three disparate differentiation pathways. By genetically eliminating Oct3/4 transcription in the ZHTc4 cell line, they documented the up-regulation of several murine trophoblast transcription factors, SOX2 and HAND1. The cells resembled undifferentiated epithelial cells and also up-regulated CDX2, ERRβ, MASH2, and TBPB [15]. To date, no growth factor mediated differentiation process has produced trophoblast in the murine system. In fact, nothing short of genetic manipulation can unlock the Oct3/4 commitment to the ICM pathway.

In addition to being "locked" out of the trophoblast fate, mESCs when treated with BMP4 form mesoderm or ectoderm depending on the differentiation protocol. EBs treated with BMP4 up-regulate brachyury (T), goosecoid (GSC), Nodal, and fibroblast growth factor 5 (FGF5) (mesodermal markers). BMP4 is also part of a longer differentiation process to specify epidermal cell fate, which expresses keratin 14 [3]. *In vivo,* through gene knock-out experiments, murine BMPs have been shown to have a local impact on the formation of Rathke's pouch of the pituitary (a gonadtropin producing tissue), on the differentiation and migration of primordial germ cells, and on the regulation of the luteal phase in the adult cycling mouse [5,16].

Human embryonic stem cells have exhibited a distinct character from mESCs from their first derivatization [17]. They spontaneously differentiate into trophoblast, evidenced by βhCG-positive immunostaining. They exhibited several markers, e.g. stage specific embryonic antigens 3 and 4 (SSEA-3 & SSEA-4) and alkaline phosphatase, which were different from their murine

counterparts. Also notably human blastocysts express SSEA-1 on their trophoblast tissues [18], while murine ES cells exhibit this marker in the undifferentiated state. However, like mESCs, hESCs expressed Oct4 and were dependant on mouse embryonic fibroblasts (MEFs) to propagate. The differences in ES cell-surface markers, the spontaneous differentiation of trophoblast tissues, and a number of macrobiological differences led Thomson to posit a species difference in the early developmental biology of mice and men in 1998 when the first human ESCs were derived [17].

In 2002, Xu and et al.[1] induced trophoblast formation in a dose-dependant manner by the addition of BMP4 to adherent hESC cultures of H1, H7, H9 cell lines. BMP4 is a member of the transforming growth factor (TGF) β-family of signaling factors which must bind to two of its type I and two type II serine/threonine kinase receptors forming a tetramer to initiate a SMAD signaling cascade. BMP4 activates SMADs 1/5/8 by phosphorylation, and the activated phospho-SMADs then recruit SMAD4 whereupon the tetramer translocates to the nucleus and acts as a transcription factor.

To form trophoblast-like cells, four human ES cell lines (H1, H7, H9, and H14) were cultured on matrigel plates and treated with conditioned medium and basic fibroblast growth factor (bFGF) to maintain pluripotency [1]. When recombinant human BMP4 was added to the cultures at 1, 10, or 100 ng/mL, a dose-dependant morphological change was noted, "a synchronous wave of differentiation occurred, characterized by flattened enlarged cells with reduced proliferation" on days 4-5 (1 ng/mL), days 3-4 (10 ng/mL), and days 2 (100 ng/mL). The response was also elicited by other TGFβ family members, including BMP2, BMP7, and GDF5, but not activin A. When a BMP4 antagonist, noggin, was added to the cultures the differentiation was heterogeneous. When hESCs were plated as single cells at low density and if

100 ng/mL BMP4 was added, syncytial cells formed. The permeabilized cells stained positive with anti-hCGβ antibodies in immunocytochemistry and in flow-cytometry experiments. The syncytial structures are large and multi-nucleated from fusion events.

Microarray analysis of 43,000 cDNAs was performed to identify the various transcripts expressed. Many transcription factors were up-regulated on every day of the seven day treatment including: transcription factor AP-2 (TFAP2), msh homeobox homolog 2 (MSX2), suppressor of cytokine signaling 3 (SSI3), GATA binding proteins 2 and 3, and Hey 1. Nine of the top ten up-regulated genes at day 7 of treatment with BMP4 are expressed in trophoblast or placenta including the alpha subunit of the gonadotropins (CGA) , hCGβ (CGB), endothelial PAS domain protein 1 (EPAS1), insulin growth factor binding protein 3 (IGFBP-3), iodothyronine deiodinase type III (DIO3), Gata binding protein 2 (GATA2), and glutamyl aminopeptidase (ENPEP). BMP4-treated cells also secreted significant amounts of placental hormones including human chorionic gonadotropin (hCG), a protein hormone, and two steroid hormones, progesterone and estradiol [1].

Two years later, the same group described a new method of deriving trophoblast-like tissues, which was spontaneous upon the formation of EBs and their culture in MEF-conditioned medium. This spontaneous differentiation was characterized by the secretion of placental hormones- hCG, progesterone, and estradiol- as well as immunohistochemistry (IHC) of expression of cytokeratins 7/8 and hCGβ. The assertion that trophoblast was derived from hESCs was met with considerable surprise (and perhaps a bit of skepticism) within the largely mouse stem cell field.

If skepticism can be overcome from the mouse embryonic stem cell field, it is harder to overcome from the hESC field. Recent papers have shown BMP-treatment led to up-regulation

of extraembryonic endoderm lineage in HES2 and HES3 hESC lines [7]. The extraembryonic endoderm lineage is derived from the ICM lineage and differentiated into visceral and parietal endoderm that eventually forms the yolk sac (Fig. 1.1). The group maintains that upon addition of BMP4 to 20% knock out serum replacement media provided to hESCs grown on MEF-feeder layers, the majority of their colonies flattened and had an abundance of eosinophilic intracellular material common to the primate yolk sac. Less than 5% of the treated cells expressed hCGβ. By RT-PCR, they also determined up-regulation of alpha fetoprotein (AFP), hepatocyte nuclear factor- 3 alpha and 4 (HNF3A & HNF4), GATA-binding proteins 4 and 6 (GATA4 & GATA6), transferrin (TRF), and vitronectin (VN). Finally, they demonstrated that the BMP receptors (BMPR1A and BMPR2) are present on undifferentiated and spontaneously differentiating HES2 & HES3 cells and that upon BMP addition, phospho-SMAD1 translocates to the nucleus. These data indicate that the BMP pathway constituents are present and that the BMP signal transduction pathway is stimulated [7]. This article argues that treatments of hESC lines with BMP4 can elicit very different responses depending on the precise culture conditions. The previous reports indicate that the studies embarked upon by Xu et al. in 2002[1] and Gerami-Niani et al. in 2004[2] need to be confirmed and elaborated upon in a different stem cell line under the same culture conditions.

The placenta is an intriguing endocrine organ as it signals in an autocrine and paracrine fashion. It is studied in a variety of models including culturing primary tissue explants, choriocarcinoma cell lines, trophoblast stem cell lines, and finally differentiating hESCs into trophoblast. The local production of hCG is the hallmark of trophoblast tissue. Human choriogonadotropin has been detected in the 6- to 8- cell blastocyst stage embryo and *in vivo* is vital to maintaining pregnancy as it prevents the regression of the corpus luteum in the ovary [19].

The trophoblast precursor cell in the human embryo following blastocyst adhesion will begin rapid proliferation into cytotrophoblast. Some of these cytotrophoblast cells will fuse to form a multi-nucleate syncytiotrophoblast, the invasive, hormone-producing, terminally differentiated, trophoblast cell [20]. The evaluation of the placenta as an endocrine organ has been difficult as much of the regulation is between layers of the trophoblast tissues, for example between the cytotrophoblast and the syncytiotrophoblast. Therefore, by isolating one population of trophoblast cells, in order to characterize the receptors for example, the signaling partners are isolated [21].

The regulation of hCG secretion in the syncytiotrophoblast is composed of a complex interplay of steroid hormones, gonadotropins, and cytokine signaling. Gonadotropin releasing hormone (GnRH) and progesterone, both of which are produced by the villous cytotrophoblast and the syncytiotrophoblast, are thought to positively regulate hCG secretion, while estradiol negatively regulates hCG secretion [22]. Activins and inhibins, both locally produced by cytotrophoblast and syncytiotrophoblast, are proposed to regulate hCG secretion [21]. Activin positively regulates GnRH-stimulated release from placenta explants and inhibin blocks the activin induced stimulation [19]. Further, the actions of activin are argued to be controlled by follistatin which is locally produced by most placental tissues, where it is proposed to bind activin and regulate its actions post-translationally [23]. Conversely, hCG is proposed to stimulate progesterone, activins, and inhibins [21]. The regulation of progesterone secretion from placental explants is enhanced by estrogens as the inhibition of aromatase activity (an enzyme vital to biosynthesis of estrogen) decreased progesterone synthesis [22]. The autocrine and paracrine signaling between hCG, progesterone, and estradiol requires the presence of their receptors, estrogen receptor α (ER1), estrogen receptor β (ER2), progesterone receptor (PR), and the hCG

receptor (LHCGR). LHCGR, ER1, ER2, PR are thought to be expressed in trophoblast tissues[24-28].

This production of estradiol and progesterone requires the presence of steroid biosynthetic enzymes, including side-chain cleavage enzyme (CYP11A1), 3- beta hydroxy steroid dehydrogenase (3β-HSD), and aromatase (CYP19A1). CYP11A1 and 3-β HSD are necessary to convert cholesterol to pregnenolone and then progesterone. CYP19A1 is necessary for the biosynthesis of estradiol. The placenta lacks CYP17 and therefore cannot then convert progesterone into estradiol. Therefore it is required that dihydroepiandrosterone be provided to the placental tissues which can then convert it to estradiol through the actions of 3β-HSD and CYP19.

This study proposes to confirm and further characterize the trophoblast-like cells derived from hESCs by both BMP4 treatment and EB formation. Both culture conditions offer unique advantages and disadvantages. The BMP4-guided differentiation induces a massive stimulus which causes a relatively homogenous, and therefore clear, wave of differentiation into a particular cell type. However, due to the notorious promiscuity of BMPs with their receptors, it also runs the risk of nonspecific signaling. The spontaneous EB differentiation utilizes less aggressive differentiation tactics as it allows the EBs to respond to presumably smaller signals within the media, but, as a consequence, the differentiation is not homogeneous. The EB has multiple cell types and analysis is much more difficult. We have utilized Real-Time RT-PCR (RT/RT-PCR), IHC, and hormone secretion analysis to focus specifically on the steroid biosynthetic enzymes and the receptors involved in the autocrine and paracrine regulation of hCG, progesterone, and estradiol in both culture conditions.

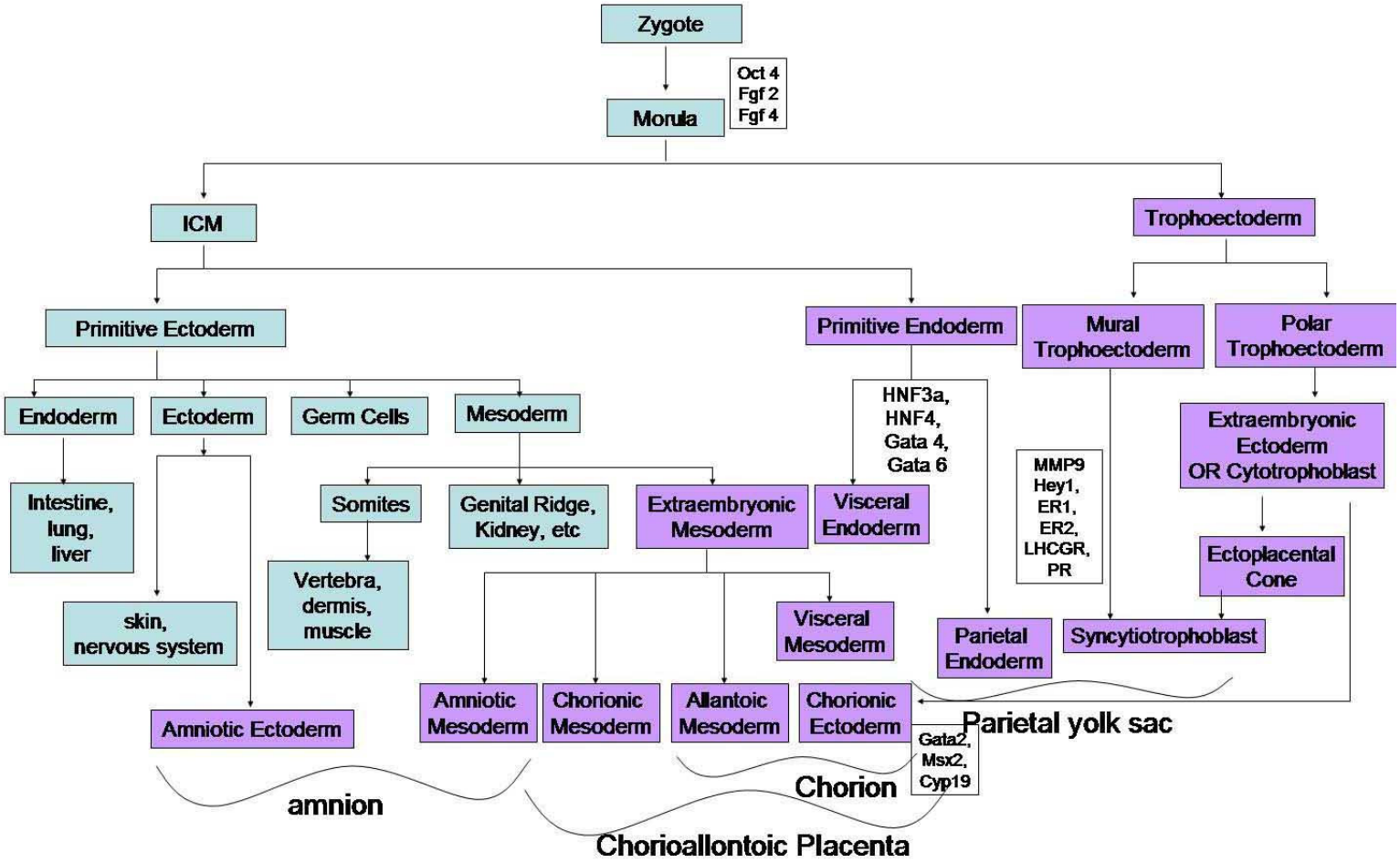


Fig 1: Schema for zygote differentiation events. Extraembryonic tissues (purple) and embryonic tissues (blue) are shown. Upregulated genes of tissue type are along 'differentiation arrows'. Adapted from [13].

Boxed genes are indicative of the cell types they are above.

Chapter 2:

Materials and Methods

I. Cell Culture

a) Maintenance of undifferentiated hESCs

Pluripotency of hESCs (BG02) was maintained by manual passaging onto mitotically inactivated MEF layers from E13.5 mouse fetuses every two to three days [29]. A hook-shaped pulled glass pipette tip was utilized to remove the MEF layer from the hESC colony and to gently disperse the colonies. The colony pieces were then 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 (FBS; Hyclone; Logan, UT) and 5% knockout serum replacement (KSR; Gibco), 1% non-essential amino acids (NEAA; Gibco), 1% penicillin/streptomycin (Gibco) , 1 mM L-Glutamine (Gibco), 0.1 mM β-Mercaptoethanol (Sigma; St. Louis, MO), 4 ng/mL basic fibroblast growth factor (bFGF; Sigma), and 10 ng/mL leukemia inhibitory factor (LIF; Chemicon; Temcula, CA). Cells were passaged every three to four days. Beginning 48 hours after passage, medium was aspirated and replaced every 24 hours.

b) Growth Factor Mediated Differentiation

The HESC line, BG02, was passaged by gentle enzymatic digestion using cell dissociation buffer (Gibco) into 10 cm matrigel-coated dishes. Cells were cultured in 50% DMEM/F12 medium which was conditioned by MEF layers and then supplemented with 4 ng/mL bFGF. Experimental groups included cells treated with 100 ng/mL BMP4 (Quest

Diagnostics, Lyndberg, NJ), and with 250 ng/mL Noggin (Quest Diagnostics). Untreated hESCs served as controls. The media were collected daily for analysis of secreted hormones. At day 7, cells were harvested and quick frozen for RNA extraction. Experimental and control experiments were each done in triplicate.

c) Formation of Embryoid Bodies

EBs were formed by manually slicing colonies into smaller pieces with glass pipette tip, which was pulled thin after brief flaming, and then removing them gently from the MEF layer. The pieces were allowed to dissociate and self-aggregate. EBs were grown on agarose dishes in 12 mL of unconditioned media as described in maintenance of pluripotency section. Each culture started with ~50 EBs and ended with ~12 EBs of various size. Loss of EBs was due to aggregation of the individuals or deterioration. Media (6 mL) was removed on alternate days by swirling the culture plate to aggregate the EBs and then pipetting from the sides. Fresh media (6 mL) was added to the culture to bring the final volume to 12 mL, and the EBs were dispersed to prevent clumping. The media samples were aliquoted and frozen for hormone analysis.

II. Hormone Assays

Media collected from cell cultures were tested by immunoflourescence-based analysis for secretion of several placental hormones hCG, hCG beta, progesterone, and estradiol. The supernatant was run on an Immulite 1000 [Diagnostic Product Corporation (DPC) Immulite, Los Angeles, CA] using DPC kits to the above hormones. Con6 (DPC), a tri-level internal control in human serum, was used to standardize all kits.

III. Immunohistochemistry of Embryoid Bodies

EBs were fixed in buffered formalin, embedded in paraffin, and 5 μM sections were prepared. Sections were stained with hemotoxylin and eosin staining following standard

procedures. 3β-HSD was detected utilizing an anti-mouse 3β-HSD polyclonal antibody (kindly provided by Dr. Anita Payne; Stanford University; Palo Alto, CA) diluted 1:800 in CAS-block (Zymed; Carlsbad, CA). Side chain cleavage (Cyp11A1) was detected utilizing a rabbit anti-mouse polyclonal antibody (gift from Dr. Payne) diluted 1: 250 in CAS-block (Zymed: Carlsbad, CA). Placental growth factor was detected utilizing a rabbit anti-human PLGF antibody (Abcam, Cambridge, UK) diluted to 2.5 μg/mL in CAS-block (Zymed) [30]. All antibodies were incubated overnight at 4°C, and all binding was visualized utilizing a streptdavidin-biotin amplification method with a Histostain-Plus kit (Zymed) following a standard protocol. The secondary antibody was a biotinylated broad spectrum anti- mouse, rabbit, guinea pig, and rat antibody. Hemotoxylin was utilized as a counterstain.

IV. Real-Time RT-PCR

Total RNA was isolated from 7 day treated or untreated BG02 cells and from EBs at day 5, day 22, and day 50 of culture. Collected hESCs were resuspended in 1 ml Trizol (Invitrogen; Carlsbad, CA) and triturated until homogenized. RNA was isolated from the crude homogenate according to the manufacturer's instructions (Trizol, Molecular Research Corporation; Albany, NY). The integrity of the RNA produced from all samples used was verified and quantified using a RNA 600 Nano Assay (Agilent Technologies; Foster City, CA) and the Agilent 2100 Bioanalyzer (Agilent Technologies; Foster City, CA). 5 ug total RNA was reverse transcribed using the cDNA Archive Kit (Applied Biosystems Inc.; Foster City, CA) according to manufacturer's protocols using the MultiScribe™ Reverse Transcriptase. Reactions were incubated initially at 25°C for 10 min and subsequently at 37°C for 120 min. Quantitative PCR (Taqman™) assays were chosen for the transcripts to be evaluated from Assays-On-Demand™ (Applied Biosystems Inc; Foster City, CA), a pre-validated library of QPCR assays and

incorporated into 384-well Micro-Fluidic Cards™. 2.0 µl (4 µg) of the cDNA samples (diluted to 50 µl) along with 50 µl of GeneAmp™ Fast PCR master mix (2x) (ABI) were loaded into respective channels on the micro-fluidic card followed by a brief centrifugation. Real-time PCR and relative quantification were carried out on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems Inc). Expression levels of all genes analyzed are represented relative to 18S rRNA expression. Data for differential expression between the treated and untreated samples are expressed as a mean of three experiments. Data with a delta Ct value greater than 31 was only analyzed if the standard deviation between the technical replicates was less than 0.5 [31,32].

Chapter 3:

Results

In order to further characterize the differentiation of hESCs to trophoblast-like cells, two experimental paradigms were examined. 1) BMP4 treatment of hESC in adherent culture maintained with MEF-conditioned media on matrigel. 2) Spontaneous differentiation of EBs into trophoblast. First, media from the cell cultures were analyzed for placental hormones, hCG, estradiol, and progesterone. Second, analysis of mRNA levels of trophoblast markers, steroidogenic enzymes, gonadotropins, various germ cell markers, and other growth factors were analyzed by RT/RT-PCR with a Taqman Low Density Array (ABI). Finally, immunohistochemistry was performed on embryoid bodies at day 22 and day 50 to examine the expression of steroidogenic enzymes and placental specific proteins.

I. BMP4-treated hESCs Secrete Hormones Indicative of Trophoblast

Media from BMP4-treated, noggin-treated, and control hESCs were kindly provided by Alison Venable. BMP4 treatment of hESCs grown on matrigel elicited the secretion of hCGβ, estradiol, and progesterone. By day 7 of treatment, all three hormones were increased significantly in BMP4-treated cells compared to treatment with its antagonist noggin (Fig. 3.1).

II. Rationale for the Genes of Interest in the Taqman Low Density Array

Ninety genes were selected for incorporation into a Taqman Low Density array for RT/RT-PCR analysis. The quantification of mRNA levels was designed to confirm and enhance the extensive microarray data published previously [1]. Several genes were also analyzed which were not previously characterized (Table 1.1). The study on gene analysis was designed to

address several questions. First, are the cells truly trophoblast-like cells since BMPs are ubiquitous in multiple developmental processes of both mouse and human? From targeted deletion of BMP4 in mouse embryos, the molecule was previously implicated in the differentiation of Rathke's pouch of the pituitary and in the differentiation and migration of primordial germ cells [5,6]. In the human stem cell field, growth factor-mediated studies like our own has shown that BMP4 leads to the differentiation of hESCs into extraembryonic endoderm and also trophoblast depending on minute changes in culture conditions [1,7]. To study these questions several genes from each cell type were selected; for the Rathke's pouch of the pituitary, follicle stimulating hormone (FSH) along with its receptor (FSHR), and luteinizing hormone β (LHB). For the investigation of primordial germ cells, several transcription factors and cell signaling molecules vital for the differentiation and migration of primordial germ cells were selected. WNT4 was chosen as it is involved in the formation of the urogenital ridge from mesoderm. Splicing factor 1 (SF1) and Wilms tumor 1 (WT1) are thought to promote differentiation from the urogenital ridge to the indifferent gonad. Sex determining region Y (SRY), Sex determining region- box 9 (SOX9), splicing factor 1 (SF1), nuclear receptor 1 (NROB1), anti-mullerian hormone (AMH) and its receptor (AMHR), and wingless-type 4 (WNT4) are implicated in the transition from the indifferent gonad to the testes or ovaries, respectively [5,33,34]. Alpha feto-protein (AFP) was selected as a marker for the formation of the extraembryonic endodermal cells [7]. Finally, trophoblast genes were selected including the β subunit of human choriogonadotropin (CGB), cAMP responsive element binding protein (CREB1), CREB binding protein (CREBBP), aminopeptidase A (ENPEP), endothelial PAS domain protein 1 (EPAS1), GATA binding protein 2 (GATA2), GATA binding protein 3 (GATA3), inhibin α (INHA), keratin 7 (KRT7), matrix metalloproteinase 9 (MMP9), placental

growth factor (PGF), and transcription factor AP-2 α (TFAP2A) [1,35,36]. As controls, gene indicators of the three germ layers were added.

The expression of BMP signal transduction and regulator machinery in hESCs during treatment was examined. Considering the promiscuity of the TGF-β family members with their cognate serine/threonine receptors and the possibility of signaling through an unanticipated pathway(s) following treatment with high concentrations of ligand, it should at least be established that the correct pathway is present. To this end, we investigated BMP4, its receptors, BMPR1B and BMPR2, signaling machinery, SMAD1, SMAD5, and ID3, inhibitory SMADs (SMAD7), negative regulators- noggin (NOG) and gremlin (GREM1), and proteins which are up-regulated in the cell-type dependant BMP cascades of differentiated chondrocytes and ovarian granulose cells, insulin-like growth factor 1 (IGF1), and runt-related transcription factor 1 and 2 (RUNX1, RUNX2) [4-6].

In order to ascertain if BMP4-treated hESCs and/or EBs were truly steroidogenic, mRNAs for several of the cytochrome P450 steroid biosynthetic enzymes necessary to produce progesterone from cholesterol and estrogen from androgen were measured to determine their expression and/or regulation during treatment. Considering the presence of fetal calf serum in the media and the possibility that some of the steroid production was not *de novo*, CYP11A1, the side-chain cleavage enzyme (the first step in steroid biosynthesis involved in the conversion of cholesterol to pregnenolone) and CYP 19A1 or aromatase (the enzyme necessary to convert testosterone into estradiol) were included as two markers of steroid biosynthesis. Cyp21A2 and SRD5A1, additional cytochrome P450 enzymes involved in the production of cortisol and the conversion of testosterone to 5-alpha-dihydrotestosterone, respectively, were also added.

Finally, assuming that the BMP4-treated cells are steroidogenic and the resulting cell differentiation is reasonably homogeneous, the possibility for autocrine signaling due to the secretion of hCG, estradiol, and progesterone was investigated. The receptors to these hormones, LHCGR, ER1, ER2, and PR were also investigated. The cytokines, activins (INHBB, INHBA) and inhibins (INHA), were also investigated as well as follistatin (FST). Considering the presence of testosterone in the media, the androgen receptor (AR) was also measured.

III. Real-time RT-PCR Analysis of Day 7 BMP-4 & Noggin-treated hESCs

The RNA from BMP4-treated, noggin-treated, and control hESCs (provided by Alison Venable), was analyzed by real time RT-PCR for mRNA quantification of cell specific markers, steroid biosynthetic enzymes, and hormone receptors. There was considerable variation between biological samples for the RT/RT-PCR in both treatment groups by day 7, particularly among the most responsive genes to treatment. Data analysis are expressed in terms of delta delta Ct which gives the relative expression of both treatment groups (Noggin and BMP4) to the control untreated cells (Fig 3.2).

Of all the genes examined, those previously described by Xu et al. 2002 [1] as trophoblast/placental-related, including hCGβ, ENPEP, EPAS1, GATA2, GATA3, INHA, KRT7, MMP9, PGF, and TFAP2A, were the most differentially regulated between BMP4 treatment and noggin treatment (Fig. 3.2A). The gene markers for pituitary and gonadal differentiation pathways did not seem to be consistently up or down regulated in the treatment populations as compared to the controls (Fig. 3.2B & C). Also, control genes for embryonic tissues, mesoderm, endoderm, and ectoderm, were mostly down regulated with the notable exception of brachyury which was up-regulated in response to BMP4 treatment and not present in the control population. RT/RT-PCR data is available that further confirm the down regulation of endodermal-related and

ectodermal-related genes (Alison Venable, unpublished results). Her data also indicate that some genes of mesodermal tissues are up-regulated. hCGβ was the only β-subunit of the gonadotropin family to be differentially expressed in BMP4 treatment. It is present at low copy number in both noggin-treated and control cells. LHβ was also present at very low copy number in all cells examined (Fig. 3.2C). The BMP signal transduction machinery tested including BMPR2, SMADS 1 and 5, and ID3, were present in all cell types tested. BMPRIB was not present at a detectable level in any of the cell types tested. Interestingly, BMPR2 is up-regulated in response to BMP4 treatment. Negative regulators typically transcribed in response to high BMP activity, NOG and SMAD7, were present in higher copy number in the BMP4-treated cells indicating that the cells are responding to a strong BMP signal (Fig. 3.2E).

Two genes for the steroid biosynthetic enzymes CYP11A1 and CYP19A1 were both up-regulated by BMP4 treatment indicating that the cells have steroidogenic potential. The gene for side-chain cleavage was present in all three cell populations, NOG, BMP4, and control hESCs. CYP19A1, however, was only expressed with consistency in BMP4-treated cells. Its expression in noggin-treated cells was sporadic. CYP19A1 expression was below detectable limits in controls. The control cytochrome P450 genes, CYP21A and SRD5A1, were not differentially regulated, although the transcripts were present in all three cell populations (cf. Fig. 3.2F and Appendix 1).

AR, ER1, and LHCGR seem to be expressed in BMP4-treated, noggin-treated, and controls cells, although at barely detectable copy number. In contrast, ER2 and PR were not detectable in any cell types. Follistatin was differentially regulated between the two treatment groups as it was higher than the controls in the BMP-treated cells. Inhibin α was also up-

regulated in the BMP-treated cells. In contrast, activin (INHBA) was present but not regulated and INHBB was not detectable in any of the cell types. (Fig. 3.2F and Appendix 1).

Several other genes were also differentially regulated between treatment conditions (Fig 3.2G). Interestingly, VEGF was up-regulated in response to BMP4 treatment. This endothelial growth factor could be indicative of a cytotrophoblast population [21]. Also DAB2, the WNT4 modulating protein is differentially regulated between treatment groups [37]. Given the up-regulation of FZD1, the WNT4 receptor, this data indicates that WNT/FZD pathway should be further investigated in trophoblast differentiation. Finally, TIMP1 is up-regulated. This protein modulates the activity of matrix metalloproteinases and considering the up-regulation of MMP9, the presence of TIMP is intriguing.

IV. Cell Culture and Hormone Secretion of Embryoid Bodies

EBs were derived from hESC colonies grown on MEF feeder layers. The EBs were grown in 12 mL suspension culture in 15% FBS/ 5% KSR media on agarose plates: approximately 50 EBs were begun in each culture. EB growth was rapid until day 25 when it leveled off. Every other day, 6 mL of supernatant was removed for hormone analysis, and the suspension culture volume was reconstituted to 12 mL. The production of placental hormones, hCG and progesterone, in the EB cultures was highly variable (Fig3.3). Estradiol secretion is less conclusive as again there is a lot of variation between biological replicates. Also because estradiol is typically active at very low concentrations (pg/mL), it is harder to discern changes in secretion concentrations. The secretion of estradiol is high at day 2, 4, and 6; it then decreases until day 26 when it begins to rise again. At day 22, the secretion of all three hormones was stabilized (Fig.3.3).

V. Immunohistochemistry of Embryoid Bodies

Day 22 and day 50 EBs were embedded in paraffin, sectioned, and stained for CYP11A1, 3β-HSD, and PGF. P450 side-chain cleavage enzyme, present in all steroidogenic tissues, catalyzes the first and rate-limiting step in steroidogenesis by converting cholesterol into pregnenolone. 3-β HSD then converts pregnenolone to progesterone through a 3β-hydroxysteroid dehydrogenation [38]. These two enzymes must be present for the EBs to produce progesterone. PGF is a member of the VEGF family of growth factors promoting angiogenesis [30,39]. It is not found exclusively in the placenta as it is present in the retina and natural killer cells in the uterus, but it is one of the more specific trophoblast markers. Sectioned human ovary was used as a control for tissue specific expression. An antibody to CYP11A1 stained the luteinized granulosa cells of the ovary darkly, as expected, since the side-chain cleavage enzyme is highly expressed in these cells. The minus antibody control did not stain the ovary (Fig. 3.4A). In the day 22 EB very little staining was present, although the cells seem to be secreting steroid hormones. There is slight staining in the epithelial-like cells about the edges of the EBs, as well as in clusters of cells in the center of the 22 day EB (Fig. 3.4B). In the day 50 EB, there is strong staining of a fibrous mass in the center of the EB. There also seems to be some cell clusters which stain both at the periphery in the epithelial-like cells and in the center (Fig.3.4C). At both EB time points, non-specific staining was not evident in the absence of the primary antibody.

High levels of 3β HSD are present in the luteinized granulosa cells and lower levels are present in the luteinized theca cells, consistent with previous reports (Fig. 3.5A) [38]. Again, the negative control in the absence of primary antibody did not stain the human ovary. In the day 22 EB, there is strong staining in the center clusters of the cells as well as some staining about

the edge (Fig. 3.5B). In the day 50 EB, there is still staining of the edges as well as some center clusters of cells. There is also staining of the fibrous mass in the center of the cell (Fig. 3.5C). Again, the negative antibody control showed no staining.

PGF staining is perplexing as it stained the negative control human ovary (Fig. 3.6A). This staining was cell type specific as it targeted the luteinized granulosa cells and capillaries running through the ovarian sections. Considering the presence of staining in the human ovary, we cannot rule out cross-reactivity of this antibody with its family member VEGF [39-41]. VEGF is present in the luteinized granulose cells of the human ovary as well as in capillaries [41]. The day 22 EBs showed very little staining (Fig 3.6B), but the day 50 EBs exhibited staining at the epithelial-like cells on the edges of the EBs and in the fibrous center (Fig 3.6C).

These data indicate that day 22 and day 50 EBs, like trophoblast, possess the steroid biosynthetic enzymes necessary to produce progesterone. They also suggest that the EBs stain positive for either PGF or VEGF; both growth factors have been observed in the placenta [42].

VI. Real-time RT-PCR Analysis of Embryoid Bodies

The Taqman Low Density array described earlier was used to analyze the mRNA expression from day 22 and day 50 EBs. Not surprisingly, the expression levels of individual genes between biological replicates varied considerably. The data are presented in terms of delta Ct, where the Ct for the 18s for the 5 day, 22 day, and 50 day embryoid body was 10.9, 12.5, and 10.8 respectively. Gene markers for all three germ layers were present and, in some cases, increased throughout the long-term culture which is to be expected as EBs are defined by their ability to differentiate into the three germ layers. The BMP-pathway related genes, BMPR1B, BMPR2, SMAD1, SMAD5, and BMP4, are expressed during the long term differentiation, although their expression levels are not significantly different in all the time points examined

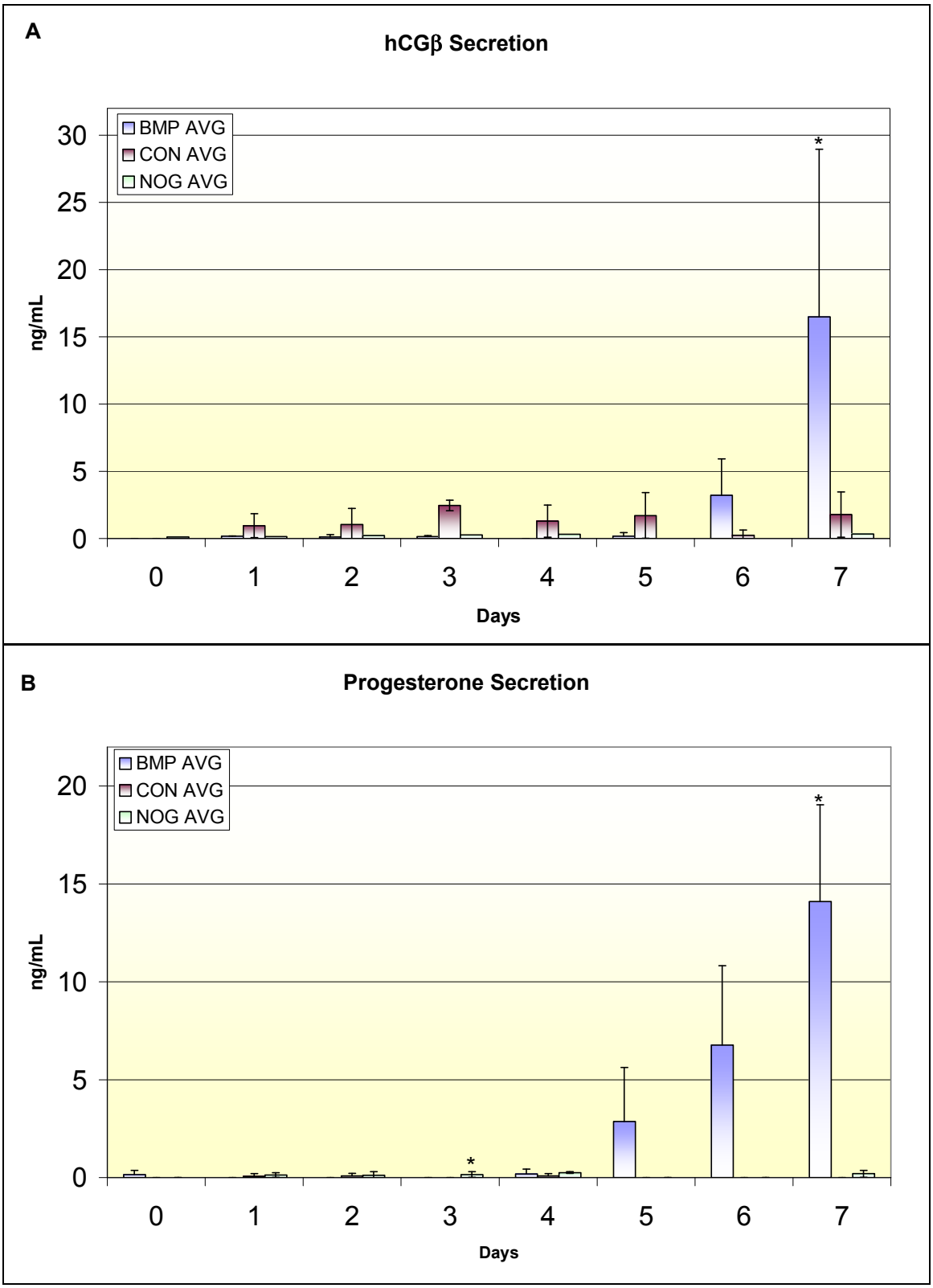
(Fig. 3.7A). Most of the gonadal-related transcription factors were expressed throughout the time course, especially WNT4 and SF1, suggesting spontaneous differentiation into gonadal tissues as has been reported in EBs[8] (Fig. 3.7B). Our goal was to track the gene expression of trophoblast-like markers through long-term culture conditions as EBs. We were also interested in the steroid biosynthetic enzyme presence and regulation throughout the culture and the presence and/or regulation of the hormone receptors.

All of the trophoblast-related markers were expressed through the 50 day culture, but only two, CGB and INHA, exhibited changes in their transcript levels. Interestingly, hCGβ was the most differentially expressed gene as a function of time. The mRNA transcript levels were highest at day 5, are slightly down-regulated by 22, and are almost gone by day 50. This is in contrast to the hormone levels in the media where hCGβ increases until day 22, suggesting another level of control between transcription and secretion. There was a dramatic loss of inhibin (INHA) from day 22 to day 50.

Many of the hormone receptors were only sporadically detectable, including both estrogen receptors and the progesterone receptor. LHCGR is present in day 5 and day 50 EBs, albeit at low copy number. Perplexingly though, it seems to be even less expressed in day 22 when hCG secretion is rising. AR is the most highly expressed of the steroid receptors. Steroid acute regulator enzyme (STAR) is present in EBs at all time points at steady levels (Fig. 3.7F).

The transcripts for the steroid biosynthetic enzymes, Cyp11A1 and CYP19A1, are present at most of the time points examined. Side-chain cleavage transcript levels remain remarkably steady throughout the long term culture. However, aromatase is expressed most highly at day 5 and then decreases throughout culture. At day 50, it appears that the transcription of CYP19A1 has decreased below detectable levels. Cyp21A2 and SRD5A1 are expressed in

EBs at all time points examined but exhibit no variation. Follistatin is stably expressed throughout the long-term culture conditions.


A
hCGβ Secretion
BMP AVG
CON AVG
NOG AVG
ng/mL
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
Days
*
B
Progesterone Secretion
BMP AVG
CON AVG
NOG AVG
ng/mL
20
15
10
5
0
0
1
2
3
4
5
6
7
Days
*
*

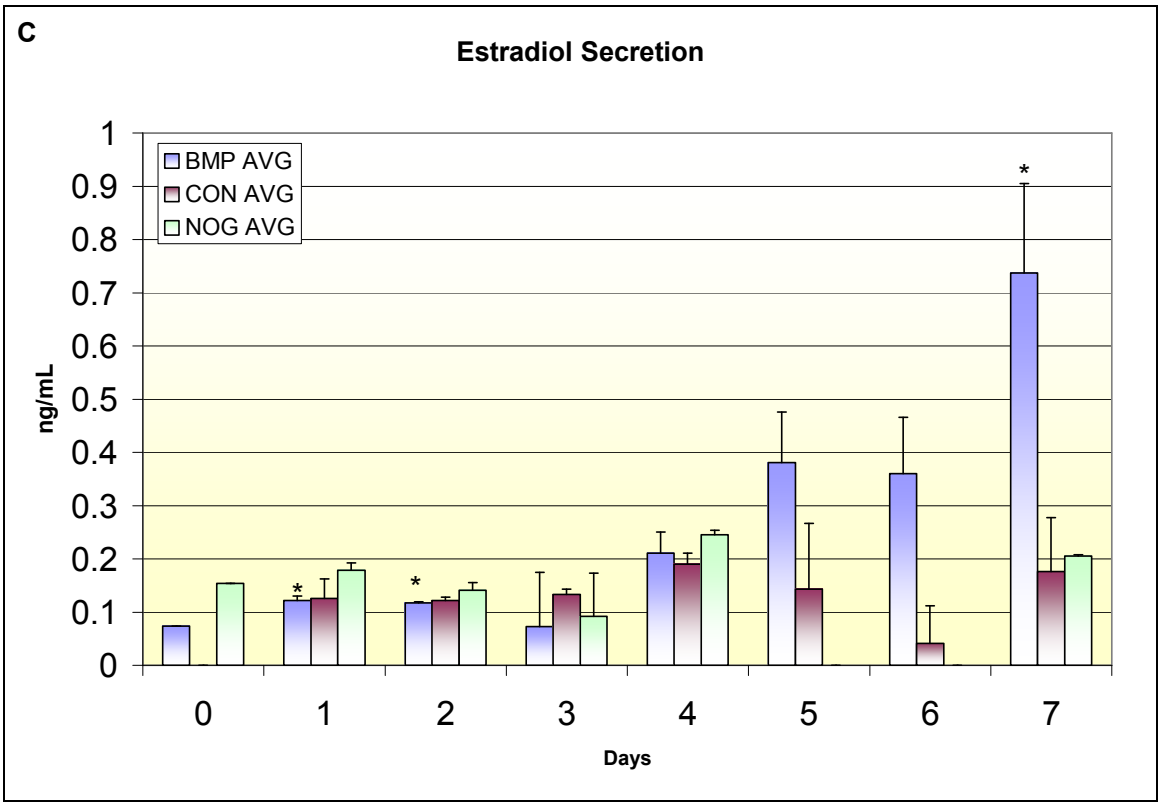
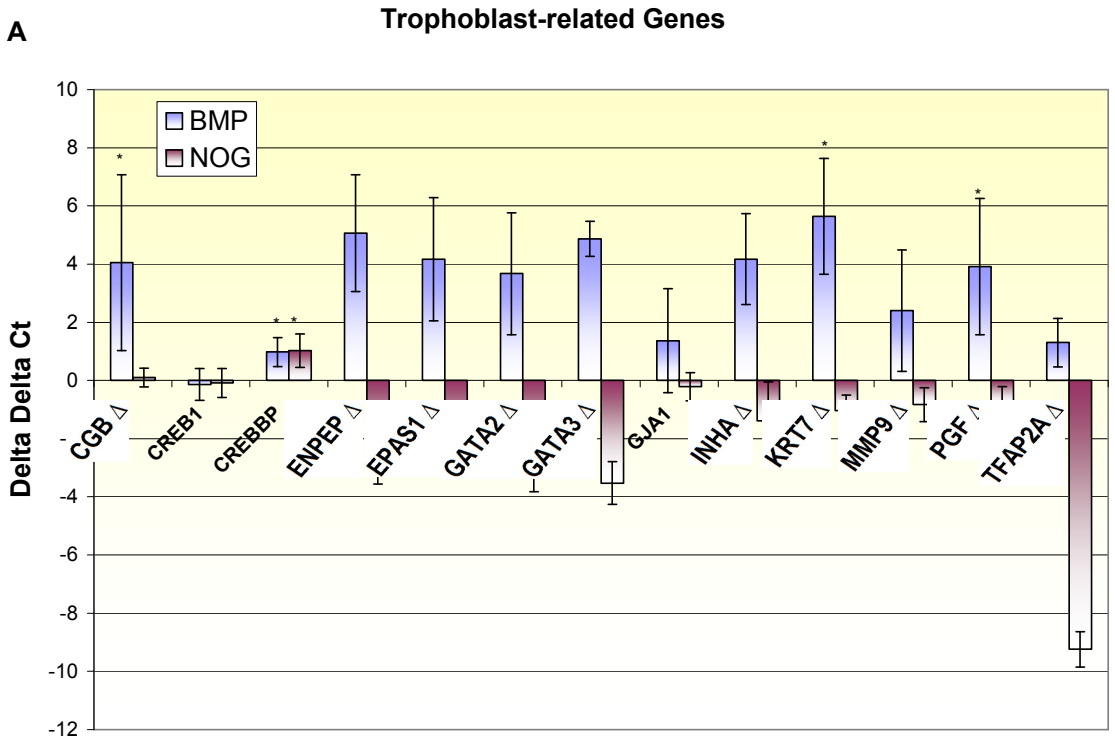


Figure 3.1: Hormone Analysis of BMP4 & Noggin-treated hESCs. Trophoblast-related hormones were secreted with BMP4 treatment (100 ng/mL) compared to control conditioned media (CON) and noggin-treated cells (NOG) (250 ng/mL) for secretion of A) hCG, B) Progesterone, and C) Estradiol. * indicates that the difference in the BMP treatment was significantly different ($p<0.05$) from noggin treatment. Noggin measurements for day 5 and day 6 are omitted.

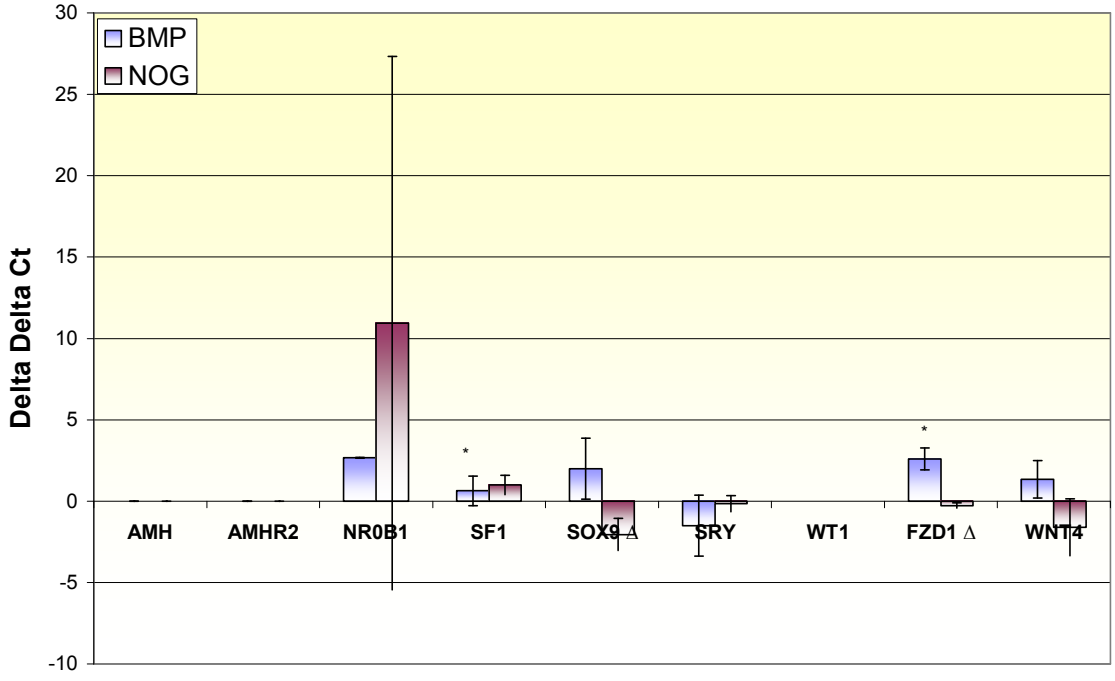
TABLE 1: Gene IDs and Protein Names	Gene ID	Protein Name
BMP Pathway-related	BMP4	Bone Morphogenic Protein 4
	BMP6	Bone Morphogenic Protein 6
	BMPR1B	BMP Receptor Type 1B
	BMPR2	BMP Receptor Type 2
	GREM1	Gremlin 1
	ID3	inhibitor of DNA binding 3
	IGF1	insulin-like growth factor 1
	NOG	Noggin
	RUNX1	Runt-related transcription factor 1
	RUNX2	Runt-related transcription factor 2
	SMAD1	mothers against DPP homolog 1 (SMAD1)
	SMAD5	SMAD5
	SMAD7	SMAD7
	SOX9	Sex determining region Y-box 9
Gonadal-related	AMH	Anti-mullerian hormone
	AMHR2	Anti-mullerian hormone receptor 2
	NROB1	Nuclear receptor subfamily 0 B1
	SF1	Splicing Factor 1
	SRY	Sex determining region Y
	WT1	Wilms tumor 1
	FZD1	Frizzled homolog 1
	WNT4	Wingless-type 4
Gonadotropin-related	CGB	Chorionic gonadotropin beta
	FSHR	Follicle Stimulating hormone receptor
	LHB	Luteinizing hormone beta
	FSHB	Follicle Stimulating hormone beta
	LHCGR	Luteinizing hormone/choriogonadotropin receptor
	TSHB	Thyroid stimulating hormone beta
ICM Derivatives	AFP	Alpha-fetoprotein
	CRABP1	Cellular retinoic acid binding protein 1
	NES	Nestin
	BAPX1	Bagpipe homeobox homolog 1
	CHRD	Chordin
	DLX5	Distal-less homeobox 5
	T	Brachyury homolog
Trophoblast-related	CGB	Chorionic gonadotropin beta
	CREB1	cAMP responsive element binding protein 1
	CREBBP	CREB binding protein
	ENPEP	glumly aminopeptidase
	EPAS1	endothelial PAS domain
	GATA2	GATA binding protein 2
	GATA3	GATA binding protein 3
	GJA1	Gap junction protein 1
	INHA	Inhibin alpha
	KRT7	Keratin 7
	MMP9	Matrix metalloproteinase 9
	PGF	Placental growth factor
	TFAP2A	Transcription factor AP-2 alpha

TABLE 1, cont'd	Gene ID	Protein Name
Steroid-related	AR	Androgen receptor
	CYP11A1	Side-Chain Cleavage Enzyme
	CYP19A1	Aromatase
	CYP21A2	Cytochrome P450 21A2
	ESR1	Estrogen receptor alpha
	ESR2	Estrogen receptor beta
	FST	Follistatin
	INHBA	Activin AB alpha
	INHBB	Activin AB beta
	PGR	Progesteron Receptor
	POMC	Proopiomelanocortin
	SRD5A1	Steroid-5-alpha-reductase
	STAR	Steroid acute regulator
Other regulated genes	CHD11	Cadherin 11
	CEBPB	CCAAT/enhance binding protein beta
	DAB2	Disabled homolog 2
	IGF1R	Insulin growth factor 1 recptor
	IL6	interleukin 6
	LAMA5	laminin 5 alpha
	NFKB1	nuclear factor kappa
	PTEM	phosphate and tensin homolog
	TIMP1	tissue inhibitor of metalloproteinase 1
	VEGF	vascular endothelial growth factor

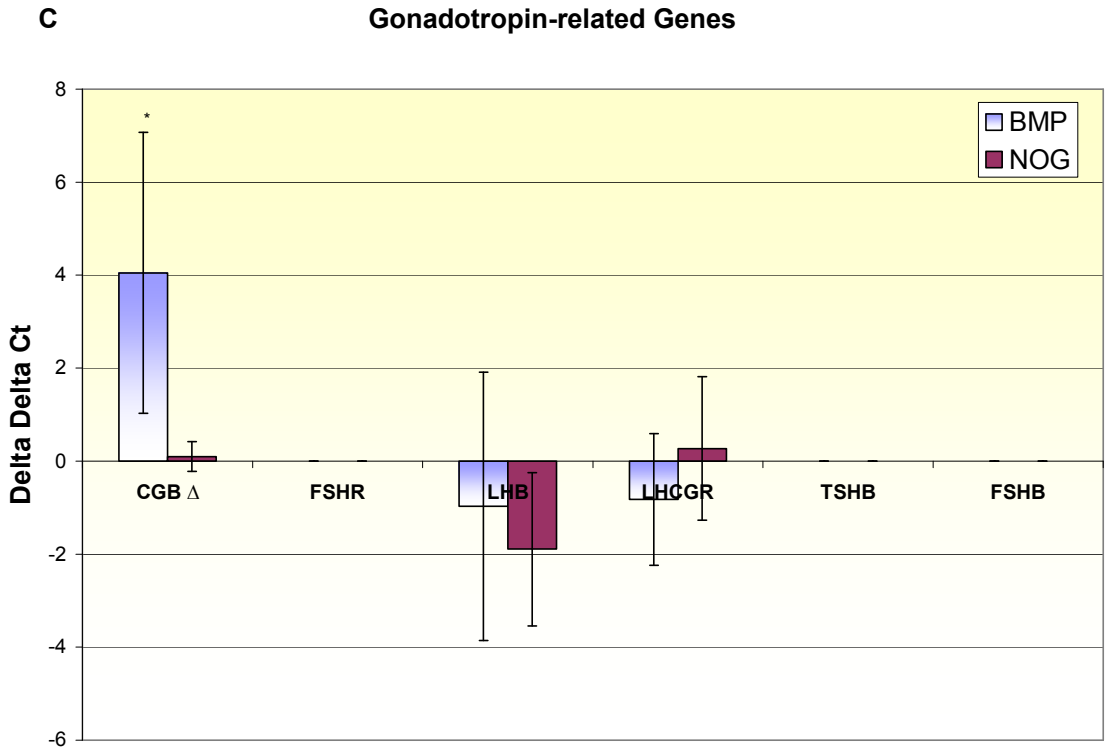
A


Trophoblast-related Genes
BMP
NOG
Delta Delta Ct
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
CGB Δ
CREB1
CREBBP
ENPEP Δ
EPAS1 Δ
GATA2 Δ
GATA3 Δ
GJA1
INHA Δ
KRT7 Δ
MMP9 Δ
PGF Δ
TFAP2A Δ

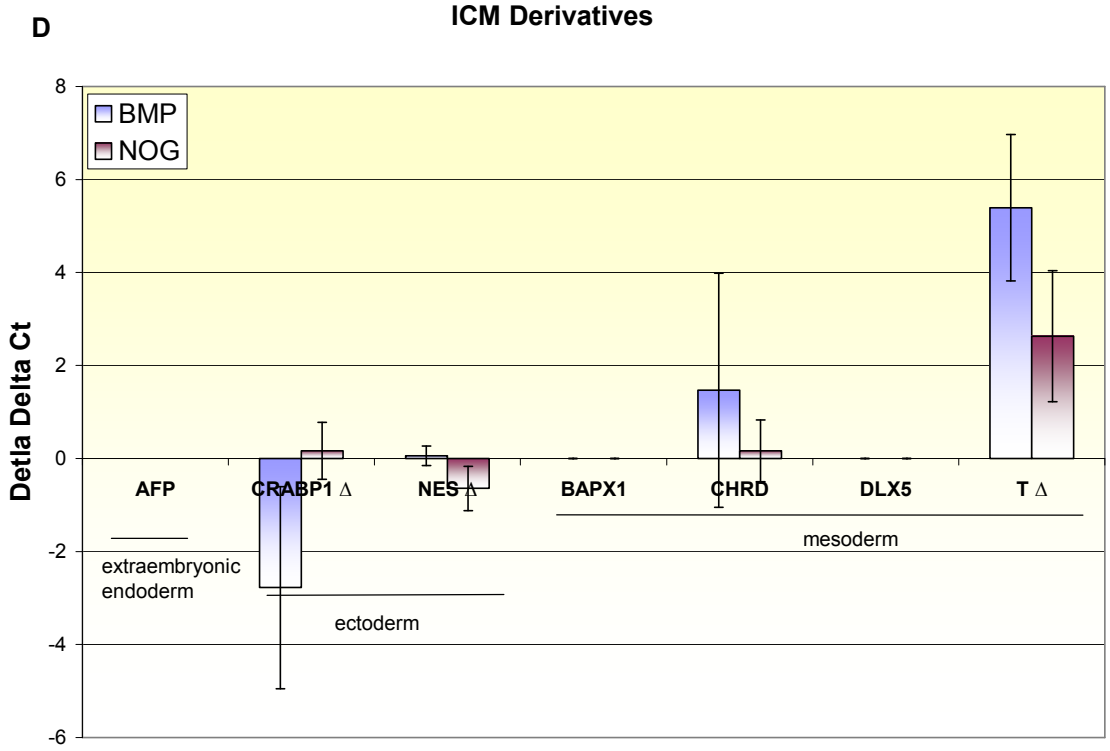
B


Gonadal-related Genes
BMP
NOG
Delta Delta Ct
30
25
20
15
10
5
0
-5
-10
AMH
AMHR2
NR0B1
SF1
SOX9 Δ
SRY
WT1
FZD1 Δ
WNT4

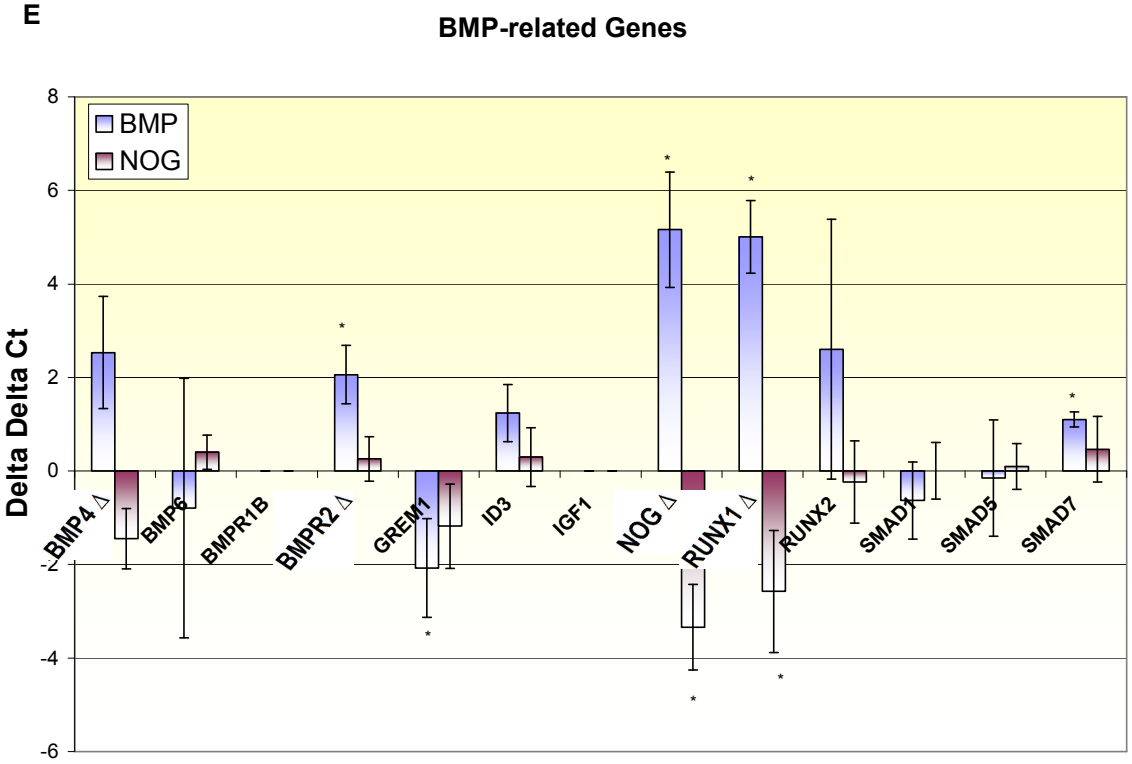
C


Gonadotropin-related Genes
BMP
NOG
Delta Delta Ct
8
6
4
2
0
-2
-4
-6
*
CGB Δ
FSHR
LHB
LHCGR
TSHB
FSHB

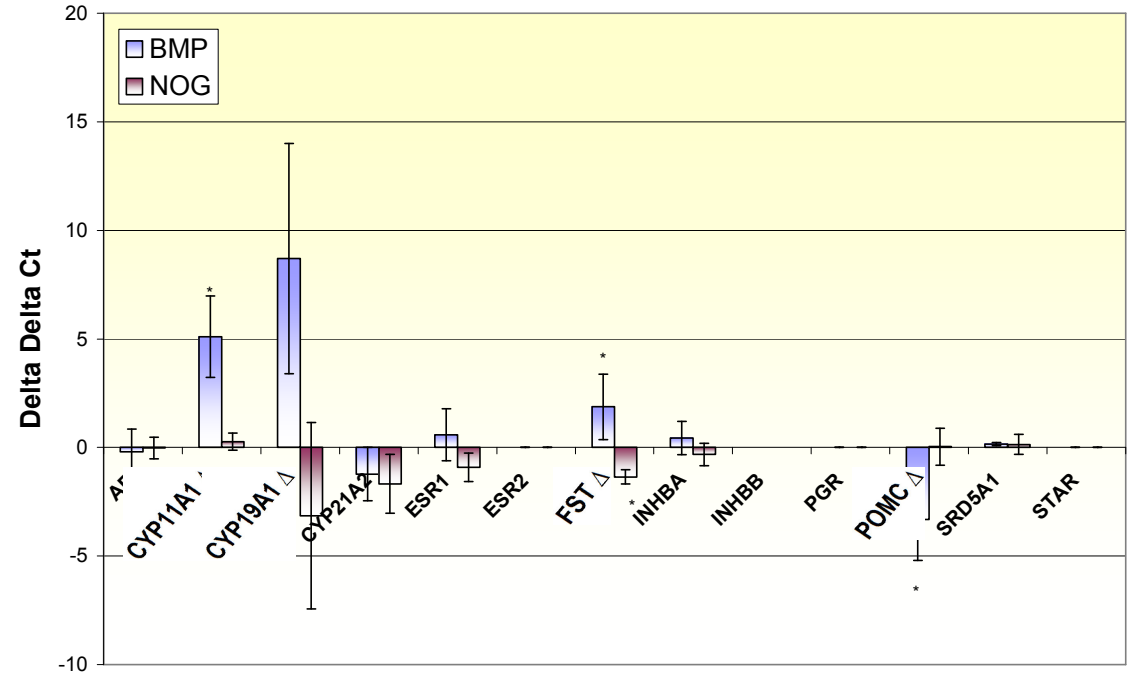
D


ICM Derivatives
BMP
NOG
Detla Delta Ct
8
6
4
2
0
-2
-4
-6
AFP
CRABP1 Δ
NES Δ
BAPX1
CHRD
DLX5
T Δ
extraembryonic endoderm
ectoderm
mesoderm

E


BMP-related Genes
BMP
NOG
Delta Delta Ct
8
6
4
2
0
-2
-4
-6
BMP4
BMP6
BMPR1B
BMPR2
GREM1
ID3
IGF1
NOG
RUNX1
RUNX2
SMAD1
SMAD5
SMAD7

F


Steroid-related Genes
BMP
NOG
Delta Delta Ct
20
15
10
5
0
-5
-10
CYP11A1
CYP19A1
CYP21A2
ESR1
ESR2
FST
INHBA
INHBB
PGR
POMC
SRD5A1
STAR

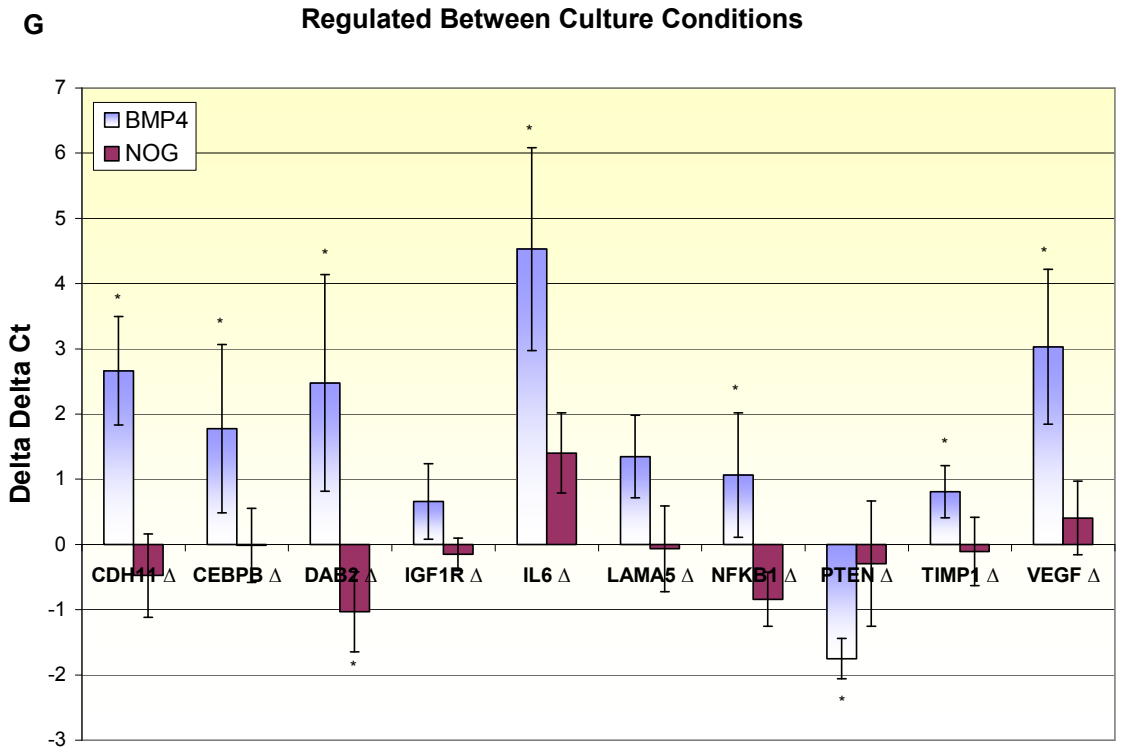
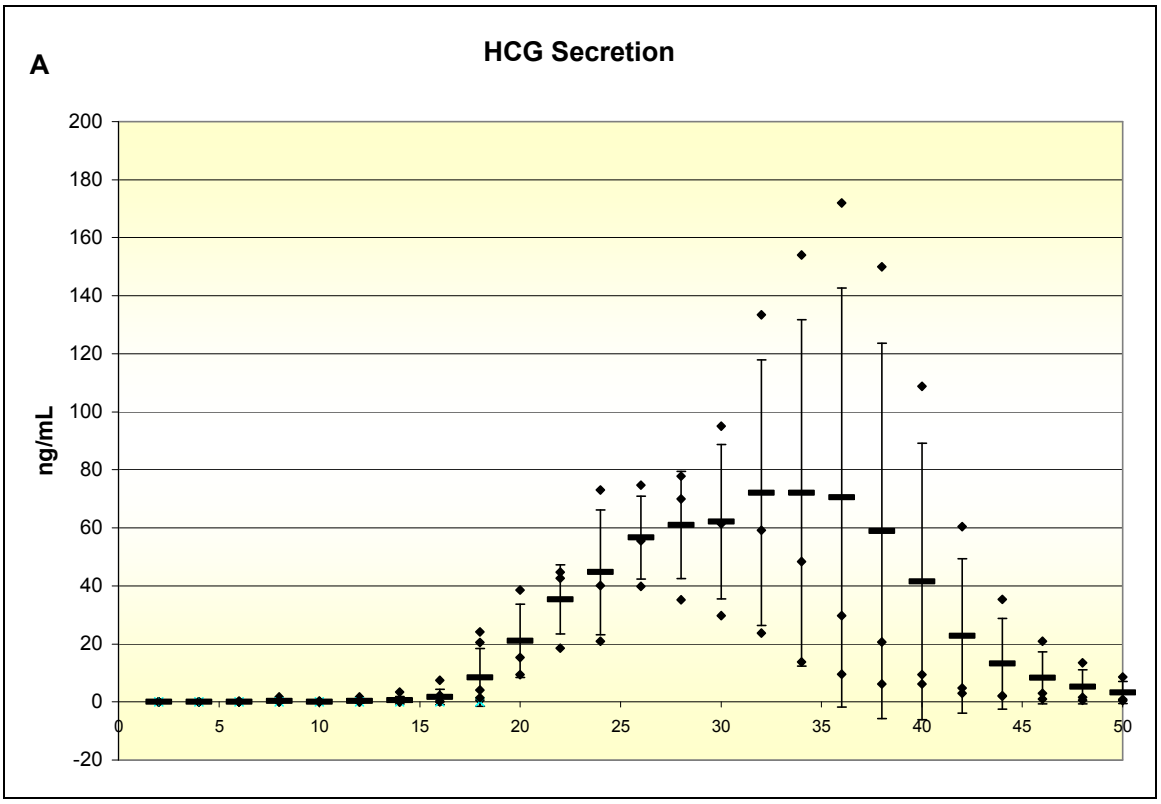
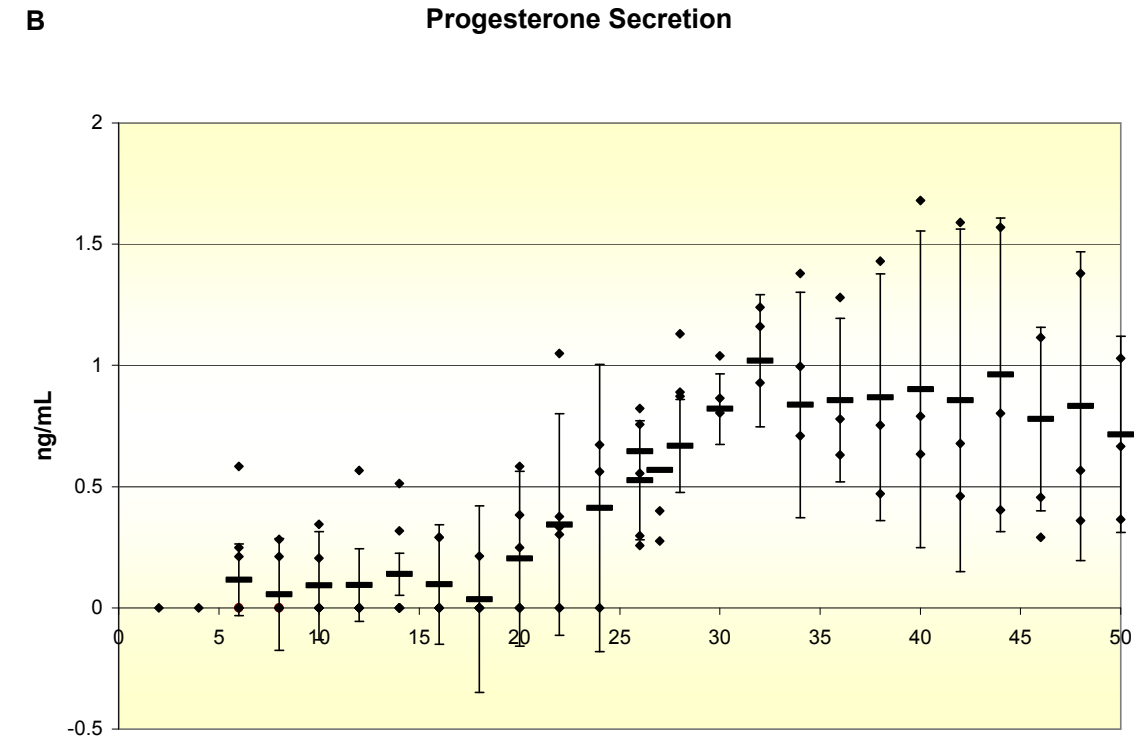


Figure 3.2A-G: RT/RT-PCR Analysis of Day 7 BMP4 & Noggin-treated hESCs.
Δ indicates a significant change between BMP4 & noggin treatment ($p < 0.10$).
* indicates a significant change between treated and control cells ($p < 0.10$). Delta Delta Ct = (Ct gene of interest (goi) – Ct 18S) untreated hESC – (Ct goi – Ct 18S) treated cells.


A
HCG Secretion
ng/mL
200
180
160
140
120
100
80
60
40
20
0
-20
0
5
10
15
20
25
30
35
40
45
50


B
Progesterone Secretion
ng/mL
2
1.5
1
0.5
0
-0.5
0
5
10
15
20
25
30
35
40
45
50

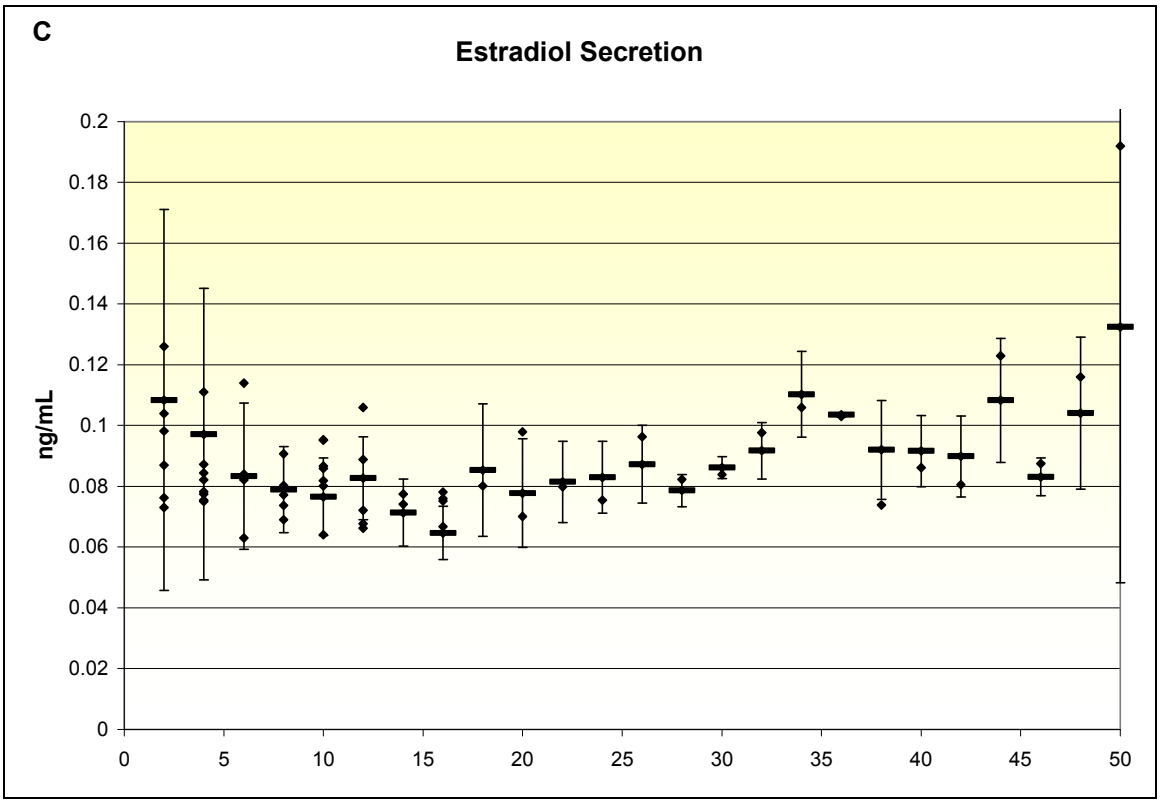
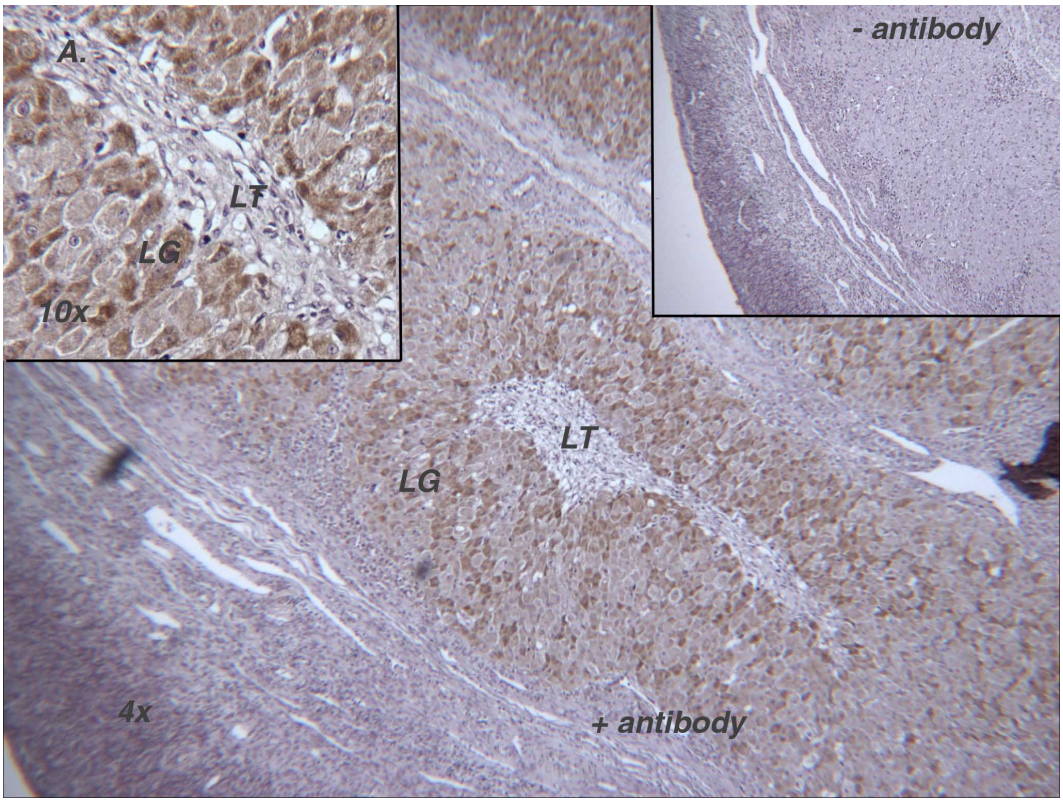
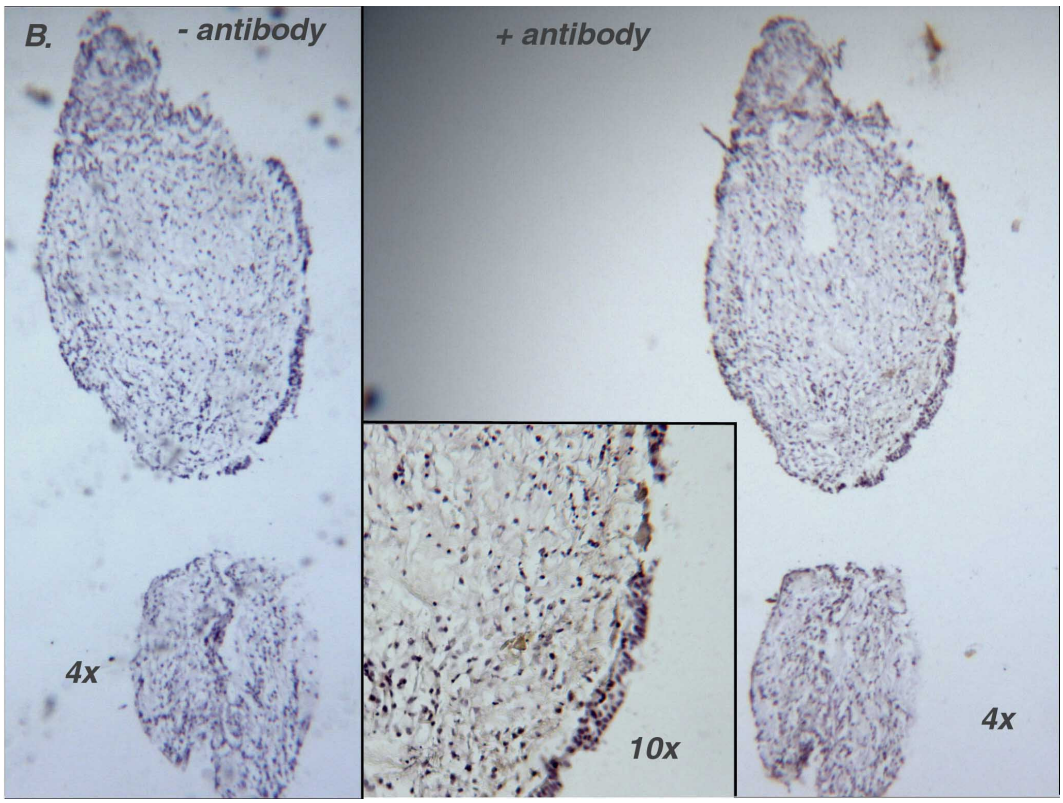


Figure 3.3 A-C: Hormone Secretion from EBs over 50 days of spontaneous differentiation. EBs derived from hESCs (BG02) were formed and allowed to differentiate spontaneously for up to 50 days in culture.

— Indicates the average of all EB data points (n=3 for each time point) with standard deviation. EBs allowed to spontaneously differentiate secrete A) hCG, B) progesterone, and C) estradiol at low levels.


A.
LT
LG
10x
- antibody
LT
LG
4x
+ antibody


B.
- antibody
4x
+ antibody
10x
4x

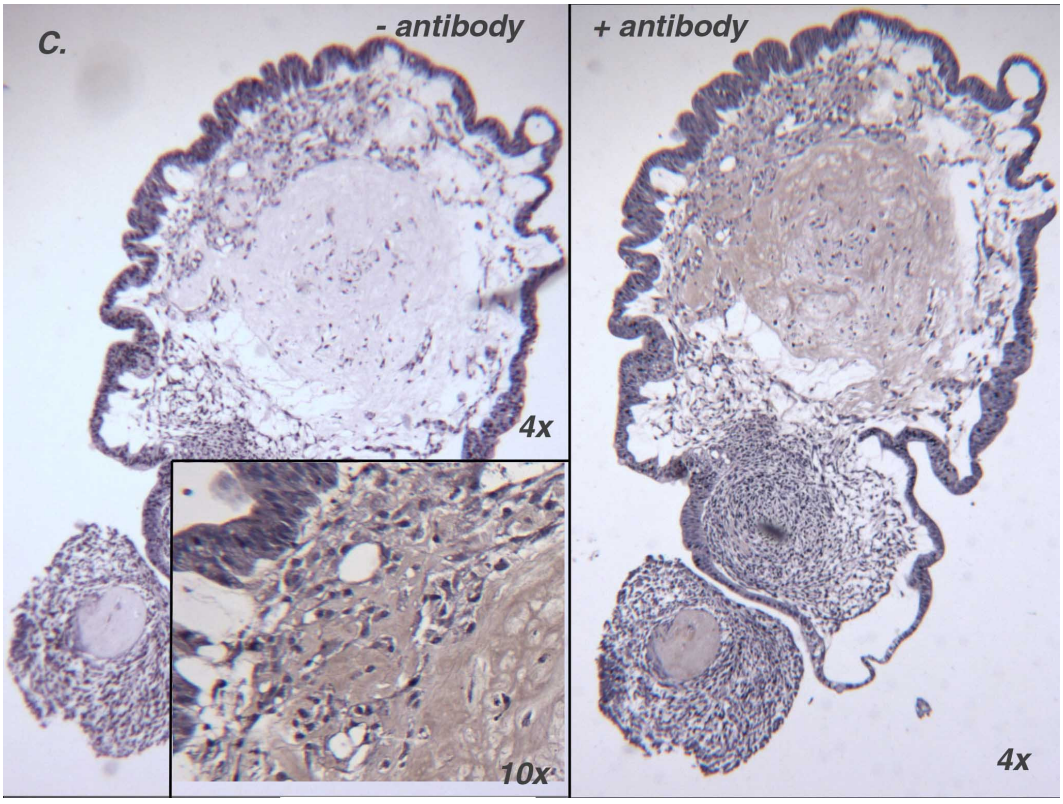
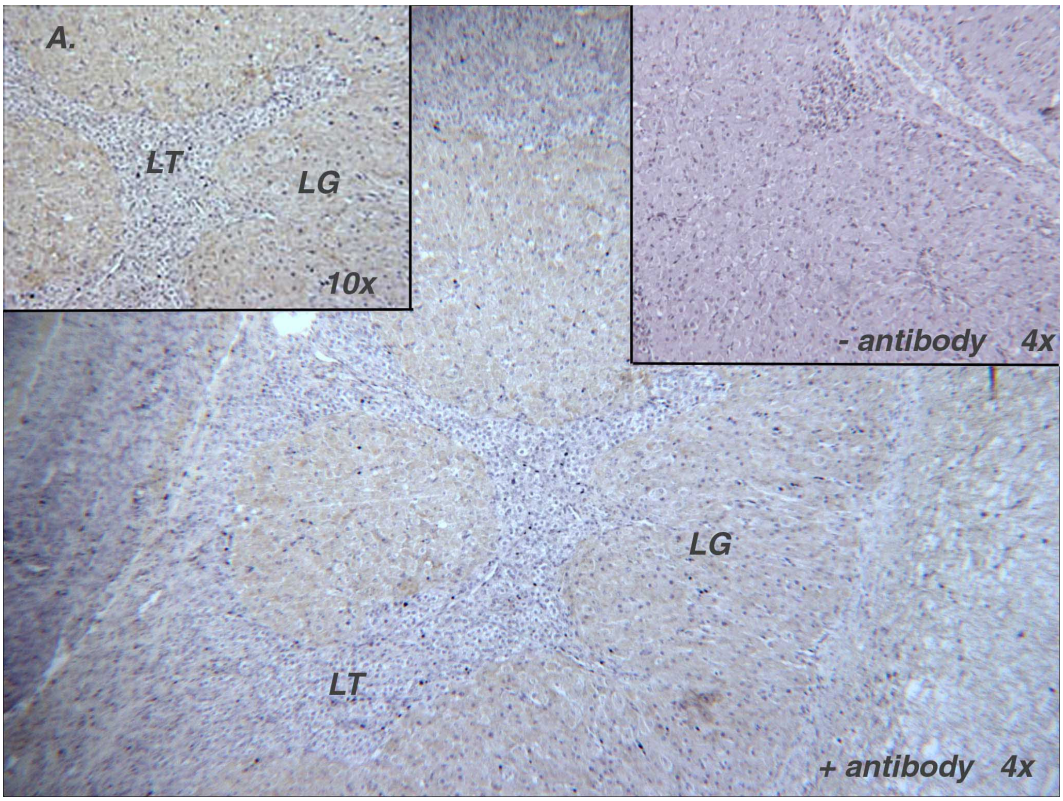
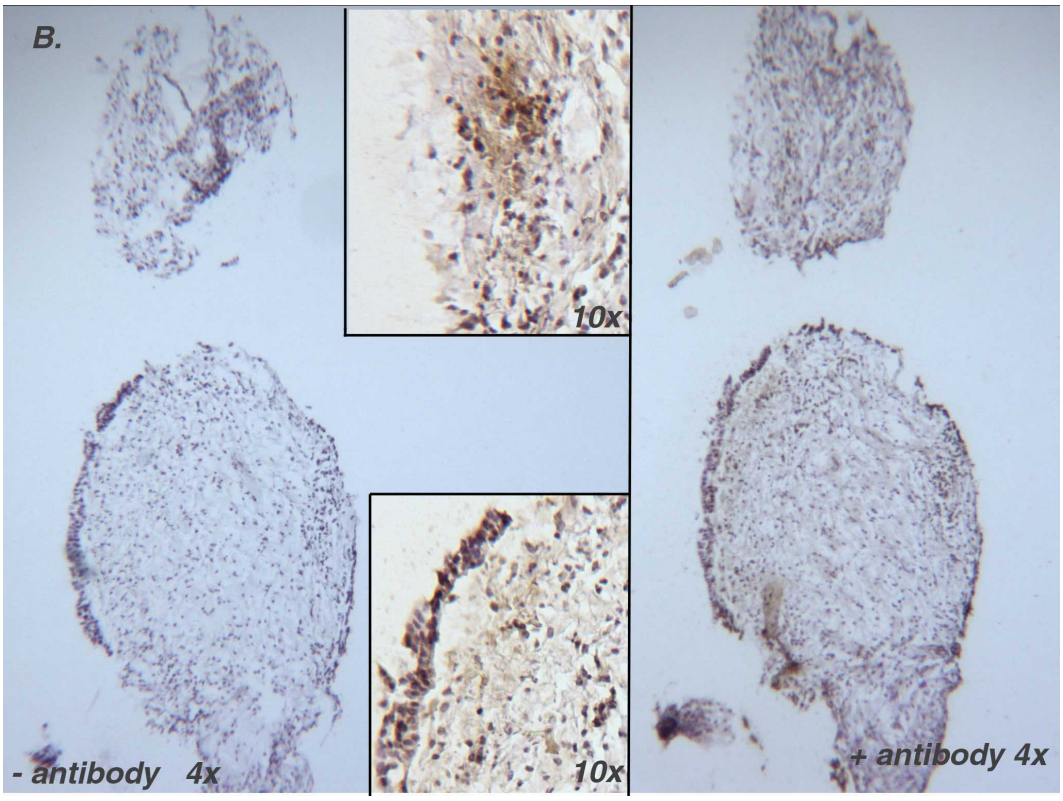


Figure 3.4: CYP11A1 expression in Day 22 and Day 50 EBs.
A) Cyp11A1 expression is specific to steroidogenic cells in human ovary. LG= luteinized granulosa cells. LT=luteinized theca cells. B. & C) stains of representative EBs at day 22 and day 50, respectively. Negative controls are without antibody. Inset (10x) shows a higher magnification of positive staining.


A.
LT
LG
10x
- antibody 4x
LG
LT
+ antibody 4x


B.
10x
10x
- antibody 4x
+ antibody 4x

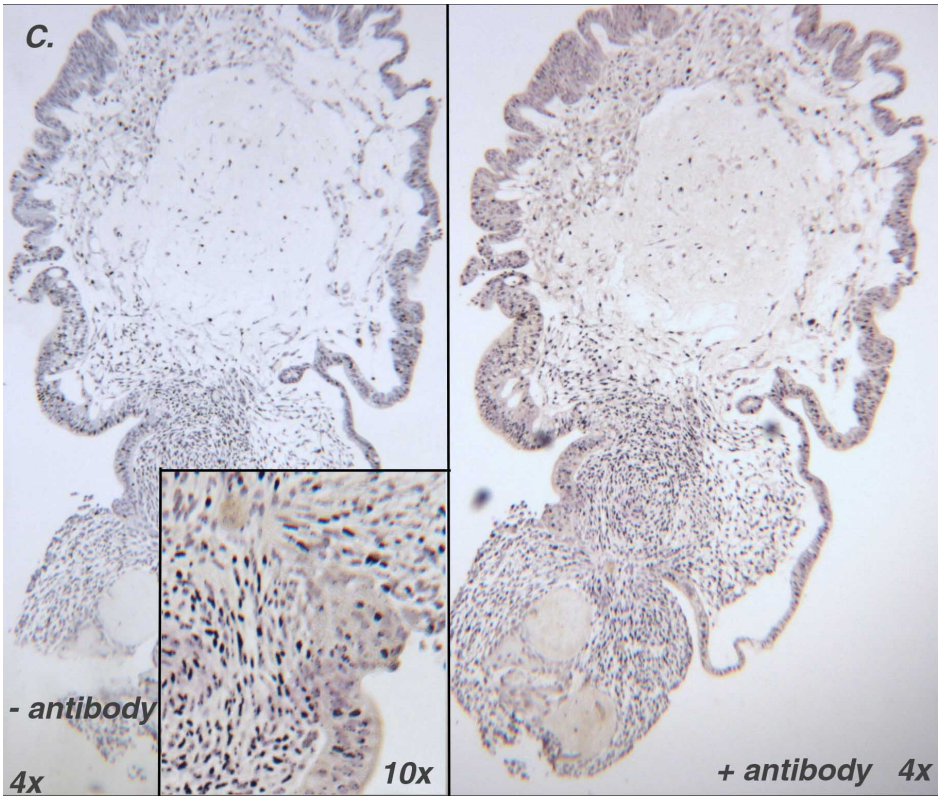
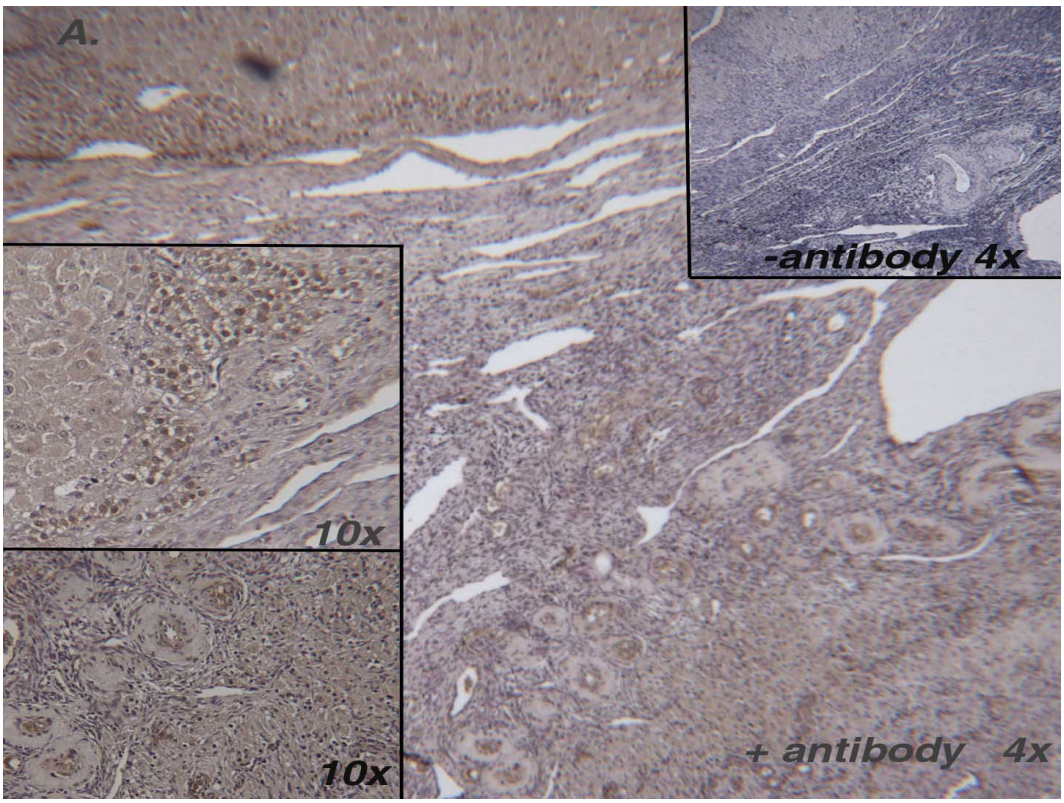
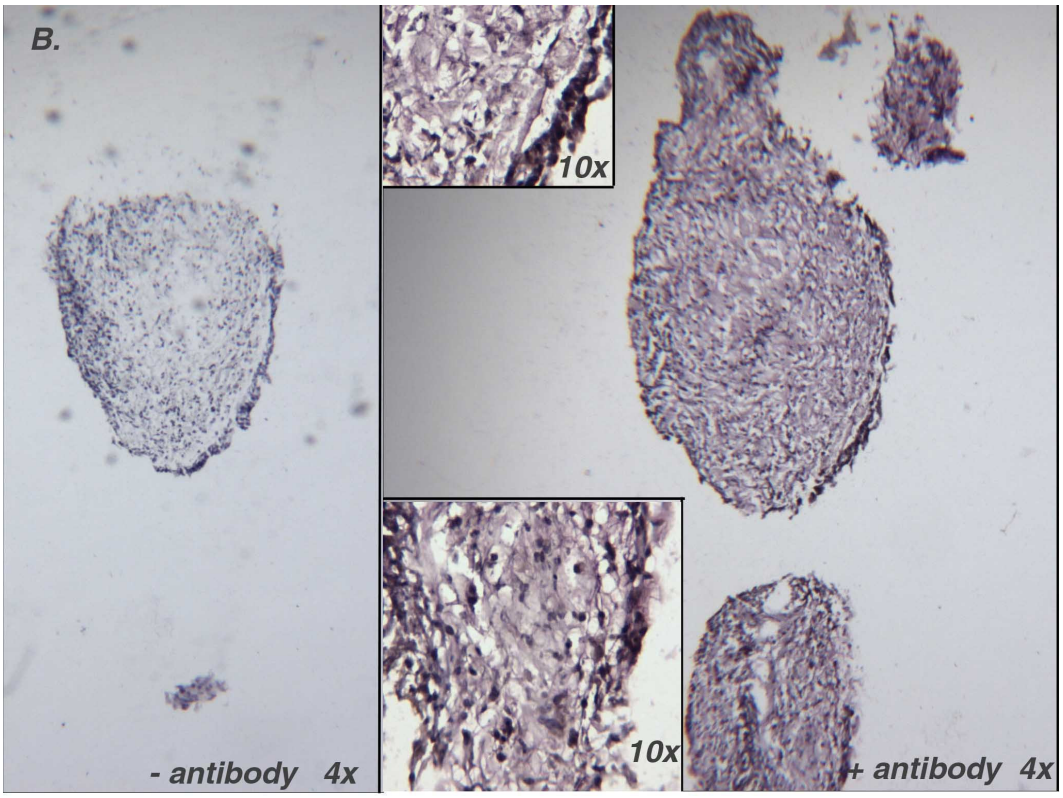


Figure 3.5: 3β-HSD expression in EBs at day 22 and day 50. A) 3β-HSD staining is specific to luteinized granulose (LG) and luteinized theca (LT) steroidogenic cells of the ovary. B&C) Positive staining of both day 22 and day 50 EBs (right panel.) next to minus antibody control (left panel). Inset shows10x magnification of positive staining.


A.
-antibody 4x
10x
10x
+ antibody 4x


B.
10x
10x
- antibody 4x
+ antibody 4x

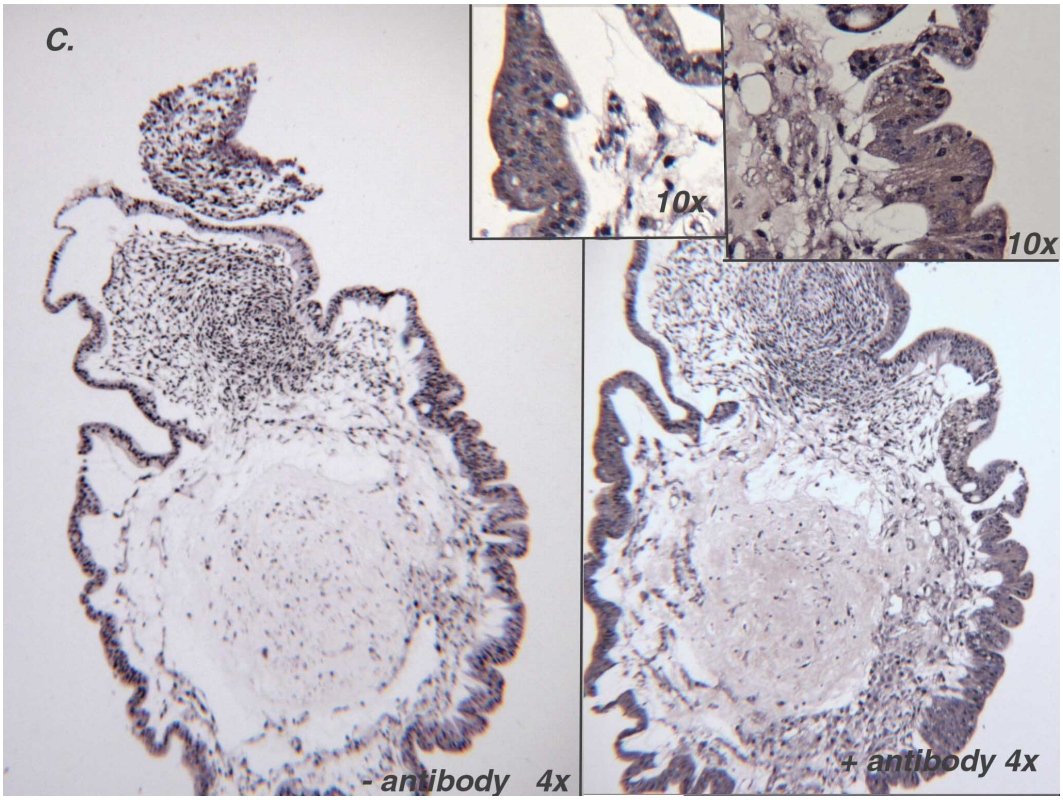
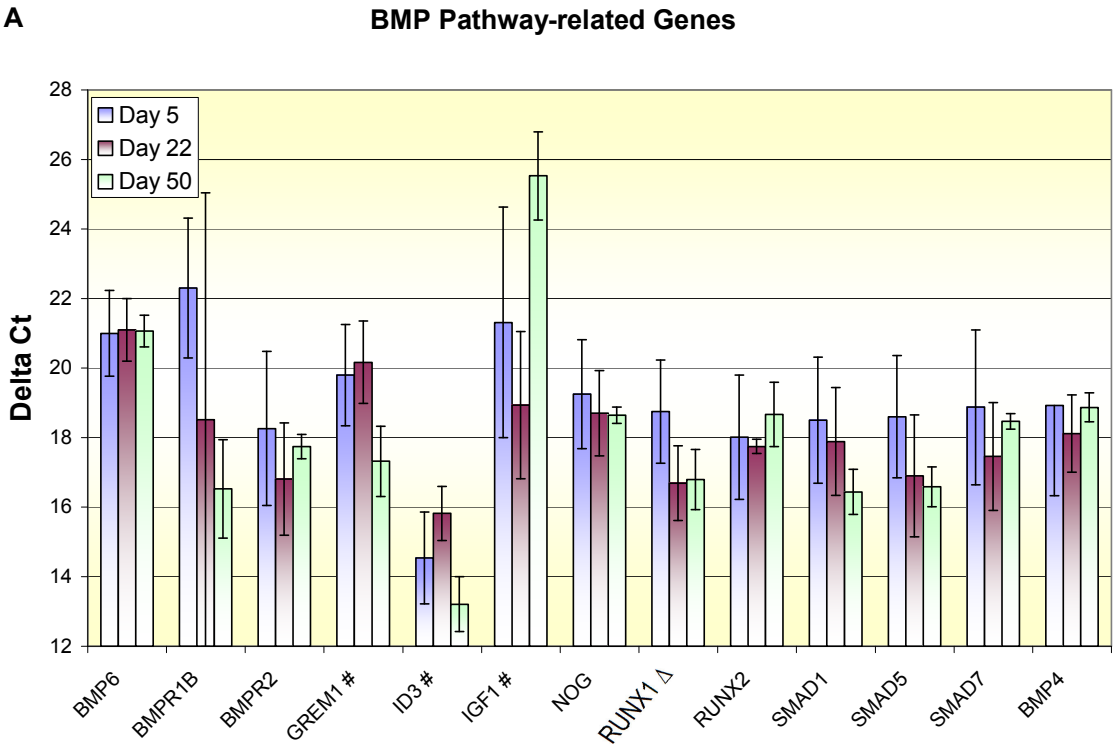
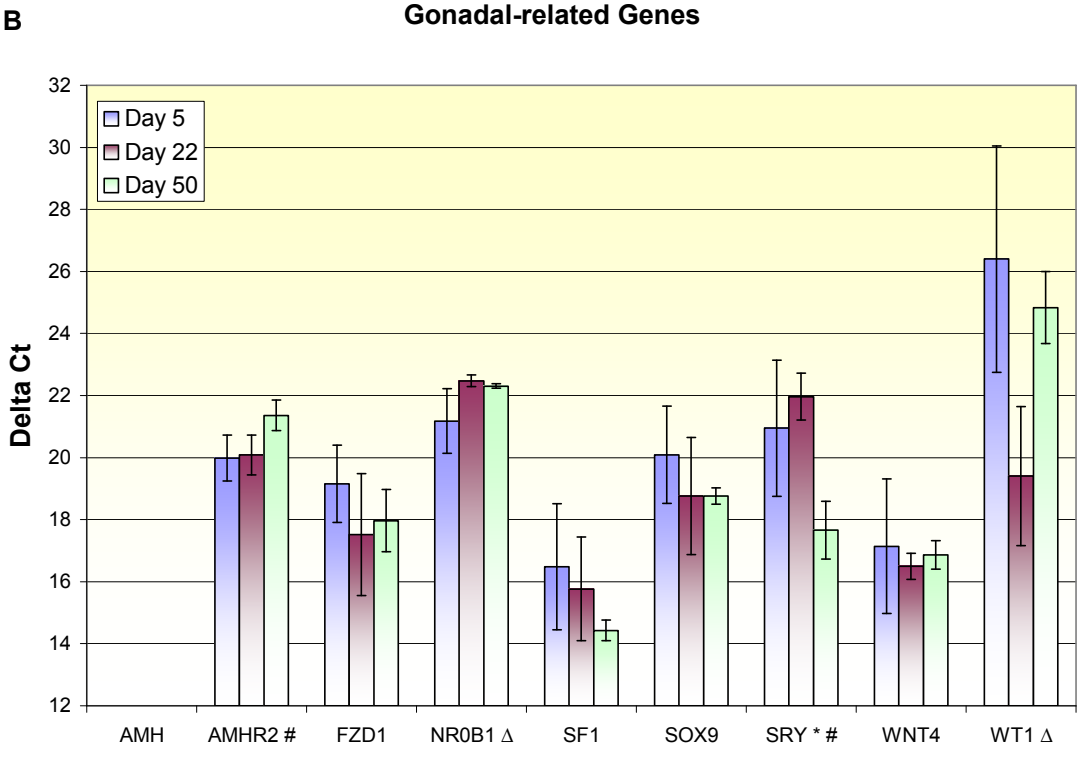


Figure 3.6: PGF expression in day 22 and day 50 EBs. A) Anti-PGF antibody stains the luteinized granulosa cells and the capillaries of the ovary specifically. B.&C) Positive staining of day 22 and day 50 EBs, respectively. Negative antibody controls are shown (-antibody). Higher magnification of positive staining

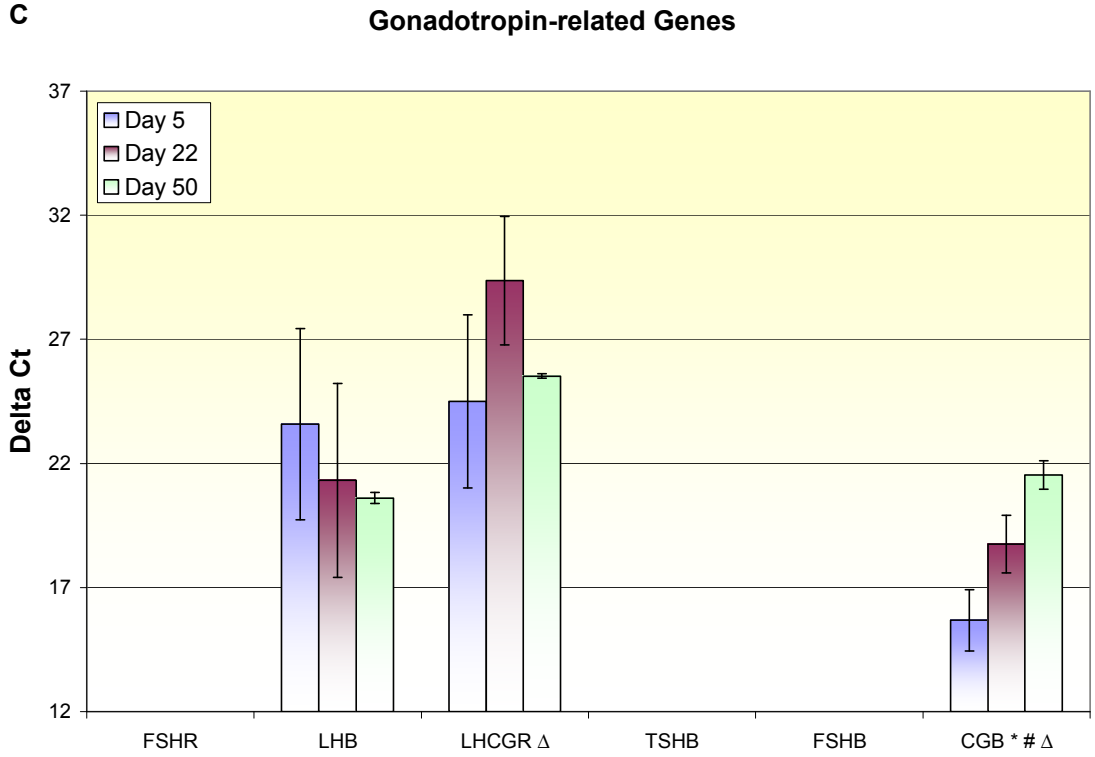
A


BMP Pathway-related Genes
Delta Ct
28
26
24
22
20
18
16
14
12
Day 5
Day 22
Day 50
BMP6
BMPR1B
BMPR2
GREM1 #
ID3 #
IGF1 #
NOG
RUNX1 Δ
RUNX2
SMAD1
SMAD5
SMAD7
BMP4

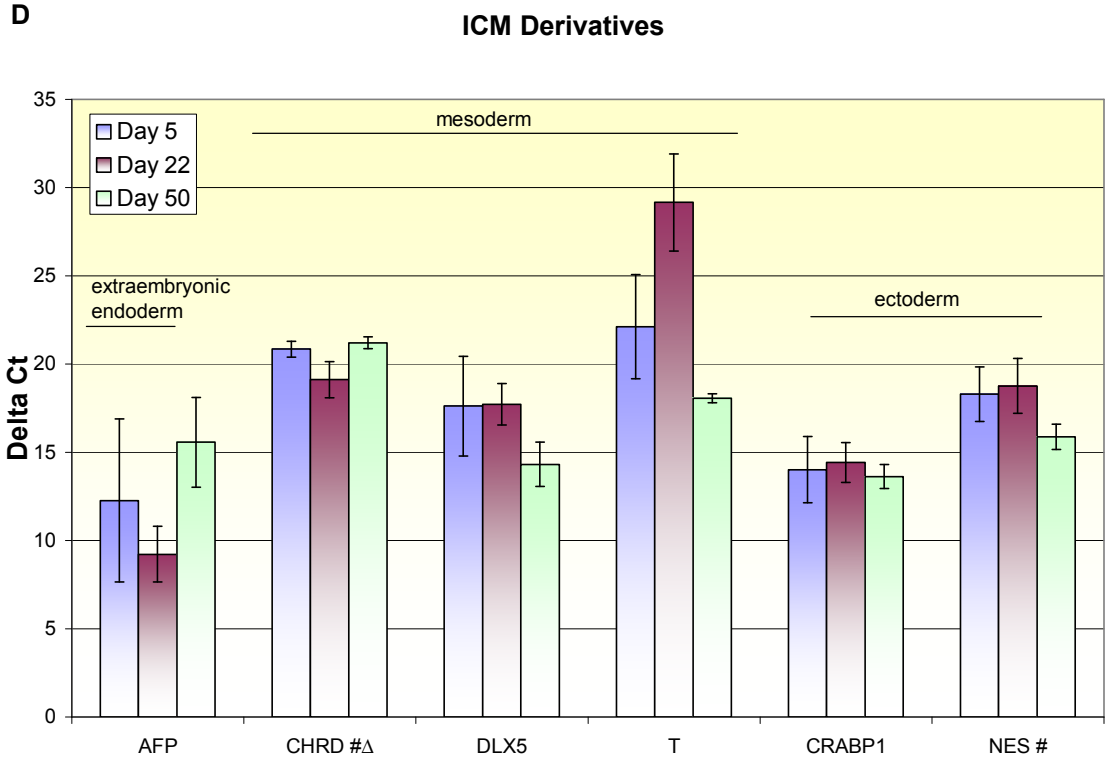
B


Gonadal-related Genes
Delta Ct
32
30
28
26
24
22
20
18
16
14
12
Day 5
Day 22
Day 50
AMH
AMHR2 #
FZD1
NR0B1 Δ
SF1
SOX9
SRY * #
WNT4
WT1 Δ

C


Gonadotropin-related Genes
Day 5
Day 22
Day 50
Delta Ct
37
32
27
22
17
12
FSHR
LHB
LHCGR Δ
TSHB
FSHB
CGB * # Δ

D


ICM Derivatives
Day 5
Day 22
Day 50
mesoderm
extraembryonic
endoderm
ectoderm
Delta Ct
35
30
25
20
15
10
5
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AFP
CHRD #Δ
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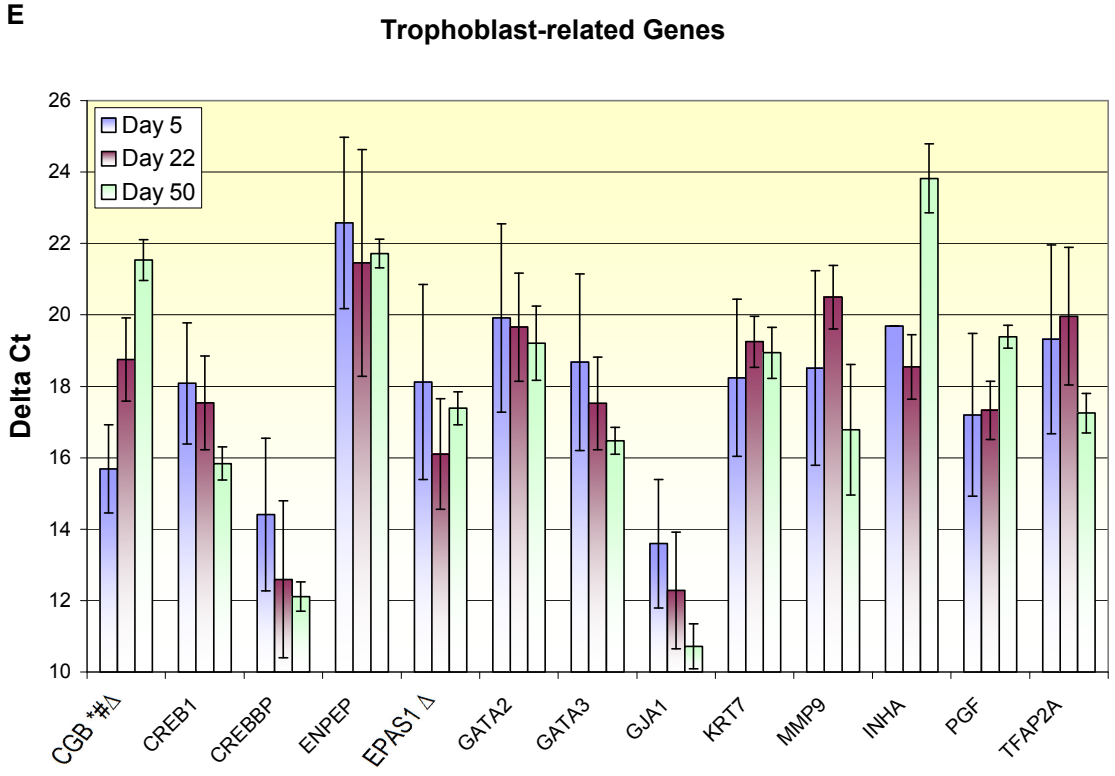
Trophoblast-related Genes
Delta Ct
Day 5
Day 22
Day 50
26
24
22
20
18
16
14
12
10
CGB *#Δ
CREB1
CREBBP
ENPEP
EPAS1 Δ
GATA2
GATA3
GJA1
KRT7
MMP9
INHA
PGF
TFAP2A

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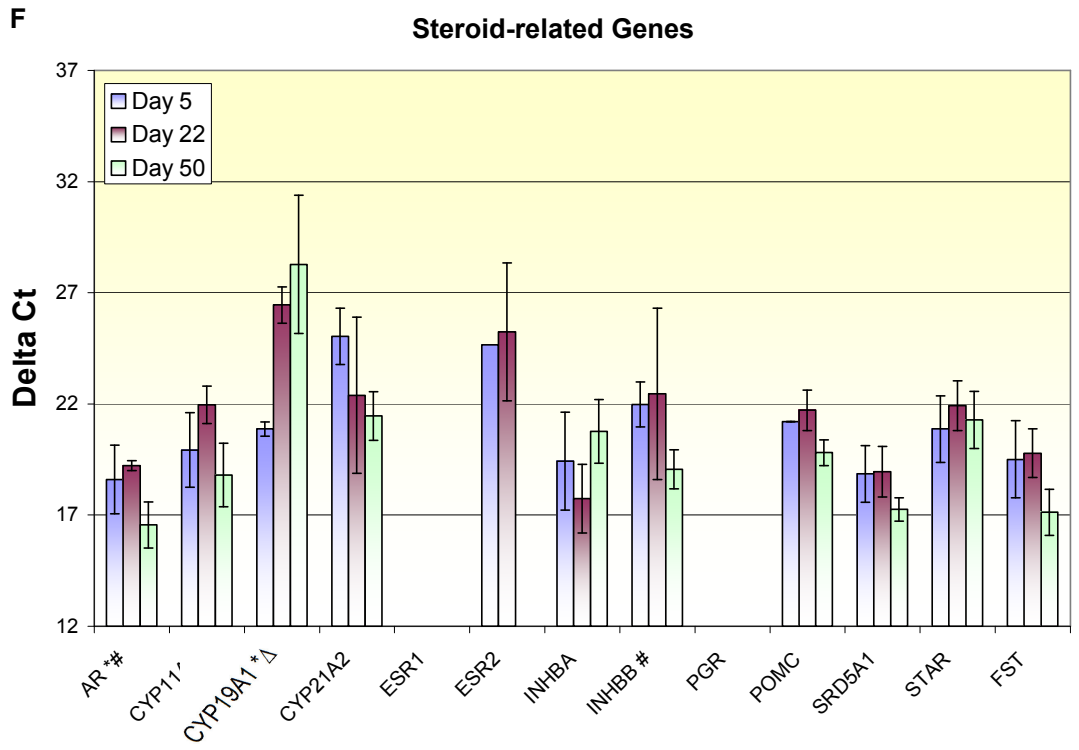
Steroid-related Genes
Delta Ct
Day 5
Day 22
Day 50
37
32
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17
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AR *#
CYP11'
CYP19A1 *Δ
CYP21A2
ESR1
ESR2
INHBA
INHBB #
PGR
POMC
SRD5A1
STAR
FST

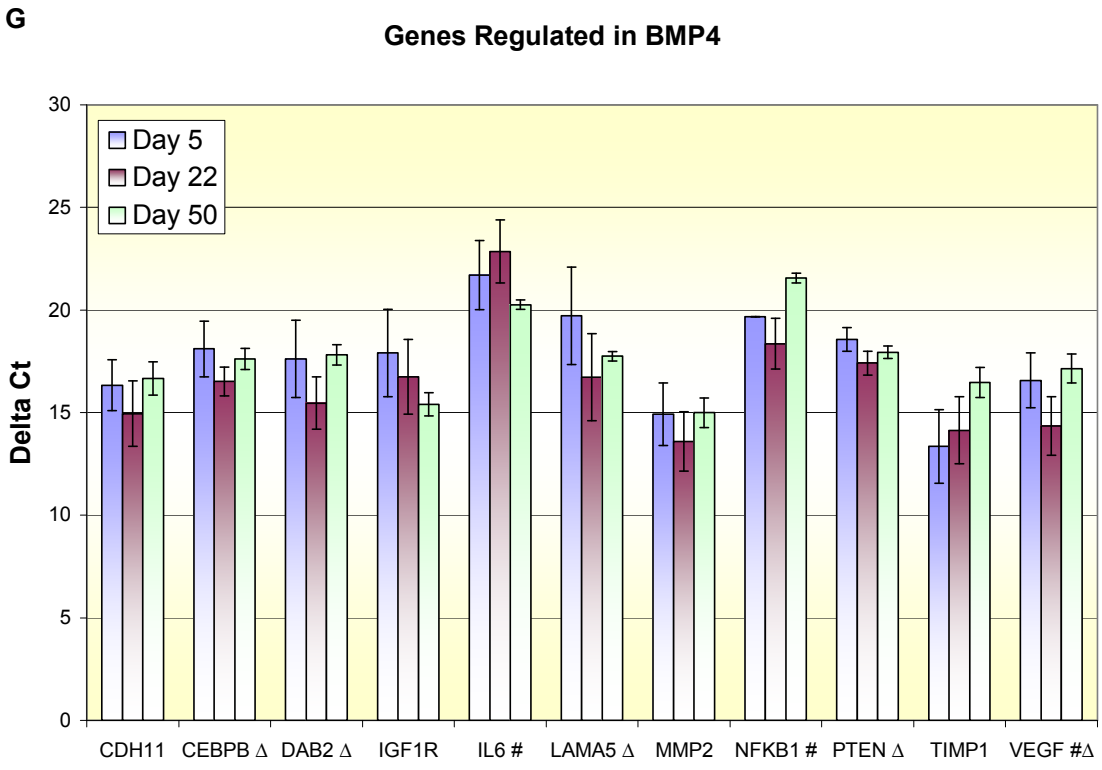

Figure 3.7A-G: RT/RT-PCR of embryoid bodies at Day 5, Day 22, and Day 50. Data presented as delta Ct= Ct gene of interest - Ct (18s endogenous control). Therefore, a lower bar indicates higher expression. *, #, Δ indicate significant difference between ($p < 0.05$) between day 5 & day 22, day 5 and day 50, and day 22 and day 50, respectively.

Chapter 4:

Discussion

Our results, like those of Xu et al. 2002[1], show that the differentiation of adherent cultures of hESCs with BMP4-treated media yields a homogenous culture of trophoblast-like cells which secrete hCGβ and are keratin 7 positive. This differentiation was abolished by the BMP4 antagonist noggin. The trophoblast-like cells have the capacity to produce progesterone from cholesterol and estradiol from testosterone. However, they exhibit some differences from cytotrophoblasts and syncytiotrophoblasts, as they express P450 enzymes not specific to these cells [21,43].

The EBs differentiated into trophoblast-like cells much like those described by Gerami-Niani et al. 2004 [2] as they secreted hCGβ and progesterone into the media and were positive for keratin 7. In contrast to their results, we found that estradiol secretion was very low, and we cannot with confidence conclude that it was synthesized by the cells. Unlike the adherent cultures, the EBs are not a homogenous set of cells; thus, conclusions are far more limited.

These finding notwithstanding, it is difficult to firmly establish that differentiation of hESCs under these conditions leads to trophoblast-like cells. The human trophoblast stem cell has yet to be isolated and cultured *in vivo,* and the murine trophoblast stem cell will most likely display a different differentiation pathway. Therefore, most of the data by which to compare our experimental results is from more differentiated cell types derived from first or third term placental explants. When assessing the results of the previous experiments [1,2] several questions should be addressed. First, do the cells resemble trophoblast-like tissues more than other tissues?

Second, are the cells steroidogenic? And third, what are the possibilities of autocrine or paracrine regulation and how do these compare to trophoblast?

I. Characterization of Trophoblast: The Maturation from Cytotrophoblast to Syncytiotrophoblast.

As placental explant cell cultures differentiate from cytotrophoblast to syncytiotrophoblast, it is possible to characterize their propensities to express peptide/glycoprotein hormones (CG, GnRH, etc.), steroid hormones (progesterone, testosterone, estradiol), hormonal receptors (PR, ER1, ER2, LHCGR), steroid biosynthetic enzymes (CYP11A1, 3β-HSD, CYP19, 17β-HSD, CYP21A2), cellular structural proteins (KRT7), and cytokines (INHA, INHBB, INHBA). Researchers have delineated three cell-types for the purpose of maturation studies including villous cytotrophoblast (VCT), young syncytiotrophoblast (yST), and mature syncytiotrophoblast (mST) [25,26]. These three cell types have remarkably similar endocrine behavior and typically differ only in the amounts of hormone and receptor expression. For example, cytotrophoblasts express relatively large amounts of ER1. However as the cell terminally differentiates, these amounts are reduced. Whereas in cytotrophoblasts, ER2 is expressed at very low levels; expression is maximal in yST and intermediate in mST. All three have been reported to express hCGβ, progesterone, inhibin, and activin. Again, there are differences in degree between the cells; typically cytotrophoblasts produce more cytokines and GnRH and the syncytiotrophoblasts produce more progesterones and hCG. Syncytiotrophoblasts are the only cells which up-regulate CYP19 and produce estrogens (Fig. 4.1)[21].

Despite the similarities in endocrine behavior, the cell biology of the maturing cells is very different. The cytotrophoblast consists of mitotically active nucleated cells. As the

cytotrophoblast differentiates into the yST, the cells fuse to form multi-nucleated epithelial-like cells (mST) at the fetomaternal interface. The mST consists of mitotically inactive terminally differentiated cells. In this fusion process, gap junctions containing connexin 43 (GJA1) are necessary [20,44].

II. BMP4-treated Cells

In our culture conditions, day 7 BMP4-treated cells most resemble sycytiotrophoblast in their morphology, their epithelial nature, the presence of gap junctions, and their hormonal capabilities (specifically the secretion of estrogens) (Fig 4.2). The cells derived were very similar to those previously described by Xu et al. 2002 [1]. We did not observe the up-regulation of genes specific to the germ layer, extraembryonic endoderm, gonadal tissues, or the pituitary. Like the results reported previously [1], the cells express high levels of CGβ, the hallmark of trophoblast tissue. CGβ is also expressed in some fetal tissues including the kidney [45], but one would expect the up-regulation of several other mesodermal genes which we did not observe. The cells express keratin 7, which is used to select specifically for trophoblast when retrieving cells from the placenta for cell culture. It is a very selective marker for trophoblast cells of all maturities, as the only other placental tissue in which it is present is the chorion [21]. The presence of keratin 7 significantly reduces the plausibility that any other extraembryonic tissue is being formed due to BMP4 treatment. In contrast to the results reported earlier of the formation of extraembryonic endoderm[7] upon treatment with BMP4, in our culture conditions the presence of keratin 7, secretion of hCGβ, as well as the lack of expression of AFP, excludes this lineage. Finally, the cells express gap junction protein GJA1, which is vital to cellular communication in the syncytiotrophoblast [43]. The concurrent up-regulation of matrix-metalloproteinase 9, a gene involved in the invasive properties in the syncytiotrophoblast, as well as the other trophoblast

related genes indicates that these cells are differentiating down a trophoblast pathway as reported earlier [1].

In our culture conditions, BMP4-treated cells expressed CYP11A1 and CYP19A1 indicating that they are likely to be capable of synthesizing both progesterone and estradiol. However, the mechanism of estradiol synthesis is still to be determined, given the minute levels of testosterone in the unconditioned media which could easily be converted into estradiol. Whether these cells convert estradiol directly from testosterone or the cells exhibit the more classical pathway converting androgens to estradiol is yet to be determined. However, there were a few differences between BMP4-treated cells and trophoblast cells described from placental explants [21,43]. We could not detect ER2 or PR in our cultures [25-27]. We also detected the stable expression of CYP21A2, which is thought not to be expressed in trophoblast cells (Fig. 4.2) [21,43].

Autocrine regulation in these cells is significantly curtailed compared to trophoblast cells described by others [21,46] as neither ER2 nor PR are expressed. The secretion of CGβ and the presence of the receptor on the cell surface indicate the possibility for autocrine regulation as CGβ may signal to the same cell through its receptor. Perhaps the up-regulation of progesterone happens in this fashion as is postulated in trophoblast tissues from placental explants[21].

Growth factor autocrine regulation in the BMP4/BMPR2 pathway and that of the WNT4/FZD1 pathway is possible, as both ligand and receptor are up-regulated. Both of these secreted proteins play essential roles in early embryonic development in the mouse [5,6,37]. Interestingly, another WNT signaling modulator, DAB2, was differentially regulated with BMP4 treatment (Fig. 4.2)[37]. This modulator is proposed to bind the Wnt signaling mediator, Disheveled-3, and prevent it from activating effecter molecules, e.g.β-catenin, and eventually c-

myc [37]. This finding is novel and shows that though the WNT4 receptor, FZD1, is significantly up-regulated in response to BMP4 treatment in our culture conditions; there is considerable regulation of the pathway which needs further study.

III. Spontaneous Differentiation in Embryoid Bodies

Because each embryoid body may be different than its counterpart and because each body is made up of several types of cells, it is not possible to determine which receptor, enzyme, or protein is up-regulated in a particular cell type. Assessing the embryoid bodies as a whole, it is possible that at each time point trophoblast-like cells (as defined by the BMP4 experiment) exist in culture as all of the trophoblast-related genes are expressed, including keratin 7 and hCG β as described previously (Fig. 4.3) [2].

The EBs secrete trophoblast hormones, like those from placental explants and those of the BMP4-treated trophoblast-like cells. Embryoid bodies secrete hCGβ in long-term spontaneous differentiation between days 22 and 38. Interestingly, though the secretion data do not exactly align with the PCR data as at day 5, the EBs had the highest transcripts of the beta subunit. There seems to be a lag between transcription and secretion in the EBs. The embryoid bodies are capable of producing progesterone on days 5, 22, and 50 as they express CYP11A1 and 3β-HSD. Estrogen secretion seems to begin immediately after EB formation, diminish until day 25, and then increase until day 35. The RT/RT-PCR data indicates that at day 5 CYP19A1 expression is highest and declines through continued culture; this indicates that the first wave of expression is substantiate by both hormonal analysis and RT/RT-PCR. Whether the estrogens are converted directly from testosterone in the media by aromatase or through the conversion of DHEA as described for canonical trophoblast is unclear[21].

The EBs do not express PR or ER1 at any time point curtailing the potential for steroid autocrine or paracrine action. However, the hCG receptor is expressed on EBs at day 5 and day 22 indicating that the hormonal glycoprotein may stimulate in an autocrine fashion. A hormonal feedback loop as described in trophoblast tissues [21], where hCG positively regulates progesterone secretion, is possible in the embryoid bodies.

Unlike trophoblast derived from placental explants, embryoid bodies, like BMP4-treated cells, stably express CYP21A2 and SRD5A1. However, the expression of genes not found in trophoblast is not particularly consequential in this system as the cultures are again made up of multiple cells types which could be 'contaminating' gene expression data. Steroid acute regulatory protein is also expressed in the EBs. This protein is involved in the intra-mitochondrial transport of cholesterol in steroidogenic tissues of the adult, including the ovaries, testes, and adrenals [47]. Its presence indicates that other steroidogenic tissues may also be spontaneously differentiating with the EBs, and care should be taken in the future to isolate the trophoblast like cells from the EB culture before more extensive hormonal investigations can be undergone.

IV. Conclusion

Both differentiation paradigms, the BMP4-treatment of adherent colonies and the formation of embryoid bodies utilizing the hESC line BGO2, yielded trophoblast-like cells similar to those reported earlier [1,2] . We have confirmed the ability of the trophoblast-like cells to produce placental hormones, progesterone, estradiol, and hCG *in vitro*. In addition, studies were initiated to characterize their potential hormonal regulation by expression analysis at set time points, day 5, day 22, and day 50. Both culture conditions yield cells with steroidogenic capabilities as both up-regulate CYP11A1 and CYP19A1. Experiments to determine the exact

endocrine nature of hESCs in different differentiation protocols are necessary and will likely take some time to complete as they are complicated by a lack of *in vivo* knowledge as well as the necessity to characterize the cells in both a homogenous single cell culture and in their signaling niche.

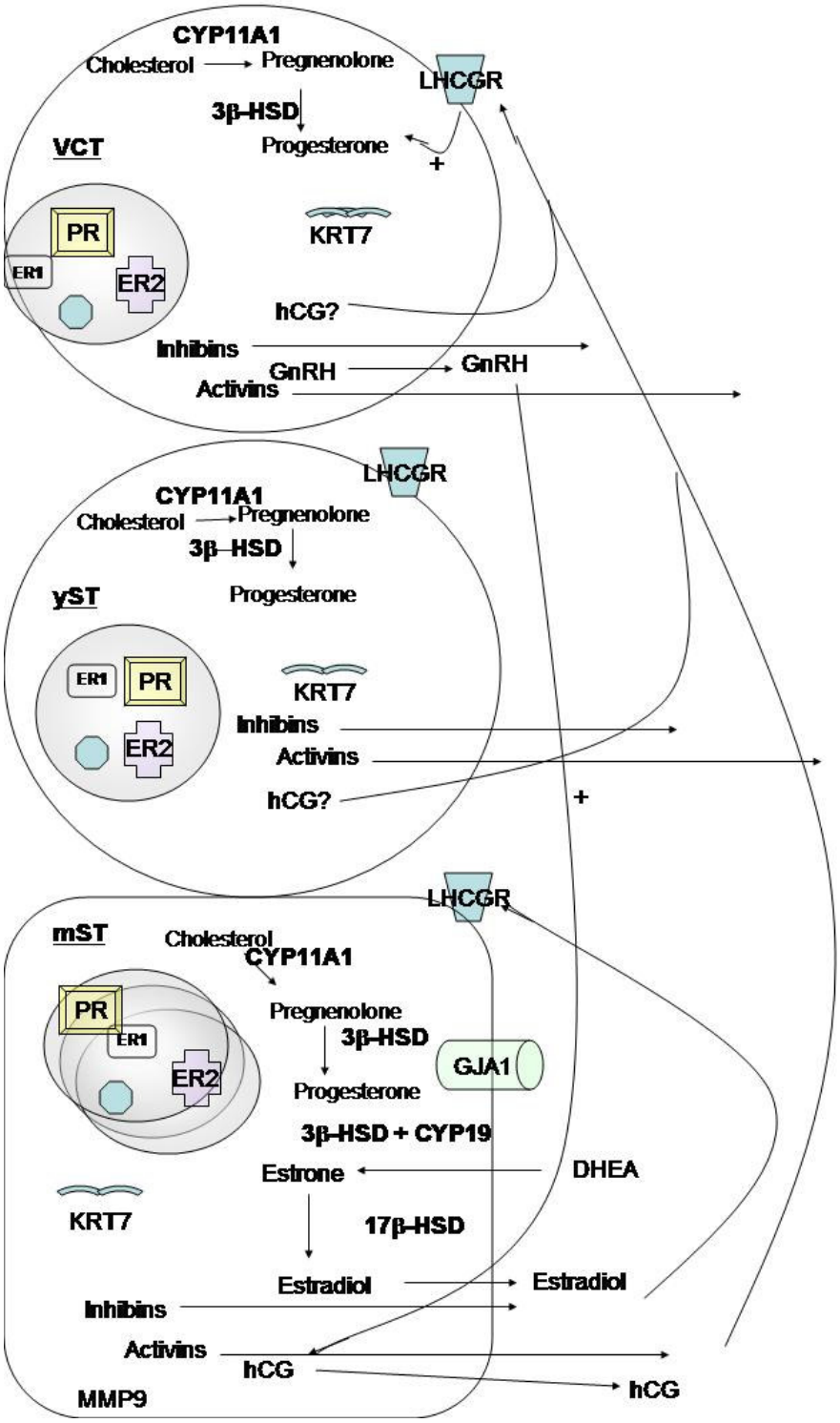

Figure 4.1: The maturation of a cytotrophoblast (VCT) to a young syncytiotrophoblast (yST) and finally to a mature syncytiotrophoblast (mST). Expected hormone receptors, steroid biosynthetic enzymes, gap junctions, and matrix-metalloproteinases are shown. [20,21,23,25-27,48]

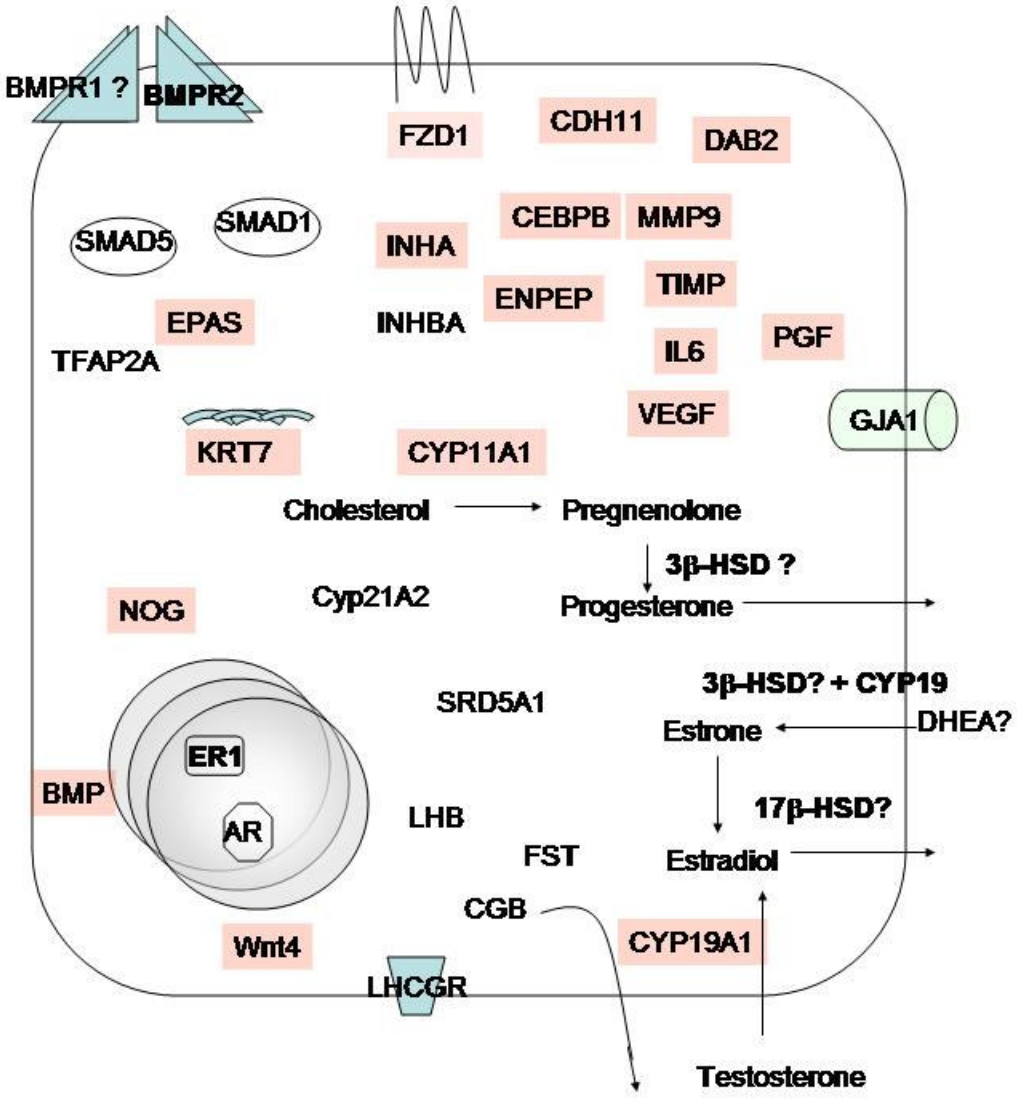

Figure 4.2: BMP4-treated cells are similar to syncytiotrophoblast in culture. Red indicates genes which are up-regulated upon BMP4 treatment. Genes which are present in all cell types, hESC control, BMP4-treated, and Noggin-treated, are shown. ? marks indicate unknowns.

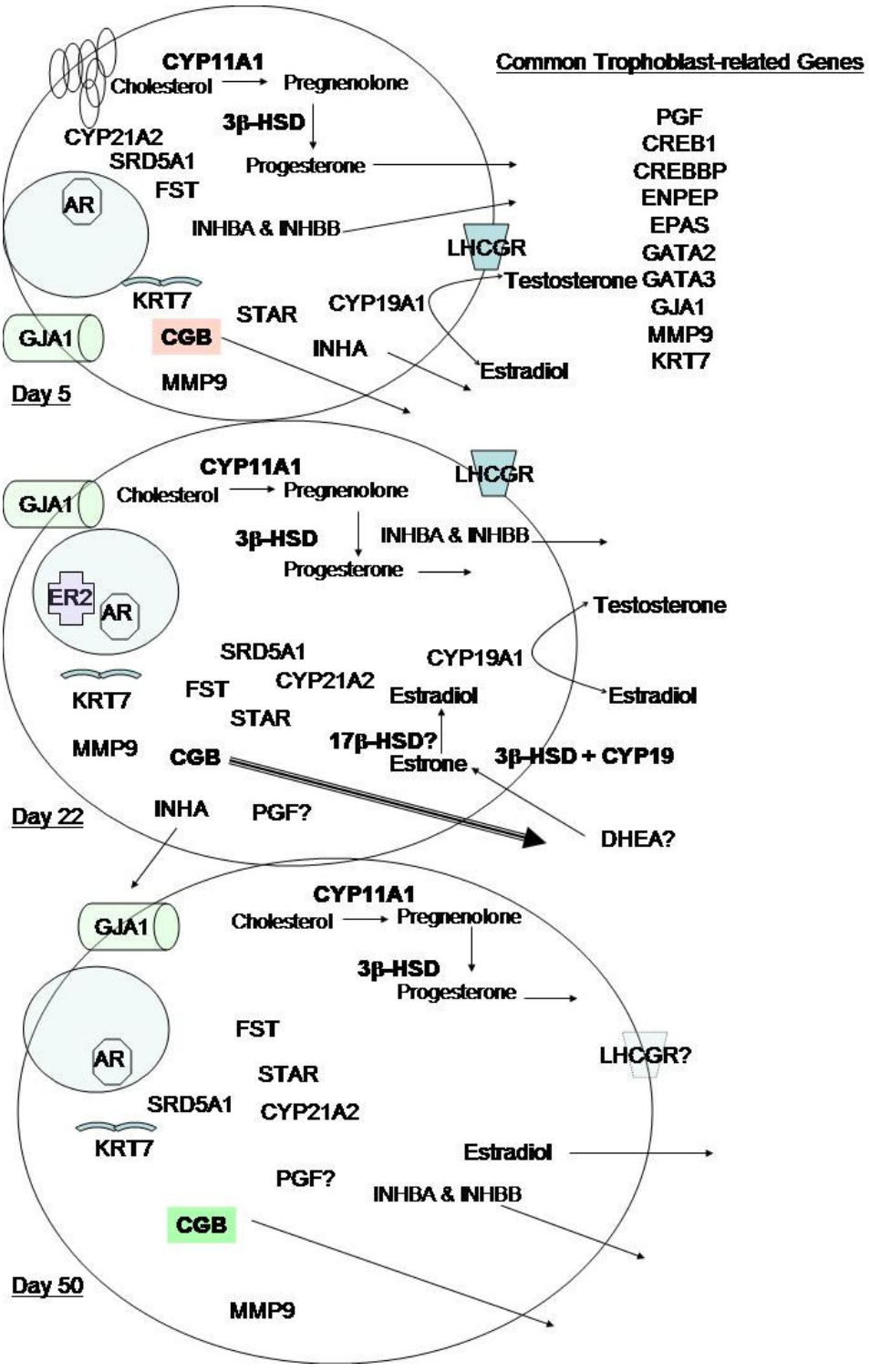

Figure 4.3: Embryoid bodies. Because of the nature of the experiment the above cannot be understood as a single cell but the product of multiple embryoid bodies over time. Up & down regulation over the long-term culture are indicated by red and green highlighting respectively.

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APPENDICES

Appendix 1: RT/RT-PCR data for BMP4 & Noggin-treated hESCs

	BMP 1 (delta Ct)	BMP 2 (delta Ct)	BMP 3 (delta Ct)	BMP 1 SD (of technical replicates)	BMP 2 SD (of technical replicates)	BMP 3 SD (of technical replicates
AFP						
AKT1	13.08432	13.64312	13.02131	0.462590572	0.677072319	0
AMH						
AMHR2						
AR	19.22353	17.74675		0.311588498	0.934272563	
BAPX1						
BMP4	15.87703	18.17859	16.46023	0.088547837	2.801116612	0
BMP6	19.74168	19.1655	24.23879	0.48740292	1.875136012	0
BMPR1B						
BMPR2	13.38585	14.63029	13.89825	0.140743153	0.447897399	0
CDH1	12.08533	12.46434	14.63475	0.182168671	0.235920542	0
CDH11	15.53357	17.19655	16.40523	0.279821215	1.032627265	0
CDH11	15.53357	17.19655	16.40523	0.279821215	1.032627265	0
CDH2	14.47337	13.36677		0.235641282	1.164219397	
CEBPB	14.71682	16.54321	14.04341	0.201321267	1.307144724	0
CEBPB	14.71682	16.54321	14.04341	0.201321267	1.307144724	0
CEBPG	14.14292	14.47128	14.19807	0.23464585	0.271808546	0
CGB	15.31808	19.1179	13.14535	0.180315492	0.739895399	0
CGB	15.31808	19.1179	13.14535	0.180315492	0.739895399	0
CHRD	18.20769	21.76992		0.739627131	4.640968251	
COL4A1	12.8903	13.90878	12.43103	0.099908636	0.217896179	0
CRABP1	17.31713	14.20066	18.37962	0.075750693	1.343884999	0
CREB1	15.6093	15.50141	16.49508	0.183823208	0.615444843	0
CREBBP	14.5252	14.61744	15.43398	1.129109488	1.129760997	0
CYP11A1	13.86163	17.3355	14.36375	0.162933408	2.023804725	0
CYP19A1	12.58313	22.125	13.31524	0.486803408	6.542979278	0
CYP21A2	21.78249	23.0755	24.23879	3.37175E-07	0.475121166	0
DAB2	13.53047	16.27588	13.28898	0.321507779	0.715519606	0
DAB2	13.53047	16.27588	13.28898	0.321507779	0.715519606	0
DCOHM	19.42626	18.99137	19.18781	0.375048611	0.150790711	0
DLX5						
EGF	18.70943	17.84662	24.23879	0.154749011	0.181532171	0
ENPEP	14.45178	18.00835	14.60863	0.435804742	2.497814233	0
EPAS1	11.30645	15.49542	12.88772	0.350712773	4.350219591	0
ESR1	21.89594	23.52801	24.23879	0.921984556	2.450853371	0
ESR2						
FASN	13.91234	13.21668	14.91053	0.142364118	0.343888921	0
FIGF						
FSHB						
FSHR						
FST	17.6144	19.74892		0.141079789	1.887507253	
FZD1	18.79096	17.83328		0.747911351	2.38419E-07	
GATA2	13.25337	17.16609	13.89403	0.379932152	3.736627919	0
GATA3	12.57324	13.58667	13.63763	0.038283302	2.38419E-07	0
GJA1	11.68	9.941006	13.51666	0.131912859	0.814416557	0

	BMP 1 (delta Ct)	BMP 2 (delta Ct)	BMP 3 (delta Ct)	BMP 1 SD (of technical replicates)	BMP 2 SD (of technical replicates)	BMP 3 SD (of technical replicates
GJA5						
GJB3						
GREM1	19.90377	18.41179		0.270826896	0.907761065	
HRAS	15.96985	16.13406	16.22999	0.261718986	0.15804363	0
HSPG2	14.31004	15.81708	15.25891	0.193688008	0.203036911	0
ID3	13.27978	14.43669	14.19206	0.370084709	2.246134368	0
IGF1						
IGF1R	13.84885	14.03226	14.9329	0.056779746	0.467835584	0
IGF1R	13.84885	14.03226	14.9329	0.056779746	0.467835584	0
IL6	14.93965	17.14058		1.043850271	0.92586802	0
IL6	14.93965	17.14058		1.043850271	0.92586802	0
INHA	17.24865	19.42311	16.38003	0.258437959	3.179634061	0
INHBA	17.76553	18.89654	17.42423	0.47403296	0.202405942	0
INHBB						
IPF1						
KRT7	11.60669	15.14098	11.7806	0.312536918	3.770739532	0
LAMA5	15.10293	15.99909		0.947437679	1.035825878	
LAMA5	15.10293	15.99909		0.947437679	1.035825878	
LAMR1						
LHB	18.71757	20.00555	24.23879	0	0.462095995	0
LHCGR	25.06904	22.31356	24.23879	0	1.190071855	0
LRP5	16.17098	16.58203	16.59132	0.142228886	0.109702826	0
MMP2	11.60972	15.44011	11.80148	0.441778118	1.383654386	0
MMP9	14.53692	17.68415	13.73831	0.692511054	2.226231504	0
NES	15.20353	15.40754	14.99257	1.176821356	1.183197216	0
NFKB1	18.02576	19.37536		0.44010768	0.935175918	
NFKB1	18.02576	19.37536		0.44010768	0.935175918	
NID	17.33404	17.11244	18.34671	0.398769653	0.31493395	0
NID2	16.14836	16.99367	19.33084	0.601774729	0.721828382	0
NOG	11.78938	14.22407	12.66322	0.429820356	3.162810185	0
NR0B1	19.82352	19.86817		0.966031855	0	
PAX8						
PGF	12.01082	15.98222	11.85341	0.254706613	2.431421074	0
PGR						
PIK3C2A	15.84879	16.19905	16.81175	0.483104008	0.184450707	0
POMC	23.21804	20.57651	24.23879	0	0.913491679	0
PTEN	19.86573	20.30085		0.067915919	0	
PTEN	19.86573	20.30085		0.067915919	0	
PTGS1						
PTGS2	16.94073	18.42794	17.33357	0.967940133	2.38419E-07	0
RUNX1	13.11083	13.7864	14.66431	0.381450746	0	0
RUNX2	17.22322	22.11545	17.38784	0.205187942	4.325540308	0
SF1	13.82276	13.81955	15.4041	0.452436078	1.079675516	0
SMAD1	16.82313	18.36129	18.0976	0.124675898	2.556773613	0
SMAD5	16.33659	16.32883	18.48595	0.205998565	0.541332583	0
SMAD7	15.21349	15.5306	15.42347	0.287032816	0.398259316	0

	BMP 1 (delta Ct)	BMP 2 (delta Ct)	BMP 3 (delta Ct)	BMP 1 SD (of technical replicates)	BMP 2 SD (of technical replicates)	BMP 3 SD (of technical replicates
SOX2	20.99111	15.04901	24.23879	0.865081634	3.41774909	0
SOX9	15.55875	18.21065		1.078099082	3.468781606	
SRD5A1	16.77984	16.67206		0.147095212	0.778125418	
SRY	22.43378	20.47333	24.23879	0.75715085	1.028822259	0
STAR						
STAT3	15.61441	15.20946	16.38605	0.487181956	0.462997436	0
T	18.42996	21.38686	18.97968	23.3832435	0.27376557	4.381159831
TFAP2A	13.81666	14.30909	15.43657	0.08205595	0	0
TIMP1	14.97106	15.7355	15.5503	0.128859035	0.201943797	0
TIMP1	14.97106	15.7355	15.5503	0.128859035	0.201943797	0
TSHB						
VEGF	13.25827	15.28509	13.19809	0.400206218	0.2840702	0
VEGF	13.25827	15.28509	13.19809	0.400206218	0.2840702	0
WNT4	16.96513	19.01547	18.90699	0.482030519	1.584577021	0
WT1						

	CON 1 (delta Ct)	CON 2 (delta Ct)	CON 3 (delta Ct)	Con 1 SD (of technical replicates	Con 2 SD (of technical replicates)	Con 3 SD (of technical replicates)
AFP						
AKT1	15.31429	15.6815	14.99092	0	0	0
AMH						
AMHR2						
AR	18.51054	18.72985	17.62782	0	0	0
BAPX1						
BMP4		17.25432	21.49106		0	0
BMP6	20.31463		20.19742	0		0
BMPR1B						
BMPR2	16.42863	16.15977	15.51563	0	0	0
CDH1	13.72654	13.43447	13.61486	0	0	0
CDH11	19.19929	18.63381	19.2866	0	0	0
CDH11	19.19929	18.63381	19.2866	0	0	0
CDH2	14.02526	14.37	12.67732	0	0	0
CEBPB	16.62622	16.94692	17.0572	0	0	0
CEBPB	16.62622	16.94692	17.0572	0	0	0
CEBPG		16.90746			0	
CGB		19.75992	20.06105		0	0
CGB		19.75992	20.06105		0	0
CHRD	21.61912	20.3008	22.45995	0	0	0
COL4A1	14.46692	15.21631	14.45029	0	0	0
CRABP1	13.85874	14.979	12.7352	0	0	0
CREB1	15.56944	16.92918	14.68138	0	0	0
CREBBP	16.31328	15.83677	15.36182	0	0	0
CYP11A1	21.32735	18.4467	21.08827	0	0	0
CYP19A1	28.64728	16.82814	28.64725	0	0	0

	CON 1 (delta Ct)	CON 2 (delta Ct)	CON 3 (delta Ct)	Con 1 SD (of technical replicates)	Con 2 SD (of technical replicates)	Con 3 SD (of technical replicates)
CYP21A2	21.80438			0		
DAB2	16.72982	16.95309		0	0	
DAB2	16.72982	16.95309		0	0	
DCOHM	19.00916	19.75958	18.80461	0	0	0
DLX5						
EGF		19.55348	18.81908		0	0
ENPEP	22.96654	16.28784	23.00205	0	0	0
EPAS1	19.84082	13.32557	19.0128	0	0	0
ESR1	23.43248		24.18014	0	0	0
ESR2						
FASN	13.29732	14.05278	13.39581	0	0	0
FIGF						
FSHB						
FSHR						
FST		19.60453	21.50925		0	0
FZD1	21.98153	19.8076		0	0	
GATA2		15.53608	21.3473		0	0
GATA3		14.84531	21.41731		0	0
GJA1	10.45165	10.87588	9.715879	0	0	0
GJA5						
GJB3						
GREM1	17.99512	17.46217	15.78989	0	0	0
HRAS	16.37021	17.36329	15.70766	0	0	0
HSPG2	16.087	16.21202	17.1879	0	0	0
ID3	15.64197	14.22987	15.75964	0	0	0
IGF1						
IGF1R	14.66679	15.31554	14.81451	0	0	0
IGF1R	14.66679	15.31554	14.81451	0	0	0
IL6	22.75139	17.74835	21.20912	0	0	0
IL6	22.75139	17.74835	21.20912	0	0	0
INHA	24.40909	18.13829	23.02097	0	0	0
INHBA	19.4512	18.36715	17.58719	0	0	0
INHBB						
IPF1						
KRT7	19.92003	15.17149	20.34282	0	0	0
LAMA5	15.85037	17.8256	17.01958	0	0	0
LAMA5	15.85037	17.8256	17.01958	0	0	0
LAMR1						
LHB	20.3827	19.61919	20.04677	0	0	0
LHCGR	23.04217	23.32775	22.78088	0	0	0
LRP5	16.98926	17.46422	16.42674	0	0	0
MMP2	15.45922	14.13085	17.39146	0	0	0
MMP9	17.20426	17.413	18.53577	0	0	0
NES	14.95932	15.86938	14.95954	0	0	0
NFKB1	20.22063	19.5611	19.51692	0	0	0
NFKB1	20.22063	19.5611	19.51692	0	0	0

	CON 1 (delta Ct)	CON 2 (delta Ct)	CON 3 (delta Ct)	Con 1 SD (of technical replicates	Con 2 SD (of technical replicates)	Con 3 SD (of technical replicates)
NID	17.76718	17.83281	17.97584	0	0	0
NID2	16.8453	18.78274	17.99875	0	0	0
NOG	19.69605	16.41598		0	0	
NR0B1	23.00656	22.2269	22.31388	0	0	0
PAX8						
PGF	18.16904	15.80388	17.62986	0	0	0
PGR						
PIK3C2A	16.56043	16.91078	15.86041	0	0	0
POMC	18.56787	20.10073	19.441	0	0	0
PTEN	17.49915	17.54647	19.95027	0	0	0
PTEN	17.49915	17.54647	19.95027	0	0	0
PTGS1						
PTGS2	18.37042	19.74247	21.31979	0	0	0
RUNX1		18.00712	19.71509	0	0	0
RUNX2	22.99807	19.80886	21.72338	0	0	0
SF1	15.02616	15.43841	14.50204	0	0	0
SMAD1	16.95284	17.98334	16.45981	0	0	0
SMAD5	17.17605	16.79144	16.73357	0	0	0
SMAD7	16.39055	16.77338	16.30945	0	0	0
SOX2	13.13486	16.01288	13.33747	0	0	0
SOX9	19.75742	17.43161	19.43874	0	0	0
SRD5A1	16.40507	17.65471	16.56301	0	0	0
SRY	20.5048		21.24606	0		0
STAR						
STAT3	14.99208	15.65032	14.28243	0	0	0
T	28.64728	28.64728	17.68968	0	0	0
TFAP2A		15.82311			0	
TIMP1	15.90589	16.20183	16.58489	0	0	0
TIMP1	15.90589	16.20183	16.58489	0	0	0
TSHB						
VEGF	16.99383	17.2251	16.61658	0	0	0
VEGF	16.99383	17.2251	16.61658	0	0	0
WNT4	20.0011	18.44395	20.47386	0	0	0
WT1						

	NOG 1 (delta Ct)	NOG 2 (delta Ct)	NOG 3 (delta Ct)	Nog 1 SD (of technical replicates)	Nog 2 SD (of technical replicates)	Nog 3 SD (of technical replicates)
AFP						
AKT1	14.22872	12.95563	13.9515	0.010768959	1.298974597	0.03478824
AMH						
AMHR2						
AR	18.38814	17.78044	18.75774	0.072893161	1.164753361	0.191238039
BAPX1						
BMP4	20.5586	20.33865	21.55349	0	1.238804307	0.104607786

	NOG 1 (delta Ct)	NOG 2 (delta Ct)	NOG 3 (delta Ct)	Nog 1 SD (of technical replicates)	Nog 2 SD (of technical replicates)	Nog 3 SD (of technical replicates)
BMP6	19.97384	19.43858	20.14396	0.144333526	1.292126068	0.302839846
BMPR1B						
BMPR2	15.88231	15.25262	16.19285	0.282980303	1.371746423	0.074926209
CDH1	13.26716	12.98871	14.01784	0.258401318	1.272407247	0.06733463
CDH11	19.50765	18.88287	20.16235	0	1.442668996	0.0944903
CDH11	19.50765	18.88287	20.16235	0	1.442668996	0.0944903
CDH2	13.83107	13.07129	13.7602	0.256982773	1.30828437	0.005709994
CEBPB	16.57744	16.54962	17.54694	0.129473833	1.028140741	0.044543728
CEBPB	16.57744	16.54962	17.54694	0.129473833	1.028140741	0.044543728
CEBPG	13.55029	13.79851	14.82725	0.084587011	0.971488823	0.052245003
CGB	20.08655	19.45696	19.89036	0	0.91917627	0.870104961
CGB	20.08655	19.45696	19.89036	0	0.91917627	0.870104961
CHRD	20.87741	20.9409	22.06533	0	1.508350672	0.455798985
COL4A1	14.20177	13.48866	14.37436	0.368832466	1.193295249	0.155869562
CRABP1	13.69127	13.0784	14.30367	0.303310625	1.217610933	0.014391033
CREB1	15.64993	15.41298	16.37705	0.286528988	1.312758835	0.149037776
CREBBP	15.1223	14.15451	15.16907	0.180664345	1.270574737	0.100552478
CYP11A1	19.99858	19.64702	20.4289	0.090375808	1.465141201	0.250437804
CYP19A1	22.89442	30.48005	30.16265	0	0	0.191486877
CYP21A2	24.27048	21.90129	24.25892	0	0.910088776	0.360974399
DAB2	18.17915	17.16464	18.2739	0	1.238076845	0.075127704
DAB2	18.17915	17.16464	18.2739	0	1.238076845	0.075127704
DCOHM	19.86263	19.06037	20.12906	1.237384129	1.19787075	0.119032305
DLX5						
EGF	18.98539	18.50397	19.12404	0.595874496	1.230177539	0.012078168
ENPEP	24.37603	22.89265	23.43086	0	0.905296769	0.135364389
EPAS1	20.22463	20.15941	20.46564	0	1.298731533	0.1383516
ESR1	25.46598	24.4116	24.27589	0.978983984	0	0
ESR2						
FASN	13.11935	12.69689	13.95412	0.196899891	1.180363758	0.009016768
FIGF						
FSHB						
FSHR						
FST	21.52975	22.13054	22.07253	0.204974287	0	0
FZD1	21.05432	21.27681		0.390763941	1.26783521	
GATA2	20.34871	21.08856	22.34385	0	0.963173824	0.186015329
GATA3	20.80998	22.07987	22.09256	0	0.761712067	0
GJA1	10.09861	9.669173	10.639	0.400255271	1.066188123	0.074063647
GJA5						
GJB3						
GREM1	17.27939	18.44391	19.05661	0	1.498105592	0.039394918
HRAS	16.31425	15.42024	16.40722	0	1.279412238	0.185661413
HSPG2	16.31949	15.41758	16.59477	0.257017372	1.241744174	0.006715738
ID3	14.71402	14.40409	15.61891	0.357799628	1.041384578	0.243709941
IGF1						
IGF1R	14.79959	15.17384	15.27145	0.126781852	1.154341427	0.074702774

	NOG 1 (delta Ct)	NOG 2 (delta Ct)	NOG 3 (delta Ct)	Nog 1 SD (of technical replicates)	Nog 2 SD (of technical replicates)	Nog 3 SD (of technical replicates)
IGF1R	14.79959	15.17384	15.27145	0.126781852	1.154341427	0.074702774
IL6	19.52489	18.45446	19.51816	0.740385902	0	0.233821663
IL6	19.52489	18.45446	19.51816	0.740385902	0	0.233821663
INHA	24.66259	22.01211	23.05234	0	1.20115645	0
INHBA	19.28466	18.27225	18.81303	0.293397595	1.284266599	0.110620889
INHBB						
IPF1						
KRT7	19.51963	18.9832	20.0463	0	1.169644095	0.21916274
LAMA5	16.48483	16.6949	17.71251	0.823958487	1.430541754	0.204559819
LAMA5	16.48483	16.6949	17.71251	0.823958487	1.430541754	0.204559819
LAMR1						
LHB	23.69298	21.59766	20.43696	2.030081025	0	0.041237243
LHCGR	24.27506	21.19572	22.86242	2.354145723	0	0.269049844
LRP5	17.09969	16.38574	17.49357	0.32650334	1.262158703	0.089365161
MMP2	17.75949	16.93269	17.79963	0.133164528	1.074176342	0.067241676
MMP9	18.93204	17.88635	18.82978	0.483489815	1.07693088	0.170367982
NES	15.85	15.45853	16.40855	0.778327643	1.31339392	0.003484309
NFKB1	20.76708	20.13691	20.91991	0.244012926	1.30691836	0.000482087
NFKB1	20.76708	20.13691	20.91991	0.244012926	1.30691836	0.000482087
NID	17.5458	16.81099	17.98372	0.479057056	1.262639058	0.014601188
NID2	17.68503	17.4327	18.50288	0.841443329	1.110968565	0.190500185
NOG	22.31068	21.39344	20.48069	0	0	0.010302816
NR0B1		0	23.1749	0	0	0.595790837
PAX8						
PGF	18.01082	17.42536	18.70379	0.068411861	1.218005841	0.046432241
PGR						
PIK3C2A	16.89294	16.71477	17.84856	0.466888142	1.21954217	0.034220705
POMC	20.00695	18.37446	19.62493	0.565119681	1.484492827	0.030323013
PTEN	17.93685	18.22043	19.7241	0.249522126	1.01345295	0.447603498
PTEN	17.93685	18.22043	19.7241	0.249522126	1.01345295	0.447603498
PTGS1						
PTGS2	24.21981	19.56544	21.88955	3.626898803	0	0.022982005
RUNX1	22.93785	20.75603	20.61191	3.898218788	0.952478988	0
RUNX2	20.78841	21.9308	22.51976	1.552765074	1.228743401	0.090354162
SF1	14.2488	13.31242	14.41524	0.059480245	1.339610241	0.00090413
SMAD1	17.20117	16.48893	17.69666	0.240321199	1.135917025	0.188530266
SMAD5	16.96367	16.25143	17.19206	0.277692906	1.155153758	0.001191651
SMAD7	15.63863	15.59803	16.83592	0.450325716	1.212597223	0.049803389
SOX2	13.2301	11.92494	13.1762	0.012248486	1.442904555	0.167507788
SOX9	20.08704	20.66895	22.01845	0	0.903621299	0.116620136
SRD5A1	16.71209	16.27891	17.19777	0.502125578	1.44240457	0.082438113
SRY	20.80303	20.67375	21.60649	0	1.271847217	0
STAR						
STAT3	15.41887	15.20159	16.46694	0.447219067	1.3304679	0.143692667
T	23.35674	21.3675		0.944145514		0.403548786
TFAP2A		25.49456	24.64086	0.63458419		0.101635009

	NOG 1 (delta Ct)	NOG 2 (delta Ct)	NOG 3 (delta Ct)	Nog 1 SD (of technical replicates)	Nog 2 SD (of technical replicates)	Nog 3 SD (of technical replicates)
TIMP1	16.19342	15.90539	16.92109	0.349952447	1.145971002	0.107140044
TIMP1	16.19342	15.90539	16.92109	0.349952447	1.145971002	0.107140044
TSHB						
VEGF	16.65297	15.92692	17.03849	0.36907569	1.270980037	0.04918216
VEGF	16.65297	15.92692	17.03849	0.36907569	1.270980037	0.04918216
WNT4	23.20305	19.84987	20.70131	3.59199336	1.524816125	0.080235522
WT1						

	BMP AVG (delta Ct)	BMP SD (delta Ct all biological reps)	NOG AVG (delta Ct)	NOG SD (delta Ct all biological reps)	CON AVG (delta Ct)	CON SD (delta Ct all biological reps)
AFP						
AKT1	13.24958205	0.342263934	13.71195272	0.669500307	15.32890317	0.345520187
AMH						
AMHR2						
AR	18.485139	1.044241354	18.30877389	0.493461239	18.289404	0.58334444
BAPX1						
BMP4	16.83861733	1.196530023	20.816912	0.647303544	19.372689	2.995823341
BMP6	21.0486578	2.777717572	19.85212817	0.368101758	20.2560245	0.08287645
BMPR1B						
BMPR2	13.97146405	0.62543965	15.77592525	0.47905729	16.034674	0.469178105
CDH1	13.06147181	1.375611851	13.42456528	0.532316461	13.5919545	0.147377756
CDH11	16.37845081	0.831812811	19.51762033	0.639798326	19.039901	0.35438589
CDH11	16.37845081	0.831812811	19.51762033	0.639798326	19.039901	0.35438589
CDH2	13.92006993	0.782483051	13.55418539	0.419702482	13.69086067	0.89451242
CEBPB	15.10114671	1.293456828	16.89133167	0.567943826	16.87677767	0.223890079
CEBPB	15.10114671	1.293456828	16.89133167	0.567943826	16.87677767	0.223890079
CEBPG	14.27075707	0.175830429	14.05868461	0.677071323	16.907455	#DIV/0!
CGB	15.8604405	3.022987163	19.811289	0.322156334	19.9104825	0.212930358
CGB	15.8604405	3.022987163	19.811289	0.322156334	19.9104825	0.212930358
CHRD	19.988803	2.518879818	21.29454383	0.668271718	21.459956	1.088339956
COL4A1	13.07670181	0.756305769	14.02159444	0.469531101	14.71117233	0.43754279
CRABP1	16.63246748	2.171976787	13.69111222	0.612634515	13.85764567	1.121898899
CREB1	15.86859786	0.545220416	15.81331822	0.502375166	15.726666	1.132115438
CREBBP	14.8588736	0.50018806	14.81529444	0.572732894	15.83728767	0.475727215
CYP11A1	15.18695843	1.877550535	20.02483289	0.39160092	20.28743733	1.598600131
CYP19A1	16.00778729	5.310289512	27.8457065	4.290872974	24.707554	6.823776155
CYP21A2	23.03226011	1.228723813	23.47689433	1.364529959	21.804377	#DIV/0!
DAB2	14.36510952	1.659174827	17.87256583	0.614905898	16.8414545	0.157872196
DAB2	14.36510952	1.659174827	17.87256583	0.614905898	16.8414545	0.157872196
DCOHM	19.20181181	0.217787718	19.6840197	0.556279091	19.191115	0.502819332
DLX5						
EGF	20.26494843	3.468383755	18.87113244	0.325443761	19.186282	0.519296392
ENPEP	15.68958534	2.009641224	23.566512	0.750939778	20.75214267	3.866242006

	BMP AVG (delta Ct)	BMP SD (delta Ct all biological reps)	NOG AVG (delta Ct)	NOG SD (delta Ct all biological reps)	CON AVG (delta Ct)	CON SD (delta Ct all biological reps)
EPAS1	13.22986216	2.115338735	20.28322717	0.161305613	17.393065	3.546797171
ESR1	23.22091229	1.20123672	24.71782533	0.651464289	23.8063125	0.528676163
ESR2						
FASN	14.01318648	0.851416784	13.25678403	0.639784287	13.581968	0.410694537
FIGF						
FSHB						
FSHR						
FST	18.68166284	1.509334354	21.91093989	0.33138882	20.5568875	1.346836893
FZD1	18.31211564	0.677182931	21.16556742	0.157324306	20.8945635	1.537198524
GATA2	14.77116469	2.098658872	21.260374	1.008605685	18.4416915	4.109150948
GATA3	13.26584986	0.600357317	21.66080167	0.736864791	18.131308	4.647108594
GJA1	11.71255514	1.788048853	10.13559656	0.485971561	10.34780467	0.586932426
GJA5						
GJB3						
GREM1	19.1577825	1.054991297	18.25997083	0.902774906	17.08238933	1.150623649
HRAS	16.11129862	0.131554878	16.04723867	0.544979328	16.48038367	0.833293788
HSPG2	15.12867824	0.761913495	16.110612	0.615766061	16.49564	0.60276422
ID3	13.96950705	0.609717937	14.91234139	0.631221355	15.21049267	0.851279931
IGF1						
IGF1R	14.27133862	0.580221024	15.08162428	0.249079729	14.9322775	0.340031379
IGF1R	14.27133862	0.580221024	15.08162428	0.249079729	14.9322775	0.340031379
IL6	16.04011506	1.556293013	19.16583883	0.616077866	20.569617	2.562092707
IL6	16.04011506	1.556293013	19.16583883	0.616077866	20.569617	2.562092707
INHA	17.68392827	1.56754	23.24234533	1.335419821	21.85611567	3.293685937
INHBA	18.02876509	0.770643795	18.78997644	0.506598426	18.46851233	0.936128338
INHBB						
IPF1						
KRT7	12.84275755	1.992219056	19.51637667	0.531557976	18.47811	2.871412467
LAMA5	15.55100693	0.633679904	16.9640824	0.656615637	16.89851633	0.993162772
LAMA5	15.55100693	0.633679904	16.9640824	0.656615637	16.89851633	0.993162772
LAMR1						
LHB	20.9873046	2.888571991	21.90920039	1.650216091	20.016224	0.38267069
LHCGR	23.87379513	1.413537451	22.777732	1.541413371	23.05026867	0.273523414
LRP5	16.44811205	0.240044854	16.99300167	0.561566737	16.96007167	0.519355851
MMP2	12.95043481	2.158248636	17.49726678	0.489353031	15.660508	1.639597728
MMP9	15.319794	2.086165441	18.54938794	0.576479346	17.717676	0.716136323
NES	15.20121295	0.207497693	15.90569478	0.477452252	15.262745	0.525362198
NFKB1	18.70056121	0.954312423	20.60796756	0.415043565	19.766217	0.39415546
NFKB1	18.70056121	0.954312423	20.60796756	0.415043565	19.766217	0.39415546
NID	17.59772852	0.658028157	17.4468355	0.592592255	17.858608	0.10669573
NID2	17.4909569	1.648486436	17.87353444	0.559437093	17.87559467	0.974572098
NOG	12.89222552	1.233391273	21.39493933	0.914994416	18.0560135	2.319361861
NR0B1	19.84584586	0.031573934	11.587449	16.38712753	22.51577767	0.427249018
PAX8						
PGF	13.28214986	2.339650616	18.04665706	0.639971047	17.20092733	1.239547124

	BMP AVG (delta Ct)	BMP SD (delta Ct all biological reps)	NOG AVG (delta Ct)	NOG SD (delta Ct all biological reps)	CON AVG (delta Ct)	CON SD (delta Ct all biological reps)
PGR						
PIK3C2A	16.28653024	0.487405803	17.15209217	0.609705839	16.443875	0.534799364
POMC	22.67778122	1.889970702	19.33544667	0.853880256	19.369867	0.768900514
PTEN	20.08328926	0.307672828	18.62713022	0.960530164	18.331965	1.401696396
PTEN	20.08328926	0.307672828	18.62713022	0.960530164	18.331965	1.401696396
PTGS1						
PTGS2	17.56740971	0.770688525	21.89160267	2.327186178	19.810896	1.475873005
RUNX1	13.85384576	0.778931431	21.43526117	1.303272455	18.861105	1.207711512
RUNX2	18.90884095	2.778227905	21.74632133	0.8802963	21.510104	1.605269145
SF1	14.34880302	0.913914108	13.99215028	0.594519367	14.98887067	0.469294982
SMAD1	17.76067433	0.822571761	17.12891989	0.607098213	17.131996	0.777405397
SMAD5	17.05045681	1.243179619	16.80238583	0.490615929	16.90035367	0.240508834
SMAD7	15.38918686	0.161307895	16.02419267	0.703268654	16.49112833	0.247777815
SOX2	20.09297019	4.660260598	12.77707817	0.738469547	14.16173533	1.606333954
SOX9	16.88470214	1.875179908	20.92481567	0.990801171	18.87592133	1.260921886
SRD5A1	16.72594957	0.076206716	16.72958906	0.459679992	16.874262	0.680488179
SRY	22.38196767	1.883265019	21.02775833	0.505348349	20.8754275	0.52415068
STAR						
STAT3	15.73663719	0.597743104	15.69579706	0.676606865	14.97494333	0.684104514
T	19.59883119	1.57268039	22.3621205	1.406600144	24.99474267	6.32637331
TFAP2A	14.52077567	0.830442297	25.06771192	0.60365482	15.823109	#DIV/0!
TIMP1	15.41895305	0.398783133	16.33996511	0.523470696	16.23086633	0.340429624
TIMP1	15.41895305	0.398783133	16.33996511	0.523470696	16.23086633	0.340429624
TSHB						
VEGF	13.91381898	1.187938443	16.53945944	0.564410702	16.94516867	0.307166344
VEGF	13.91381898	1.187938443	16.53945944	0.564410702	16.94516867	0.307166344
WNT4	18.29586433	1.153723408	21.25140767	1.742959999	19.63963667	1.062128683
WT1						

	BMP (delta delta Ct)	NOG (delta delta Ct)	BMP SD (delta delta Ct)	Nog SD (delta delta Ct)
AFP				
AKT1	14.98663923	1.616950444	0.342263934	0.669500307
AMH	0	0		
AMHR2	0	0		
AR	17.24516265	-0.019369889	1.044241354	0.493461239
BAPX1	0	0		
BMP4	18.17615898	-1.444223	1.196530023	0.647303544
BMP6	17.47830693	0.403896333	2.777717572	0.368101758
BMPR1B	0	0		
BMPR2	15.40923435	0.25874875	0.62543965	0.47905729
CDH1	12.21634265	0.167389222	1.375611851	0.532316461
CDH11	18.20808819	-0.477719333	0.831812811	0.639798326
CDH11	18.20808819	-0.477719333	0.831812811	0.639798326

	BMP (delta delta Ct)	NOG (delta delta Ct)	BMP SD (delta delta Ct)	Nog SD (delta delta Ct)
CDH2	12.90837762	0.136675278	0.782483051	0.419702482
CEBPB	15.58332084	-0.014554	1.293456828	0.567943826
CEBPB	15.58332084	-0.014554	1.293456828	0.567943826
CEBPG	16.73162457	2.848770389	0.175830429	0.677071323
CGB	16.88749534	0.0991935	3.022987163	0.322156334
CGB	16.88749534	0.0991935	3.022987163	0.322156334
CHRD	18.94107618	0.165412167	2.518879818	0.668271718
COL4A1	13.95486656	0.689577889	0.756305769	0.469531101
CRABP1	11.68566888	0.166533444	2.171976787	0.612634515
CREB1	15.18144558	-0.086652222	0.545220416	0.502375166
CREBBP	15.33709961	1.021993222	0.50018806	0.572732894
CYP11A1	18.4098868	0.262604444	1.877550535	0.39160092
CYP19A1	19.39726449	-3.1381525	5.310289512	4.290872974
CYP21A2	20.57565319	-1.672517333	1.228723813	1.364529959
DAB2	15.18227967	-1.031111333	1.659174827	0.614905898
DAB2	15.18227967	-1.031111333	1.659174827	0.614905898
DCOHM	18.97332728	-0.4929047	0.217787718	0.556279091
DLX5	0	0		
EGF	15.71789825	0.315149556	3.468383755	0.325443761
ENPEP	18.74250144	-2.814369333	2.009641224	0.750939778
EPAS1	15.27772626	-2.890162167	2.115338735	0.161305613
ESR1	22.60507578	-0.911512833	1.20123672	0.651464289
ESR2	0	0		
FASN	12.73055122	0.325183972	0.851416784	0.639784287
FIGF	0	0		
FSHB	0	0		
FSHR	0	0		
FST	19.04755315	-1.354052389	1.509334354	0.33138882
FZD1	20.21738057	-0.271003917	0.677182931	0.157324306
GATA2	16.34303263	-2.8186825	2.098658872	1.008605685
GATA3	17.53095068	-3.529493667	0.600357317	0.736864791
GJA1	8.559755814	0.212208111	1.788048853	0.485971561
GJA5	0	0		
GJB3	0	0		
GREM1	16.02739804	-1.1775815	1.054991297	0.902774906
HRAS	16.34882879	0.433145	0.131554878	0.544979328
HSPG2	15.73372651	0.385028	0.761913495	0.615766061
ID3	14.60077473	0.298151278	0.609717937	0.631221355
IGF1	0	0		
IGF1R	14.35205648	-0.149346778	0.580221024	0.249079729
IGF1R	14.35205648	-0.149346778	0.580221024	0.249079729
IL6	19.01332399	1.403778167	1.556293013	0.616077866
IL6	19.01332399	1.403778167	1.556293013	0.616077866
INHA	20.28857567	-1.386229667	1.56754	1.335419821
INHBA	17.69786854	-0.321464111	0.770643795	0.506598426
INHBB	0	0		
IPF1	0	0		

	BMP (delta delta Ct)	NOG (delta delta Ct)	BMP SD (delta delta Ct)	NOG SD (delta delta Ct)
KRT7	16.48589094	-1.038266667	1.992219056	0.531557976
LAMA5	16.26483643	-0.065566067	0.633679904	0.656615637
LAMA5	16.26483643	-0.065566067	0.633679904	0.656615637
LAMR1	0	0		
LHB	17.12765201	-1.892976389	2.888571991	1.650216091
LHCGR	21.63673122	0.272536667	1.413537451	1.541413371
LRP5	16.72002681	-0.03293	0.240044854	0.561566737
MMP2	13.50225936	-1.836758778	2.158248636	0.489353031
MMP9	15.63151056	-0.831711944	2.086165441	0.576479346
NES	15.05524731	-0.642949778	0.207497693	0.477452252
NFKB1	18.81190458	-0.841750556	0.954312423	0.415043565
NFKB1	18.81190458	-0.841750556	0.954312423	0.415043565
NID	17.20057984	0.4117725	0.658028157	0.592592255
NID2	16.22710823	0.002060222	1.648486436	0.559437093
NOG	16.82262223	-3.338925833	1.233391273	0.914994416
NR0B1	22.48420373	10.92832867	0.031573934	16.38712753
PAX8	0	0		
PGF	14.86127672	-0.845729722	2.339650616	0.639971047
PGR	0	0		
PIK3C2A	15.9564692	-0.708217167	0.487405803	0.609705839
POMC	17.4798963	0.034420333	1.889970702	0.853880256
PTEN	18.02429217	-0.295165222	0.307672828	0.960530164
PTEN	18.02429217	-0.295165222	0.307672828	0.960530164
PTGS1	0	0		
PTGS2	19.04020747	-2.080706667	0.770688525	2.327186178
RUNX1	18.08217357	-2.574156167	0.778931431	1.303272455
RUNX2	18.73187609	-0.236217333	2.778227905	0.8802963
SF1	14.07495656	0.996720389	0.913914108	0.594519367
SMAD1	16.30942424	0.003076111	0.822571761	0.607098213
SMAD5	15.65717405	0.097967833	1.243179619	0.490615929
SMAD7	16.32982044	0.466935667	0.161307895	0.703268654
SOX2	9.501474735	1.384657167	4.660260598	0.738469547
SOX9	17.00074143	-2.048894333	1.875179908	0.990801171
SRD5A1	16.79805528	0.144672944	0.076206716	0.459679992
SRY	18.99216248	-0.152330833	1.883265019	0.505348349
STAR	0	0		
STAT3	14.37720023	-0.720853722	0.597743104	0.676606865
T	23.42206228	2.632622167	1.57268039	1.406600144
TFAP2A	14.9926667	-9.244602917	0.830442297	0.60365482
TIMP1	15.8320832	-0.109098778	0.398783133	0.523470696
TIMP1	15.8320832	-0.109098778	0.398783133	0.523470696
TSHB	0	0		
VEGF	15.75723022	0.405709222	1.187938443	0.564410702
VEGF	15.75723022	0.405709222	1.187938443	0.564410702
WNT4	18.48591326	-1.611771	1.153723408	1.742959999
WT1	0	0		

Appendix 2: RT/RT-PCR data for EBs at day 5, day 22, and day 50

	Day 22a (delta Ct)	Day 22a SD (of technical replicates)	Day 22b (delta Ct)	Day 22b SD (of technical replicates)	Day 22c (delta Ct)	Day 22c SD (of technical replicates)
AFP	11.618757	0	13.9213115	0.759041822	11.37468183	1.355393134
AKT1	15.375555	0.188577609	17.610209	0.357759713	16.91375867	0.998163201
AMH						
AMHR2	19.362246	0.541787802	20.435233	0.264374462	20.34606	0
AR	18.5464475	0.262604883			26.96082	0
BAPX1	21.717624	0.201788538	27.642984	0	23.9787915	3.569224221
BMP4	17.669434	0.285776836	19.7619615	0.285776836	19.779472	0.328314272
BMP6	20.633265	0.216500577	22.083271	0	20.8242735	1.183854995
BMPR1B	16.2181365	0.03664038	27.642984	0	29.130383	0
BMPR2	16.709388	0.262311012	20.115843	0.74529338	18.06050133	1.284452316
CDH1	15.9007405	0.15478703	18.5775455	0.765953859	18.271507	0
CDH11	15.25347225	0.288266659	18.476188	0.439412052	15.62503767	1.107599759
CDH11	15.25347225	0.288266659	18.476188	0.439412052	15.62503767	1.107599759
CDH2	13.705883	0.121457176	17.897098	0.749102737	16.29845567	1.532200637
CEBPB	17.3590535	0.15337945	18.781362	0.429031294	18.168749	0.481666009
CEBPB	17.3590535	0.15337945	18.781362	0.429031294	18.168749	0.481666009
CEBPG	17.1849675	0.129731183	19.3783275	0.638314416	18.53117033	0.632985413
CGB	17.0881255	0.097943432	15.7250685	0.371218325	14.72372433	0.869135331
CGB	17.0881255	0.097943432	15.7250685	0.371218325	14.72372433	0.869135331
CHRD	19.74378	0.000713605	21.900006	0.203497495	20.923355	0
COL4A1	14.064677	0.187292427	17.4216355	0.478163225	14.85634633	1.034305928
CRABP1	12.70859425	0.087188262	14.338354	0.349175669	15.013515	0.911574876
CREB1	16.6892575	0.317208362	19.391374	0.899326967	18.747362	0.450360923
CREBBP	11.8945305	0.201801817	15.990075	0.58355332	16.281036	0
CYP11A1	20.2478085	0.182328947	20.5871785	0.51379382	18.95685	0.576726731
CYP19A1	21.3975015	0.078875862			19.83036	0
CYP21A2	20.873721	0.345390561	27.788426	0.167941956	25.99224567	3.313194329
DAB2	16.7126295	0.196567559	19.068534	0.051086261	17.08707725	1.320226572
DAB2	16.7126295	0.196567559	19.068534	0.051086261	17.08707725	1.320226572
DCOHM	19.292278	0.161710037	21.3709495	0.086424139	21.9378565	0.372673248
DLX5	17.067644	0.582484068	19.405222	0	16.934626	0
EGF	19.819684	0.238144284	24.7455395	3.345680724	19.39536	0
ENPEP	21.2190455	0.379246958	27.642984	0	20.20281	0
EPAS1	16.814612	0.118786354	19.9176205	0.582309131	18.6896775	0.085538484
ESR1	30.4126	0	27.788426	0.167941956	28.035315	1.264475609
ESR2	21.5847475	0.347189007	27.788426	0.167941956	24.574038	2.732262806
FASN	16.063945	0.043884394	19.391026	19.6651385	17.135975	0
FIGF	19.796919	0	21.266525	0	20.372978	0.387044073
FSHB						
FSHR	20.391843	0.257468198	21.88597	0.531635675	24.446053	0
FST	18.342574	0.240159237	20.715264	0.68030106	19.964891	0.198976842
FZD1	17.0271065	0.196818129	19.369812	0	21.173117	0.120447968
FZD1	17.0271065	0.196818129	19.369812	0	21.173117	0.120447968
GATA2	18.8572265	0.268526188	21.46758	0.906300204	20.568586	0.469274918
GATA3	17.245101	0.224697797	20.190636	0	19.3526685	0.364534919

	Day 22a (delta Ct)	Day 22a SD (of technical replicates)	Day 22b (delta Ct)	Day 22b SD (of technical replicates)	Day 22c (delta Ct)	Day 22c SD (of technical replicates)
GJA1	11.96224	0.056787018	15.3800525	0.421144112	13.274959	0
GJA5	21.4000425	0.109003153	23.37182	0	22.654789	0
GJB3	20.9486315	0.197426657	20.557316	0	24.7155965	2.56880513
HRAS	17.113086	0.205774564	17.5953275	0.251870787	17.889309	0.827744572
HSPG2	15.70469	0.247429231	20.251331	0	18.16744367	1.767545379
ID3	13.63608	0.116710202	14.886996	0.356063458	15.082188	0.656668959
IGF1	20.533491	0	23.971544	0	19.434702	0
IGF1R	15.789217	0.272877677	19.4879485	0.443688486	18.25602533	1.654186655
IGF1R	15.789217	0.272877677	19.4879485	0.443688486	18.25602533	1.654186655
IL6	23.335128	0.334479796			20.59986433	0.784971286
IL6	23.335128	0.334479796			20.59986433	0.784971286
INHA	20.361771	0.265334018	20.498796	0	18.95756967	0.696903566
INHBA	19.5278855	0.286135371	22.18615	0	18.43318667	0.965478294
INHBB	19.502699	0.20635076			26.940247	0
IPF1	21.386745	0	27.788426	0.167941956	17.85864	0
KRT7	18.663697	0.226709285	18.8446905	0.373530036	17.550301	0.441042149
LAMA5	17.048117	0	21.41743	0.537543123	19.3344725	2.108994138
LAMA5	17.048117	0	21.41743	0.537543123	19.3344725	2.108994138
LAMR1						
LHB	20.5932815	0.237575594	20.650627	0	28.035315	1.264475609
LHCGR	22.842906	0.536067416	27.933868	0	24.4465085	2.879521189
LRP5	16.370296	0	19.402228	0.213866706	18.701955	1.459677159
MMP2	13.6238845	0.157924351	16.6359535	0.577815036	14.645704	1.208762019
MMP2	13.6238845	0.157924351	16.6359535	0.577815036	14.645704	1.208762019
MMP9	17.7627205	0.171127197	19.641105	0.406229423	18.26094567	0.634656089
NES	16.0117235	0.152848288	19.341718	0.396048428	19.124378	0.564540992
NFKB1	18.792344	0.214150762	21.452488	0	19.657332	1.210771777
NFKB1	18.792344	0.214150762	21.452488	0	19.657332	1.210771777
NID	16.840169	0.206790701	20.6641645	0.94922331	19.352009	0
NID2	14.566761	0.256389708	19.929921	0.897930934	16.930498	1.987392047
NOG	17.8897965	0.297369452	19.176622	0.009916568	20.6753905	0.490854539
NR0B1	21.103804	0.077300273	20.92062	0	21.373232	0.015507628
PAX8	20.247631	0.528403668			21.256514	0
PGF	16.99198	0	18.115146	0.271384649	16.65793033	0.610518231
PGR	23.3678965	0.001624086	27.788426	0.167941956	23.137197	0
PIK3C2A	17.8976985	0.268356447	21.99043	0	21.1423	0
POMC	20.736768	0.616353744	20.284775	0	22.14433	0.309429722
PTEN	17.741258	0.162803538	18.8897235	0.222720372	18.91584933	0.267831799
PTEN	17.741258	0.162803538	18.8897235	0.222720372	18.91584933	0.267831799
PTGS1	19.190629	0.337803024			21.703383	1.630405983
PTGS2	19.3349535	0.23855478	22.4806795	1.033555709	18.346458	1.392898121
RUNX1	18.056328	0.093400262			20.128422	0
RUNX2	18.010815	0.201576073				
SF1	14.756254	0.229198819	17.912378	0.536442693	16.7756375	1.764105872
SMAD1	16.864331	0.157747105	20.670404	0	18.86671733	1.042663411
SMAD5	16.3656515	0.148602454	20.3343135	0.510785825	19.1034775	0.173383482

	Day 22a (delta Ct)	Day 22a SD (of technical replicates)	Day 22b (delta Ct)	Day 22b SD (of technical replicates)	Day 22c (delta Ct)	Day 22c SD (of technical replicates)
SMAD7	17.1350035	0.175894956	20.0103635	0.665119634	20.071129	0
SOX2	16.067767	0.193622496	18.8525705	0.769163926	20.865864	0
SOX9	18.3274335	0.183033314			21.853178	0.097507532
SRD5A1	17.7963925	0.106533249	19.743025	1.116691261	19.034362	0.969936905
SRY	20.323087	0.177127598	21.139236	0	21.996555	0
STAR	20.01182	0.325735248	22.3347615	0.273732732	20.268958	0.256172624
T	20.9124425	0.465548122			22.93408233	3.391388817
TFAP2A	17.6078935	0.140349809	20.258354	0	20.54745	2.142983326
TIMP1	13.9358235	0.178677784	15.23714425	0.255966798	11.69350933	0.705124785
TIMP1	13.9358235	0.178677784	15.23714425	0.255966798	11.69350933	0.705124785
TSHB	24.8605795	0.702891044	27.788426	0.167941956	27.22227333	1.595564898
VEGF	14.953103	0.440708781	18.091626	0.662668783	16.661355	0.50601287
VEGF	14.953103	0.440708781	18.091626	0.662668783	16.661355	0.50601287
WNT4	17.260816	0.250235154	17.023163	0.552346384		
WNT4	17.260816	0.250235154	17.023163	0.552346384		
WT1	25.386395	0	25.270852	2.739102098	28.035315	1.264475609

	Day 50a (delta Ct)	Day 50a SD (of technical replicates)	Day 50b (delta Ct)	Day 50b SD (of technical replicates)	Day 50c (delta Ct)	Day 50c SD (of technical replicates)
AFP	6.082665	0.072632973	9.5845185	0.244428742	12.002715	0.033313111
AKT1	16.217045	0.042083061	15.5576898	0.126220605	15.4987673	0.180865075
AMH						
AMHR2	19.6773075	0.337300152	20.579735	0	20.394981	0
AR	31.471134	0	19.310343	0.352785263	18.2846825	0.095546273
BAPX1	21.2016725	0.392843557	20.4883425	0.630146065	21.4132985	1.176602321
BMP4	18.35059	0	18.4741725	0.009076524	17.6408015	0.058842962
BMP6	20.669843	0.090238692	21.291479	0.595923627	21.3437815	0.147391173
BMPR1B	20.271122	0	19.286491	0	17.245532	0.282846206
BMPR2	17.202656	0.044012566	16.8184265	0.043442721	16.394658	0.113973562
CDH1	14.770444	0	14.8739845	0.159352716	14.341479	0.008467419
CDH11	16.0079555	0.004424235	14.164394	0.112153754	14.6556295	0.016003572
CDH11	16.0079555	0.004424235	14.164394	0.112153754	14.6556295	0.016003572
CDH2	15.7025485	0.031931511	15.5175895	0.149081654	15.6619525	0.133420451
CEBPB	17.1554365	0.015092513	16.0075485	0.199520129	16.398843	0.064963452
CEBPB	17.1554365	0.015092513	16.0075485	0.199520129	16.398843	0.064963452
CEBPG	17.648989	0.021118318	17.137215	0.068627317	17.132561	0.048071338
CGB	19.58715	0	18.9310745	0.064815073	18.158852	0.054910629
CGB	19.58715	0	18.9310745	0.064815073	18.158852	0.054910629
CHRD	19.320402	0.300935745	19.2713125	0.234672677	18.7574865	0.055278402
COL4A1	14.58246	0.044465208	13.1337465	0.154949843	14.688615	0.06564588
CRABP1	15.451249	0	14.598387	0.053512287	13.728187	0.014744371
CREB1	18.0400505	0.07076409	17.5813255	0.155023743	16.9811315	0.084052384
CREBBP	12.998179	0.054855781	12.7072165	0	11.965757	0
CYP11A1	23.954304	0	22.321724	0	20.7702665	0.394998228

	Day 50a (delta Ct)	Day 50a SD (of technical replicates)	Day 50b (delta Ct)	Day 50b SD (of technical replicates)	Day 50c (delta Ct)	Day 50c SD (of technical replicates)
CYP19A1	23.602272	0.970305255	29.294618		29.294618	0.060591756
CYP21A2	23.3424855	0.419940914	21.801403	0	21.074902	0
DAB2	14.8063985	0.033658943	15.7191725	0.049514136	15.8693965	0.044022381
DAB2	14.8063985	0.033658943	15.7191725	0.049514136	15.8693965	0.044022381
DCOHM	20.412735	0.098505194	20.0206235	0.086089276	19.694242	0.042241255
DLX5	19.3146455	0.089182719	17.339898	0.505981693	16.488848	0.024559326
EGF	17.0795655	0.047173558	18.8978655	0.066671832	16.90067	0.057589535
ENPEP	21.784364	0.080293257	21.6578215	0.057773709	20.9162005	0.009877886
EPAS1	16.186	0.003419068	15.543768	0.229669937	16.5718495	0.079221117
ESR1			28.829657	0.049371531	24.9524165	0.348566565
ESR2	24.463116	0	28.7869	0	22.4699755	0.518480749
FASN	17.135975	0	16.637006	0.051460384	15.961399	0.016224697
FIGF	22.845608	0	21.140427	0	20.5624135	0.451642063
FSHB						
FSHR	22.404243	0	21.990942	0.080720496	22.7647925	0.503570101
FST	21.122249	0.058117233	18.790369	0.078663974	19.445495	0.222689772
FZD1	18.5619275	0.110513502	17.74195	0.211582708	16.2575975	0.023449081
FZD1	18.5619275	0.110513502	17.74195	0.211582708	16.2575975	0.023449081
GATA2			20.6254215	0.108373842	18.6882965	0.004247566
GATA3	18.0029015	0.075457948	17.432325	0.028809778	17.136302	0.045447858
GJA1	13.444238	0	11.8699705	0.11223747	12.1171655	0.133473567
GJA5			24.238903	0	23.890076	0
GJB3	23.3011035	1.317324521	21.385956	0	22.207183	0.26721618
GREM1	20.230076	0.501115785	21.27578	0.193975834	18.997016	0.010900373
HRAS	18.4402275	0.046814447	17.4411645	0.081403501	17.719226	0.017917488
HSPG2	15.836144	0.090089736	14.7650555	0.144636057	14.8717075	0.093777272
ID3	16.781721	0.060361971	15.7311885	0.049507208	14.9533115	0.093807294
IGF1	20.06786	0.098201507	19.4808255	0.041486658	17.2629345	0.146111765
IGF1R	17.442909	0.094228183	16.5969835	0.193220083	16.169526	0.062699085
IGF1R	17.442909	0.094228183	16.5969835	0.193220083	16.169526	0.062699085
IL6	22.826847	0	22.9672855	0.173718345	22.7598315	0.296274796
IL6	22.826847	0	22.9672855	0.173718345	22.7598315	0.296274796
INHA	18.7383975	0.023752767	17.334713	0.062826102	19.5534385	0.211447031
INHBA	19.20397	0.008551712	18.078498	0.330011104	15.9327275	0.109863405
INHBB			22.483049	1.144452571	22.402668	0
IPF1	18.089184	0.07202791	17.706047	0.140932355	16.895487	0.06084579
KRT7	20.0123845	0.054256492	18.350199	0.033787692	19.3782205	0.034854058
LAMA5	16.7725185	0.216827935	16.93602	0	16.577481	0.210970717
LAMA5	16.7725185	0.216827935	16.93602	0	16.577481	0.210970717
LAMR1						
LHB	21.503523	0.19833021	21.015953	0	21.2740165	0.032662437
LHCGR	29.358126	0.094322868				
LRP5	16.2855605	0.083211762	16.398206	0.056832051	15.4730365	0.140938706
MMP2	14.5055775	0.006158595	13.3995995	0.112994953	12.867441	0.043629205
MMP2	14.5055775	0.006158595	13.3995995	0.112994953	12.867441	0.043629205
MMP9	21.559515	0.073285379	18.052244	0.097041033	21.8583785	0.558696659

	Day 50a (delta Ct)	Day 50a SD (of technical replicates)	Day 50b (delta Ct)	Day 50b SD (of technical replicates)	Day 50c (delta Ct)	Day 50c SD (of technical replicates)
NES	19.671774	0.006570246	18.399676	0.375035188	18.2001655	0.068306888
NFKB1	18.6142675	0.124998065	18.3023615	0.224074836	18.155137	0.059871223
NFKB1	18.6142675	0.124998065	18.3023615	0.224074836	18.155137	0.059871223
NID	17.385582	0	17.195052	0.087835761	15.9210505	0.043390759
NID2	16.927619	0.08434741	15.996023	0.243514797	16.0826115	0.0763182
NOG	19.13229	0	18.5578155	0.046316771	18.62998	0.032828136
NR0B1	22.568588	0	22.440487	0	22.42476	0
PAX8	29.27644	0	25.745482	3.610670064		
PGF	17.5990937	0.109569723	17.3772795	0.093838471	16.940343	0.02909268
PGR			20.443794	0.193627114	23.478014	0
PIK3C2A	18.058811	0	18.2123345	0.331803777	17.744374	0.083604938
POMC	21.4946175	0.108645196	22.467645	0.051182101	21.1756565	0.11030912
PTEN	17.65062	0.078762124	17.5663045	0.132676824	17.013546	0.090444229
PTEN	17.65062	0.078762124	17.5663045	0.132676824	17.013546	0.090444229
PTGS1	20.779545	0	18.9590645	0.298048416	19.6197375	0.209875483
PTGS2	22.140343	0	20.17762	0.009148692	19.906423	0.191166448
RUNX1	18.075096	0	16.159278	0.119607346	16.36533	0
RUNX2	18.555107	0	18.409176	0.090708655	16.6808905	0.154357481
SF1	16.1283275	0.057576256	15.8029215	0.00164718	15.3694665	0.080344641
SMAD1	18.6359115	0.026065633	17.8767215	0.196196901	17.1531495	0.135625929
SMAD5	17.688095	0	17.1154115	0.107960459	16.2940805	0.032170534
SMAD7	17.821938	0	17.2785185	0.106689134	17.451934	0.061404665
SOX2	22.927692	0	20.047125	0.148656147	19.7830835	0.039210744
SOX9	18.750465	0	19.063053	0	18.481173	0
SRD5A1	19.638231	0.015146207	18.7327275	0.139069246	18.4831455	0.123428827
SRY	23.125221	0			21.387576	0.315665105
STAR	23.341406	0.123125718	20.37118	0.107929859	22.0486575	0.252074014
STAT3	15.976957	0.246647499	15.7631375	0.127185068	15.9184415	0.053530185
T	29.358126	0.094322868	28.829657	0.049371531	29.294618	0.060591756
TFAP2A	20.6775395	0.241450769	19.57934	0.141732563	19.624694	0.076915758
TIMP1	14.3002925	0.004378047	13.2052305	0.107266484	14.895846	0.01534597
TIMP1	14.3002925	0.004378047	13.2052305	0.107266484	14.895846	0.01534597
TSHB	22.4862975	0.370107503	28.872414	0	23.1744205	0.446818879
VEGF	15.659223	0	14.1688925	0.116037589	13.888085	0.138398942
VEGF	15.659223	0	14.1688925	0.116037589	13.888085	0.138398942
WNT4	17.0187575	0.314482114	16.456315	0.004208883	16.0133795	0.061509166
WNT4	17.0187575	0.314482114	16.456315	0.004208883	16.0133795	0.061509166
WT1	20.7879502	0.009682092	18.7241505	0.189990385	18.340727	0.210258266

	Day 5a (delta Ct)	Day 5a SD (of technical replicates)	Day 5b (delta Ct)	Day 5b SD (of technical replicates)
AFP	12.7121713	1.725349214	21.278238	0
AKT1	14.151377	1.626160149	17.0612025	0.423547044
AMH				
AMHR2	20.718899	0	22.004503	0

	Day 5a (delta Ct)	Day 5a SD (of technical replicates)	Day 5b (delta Ct)	Day 5b SD (of technical replicates)
AR	15.377627	1.319685306	17.7426005	0.208376682
BAPX1	20.456396	2.281538153	28.269866	0.318833603
BMP4	16.594744	1.381890179	21.1414365	0.275300816
BMP6	21.0662575	1.231965593		
BMPR1B	14.780843	0	18.271463	0
BMPR2	15.87842	1.295528971	19.6011725	0.767136272
CDH1	12.4630305	1.390964393	16.6607655	0.332704443
CDH11	15.7808625	1.10727641	17.552989	0.503753121
CDH11	15.7808625	1.10727641	17.552989	0.503753121
CDH2	12.188743	1.671770821	14.8973225	0.391236214
CEBPB	16.535192	0.920328661	18.6998575	0.608243705
CEBPB	16.535192	0.920328661	18.6998575	0.608243705
CEBPG	15.5322365	0.409052088	17.132752	0.430467742
CGB	20.465118	0	22.606411	0
CGB	20.465118	0	22.606411	0
CHRD	20.823235	0	21.589375	0
COL4A1	14.1312205	1.196160639	16.862874	0.411260453
CRABP1	12.144477	1.536382183	15.111511	0.30508112
CREB1	14.5908675	1.536357356	17.083832	0.442762993
CREBBP	10.321362	1.404017705	13.896118	0.340973831
CYP11A1	18.7967075	1.67837744		
CYP19A1			28.269866	0.318833603
CYP21A2	20.980562	1.320452027	22.414547	0
DAB2	16.7883035	1.307812098	19.858622	0
DAB2	16.7883035	1.307812098	19.858622	0
DCOHM	17.847328	1.56897476	21.4734905	0.561976009
DLX5	12.786568	1.975435123	17.38478	0
EGF	17.396625	1.656983726	19.65805	0.112814243
ENPEP	19.8130035	1.792982623	23.624553	0.701221924
EPAS1	14.9849805	1.382854931	19.782899	0.326928054
ESR1	23.501228	0	28.269866	0.318833603
ESR2				
FASN	13.8414255	0.562040672	16.617729	0.27837636
FIGF	26.647314	2.066635681		
FSHB				
FSHR	20.884033	0	28.545984	0
FST	15.7771	1.434036455	18.4839985	0.113909476
FZD1	17.104647	1.142056567	18.826195	0.602424591
FZD1	17.104647	1.142056567	18.826195	0.602424591
GATA2	17.663496	1.425388903	22.294653	0
GATA3	15.0548005	1.439607308	19.327652	0
GJA1	9.233218	1.2839046	12.2019215	0.18875832
GJA5	25.123247	0.306794695	28.545984	0
GJB3	19.190197	1.898870394	23.125317	0
GREM1	16.298753	1.402212908	18.3396485	0.439685139
HRAS	15.060342	1.425360035	17.3258075	0.33817426

	Day 5a (delta Ct)	Day 5a SD (of technical replicates)	Day 5b (delta Ct)	Day 5b SD (of technical replicates)
HSPG2	14.4989583	1.404274337	18.809552	0.365148642
ID3	12.235209	1.23346959	14.1844415	0.162173649
IGF1	22.786558	2.341400138	28.269866	0.318833603
IGF1R	13.6381775	1.493643829	17.1607105	0.255411099
IGF1R	13.6381775	1.493643829	17.1607105	0.255411099
IL6	20.258089	1.685771565		
IL6	20.258089	1.685771565		
INHA	23.820171	0		
INHBA	19.6928005	1.854533935	22.918175	0
INHBB	19.0634515	1.009475584		
IPF1			28.269866	0.318833603
KRT7	17.0303415	1.200150129	20.8461045	0.317307666
LAMA5	15.761731	1.178219479	19.733255	1.155468414
LAMA5	15.761731	1.178219479	19.733255	1.155468414
LAMR1				
LHB	17.268467	0	23.946882	0
LHCGR	22.495308	0	28.545984	0
LRP5	15.699349	1.268679874	18.593936	0.49972668
MMP2	13.7589143	1.100347918	16.2235935	0.437654021
MMP2	13.7589143	1.100347918	16.2235935	0.437654021
MMP9	15.1530515	1.292595454	20.050922	0
NES	14.701842	1.279301964	17.038805	0.475272432
NFKB1	21.563555	0	21.563555	2.563052097
NFKB1	21.563555	0	21.563555	2.563052097
NID	16.605063	1.179884557	18.751195	0.457394204
NID2	17.343494	0	20.559634	0.814567329
NOG	17.3814945	1.191011829	19.902211	0.269094878
NR0B1	21.404377	0	23.20997	0
PAX8	22.502068	0	17.905274	0.033493244
PGF	17.5232105	1.573056049	21.249984	0.581793557
PGR	26.647314	2.066635681	27.993748	0
PIK3C2A	15.6943185	1.553997717	18.7746135	0.324429859
POMC	19.808146	0		
PTEN	17.703638	0.596812747	18.1730755	0.521555717
PTEN	17.703638	0.596812747	18.1730755	0.521555717
PTGS1	19.191309	1.903969552	21.629148	0
PTGS2	21.355099	0	23.7760365	0.557203632
RUNX1	16.794476	1.484798245		
RUNX2	17.826723	1.584451211	20.348019	0
SF1	12.7707045	1.43208097	16.0902815	0.482808585
SMAD1	15.0767665	1.570259364	17.799542	0.494245317
SMAD5	15.178163	1.319210724	17.9928395	0.488771459
SMAD7	16.570683	1.354218935	20.369672	0.3757996
SOX2	13.413672	1.036828707	15.448002	0.413584865
SOX9	17.333248	0.42875763	20.19399	0.253948671
SRD5A1	16.277801	0.955553955	18.238234	0.559976645

	Day 5a (delta Ct)	Day 5a SD (of technical replicates)	Day 5b (delta Ct)	Day 5b SD (of technical replicates)
SRY	15.849718	1.499273571	19.4817135	0.476330715
STAR	20.4664385	1.051610606	22.90451	0
STAT3	14.938519	1.428676335	17.5498195	0.59079387
T	16.3266635	1.56499393	21.543612	0
TFAP2A	14.9538465	1.504201256	19.5427085	0.267596654
TIMP1	15.011455	1.308161395	17.9391205	0.109005463
TIMP1	15.011455	1.308161395	17.9391205	0.109005463
TSHB	24.6692465	0.633609011	28.269866	0.318833603
VEGF	16.196328	1.286307532	18.093355	0.329252466
VEGF	16.196328	1.286307532	18.093355	0.329252466
WNT4	14.998775	1.214344285	18.7252645	0.474509752
WNT4	14.998775	1.214344285	18.7252645	0.474509752
WT1	21.67302	0	27.993748	0

	Day 22 AVG (delta Ct)	Day 50 AVG (delta Ct)	Day 5 AVG (delta Ct)	Day 5 SD (of all biological replicates)	Day 22 SD (of all biological replicates)	Day 50 SD (of all biological replicates)
AFP	12.26423758	9.2232995	15.56752683	4.620977295	1.580523763	2.54192574
AKT1	16.67325771	15.757834	15.60628975	1.905088512	1.111720562	0.35972674
AMH						
AMHR2	19.9882036	20.08233275	21.361701	0.742243815	0.642308237	0.49103562
AR	18.602064	19.2201994	16.56011375	1.537213144	0.220908268	1.03555024
BAPX1						
BMP4	18.9284526	18.1161076	18.86809025	2.599479574	1.112370028	0.41326667
BMP6	20.9996696	21.10170117	21.0662575	1.231965593	0.903917642	0.45521787
BMPR1B	22.30241	18.51216925	16.526153	2.015310397	6.528656124	1.41645753
BMPR2	18.26170943	16.80524683	17.73979625	2.220632229	1.611374408	0.35125836
CDH1	17.4456158	14.6402742	14.561898	2.431295387	1.409008466	0.2760595
CDH11	16.3334905	14.94265967	16.66692575	1.237529187	1.593357087	0.81632872
CDH11	16.3334905	14.94265967	16.66692575	1.237529187	1.593357087	0.81632872
CDH2	16.01447557	15.6273635	13.54303275	1.832892179	1.950251461	0.1344132
CEBPB	18.10305483	16.52060933	17.61752475	1.363946438	0.700039157	0.50963667
CEBPB	18.10305483	16.52060933	17.61752475	1.363946438	0.700039157	0.50963667
CEBPG	18.38858586	17.306255	16.33249425	0.939694327	1.00427179	0.2571244
CGB	15.68536586	18.7534006	21.5357645	1.23627609	1.165033482	0.57272728
CGB	15.68536586	18.7534006	21.5357645	1.23627609	1.165033482	0.57272728
CHRD	20.8421854	19.11640033	21.206305	0.442331135	1.024116291	0.33354872
COL4A1	15.36309486	14.1349405	15.49704725	1.678590735	1.553526454	0.74637075
CRABP1	14.02015442	14.4208794	13.627994	1.888582825	1.132748378	0.68132981
CREB1	18.08009643	17.53416917	15.83734975	1.694488671	1.31548107	0.46360988
CREBBP	14.4100494	12.59459225	12.10874	2.13207972	2.197023795	0.40994711
CYP11A1	19.93061233	21.95414025	18.7967075	1.67837744	0.842568986	1.43157479
CYP19A1	20.875121	26.448445	28.269866	0.318833603	0.81157142	3.10853886
CYP21A2	25.04300443	22.390319	21.458557	1.262737606	3.512791898	1.08954926
DAB2	17.62274692	15.46498917	17.811743	1.881502504	1.285406353	0.49213377
DAB2	17.62274692	15.46498917	17.811743	1.881502504	1.285406353	0.49213377

	Day 22 AVG (delta Ct)	Day 50 AVG (delta Ct)	Day 5 AVG (delta Ct)	Day 5 SD (of all biological replicates)	Day 22 SD (of all biological replicates)	Day 50 SD (of all biological replicates)
DCOHM	20.74124557	20.0425335	19.66040925	2.224236802	1.15586427	0.31508174
DLX5	17.618784	17.71446383	14.31930533	2.824834033	1.168133435	1.26511886
EGF	21.7051614	17.62603367	18.5273375	1.625820778	3.259463789	0.94384637
ENPEP	22.57097125	21.45279533	21.71877825	2.395690053	3.171509325	0.40331324
EPAS1	18.12070786	16.10053917	17.38393975	2.728095961	1.54672713	0.4607141
ESR1	0	0	0	0	0	0
ESR2	0	0			0	0
FASN	18.37336983	16.466557	15.22957725	1.539752837	1.720566988	0.47631981
FIGF	20.1402252	21.2777155	28.269866	0.318833603	0.853517303	1.04285594
FSHB						
FSHR						
FST	19.50951057	19.78603767	17.13054925	1.72638792	1.099856476	1.03346784
FZD1	19.1540518	17.52049167	17.965421	1.249518811	1.962281058	1.00393441
FZD1	19.1540518	17.52049167	17.965421	1.249518811	1.962281058	1.00393441
GATA2	19.91416957	19.656859	19.207215	2.63408507	1.513307071	1.03786837
GATA3	18.677235	17.52384283	16.47908433	2.472262966	1.297851319	0.37875522
GJA1	13.5919088	12.283702	10.71756975	1.799941393	1.638284635	0.63074538
GJA5	22.2066735	24.0644895	26.26415933	1.783397902	0.906721494	0.20139536
GJB3	22.3771544	22.4805058	20.50190367	2.508546148	2.50716288	1.0958846
GREM1	19.7971992	20.167624	17.31920075	1.454504386	1.188483002	1.01244617
HRAS	17.58353629	17.86687267	16.19307475	1.544700944	0.654880695	0.4425616
HSPG2	17.69384033	15.15763567	16.65425513	2.492227827	2.018802807	0.51332078
ID3	14.535088	15.82207367	13.20982525	1.32245752	0.776960387	0.78511182
IGF1	21.31324567	18.93720667	25.528212	3.314142028	2.116931145	1.265144
IGF1R	17.903201	16.73647283	15.399444	2.128215135	1.822170189	0.56489617
IGF1R	17.903201	16.73647283	15.399444	2.128215135	1.822170189	0.56489617
IL6	21.6939698	22.8562162	20.258089	1.685771565	1.541014506	0.22163563
IL6	21.6939698	22.8562162	20.258089	1.685771565	1.541014506	0.22163563
INHA	19.68250783	18.542183	23.820171	0	0.903077463	0.9640893
INHBA	19.42358017	17.7383985	20.76792533	2.199482177	1.540328782	1.42889698
INHBB	21.98188167	22.45625533	19.0634515	1.009475584	3.84405781	0.88746041
IPF1	23.70555925	17.56357267	28.269866	0.318833603	4.565249885	0.52724629
KRT7	18.23823971	19.24693467	18.938223	2.19555486	0.711450313	0.71636949
LAMA5	19.7103844	16.7272038	17.747493	2.381953909	2.124121251	0.22599379
LAMA5	19.7103844	16.7272038	17.747493	2.381953909	2.124121251	0.22599379
LAMR1						
LHB	23.581564	21.3142064	20.6076745	3.855784698	3.904551855	0.22334311
LHCGR	24.5025394	29.358126	25.520646	3.493359417	2.58876104	0.09432287
LRP5	18.5467695	16.05226767	17.1466425	1.786260817	1.456531058	0.43988468
MMP2	14.92239829	13.59087267	14.99125388	1.528594469	1.449959032	0.71545426
MMP2	14.92239829	13.59087267	14.99125388	1.528594469	1.449959032	0.71545426
MMP9	18.51292686	20.49004583	16.785675	2.720217746	0.890989574	1.82947321
NES	18.29714529	18.75720517	15.8703235	1.535777343	1.55655455	0.70930869
NFKB1	19.66819533	18.35725533	21.563555	0	1.239647826	0.24270024
NFKB1	19.66819533	18.35725533	21.563555	0	1.239647826	0.24270024
NID	18.8721352	16.7235574	17.678129	1.415010156	1.904758433	0.69686956
NID2	17.14239333	16.33541783	19.48758733	1.776624241	2.563052097	0.46082177

	Day 22 AVG (delta Ct)	Day 50 AVG (delta Ct)	Day 5 AVG (delta Ct)	Day 5 SD (of all biological replicates)	Day 22 SD (of all biological replicates)	Day 50 SD (of all biological replicates)
NOG	19.24726967	18.7015762	18.64185275	1.566651891	1.226132491	0.23186926
NR0B1	21.1749384	22.477945	22.3071735	1.042459605	0.190182653	0.07056316
PAX8	20.58392533	26.922468	19.43753867	2.373915984	0.662534203	3.33869338
PGF	17.19934383	17.32899667	19.38659725	2.274600394	0.813754311	0.31718415
PGR						0.19362711
PIK3C2A	19.73203175	17.9944456	17.234466	1.947046316	1.994764498	0.29806448
POMC	21.2093942	21.71263967	19.808146	0	0.914493138	0.58023859
PTEN	18.57278729	17.41015683	17.93835675	0.576363054	0.585773973	0.3095421
PTEN	18.57278729	17.41015683	17.93835675	0.576363054	0.585773973	0.3095421
PTGS1	20.447006	19.5874298	20.003922	1.939039908	1.729775749	0.73215557
PTGS2	19.81009143	20.4616858	22.96905733	22.96905733	2.063618683	0.90072227
RUNX1	18.747026	16.6897455	16.794476	1.484798245	1.072467767	0.86333106
RUNX2	18.010815	17.747048	18.667155	1.789266234	0.201576073	0.92511277
SF1	16.48142317	15.76690517	14.430493	2.031574443	1.673285473	0.32874999
SMAD1	18.49986967	17.88859417	16.43815425	1.810958371	1.550373449	0.64460793
SMAD5	18.6011475	16.9014158	16.58550125	1.764023446	1.756900907	0.5710031
SMAD7	18.8723726	17.4565686	18.4701775	2.229350887	1.547323927	0.22094297
SOX2	18.1413078	20.5176218	14.430837	1.310136074	1.99876589	1.27938528
SOX9	20.09030575	18.764897	18.763619	1.56354262	1.889473672	0.26046465
SRD5A1	18.8579265	18.951368	17.2580175	1.274282247	1.14268074	0.52740187
SRY	20.94549125	21.966791	17.66571575	2.197625966	0.749115176	0.93003372
STAR	20.8718465	21.9204145	21.279129	1.499549585	1.116526029	1.27970923
STAT3	17.86109114	15.88617867	16.24416925	1.724124664	1.260257101	0.17511358
T	22.1254264	29.16080033	18.06564633	2.954195442	2.748063196	0.25424024
TFAP2A	19.3138082	19.9605245	17.2482775	2.648935017	1.924942382	0.55117106
TIMP1	13.34663764	14.13378967	16.47528775	1.785335336	1.63753941	0.73348378
TIMP1	13.34663764	14.13378967	16.47528775	1.785335336	1.63753941	0.73348378
TSHB						
VEGF	16.56869467	14.3546356	17.1448415	1.335579363	1.4276611	0.70792603
VEGF	16.56869467	14.3546356	17.1448415	1.335579363	1.4276611	0.70792603
WNT4	17.1419895	16.49615067	16.86201975	2.167052726	0.416802519	0.46114397
WNT4	17.1419895	16.49615067	16.86201975	2.167052726	0.416802519	0.46114397
WT1	26.3997458	19.39994315	24.833384	3.649274012	2.239951576	1.16148776