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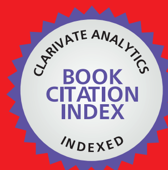

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Dynamic Changes in Gene Expression during Early Trophoblast Differentiation from Human Embryonic Stem Cells Treated with BMP4

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1. Introduction

Embryonic stem cells (ESC) have routinely been established from the blastocyst stage embryo in rodents and primates, including the human. Whereas mouse (m) ESC are dependent on LIF and BMP4 for maintenance of pluripotency and represent the "*ground*" or "*naïve*" pluripotent state, human (h) ESC require FGF2 and TGFB/ACTIVIN/NODAL signaling and are analogous to stem cells derived from advanced epiblast stage of mouse embryos. Such, epiblast-derived stem cells (EpiSC) are often categorized as "*primed*". Moreover, while the former are not generally prone to spontaneous trophoblast differentiation *in vitro* except in the face of perturbations of *Pou5f1* expression and Ras signaling, hESC are highly susceptible to both spontaneous and BMP4-mediated conversion to trophoblast (TR), particularly under high oxygen (O_2) conditions. Spontaneous differentiation in hESC has generally been counteracted by a variety of strategies to overcome or quench BMP4 activity, a growth factor that is present in most medium formulations. Here, we shall review these phenomena, as well as the opposing roles of FGF2 and BMP4 in mediating differentiation of hESC into TR. We shall describe how omission of FGF2 not only accelerates TR differentiation but drives the process unidirectionally, avoiding up-regulation of lineages representing endoderm, ectoderm, and mesoderm. In addition we shall summarize data from microarray analyses that have allowed gene expression changes associated with temporal changes in TR differentiation to be examined from early time points after BMP4 exposure for several days when extensive differentiation into specialized sub-lineages has occurred. Such analyses have also permitted the examination of the contrasting effects of low (4 %) and high (20 %) O_2 on gene regulation, colony morphology, and the production of progesterone and chorionic gonadotropin over time. These experiments illustrate the value of the BMP4/hESC model for studying emergence of TR from pluripotent precursor cells to fully differentiated syncytiotrophoblast and extravillous trophoblast and possibly for investigating the etiology of pregnancy disorders such as preeclampsia.

2. Embryonic stem cells (ESC) and trophoblast stem cells (TSC)

Mouse embryonic stem cells (mESC) were established from d 3.5 blastocyst stage embryos of certain strains almost 30 years ago (Evans and Kaufman 1981; Martin 1981). They require LIF/STAT3 signaling for maintenance of pluripotency (Hall, Guo et al. 2009), while BMP4 provides additional support for self renewal and resistance to differentiation (Ying, Nichols et al. 2003). However, mESC are generally recognized as being pluripotent rather than totipotent and only capable of contributing to the embryonic lineage and not to elements of extraembryonic tissues derived from trophectoderm (TE) (Beddington and Robertson 1989; Nagy, Gocza et al. 1990), presumably because they are already stably committed offshoots of the ICM and/or its derivative, the epiblast (Rossant and Spence 1998; Hemberger, Nozaki et al. 2003).

ESC established from monkey and human blastocysts (Thomson, Kalishman et al. 1995; Thomson, Itskovitz-Eldor et al. 1998), have markedly different characteristics to mESC. They exhibit a more flattened colony morphology and are dependent on FGF2 and TGFB/ ACTIVIN/NODAL signaling rather than LIF/STAT3 for maintenance of their pluripotency (Dvorak and Hampl 2005). These and other differences suggest a departure of hESC from the ground state pluripotency evident in LIF-dependent mESC, and, as we shall see, readily give rise to cells with the features of trophoblast. Intriguingly, however, ESC derived from a later gastrulation (egg-cylinder) stage mouse embryos, so-called epiblast stem cells (EpiSC) share striking similarities to hESC in terms of their flattened morphology, lack of dependence on LIF, but a requirement for FGF2 and ACTIVIN signals to maintain pluripotency (Brons, Smithers et al. 2007; Tesar, Chenoweth et al. 2007). Additionally, the EpiSC from mice lack demonstrated competence to contribute to fetal tissues after being introduced into pre-implantation embryos, although they can donate to the three main germ layers, ectoderm, mesoderm, and endoderm, in embryoid bodies and teratomas, and are, therefore regarded as pluripotent. EpiSC therefore, may represent a more "advanced" state and are frequently termed "*primed*" ESC. Based on their similarities, hESC and mEpiSC may embody physiologically equivalent states.

In addition to their abilities to give rise to pluripotent ESC, blastocysts have provided a source of multipotent TSC, most likely from actively proliferating polar TE. Such cells are, therefore, of extra-embryonic ectoderm origin. Such TSC can be cultured in a continuously self-renewing state *in vitro* in the presence of FGF4 plus heparin, but also require NODAL signaling, which is usually derived from a supporting feeder layer of primary embryonic fibroblasts (Tanaka, Kunath et al. 1998; Rossant 2001; Rossant 2007). However, it should be noted that dependency on FGF4 may not be a universal feature of TSC from all species (Vandevoort, Thirkill et al. 2007; Grigor'eva, Shevchenko et al. 2009).

There have been many excellent reviews on embryonic and trophoblast stem cells (TSC), and the differences that distinguish them. Our goal here is rather different from those of such earlier publications and is intended to illustrate the various ways whereby ESC can be diverted towards the trophoblast (TR) lineage. In the second half of the manuscript, we concentrate on work from our own laboratory on a unique hESC-based model to derive TR *de novo*. As well as reviewing past work, we include some previously unpublished data to emphasize the value of this model system for studying both the origins of TR and the differentiation of its sub-lineages.

3. Differentiation potential of murine and human ESC into trophoblast

3.1 Genetic manipulation

TE, the precursor of placental TR becomes apparent as the blastocyst emerges and the blastocoel cavity expands. The formation of TE and its segregation from the pluripotent inner cell mass marks the first visual evidence of separation of cell lineages during mammalian conceptus development. Although, *naïve* mESC are incapable of differentiating into TR when introduced into early mouse embryos (they instead populate the inner cell mass) and do not form TR when permitted to form embryoid bodies or teratomas, genetic manipulations of some of the key transcription factors that maintain pluripotency can result in TR differentiation *in vitro*. For example, down regulation of master regulators of pluripotency such as *Pou5f1* (Niwa, Miyazaki et al. 2000; Velkey and O'Shea 2003; Hay, Sutherland et al. 2004; Hough, Clements et al. 2006; Ivanova, Dobrin et al. 2006) and *Sox2* (Masui, Nakatake et al. 2007) in mESC by RNAi or by other method leads to TR differentiation (Table 1). Alternatively, upregulation of key TSC markers such as *Cdx2* (Niwa, Toyooka et al. 2005; Tolkunova, Cavaleri et al. 2006) and *Eomes* (Niwa, Toyooka et al. 2005), as well as an upstream transcription factor of *Cdx2* known as *Tead4* (Nishioka, Inoue et al. 2009), *Lef1* a component of WNT signaling pathway (He, Pant et al. 2008), and finally, Ras (Lu, Yabuuchi et al. 2008) a signaling molecule can lead to TR differentiation in mESC (Table 1).

Primed ESC, on the other hand, represented by primate ESC and mEpiSC are susceptible for spontaneous differentiation into TR *in vitro* (Thomson, Kalishman et al. 1995; Thomson, Itskovitz-Eldor et al. 1998; Brons, Smithers et al. 2007; Tesar, Chenoweth et al. 2007). For instance, chorionic gonadotropin (CG), a secreted protein produced by TR is readily detectable in the culture media of primate ESC (Thomson, Kalishman et al. 1995; Thomson, Itskovitz-Eldor et al. 1998). Although hESC are clearly distinct from mESC, down regulation of *POU5F1* still leads to TR differentiation (Hay, Sutherland et al. 2004; Matin, Walsh et al. 2004; Zaehres, Lensch et al. 2005; Babaie, Herwig et al. 2007) (Table 1). Additionally, silencing of the expression of NANOG, a component of the triad of transcription factors that also includes POU5F1 and SOX2 and is essential for maintenance of the pluripotent state (Boyer, Lee et al. 2005; Loh, Wu et al. 2006), induces TR and extraembryonic endoderm-associated genes (Hyslop, Stojkovic et al. 2005; Zaehres, Lensch et al. 2005). This similarity in outcomes in both mESC and hESC suggests that TR is the default state when the core transcriptional circuitry associated with pluripotency is disturbed. The conversion of hESC and especially mEpiSC to TR is intriguing, given that both cell types are assumed to represent a more “advanced” ESC state representing the later forming epiblast rather than the inner cell mass, a stage in embryogenesis that occurs well after TE and its accompanying TSC have been specified.

3.2 Manipulation of culture conditions

In addition to the genetic manipulations, TR differentiation from ESC has also been accomplished by modifying the culture conditions in which the cells are grown. As mentioned earlier, hESC, in particular, are relatively more susceptible for manipulation compared to mESC. There are two principal means of achieving TR differentiation from hESC (Table 1). They are:

	Grouud, Naïve type	Primed, Epiblast type
Genetic manipulation, down-regulation	Oct4 (Niwa 2000 Velkey 2003 Hay 2004 Hough 2006 Ivanova2006)	OCT4 (Hay 2004 Matin 2004 Zaehres 2005 Babaie 2007)
	NANOG (Hough 2006*)	NANOG (Zaehres 2005, Hyslop 2005)
	Sox2 (Masui 2007)	
Genetic manipulation, over-regulation	Cdx2 (Niwa 2005, Tolkunova2006)	
	Eomes (Niwa 2005)	
	Tead4 (Nishioka 2009)	
	Ras (Lu 2008)	
	Wnt3a (He 2008)	
Culture condition modifcation	BMP4-Laminin (Hayashi 2010)	BMP4-Matrigel (Xu 2002, Das 2007, Wu 2008, Chen 2008)
	Collagen IV (Schenke-Layland 2007)	EB in semi-solid medium (Golos2006, Gerami-Naini 2004, Peiffer 2007)
		Enrichment of hCGb producing cells (Harun 2006)
		inhibition of Activin/Nodal signaling (Wu 2008)

Table 1. Differentiation potential of murine and human ESC into trophoblast by manipulations of genetic (upper) and culture conditions (lower). *Reduction in *Nanog* expression correlated with induction of extraembryonic endoderm genes *GATA4*, *GATA6*, and *Lamb1*, with subsequent generation of groups of cells with parietal endoderm phenotype.

Embryoid body (EB) based differentiation into TR: This approach has been used with hESC, which are first dissociated and converted into EB in a semi-solid medium (Gerami-Naini, Dovzhenko et al. 2004; Golos, Pollastrini et al. 2006) when they spontaneously differentiate and form TR, particularly in their outer layers. It has proved possible to select EBs secreting the highest levels of CG to provide multicellular structures for TR (Harun, Ruban et al. 2006). Alternatively, EB that attach readily to the substratum are also enriched in TR (Peiffer, Belhomme et al. 2007).

Perturbation of BMP4 and/ or ACTIVIN/NODAL signaling axis: Treatment of hESC with BMP4 on a medium conditioned by mouse embryonic fibroblasts and on a Matrigel substratum under otherwise standard feeder-free culture conditions for ESC maintenance induces differentiation of hESC towards the TR lineage (Xu, Chen et al. 2002; Das, Ezashi et al. 2007; Chen, Ye et al. 2008; Wu, Zhang et al. 2008), as does inhibition of ACTIVIN/NODAL signaling through use of SB-431542, which binds to the activin receptor-like kinase ALK5/4/7 (Wu, Zhang et al. 2008). In the inhibitor approach, it is unclear whether the outcome is either the direct result of interfering with the signaling pathway or due to the observed up-regulation of BMP4 that occurs concurrently (Wu, Zhang et al. 2008). Not just hESC are susceptible to BMP4 driven differentiation towards TR, mouse EpiSC (Brons, Smithers et al. 2007), as well as the recently reported porcine EpiSC (Alberio, Croxall et al. 2010) also respond in a somewhat similar manner. These results indicate that BMP4 mediated differentiation into TR may be a conserved phenomenon across all FGF2-dependent, i.e. "*primed*" stem cell lines and not just restricted to hESC. Confusing this straightforward interpretation, however, a recent report has demonstrated that *naïve* mESC are also prone to TR differentiation in response to BMP4 provided that they are grown on a laminin or fibronectin rather than a collagen I-, or poly-D-lysine (PDL)-coated culture surface (Hayashi, Furue et al.). At first glance, such an outcome is counterintuitive since it contradicts the established dogma that exposure of mESC to BMP4 favors pluripotency. On the other hand, it is becoming increasingly clear that the extracellular matrix provides directional cues and plays an important role in controlling the fate of stem cells and their differentiation (Gerecht-Nir, Ziskind et al. 2003; Schenke-Layland, Angelis et al. 2007; Xiao, Zeng et al. 2007). Conceivably, the laminin substratum shifted the phenotype of the mESC from the *naïve* to the *primed* state, and hence made the cells susceptible to BMP4-driven differentiation to TR.

The general features of TR differentiation in colonies of hESC responding to BMP4 were first described by Xu et al. (Xu, Chen et al. 2002). The process was strongly dependent on the concentration of BMP4 supplied, and occurred much more rapidly at 100 ng/ml than at 10 ng/ml. As differentiation proceeded, cells within the colonies gradually changed their morphologies to a more flattened epithelioid form. Differentiation proceeded from the periphery inwards, so that a central core of smaller cells in the larger colonies remained for as long as 5 days. These overt changes in gross morphology correlated with changes in gene expression reflecting the emergence and later differentiation of TR. Several TR expressed genes, such as *TCFAP2C, HERVW, GATA3, MSX2, CGA, CGB, GCM1, HASH2, HLA-G, MMP9* and *KRT7*, were highly up-regulated by the time that multinuclear cells presumed to correspond to syncytial TR, became evident, approximately a week after addition of BMP4. Accompanying these changes in gene expression, the colonies markedly increased their production of the placental hormones hCG, progesterone, and estradiol-17β. Based on the weight of evidence, this model system appears to provide a more powerful means of assessing the emergence and differentiation of human TR than the one based on EB. In the sections that follow we summarize some of our results employing this model system.

3.3 Role of FGF2 in the BMP-driven differentiation of trophoblast from hESC

As BMP signaling activity is detectable in the "serum replacement", a component of standard hESC culture medium (Xu, Peck et al. 2005), it is perhaps no surprise that hESC maintained under such conditions tend to undergo some differentiation toward TR over time.

Consequently, media with elevated FGF2 concentrations and containing the BMP antagonist, NOGGIN, provide better sustained proliferation of undifferentiated hESC than standard conditions (Wang, Zhang et al. 2005; Xu, Peck et al. 2005). Such high concentrations of FGF2 appear to conteract BMP signaling activity and also support feeder-independent growth of hESC (Ludwig, Bergendahl et al. 2006). Keeping in mind the ability of FGF2 to antagonize differentiation of hESC, we hypothesized that elimination of this growth factor from the differentiation medium would expedite TR formation, as it proved to do (Das, Ezashi et al. 2007). Complete elimination of FGF2 from the differentiation medium accelerated the response of hESC to BMP4 as evident from earlier changes in cell morphology and elevated hCG and progesterone release over time (Das, Ezashi et al. 2007). In absence of of FGF2, 10 ng/ml BMP4 was about as effective as the 50 ng/ml in inducing morphological differentiation of the cells over time (Das, Ezashi et al. 2007). Furthermore, these culture conditions led to unidirectional TR differentiation as evidenced by microarray analysis of the transcripts present (GEO GSE10469), with complete lack of up-regulation of gene markers for primitive (yolk sac) and definitive endoderm, e.g. *AFP* (alpha-fetoprotein), *RBP4* (retinol binding protein 4), *FGG* (fibrinogen-γ), which had been noted to increase in expression when FGF2 was present (Xu, Chen et al. 2002; Zhang, Li et al. 2007). Expression of lineage markers for mesoderm, e.g. *WNT3A*, *T* (Brachyury), *MEOX2*, *MIXL1*; for endoderm, e.g. *FOXA2*, *SOX7, ONECUT1, ATBF1*, *DPF3*; and for ectoderm, e.g. *PAX6, MEIS1, HOXB1, OTX1*, *SOX1*, *ZIC1* (Cai, Chen et al. 2006; Zhang, Li et al. 2007) was either absent or very low in both control hESC and in the cells after BMP4 addition in absence of FGF2 (GEO GSE10469). There was a modest 2-fold up-regulation of WNT3, a potential mesoderm marker, after 24 h, but since WNT signaling is probably involved in directing TR emergence, such a change was not unanticipated. Taking these results into consideration, our laboratory now routinely omits FGF2 and employs 10 ng/ml BMP4 in the differentiation medium.

3.4 Effects of oxygen and time on the BMP-driven differentiation of TR from hESC

Human pre-implantation embryos only experience a low O_2 environment *in utero* (as discussed by Ezashi et al. 2005), while hESC are routinely cultured by most groups in 20% (atmospheric) O_2 conditions. We were concerned that hESC cultured under such non-physiological conditions might be either poised for differentiation, or, worse, already cryptically differentiated. Such a concern was justified by the fact that cells maintained under 20 % O_2 produced considerable amounts of hCG and progesterone after about five days in a standard culture condition, suggesting that lineage commitment had indeed progressed even before signs of overt differentiation became evident. By contrast, hESC which grow equally as efficiently under 4% O_2 conditions as under ~20 % O_2 (Ezashi, Das et al. 2005), produced very little of the two placental hormones. In addition, growth under low O_2 provided significant protection against subsequent differentiation when the same cells were passaged into high O_2 conditions (Ezashi, Das et al. 2005). Since O_2 tension slows the proliferation and enhances differentiation of villous and extra-villous TR (Genbacev, Joslin et al. 1996; Genbacev, Zhou et al. 1997; James, Stone et al. 2006), we included O_2 tension as an additional variable in our experiments. As described below, the expression of TR markers was markedly accelerated under 20 % relative to low O_2 conditions (Das, Ezashi et al. 2007; Schulz, Ezashi et al. 2008).

Over the initial days of exposure to BMP4, smaller ESC colonies rapidly differentiate to TR. In the case of larger colonies, however, cells in the center begin to pile up, possibly because they continue to divide in space that is progressively restricted in area (Fig 1, also see Fig 2

and 5 of Das et al 2007). These cells remain POU5F1-positive and are negative for KRT7, a common marker of TR. Whether such cells have retained their original pluripotent potential remains unclear. However, the cells that ring this core demonstrate overt signs of

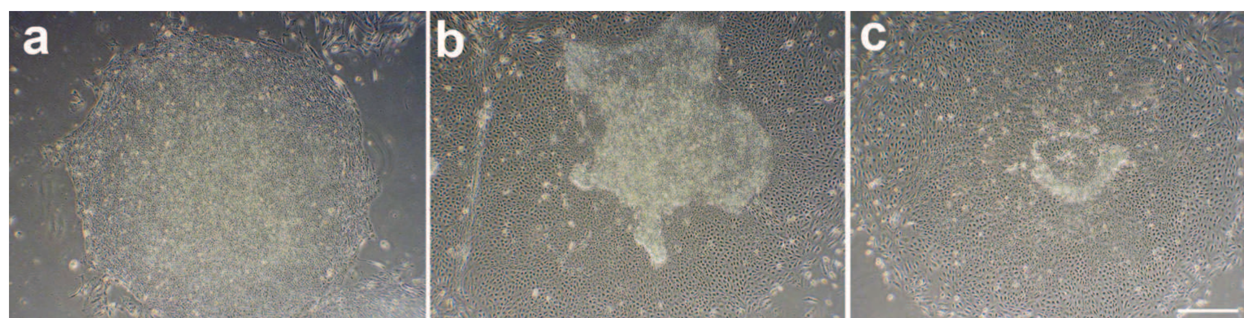

Fig. 1. Effects of treatment with 10 ng/ml BMP4 on hESC colony morphology under a low (4%, **a** & **b**) and high (20%, **c**) O_2 atmosphere at day 3. (**a**) Control cells on standard medium under 4 % O_2. (**b**) BMP4 treated cells under 4 % O_2. (**c**) BMP4 treated cells under 20 % O_2. Bar, 0.5 mm. Note that the colonies treated with BMP4 are larger than ones without treatment (**a**), due to spreading of differentiated cells at periphery.

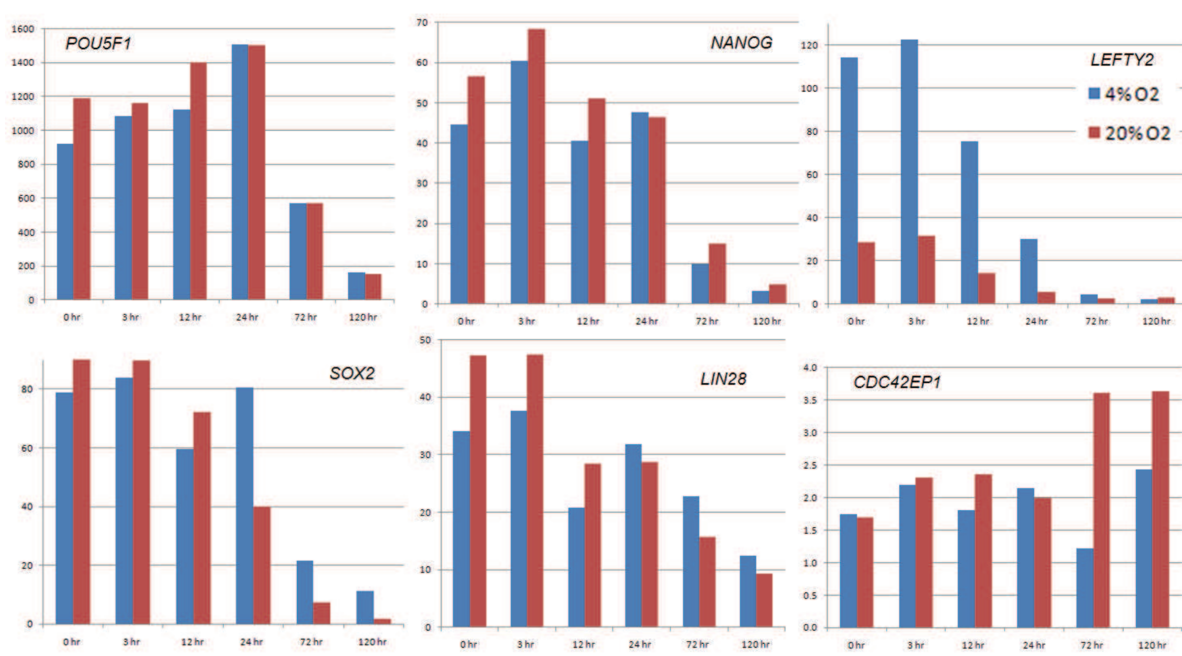

Fig. 2. Changes in gene expression as assessed by microarray (with values normalized to the median intensity of the array) for five genes associated with a pluripotent phenotype (*POU5F1, NANOG, SOX2, LIN28* and *LEFTY2*) and one implicated with cell morphogenesis (*CDC42EP1*). H1 hESC were exposed to BMP4 (10 ng/ml) under either 4% (blue) or 20% (red) oxygen. RNA isolated at 0, 3, 12, 24, 72 and 120 h. These data were obtained on Agilent Whole Human Genome Oligo microarrays. Genes that were changing significantly over time within each oxygen condition were identified by ANOVA. The value represents a normalized expression (1.0 being the signal at the 50th percentile). The genes shown here were selected from 33,814 genes of which 5,194 changed expression more than 2-fold at $P<0.01$ in at least one time point. Changes in gene expression have been verified by real-time PCR with cDNA from H1 and H9 cells for *POU5F1, NANOG, SOX2, LEFTY2, CGA* and *CGB*.

morphological differentiation, taking on a more flattened appearance and becoming negative for POU5F1 and positive for KRT7. Why differentiation proceeds in this directional manner is uncertain, as all the cells in the colony are exposed to BMP4. However, one explanation, albeit unexpected, for the apparent directional differentiation from the periphery to the center of the colony is that it is due to migration of differentiating TR outwards from the interior. In this regard, the product of the *Cdc42ep1* (*Borg5*) gene, CDC42EP1, sometimes known as either CDC42 effector protein or binder of Rho guanosine 5′-triphosphatase 5, has been proposed as directing such movement (Vong, Liu et al. 2010). Reducing *Cdc42ep1* expression by using an RNAi approach lowered the number of colonies with peripheral TR and led to an increase in patches of TR scattered within the interior of the colonies. Our microarray data (Fig. 2) are somewhat consistent with this hypothesis in the sense that under high O_2 there was a increase in expression of *CDC42EP1* at 72 h when many of the main TR markers were being up-regulated (Schulz, Ezashi et al. 2008). On the other hand, visible differentiation had also occurred in the outer parts of the colony by this stage and even a modest up-regulation of gene expression was not observed under physiological O_2 conditions. Obviously, clarifying a role for CDC42EP1 in hESC differentiation will require a much more sophisticated approach than transcript profiling.

In order to explore the temporal changes in gene expression following BMP4 supplementation of hESC under both low and high O_2 environments, microarray analysis was performed on BMP4-treated H1 hESC with RNA collected at 3, 12, 24, 72, and 120 h of BMP4 treatment under both sets of O_2 conditions. Biotinylated cRNA was prepared and hybridized to Agilent-014850 Whole Human Genome 4x44K Microarrays. Slides were washed and scanned on an Agilent G2565 Microarray scanner, and data analyzed with Agilent Feature Extraction and GeneSpring GX v7.3.1 softwares. Approximately 32,000 probe sets (out of 40,391) provided a signal above background at one of the time points. Of these, 14,478 showed a ≥2-fold (plus or minus) change ($p<0.05$) under 4 % O_2, and 18,580 under 20 % O_2. When more stringent selection was made (2-fold; $p<0.005$) 4547 genes were found to be regulated over time of exposure to BMP4 and 739 by O_2. Adjustment of the data to $p<0.05$ with the extremely stringent Beniamini & Hochberg False Discovery Rate in a two-way ANOVA indicated that 5,173 genes were differentially regulated on at least one time-point. Full access to the microarray data set is available at GEO GSE10469.

As noted by Xu et al. (Xu, Chen et al. 2002), we observed a decline in the expression of transcription factors associated with pluripotency (Boyer, Lee et al. 2005; Cai, Chen et al. 2006; Loh, Wu et al. 2006; Wang, Rao et al. 2006) over time after exposure to BMP4 (Fig. 2). In general, the decrease did not become obvious in the first 24 h but became pronounced between 24 and 72 h. The O_2 atmosphere had no affect the changes observed. Interestingly, one gene *LEFTY2*, which is highly expressed in hESC and is considered to be under the transcriptional control of the POU5F1/NANOG/SOX2 triad (Boyer, Lee et al. 2005; Loh, Wu et al. 2006; Babaie, Herwig et al. 2007), showed a much more rapid drop in expression than POU5F1 and the other pluripotency genes. It also had a much lower expression in cells under 20% O_2 than in those under 4% O_2 (Fig. 2). We have previously hypothesized that the *LEFTY* genes may play a central role guarding against differentiation and that their low concentrations in ESC maintained under 20% O_2 could be a reflection of the cryptically differentiated state of such cells (Westfall, Sachdev et al. 2008).

During the first 3 h of BMP4 exposure more than 500 genes showed greater than a 2-fold up-regulation. Since transcription factors likely underpin emergence of new cell lineages from pluripotent precursors, we examined expression of transcription factor genes that were up-

regulated during the first 3 h of exposure to BMP4 under both O_2 conditions. An additional criterion was that the genes should have a normalized expression of > 0.1 at 3 h (1.0 being the signal at the 50th percentile) under at least one of the two conditions. The following 15 transcription factor genes were identified as significantly up-regulated at 3 h: *ID2*, 10.2-fold; *MSX2*, 11.9-fold; *GATA5*, 8.4-fold; *GATA3*, 8.4-fold; *LEF1*, 6.4-fold (under high O_2 only); *DLX2*, 3.0-fold; *TBX3*, 3.0-fold; *FOXF2*, 5.8-fold; *HEY1*, 4.9-fold; *GATA2*, 4.9-fold; *TCFAP2A*, 3.3-fold; *TCFAP2C*, 2.2-fold; *HHEX*, 9.4-fold; *RUNX3*, 2.7-fold; *CDX2*, 3.6-fold. A DAVID analysis of the full list of up-regulated genes suggested that WNT as well as NOTCH signaling was an immediate early response following BMP4 treatment as early as 3 h of exposure, as evidenced by up-regulation of *WIF1, FRCB, MEGF10, MYL4, LEF1, LUM, WISP1* and *FRAT1*.

We also examined the temporal expression of a number of genes encoding transcription factors that have been implicated in the emergence of TR in genetic studies in mice and mouse TSC. The caudal-related transcription factor CDX2 is one such gene product. It is the best marker for distinguishing TE from ICM in a variety of species (Roberts, Yong et al. 2006; Kuijk, Du Puy et al. 2008; Harvey, Armant et al. 2009; Katayama, Ellersieck et al. 2010). *Cdx2* -/- mouse conceptuses fail to implant (Chawengsaksophak, James et al. 1997), and ectopic expression of CDX2 in mouse ESC down-regulates POU5F1 (Niwa, Toyooka et al. 2005) and suppresses the expression of genes that are components of pluripotency network, thereby causing the cells to adopt a TR phenotype. The gene is up-regulated immediately in ESC in response to BMP4 (Fig. 3), reaching maximal expression between 24 and 72 h, the stage at which *POU5F1* transcripts are in rapid decline, an observation consistent with the reciprocal relationship observed for these transcription factors in embryos. Unlike *LEFTY2*, its expression is higher under 20% O_2 than under 4 % O_2 (Fig. 3).

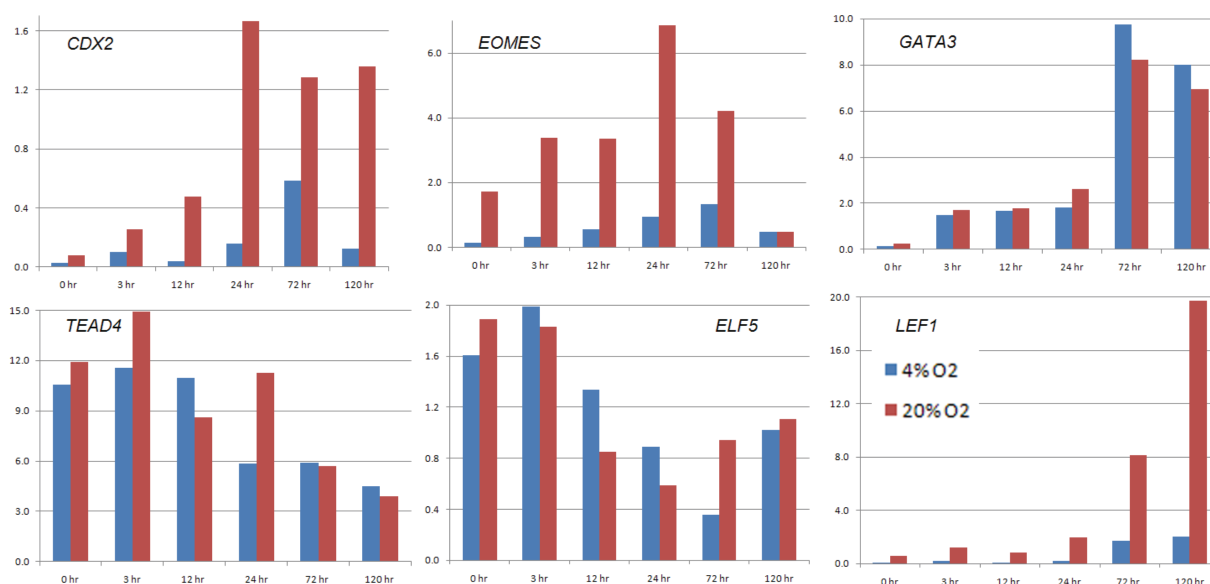

Fig. 3. Changes in gene expression as assessed by microarray (with values normalized to the median intensity of the array) for six genes implicated in the lineage decisions leading to acquisition of a trophoblast phenotype (*CDX2, EOMES, GATA3, TEAD4, ELF5* and *LEF1*). H1 hESC were exposed to BMP4 (10 ng/ml) in either 4% (blue) or 20% (red) oxygen and RNA collected after 0, 3, 12, 24, 72 and 120 h as described in Fig 2.

The product of the T-box gene, *Eomes*, is the earliest acting transcription factor known to be required for immediate post-implantation lineage commitment steps, as mice lacking the *Eomes* gene implant but arrest at a blastocyst-like stage of development (Russ, Wattler et al. 2000). Also such conceptuses are unable to form TSC. *EOMES* is not, however, markedly responsive to treatment of ESC with BMP4 and its expression remains low under 4 % O_2 (Fig. 3).

Like *Cdx2*, the *Gata3* gene is transcribed in TE, but not in the ICM of mouse blastocysts (Home, Ray et al. 2009), and can force emergence of TR when expressed ectopically in mouse ESC (Ralston, Cox et al. 2010). Although both genes are regulated by TEAD4 (see below), they appear to operate semi-independently, specifying TR fate via different pathways and targets (Ralston, Cox et al. 2010). *GATA3* expression is barely detectable in control hESC, but is highly responsive to BMP4 (Fig. 3) and exhibits a further up-regulation between 24 and 72 h to a level that is maintained until 120 h. It seems that GATA3 has a dual role, one in TR emergence, the other in driving the differentiation of on or more specialized sublineages.

Mouse embryos mutant for the *Tead4* cannot form blastocysts (Yagi, Kohn et al. 2007; Nishioka, Yamamoto et al. 2008) and are unable to maintain CDX2 expression. Instead, the entire conceptus consists of ICM-like cells expressing POU5F1 and NANOG. Importantly, TSC cannot be isolated from either *Cdx2*$^{-/-}$ or *Tead4*$^{-/-}$ blastocysts, while ES cells can. Moreover, forcing *Tead4* transcription in mouse ESC induces *CDX2* expression and a TR phenotype (Nishioka, Inoue et al. 2009). Thus, *Tead4* is probably the earliest known gene that is required for trophoblast specification. On the other hand, *TEAD4* is expressed in hESC before BMP4 treatment, shows only minor responsiveness to BMP4 at early time points, and then declines in activity over time (Fig. 3), Although these results do not rule out a role for TEAD4 in directing the TR lineage, e.g. by controlling expression of the *CDX2* and *GATA3* genes, it is clear that the microarray results are not easily interpreted and that TEAD4, like many other transcription factors, is probably pleiotropic in action and involved in multiple developmental events.

ELF5, an ETS-domain transcription factor, like all the other transcription factors discussed in this section, is implicated in early placental development of the mouse. *Elf5*$^{-/-}$ conceptuses implant, form an ectoplacental cone, but extraembryonic ectoderm, the site of the TSC niche, is not detectable. TSC cannot be derived from these embryos suggesting a fundamental defect in their generation or self-renewal (Donnison, Beaton et al. 2005). One proposed role for ELF5 is in transcriptional control of *Cdx2* and *Eomes*, with the three transcription factors forming a positive feedback loop that reinforces the early stages of TSC commitment (Ng, Dean et al. 2008). Interestingly, ELF5/CDX2 double-positive cells have been identified amongst human villous cytotrophoblasts and may define a TSC-like subpopulation (Hemberger, Udayashankar et al. 2010). *ELF5* is expressed in hESC and transcript levels are only barely affected by exposure to BMP4 (Fig. 3). As with *TEAD4*, the data do not allow inferences to be made about any role for ELF5 in TR emergence, but suggests that this transcription factor is also pleiotropic in its actions.

Lymphoid enhance factor-1 (LEF1), a downstream component of the WNT signaling pathway, has been implicated in modulating *Cdx2* expression (He, Pant et al. 2008). However, *Lef1*$^{-/-}$ mouse conceptuses are born alive, suggesting that the placenta is functional despite the fact that several other organs are defective in the pups (van Genderen, Okamura et al. 1994). *LEF1* transcripts are not detectable in hESC grown under physiological O_2 conditions and only barely so under 4 % O_2 (Fig. 3). Expression rose markedly over time

(over 200-fold by 5 days) and was again much more pronounced at high O_2. Such data suggest that LEF1 likely plays an important role in human TR development, particularly in later lineage differentiation.

Clearly microarray data on these transcription factors implicated in TR lineage determination have only limited value, but they form a starting point for genetic manipulation studies in which their expression can either be silenced or over-expressed and effects on cellular phenotype evaluated. Several other transcription factors, such as GCM1, HAND1, and DLX3, although not up-regulated immediately and therefore not implicated in "early" responses, show major increases in expression over time of exposure to BMP4 (data available at GEO GSE10469).

Finally, this model system for studying TR emergence, permits a study of the events accompanying differentiation of more advanced lineages, such as syncytiotrophoblast and extravillous TR. As BMP4 treatment progressed beyond 72 h, many genes considered general TR markers such as *KRT7* and *KRT8*, as well as ones whose expression is associated with syncytiotrophoblast, e.g. *CGA, CGB, HERVW*, became highly up-regulated, especially under 20 % O_2 (Fig. 4). These changes correlated well with the emergence of extensive patches of syncytial TR appearing on the periphery of the colonies. These hCG-positive syncytial cells have multiple nuclei in a continuous cytoplasm and materialize much more rapidly under 20 % O_2 than under 4 % O_2 (Das, Ezashi et al. 2007). Consistent with the immunohistochemistry and gene expression studies, abundant hCG is released into the medium under these condition (Das, Ezashi et al. 2007). Also evident by 72 h is the expression of HLA-G, a marker of extravillous TR.

Fig. 4. Changes in in gene expression as assessed by microarray of markers associated with trophoblast differentiation (*CGA, CGB, HERVW,* and *KRT7)* after 0, 3, 12, 24, 72 and 120 h of BMP4 treatment as described in Fig. 2.

4. Concluding remarks

These results strongly suggest that only TR emerges when hESC are driven to differentiate by BMP4 in absence of FGF2, that high O_2 promotes and low oxygen impedes TR differentiation, and that markers representing all specialized TR cells are represented in BMP4 supplemented hESC culture by 3-5 days of treatment. This model system also affords an opportunity to follow the origin or "birth" of TR *in vitro*, obviating the need for obtaining first trimester primary human TR from selective or spontaneous abortions, which until recently have represented the only credible means of investigating early differentiation events (Golos, Pollastrini et al. 2006). The model also affords a means to investigate both extrinsic factors, such as O_2 and various hormones and growth factors, and intrinsic genetic and epigenetic mechanisms that control the emergence and differentiation of TR. Therefore, the BMP4/hESC model system may prove useful for investigating the etiology of pregnancy disorders, such as preeclampsia (Golos, Giakoumopoulos et al. 2010; Das, Ezashi et al. 2007; Schulz, Ezashi et al. 2008).

5. Acknowledgements

This work was supported by HD055231 and NIH HD21896.

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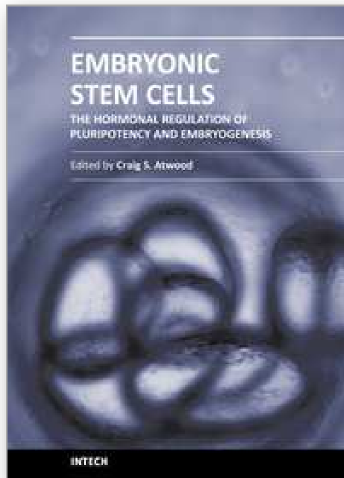

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Edited by Prof. Craig Atwood

ISBN 978-953-307-196-1
Hard cover, 672 pages
Publisher InTech
Published online 26, April, 2011
Published in print edition April, 2011

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