DEBATE

Differentiation of trophoblast cells from human embryonic stem cells: to be or not to be?

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Abstract

It is imperative to unveil the full range of differentiated cell types into which human pluripotent stem cells (hPSCs) can develop. The need is twofold: it will delimit the therapeutic utility of these stem cells and is necessary to place their position accurately in the developmental hierarchy of lineage potential. Accumulated evidence suggested that hPSC could develop *in vitro* into an extraembryonic lineage (trophoblast (TB)) that is typically inaccessible to pluripotent embryonic cells during embryogenesis. However, whether these differentiated cells are truly authentic TB has been challenged. In this debate, we present a case for and a case against TB differentiation from hPSCs. By analogy to other differentiation systems, our debate is broadly applicable, as it articulates higher and more challenging standards for judging whether a given cell type has been genuinely produced from hPSC differentiation.

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Introduction

Case for differentiation of human embryonic stem cells into trophoblast cells (proposers: R M Roberts, M Amita, K Adachi, A P Alexenko, D J Schust, L C Schulz, B P V L Telugu, and T Ezashi).

Ethical and regulatory concerns limit our ability to move from animal models to humans in an effort to follow trophoblast (TB) development from the blastocyst to the fully functional placenta (Enders 2000, Carter 2007). As a result, limited morphological information is available on initial stages of human TB invasion and placentation (Enders 2000). What is generally inferred is that cytotrophoblast (CTB) cells derived from blastocyst trophectoderm differentiate into either villous CTB or extravillous CTB. The former provides the multilayered structures that ultimately form the chorionic villi and syncytio TB (Georgiades *et al*. 2002, Malassine & Cronier 2002), a cell layer that makes direct contact with maternal blood and is characterized by the production of human chorionic gonadotropin (hCG) and other placental hormones. Extravillous CTB, by contrast, assumes an HLA-G+ phenotype, and a subpopulation modifies maternal spiral arteries to increase blood flow through the placental bed.

In absence of a ready source of trophoblast stem cells (TSCs), the best *in vitro* models to study the development of these TB lineages have, until recently, been choriocarcinoma cells, such as JAr and JEG3 lines, immortalized extravillous CTB cell lines, and primary CTB cultures from placenta (Ringler & Strauss 1990, Schulz *et al*. 2008). However, there are major shortcomings to each of these models. Importantly, none of them allows cell lineages to be followed from their stem

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cell progenitors to fully differentiated syncytioTB and extravillous TB cell types (Schulz *et al*. 2008, Genbacev *et al*. 2013).

An experimental system in which human embryonic stem cell (hESC) are driven toward TB in response to bone morphogenetic protein 4 (BMP4) was first described by Thomson's group (Xu *et al*. 2002, Xu 2006) and later by others (Besser 2004, Gerami-Naini *et al*. 2004, Golos *et al*. 2006, Chen *et al*. 2008, Wu *et al*. 2008, Zhang *et al*. 2008, Douglas *et al*. 2009, Erb *et al*. 2011, Marchand *et al*. 2011, Sudheer *et al*. 2012) including ourselves (Das *et al*. 2007, Schulz *et al*. 2008, Gupta *et al*. 2012, Amita *et al*. 2013, Telugu *et al*. 2013). This phenomenon has generally been observed with the FGF2-dependent 'primed' type of ESC, such as those derived from the inner cell mass of human (Xu *et al*. 2002) and rabbit (Tan *et al*. 2011) blastocysts, and generally not in naïve type ESC as represented, for example, by the LIF/STAT3-dependent mouse ESC (mESC). However, there is at least one exception where BMP4 exposure generated TB from naïve type cells, namely when mESC were cultured on a laminin substratum (Hayashi *et al*. 2010).

This BMP4-dependent model system has allowed the very early stages of TB lineage specification and expansion from pluripotent progenitors to be examined over time and subsequent differentiation of these early progenitor cells to be followed along specific sub-lineages. Morphologic differentiation in response to BMP4 is readily visible as the spread of larger, flattened cells proceeding from the periphery inwards (Xu *et al*. 2002, Das *et al*. 2007, Amita *et al*. 2013). Microarray analysis performed over time following exposure to BMP4 revealed immediate upregulation of transcription factors associated with TB emergence, with little evidence for major upregulation of lineage markers for mesoderm, endoderm, or ectoderm (GEO GSE10469; Marchand *et al*. 2011, Ezashi *et al*. 2012, Sudheer *et al*. 2012). There was a gradual loss of expression of 'stemness genes', increased expression of genes associated with differentiated TB, and emergence of morphologically recognizable syncytium-like cells, which was accompanied by release of placental hormones (Das *et al*. 2007, Sudheer *et al*. 2012, Amita *et al*. 2013). When the ability of cells to migrate through Matrigel was assessed, cultures maintained under conditions designed to minimize differentiation, i.e. with FGF2 present and BMP4 absent, showed little or no invasiveness (Amita *et al*. 2013, Telugu *et al*. 2013), but significant migration was observed in cultures treated with either BMP4 (Telugu *et al*. 2013) or BMP4 plus the Activin A signaling inhibitor A-83-01 and FGF2 signaling inhibitor PD173074) (BMP4/AP treatment) (Amita *et al*. 2013). By day 5 of treatment, invasive potential had been raised ~160-fold by BMP4/AP and 65-fold by BMP4 alone and the cells that had migrated to the underside of the invasion chamber membrane were all positive for KRT7, while a majority were positive for HLA-G (Amita *et al*. 2013, Telugu *et al*. 2013).

The original medium used to observe BMP4-driven differentiation consisted of 80% DMEM/F12, 20% knockout serum replacement (KOSR; Invitrogen), and FGF2 (4 ng/ml) (Amit *et al*. 2000, Xu *et al*. 2002). It was defined, but for the fact that the cells were grown on either a 'feeder' layer of irradiated mouse embryonic fibroblasts (MEF) or on MEF-conditioned medium. Initially, BMP4 was added to this medium in the continued presence of FGF2 (Xu *et al*. 2002, Das *et al*. 2007), but it was quickly realized that removing FGF2 enhanced differentiation to TB (Das *et al*. 2007, Marchand *et al*. 2011, Sudheer *et al*. 2012). Indeed, in presence of high FGF2 concentrations, BMP4 induced expression of genes characteristic of mesoderm (Vallier *et al*. 2009*a*, Yu *et al*. 2011). BMP4-directed TB differentiation was further improved by inclusion of the Activin inhibitor SB431542 (Wu *et al*. 2008, Erb *et al*. 2011). Activin A promotes transcription of key pluripotency genes (Xu *et al*. 2008, Vallier *et al*. 2009*b*), and its own signaling probably competes with that of BMP4 for SMAD intermediates. Recently, the Roberts's lab has employed the chemically defined mTeSR1 medium supplemented with FGF2 (100 ng/ml) and TGFβ (0.6 ng/ml) to grow hESC (Amita *et al*. 2013), while differentiation was induced, as before, with 10 ng/ml BMP4 in DMEM/F12/KOSR medium, with or without MEF conditioning and in the absence of FGF2. As it turns out, MEF conditioning is unnecessary to drive TB differentiation (Amita *et al*. 2013), so that it is now possible to conduct both the hESC growth phase and differentiation phase under chemically defined conditions. Addition of inhibitors of Activin (A-83-01) and FGF2 signaling (PD173074) further accelerates TB differentiation (Amita *et al*. 2013).

Despite the compelling evidence supporting the BMP4/hESC model, Bernardo *et al*. (2011) concluded: 'we have no evidence to support the thesis that hESCs have the capacity to generate TB simply by addition of BMP to their growth medium in the presence or the absence of FGF2 or with Activin/Nodal inhibition' and that 'BMP induces human pluripotent stem cell (hPSC) and mouse PSC primarily to form mesoderm'. In that study, the hESCs were cultured on a chemically defined, serum-free, F12-based medium, supplemented with 10 ng/ml recombinant Activin A and 12 ng/ml recombinant FGF2, on a gelatin-coated substratum soaked in FBS. During differentiation, the BSA in this medium was replaced with polyvinyl alcohol, and the content of FGF2, Activin A, BMP4, and various inhibitors adjusted according to the differentiation protocol employed. The fact that Bernardo *et al*. chose to use this medium rather the one used by earlier investigators to draw inferences about the BMP4/hESC model clearly complicates the present debate on the validity of the BMP4/hESC model.

The results from the Roberts's lab differed most significantly from those of Bernardo *et al.* (2011) can be summarized as follows:

i) They showed that three genes associated with embryonic and extra-embryonic mesoderm, namely *KDR* (*FLK1*), *VCAM1*, and *TBX4*, were quickly upregulated by BMP4 (Ezashi *et al.* 2012, Amita *et al.* 2013). Our data, however, indicated that, with the exception of *KDR*, which is expressed in human extravillous CTB (Iacob *et al.* 2008), these genes are expressed relatively weakly in hESC, with very little upregulation by BMP4 over the initial 3 days of exposure.
ii) Bernardo *et al.* (2011) reported that only 4–8% of BMP-treated cells expressed KRT7 at day 7, whereas the Roberts's lab showed that virtually all cells in the colonies of BMP-treated cells became positive for KRT7 by day 2 (BMP4/AP) or day 4 (BMP4). Even when the Roberts's lab used the chemically defined medium (CDM) medium of Bernardo *et al.*, BMP4 converted over 40% and BMP4/AP over 70% of the cells to a KRT7+ state within 5 days (Amita *et al.* 2013).
iii) Bernardo *et al.* (2011) noted only 'sporadic' multinucleated cells and suggested that these were not syncytio TB but some mesoderm derivative. In the BMP4/hESC model, large areas of the colonies form syncytia, and these multinucleated cells are strongly positive for syncytio TB markers.
iv) In the hands of the Roberts's lab, WA01 and WA09 produced large quantities hCG by day 9 in response to BMP4 with or without A-83-01 and PD, but when the same cells were treated with BMP4/AP on the CDM medium and substratum, described by Bernardo *et al.*, in the absence of the pluripotency factors, FGF2, and Activin A, the cells produced only about 1% of that amount of hormone.
v) According to Bernardo *et al.* (2011), BMP4-treated hESC did not express HLA-G, while in the Roberts's lab strong signals for both surface and internal HLA-G were observed (Das *et al.* 2007, Amita *et al.* 2013).
vi) The Roberts's lab observed no upregulation in *T* (Brachyury; marker of early-stage mesoderm and endoderm) under BMP4/AP conditions and only weak expression with *BMP4* alone and then only under high O_2 conditions (Amita *et al.* 2013).

What then can explain the discrepancies between our two papers? The nature of the cells is not an issue. In our hands, for example, WA09 cells provide qualitatively similar outcomes to WA01 cells (Amita *et al.* 2013). Possibly, it is the nature of the culture medium and substratum that influences the directionality of differentiation. For example, does either the continued presence of Activin A in the CDM medium or the soaking of the matrix in FBS inhibit TB formation and bias differentiation in favor of mesoderm even when a potent Activin A inhibitor is present? The Roberts's lab do not quarrel with the notion that FGF2 and Activin A in combination with BMP4 can promote both endoderm and mesoderm in proportions that depend on the relative concentrations of the three growth factors (Zhang *et al.* 2008, Vallier *et al.* 2009*a*, Yu *et al.* 2011). However, it is unambiguously clear that BMP4 in the absence of FGF2 and Activin A signaling induces hESC to form TB. Our concern is that there may be a broad perception that the BMP4/hESC model for TB differentiation is somehow flawed and that this slant might be reflected in the manner in which manuscripts and grant proposals that incorporate the model are reviewed.

Case against differentiation of hESC into TB cells (opposers: K M Loh, A S Bernardo, and R A Pedersen)

The developmental competence of cells – what repertoire of committed fates a particular progenitor can access – is pertinent to both developmental and stem cell biology. For example, the mouse embryo's pluripotent epiblast cells, which can form all cell types of the body proper, have lost the ability to form any extraembryonic lineage, such as TB or hypoblast (Gardner & Rossant 1979). Unexpectedly, hESCs, which are derived from and are thought to correspond to pluripotent epiblast, have been reported to harbor TB potential (Xu *et al.* 2002), a view supported by other studies (Erb *et al.* 2011, Drukker *et al.* 2012, Sudheer *et al.* 2012, Amita *et al.* 2013). The unanticipated TB competence of hESC has led to suggestions that hESC are either 'totipotent' in multilineage potential or altered *in vitro* derivatives of epiblast with aberrantly expanded lineage potential (Silva & Smith 2008).

Studies reporting the derivation of TB from hESCs *in vitro* attribute this outcome to BMP signaling (Xu *et al.* 2002, Erb *et al.* 2011, Drukker *et al.* 2012, Sudheer *et al.* 2012, Amita *et al.* 2013). Such a role of BMP was surprising, as genetic perturbations in mouse ($Bmpr1a^{-/-}$ and $Bmpr2^{-/-}$ mutants) do not reveal any defect in TB development *in vivo* (Mishina *et al.* 1995, Beppu *et al.* 2000). Rather such *in vivo* genetic studies of $Bmpr1a^{-/-}$ and $Bmpr2^{-/-}$ mutants demonstrate a critical role for BMP signaling in the mesoderm development, a conclusion recently corroborated *in vitro* using both hESC differentiation and mouse epiblast explant approaches (Bernardo *et al.* 2011, Loh *et al.* 2014).

Examining BMP signaling from an embryonic perspective could provide insights into its possible role in TB development. To the best of our knowledge, there is no evidence for robust BMP signaling in pre-implantation trophectoderm, as would be evidenced by expression of the appropriate receptors. The extraembryonic ectoderm

of post-implantation mouse embryos actively transcribes *Bmp4* and *Bmp8b*; however, significant amounts of pSmad1/5/8 signaling have not been observed there (Di-Gregorio *et al*. 2007).

Why then would BMP be expected to induce hESCs to differentiate into TB? In retrospect, from a developmental perspective, BMP-induced differentiation of hESCs into TB would not have been anticipated. Nevertheless, a number of studies report BMP-based techniques to apparently generate TB from hESCs, most prominently the application of BMP signaling in conjunction with inhibitors of TGFβ and FGFR/MAPK signaling (Erb *et al*. 2011, Sudheer *et al*. 2012, Amita *et al*. 2013). Presumably, combined TGFβ and FGFR/MAPK inhibition blocks the expression of pluripotency factors and primitive streak genes, thus diverting hESC from other mutually exclusive prospective fates to help consolidate putative TB commitment. In these various culture conditions, cells have emerged that express i) TB-associated genes (e.g. *CDX2* and *HAND1*) and keratins, ii) secrete placental hormones (e.g. hCG), and/or iii) are multinucleated (Xu *et al*. 2002, Erb *et al*. 2011, Drukker *et al*. 2012, Sudheer *et al*. 2012, Amita *et al*. 2013).

At the crux of this matter is the question of how markers can be used to identify with confidence the developmental lineages that arise during hESC differentiation *in vitro*. Precisely defining cell identity by marker analysis alone can be confounded by three factors: i) TB 'marker genes' may be expressed in other embryo-derived lineages (e.g. mesoderm), besides TB, thereby confounding the diagnostic utility of these markers; ii) a large number of potential lineage fates must be examined and excluded in order to rule out a developmentally 'mixed' phenotype and thereby identify cells as bona fide TB, and iii) bona fide TB cells should manifest a substantial set of additional expected characteristics. These issues lead us to identify eight decisive areas that could be addressed in future work to ascertain whether hESC truly harbor TB competence, thus forming a framework for future progress and dialogue.

i) It has been recently appreciated that *CDX2*, *HAND1*, and *GATA3* – typically viewed as salient hallmarks of TB – are also expressed in posterior mesoderm, warranting caution in use of these in assigning TB identity to cells formed during BMP-induced differentiation of hESCs (Bernardo *et al*. 2011). This caveat applies equally to differentiation of mESCs induced by BMP treatment (Hayashi *et al*. 2010). Keratin induction by TB differentiation protocols has been cited as key evidence for TB identity (Amita *et al*. 2013), yet keratins have broad embryonic expression in a variety of lineages. Similar issues apply to using an epithelial-like morphology of these differentiated cells to definitively assign TB identity (Erb *et al*. 2011, Amita *et al*. 2013).

ii) Another issue is that candidate hESC-derived TB does not detectably express *SOX2* and *EOMES* (Sudheer *et al*. 2012, Amita *et al*. 2013), which are highly expressed in the mouse extraembryonic ectoderm *in vivo* and TSCs *in vitro*, and are essential for TSC specification (Russ *et al*. 2000, Avilion *et al*. 2003, Keramari *et al*. 2010). The absence of *SOX2* and *EOMES* during *in vitro* differentiation of hESCs toward putative TB raises the question of whether they actually acquire a bona fide TB identity.

iii) Placental hormone secretion following hESC differentiation is compelling, but it is unclear that this precisely identifies the TB lineage, since the placental expression domains of these hormones have not been specifically mapped *in vivo*. Thus, it remains formally possible that these hormones could be expressed by the extraembryonic mesoderm component of the chorio-allantoic placenta as well as by TB. Indeed, there is evidence to suggest that such BMP-induced hESC populations actually correspond to extra-embryonic mesoderm (Bernardo *et al*. 2011).

iv) Though cardinal TSC markers *SOX2* and *EOMES* are not expressed, a variety of other TB genes (e.g. *ELF5* and *AP2* family members) are transcriptionally upregulated to some extent during hESC differentiation (Xu *et al*. 2002, Amita *et al*. 2013). However, these genes are minimally expressed in undifferentiated hESCs, which may exaggerate their fold-change of upregulation, and they may not actually be detectable at the level of protein.

v) BMP-containing culture conditions have also induced the formation of multinucleated cells, which are thought to indicate fused syncytiotrophoblast (e.g. Drukker *et al*. (2012)). Nevertheless, it has been shown that even undifferentiated ESCs can readily fuse with heterologous cell types to form multinucleated syncytia (Ying *et al*. 2002).

vi) HLA-G expression by putative hESC-derived TB has been proclaimed as one measure of TB identity (Amita *et al*. 2013), as HLA-G is expressed by human extravillous TB *in vivo* (Bernardo *et al*. (2011) and references therein). Therefore, it would be a good measure of TB identity. However, other attempts have not detected HLA-G expression, even using sensitive flow cytometry methods (Bernardo *et al*. 2011, Sudheer *et al*. 2012), regardless of the presence or absence of KOSR in the medium (Supplementary Fig. 4 of Bernardo *et al*. 2011). A complementary approach to assess the identity of these cells would be to characterize their expression of HLA-A/HLA-B, as these HLAs are expressed in fetal lineages such as mesoderm but not in extravillous TB (Bernardo *et al*. (2011) and references therein).

However, putative hESC-derived TB, differentiated in either CDM or KOSR, continuously express HLA-A and/or HLA-B (Bernardo *et al.* 2011). This remains to be tested by others.

vii) If differentiating hESCs have decisively entered the TB lineage, this should be reflected at the levels of both gene expression and chromatin. However, the promoter of *ELF5* (a definitive TB hallmark) remains largely methylated in hESC-differentiated TB populations (Bernardo *et al.* 2011), raising the question of whether observed *ELF5* mRNA induction upon hESC differentiation (Amita *et al.* 2013) reflects leaky expression instead of robust upregulation. Although it remains possible that a small fraction of cells that emerge from these culture conditions are bona fide TB with complete *ELF5* demethylation, such cells were not abundant enough to be captured by bisulfite sequencing. In any case, comprehensive chromatin state mapping of putative hESC-derived TB (Xie *et al.* 2013) should enable chromatin-level assessment of *ELF5* and other hallmark TB genes, and thereby determine whether their chromatin state resembles that of placenta.

viii) To fully address whether hESC have TB competence, systematic protocols to elicit putative TB from hESC must be more widely adopted so that these cells can be analyzed by different groups. The use of complex culture systems containing, for example, mouse feeder cells or feeder-conditioned medium (Erb *et al.* 2011, Amita *et al.* 2013), or animal serum (Drukker *et al.* 2012) or even KSR (Xu *et al.* 2002, Amita *et al.* 2013) complicates reproducibility by other groups. Moreover, neither feeder cells nor animal serum represent a physiologic context for TB development, as these contain undefined factors that may influence differentiation in various ways. Ultimately, the adoption of CDM for differentiation in the complete absence of serum or feeders (Bernardo *et al.* 2011) would be ideal to ensure that differentiation protocols can be consistently evaluated throughout different laboratories. Such CDM offer highly controllable culture environs to precisely define the effects of developmental signaling-perturbations (e.g. BMP treatment) on hESC differentiation without being confounded by undefined factors (Bernardo *et al.* 2011, Loh *et al.* 2014).

Overall, these issues emphasize the need to precisely map molecular markers throughout multiple stages of human TB development *in vivo* (Niakan *et al.* 2012) and to establish TSC lines *in vitro* from human embryos. Accurately defining the molecular portrait of bona fide human TB is obligatory to fully evaluate whether putative hESC-derived TB is akin to its *in vivo* counterpart. This is especially important because significant *cis*-regulatory evolution between the human and mouse lineages has markedly distinguished TB development in these two species (e.g. Niakan *et al.* (2012)), therefore hESC differentiation efforts must reciprocally be guided by the knowledge of the human developmental process.

Altogether, it remains possible that hESCs harbor TB potential, but that the necessary extrinsic signaling conditions to expose such competence have so far remained elusive. Alternatively, presumptive epigenetic perturbations could resuscitate dormant TB potential, as DNA methyltransferase disruption reportedly enhances mESC differentiation toward TB (Ng *et al.* 2008), which is a developmentally inaccessible fate for mESCs. Indeed, from a developmental perspective, the peri-implantation epiblast *in vivo* (Gardner & Rossant 1979) and mESCs *in vitro* (Silva & Smith 2008) are generally refractory to TB differentiation. Because hESCs are thought to reflect an even later stage of development (reminiscent of the post-implantation epiblast), it is difficult to reconcile the known sequence of lineage commitments during early mammalian embryogenesis with an apparent TB competence of hESC. Nevertheless, such theoretic considerations should not deter experimental work to more fully explore whether hESC can indeed have close encounters of the TB kind.

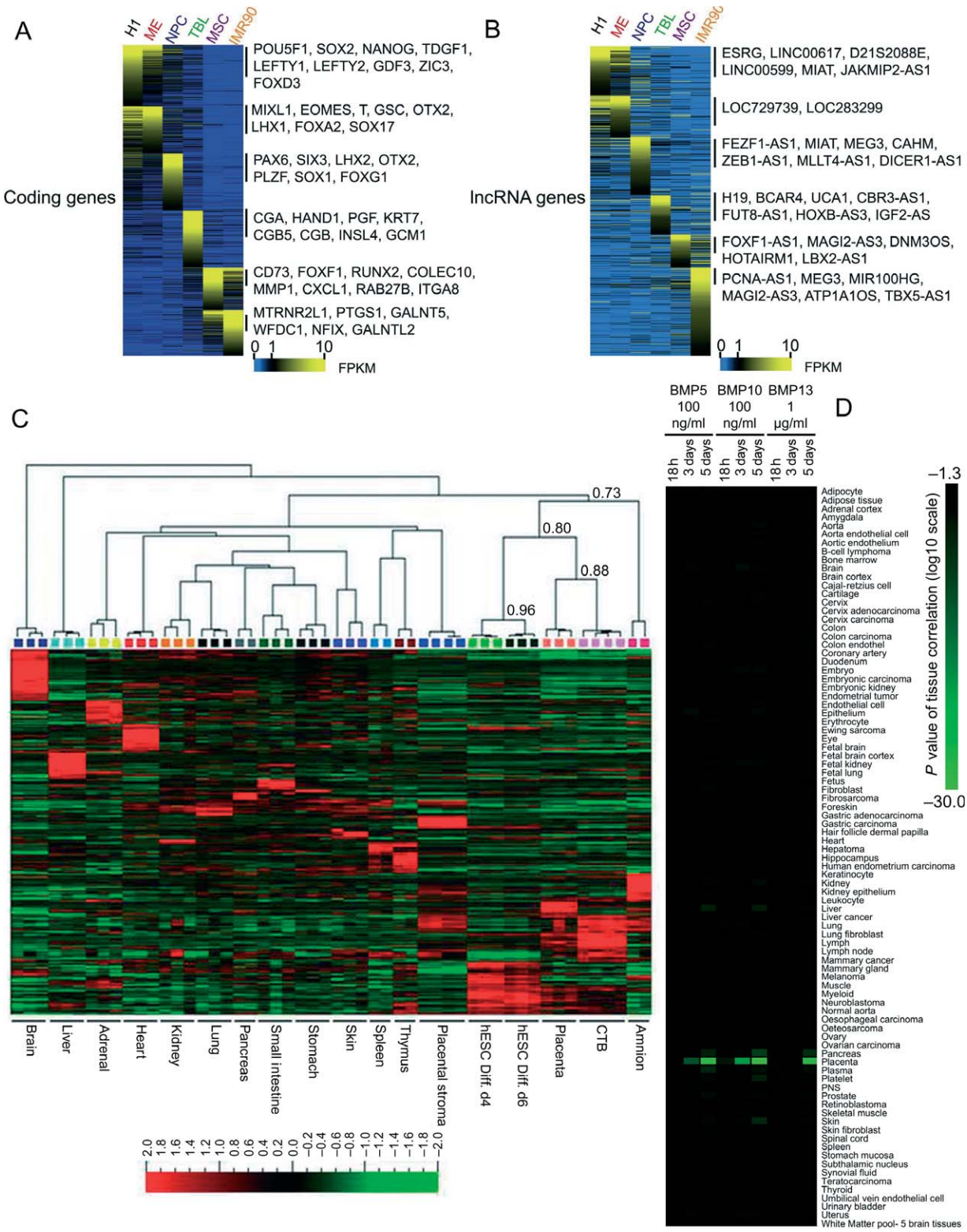
Debate

Response to case against case for differentiation of hESC into TB cells (proposers: R M Roberts, M Amita, K Adachi, A P Alexenko, D J Schust, L C Schulz, B P V L Telugu, and T Ezashi)

Q5 Q6 Pedersen *et al.* raise several issues that they believe to argue against the TB-like properties of the cells generated by BMP4 treatment of hESC (points 1, 3, 5, and 6) and suggest that additional criteria (points 2, 4, and 6) need to be met before TB status can be accepted. What Pedersen's arguments lack is any evidence that the cells are not TB, other than the preconceived notion, based largely on mouse data, that epiblast-derived cells should not be able to embark on this differentiation pathway.

Q7 Just as 'one swallow does not a summer make', one marker gene product does not TB make. Certainly, the expression of any one marker or even several together would not constitute proof that the BMP–hESC had converted to TB, but the combined weight of evidence from numerous studies employing a wide range of early- and late-gene markers, including hundreds from microarray analyses, implies that these cells are TB (Fig. 1 and GEO GSE10469). For example, recent genome-wide expression profiling shows that BMP–hESC cluster with early-gestation placenta and away from all other fetal

tissues, including mesoderm-derived amnion and placental stroma (Li *et al*. 2013). That study also confirms with a different antibody that HLA-G is expressed (point 6 of Loh *et al*.). Another recent paper has shown that BMP–hESC resemble placental TB not just in the expression of coding genes but also in the composition of long non-coding RNA species and in 'epigenetic status' (Xie *et al*. 2013; Fig. 1A and B). Figure 1D also illustrates that the gene expression profiles of H1 hESC exposed to different members of the BMP family for



increasing periods of time correlated most closely with that of the placenta and not with a broad and representative selection of other organs (Lichtner *et al*. 2013). Finally, the arguments against are mistaken in that BMP–hESC do not express *SOX2* and *EOMES* (point 2). They do so robustly for the initial 24 h after BMP4 exposure and only later does expression decline (GEO GSE10469). Moreover, with the exception of placental hormone transcripts, the qPCR data of Amita *et al*. (2013), and all the array data of Telugu *et al*. (2013), were presented as normalized values and not as fold-change (see point 4 of Case against differentiation of hESC into TB cells).

This returns us to the issue as to why the results of (Bernardo *et al*. 2011) differ so dramatically from those of us who have employed the BMP–hESC model. The case against comments that the use of MEF-conditioned medium (Xu *et al*. 2002, Amita *et al*. 2013) is a confounding feature of the differentiation protocol. However, the generation of TB from hESC can be readily performed in a CDM in the absence of products of MEF (Amita *et al*. 2013; Fig. S6B). Moreover, the Roberts's lab have argued that the medium used by Bernardo *et al*. (2011) is not only sub-optimal for culturing hESC, but far from appropriate for testing the soundness of the BMP–hESC model (Amita *et al*. 2013). In essence, the line of reasoning of Pedersen's lab boils down to the idea that TB emergence from hPSCs with the features of epiblast simply should not happen, a premise based entirely on what is perceived to occur during mouse development. Given the differences in early embryonic development between the species, is it not likely that this BMP-directed differentiation to TB may not be homologous to the signaling that generates trophectoderm and its immediate lineage descendants in mouse but, instead, reflects a different stem cell niche found in the early human placenta?

In summary, the counter arguments of Pedersen *et al*. fail to weaken our confidence in the validity of the BMP–hESC model of TR. This controversy could have been averted if Bernardo *et al*. (2011) had reproduced the culture conditions used in the prior studies that had employed the model to study TB development, and by describing their own culture procedures in greater detail such that they could be readily reproduced by others.

Note Added in Proof: two recent reports have shown that two kinds of mouse PSC generated in hitherto unconventional ways, namely by *in vivo* reprograming (Abad *et al*. 2013) and 'stimulus-triggered acquisition of pluripotency' (STAP) (Obokata *et al*. 2014), respectively, can contribute to both the placenta and embryo proper after injection into blastocysts. The former can also give rise to regions of TB-like cells in teratomas, while the STAP cells differentiate along the TB lineage in response to FGF4, the standard growth factor used to grow mouse TSCs. Although it is not clear whether the epigenetic states of these cells are similar and how they acquired totipotent characteristics during their derivation, it is apparent that the dogma that says pluripotent cells derived from anything, but a totipotent blastomere cannot become TB should be rejected.

Response to case for differentiation of hESC into TB cells (opposer: K M Loh, A S Bernardo, and R A Pedersen)

To consider TB induction from hESCs as a valid model of human TB development, we must determine whether hESC-derived progeny truly resemble bona fide TB. Roberts *et al*. enumerate various accounts over the past decade that reported TB formation from hESCs, principally based on the expression of a few marker genes and cell morphology. Importantly, they have not addressed the more rigorous question of whether the chosen markers are sufficient to validate a TB identity, nor have they engaged in distinguishing between circumstantial and direct evidence for a bona fide placental TB phenotype. In the published reports, key markers of TB remained absent, genes from alternate lineages continued to be expressed and TB genes appeared inactive at the chromatin level (Bernardo *et al*. 2011). The current debate thus exemplifies the challenge of correctly identifying and validating the lineages that arise from hESC differentiation *in vitro*, owing to a shortage of the fundamental knowledge of human developmental biology that is needed as a benchmark to evaluate *in vitro* differentiation (McKnight *et al*. 2010, Mascetti & Pedersen 2014). Taking into account this uncertainty, we advocated using more complex and function-based

Figure 1 Proposer's view: examples of TB lineage restricted transcripts present in hESC treated with bone morphogenetic proteins (BMPs). The data were derived from three independent laboratories by different microarray methods. The top panel (Xie *et al*. 2013) compared heat maps for transcripts of coding genes (A) and long non-coding RNA (B) from H1 cells (lane 1) and H1 cells differentiated along various lineages by standardized protocols (ME, mesoendoderm; NPC, neural progenitor cells; TBL, trophoblast-like cells; MSC, mesenchymal stem cells; IMR90, primary human fetal lung fibroblast cells). Differentiation to TBL was achieved with 50 ng/ml BMP4 in FGF2-minus medium for 5 days (TBL). The study demonstrated that known lineage markers were strongly associated with the anticipated lineages, and that the trophoblast-like cells were distinct from the other lineages, including mesoendoderm, where differentiation had been driven with FGF2 and BMP4 in combination for 48 h. (C), Li *et al*. (2013) compared expression profiles of H9 hESC that had been treated with 10 ng/ml BMP4 in FGF2-minus feeder-conditioned medium for either 4 or 6 days (hESC Diff.d4 and hESC Diff.d6 respectively) with whole, first-trimester placenta (Placenta), isolated cytotrophoblast (CTB) from such placentas, and a number of other tissues. Note that the differentiated hESC at both time points clustered most closely with placenta and CTB and were distinct from placental stroma, amnion, and other representative tissues on the array. (D) Lichtner *et al*. (2013) showed that, in addition to BMP4, BMP5, BMP10, and BMP13 in the absence of FGF2 drove hESC to the TB lineage. The transcriptional signatures of H1 hESC treated with the various BMP sub-types for 18 h, 3, and 5 days were compared with those of various tissues and anatomical compartments. The analysis identified placenta as the tissue that most resembled the BMP-treated hESC ($P<0.05$).

criteria (Bernardo *et al*. (2011) and in this debate) as a higher standard for TB identity than has been used in previous literature or by Roberts *et al*. (Amita *et al*. 2013).

Induction of TB-associated markers after differentiation of hESCs using BMP, together with TGFβ and FGF/MAPK antagonists (Erb *et al*. 2011, Sudheer *et al*. 2012, Amita *et al*. 2013), had been regarded as a pivotal advance in modeling TB development. TGFβ and FGF/MAPK inhibition likely suppresses primitive streak/mesoderm induction (Bernardo *et al*. 2011, Loh *et al*. 2014), therefore minimizing the expression of chosen mesoderm genes (e.g. *BRACHYURY* and *TBX4*) and enhancing the expression of a few TB-associated markers (e.g. *KRT7*) (Amita *et al*. 2013). Rather than qualifying as a definitive marker of TB identity, however, *Krt7* is promiscuously expressed in diverse epithelial tissues (Ramaekers *et al*. 1987). Indeed, hESCs induced to express *Krt7* by BMP treatment express not only TB-associated markers but also *Isl1* and other mesodermal markers, possibly reflecting a mesodermal identity (Bernardo *et al*. 2011) or even a developmentally ambiguous mixed phenotype. The *KRT7*-expressing cell populations analyzed by Roberts *et al*. lacked expression of three particular mesoderm genes (*TBX4*, *FLK1*, and *VCAM1*; Amita *et al*. 2013), which signified to them that these cells do not belong to the mesoderm lineage. Their assertion relies on the assumption that presence or absence of a marker associated with a particular cell lineage is sufficient to define and validate cells as belonging to that lineage.

Expression of additional TB markers would perhaps lend confidence to an assignment of TB identity, provided that these were genes with known key functions in TB development, rather than genes expressed by a wide variety of cells and perhaps incidental to TB function. From this perspective, it is significant that transcription factors *SOX2* and *EOMES* (key markers of mouse TSCs and essential for TB specification *in vivo* are not detectably expressed during BMP-induced hESC differentiation (Sudheer *et al*. 2012, Amita *et al*. 2013). While Roberts *et al*. assert that these markers are expressed during the first 24 h of putative TB induction *in vitro*, both transcription factors are expressed in undifferentiated hESCs to some extent and their 'expression during the first day of differentiation' probably reflects residual expression from hESCs undergoing incipient differentiation. Indeed, both *SOX2* and *EOMES* are acutely downregulated within 3 h of BMP4 + TGFβ inhibitor + FGFR/MAPK inhibitor treatment of hESCs and are progressively suppressed with prolonged treatment; at no stage is either gene upregulated (Sudheer *et al*. 2012). Additional key transcriptional regulators of TB (e.g. *ELF5*; Ng *et al*. 2008) are modestly upregulated (approximately tenfold) upon putative TB induction (Amita *et al*. 2013, Li *et al*. 2013), yet to the best of our knowledge, very low expression is typically observed for this gene in hESCs, and robust expression of Elf5 protein has not been reported. Finally, expression of *CDX2*, *GATA3*, and *HAND1* has been cited as a proxy for TB commitment (Sudheer *et al*. 2012, Amita *et al*. 2013); however, these transcription factors are also expressed in posterior mesoderm (Bernardo *et al*. 2011), complicating their use as diagnostic markers. These ambiguities about the significance of expression of TB-associated marker genes (even multiple ones) by BMP-treated hESCs raises the questions of whether i) their TB identity is truly established and ii) the resultant cells share functional properties of bona fide TB. A partial TB identity or a mixture of extraembryonic identities would be reminiscent of the 'partial programing' that occurs when fetal progenitors are exposed to incomplete signaling regimens, leading to lineage infidelity whereby some but not all characteristic lineage markers are expressed (Wandzioch & Zaret 2009).

Instead of addressing the specificity of cardinal TB markers and/or the functionality of putative hESC-derived TB, Roberts *et al*. have instead argued that their microarray profiles broadly cluster with placenta and they share placental gene ontology terms (Fig. 1C and D), therefore, constituting evidence of their identity. However, these cells clearly lack expression of a cluster of signature genes expressed in first-trimester CTB (Li *et al*. 2013) and there are marked transcriptional discrepancies between BMP-treated hESCs and either placenta or CTB (Fig. 1C). Furthermore, it was never assessed whether BMP-treated hESCs might cluster even closer to first-trimester amnion or early hESC-derived mesoderm or extraembryonic mesoderm populations – therefore the relevant biological comparators are missing. To suggest that putative TB does not belong to a mesodermal lineage, Roberts *et al*. have cited RNA-seq analyses in which $Eomes^+$ $Brachyury^+$ anterior primitive streak/'mesendoderm' populations (Supplementary Fig. 1 of Xie *et al*. 2013) were shown to express a cohort of different genes than BMP-treated hESCs (Fig. 1A). However, in this study, any genes commonly expressed between the two cell populations were omitted from the analysis (Supplementary Methods of Xie *et al*. 2013), therefore obscuring any similarities between these lineages (Fig. 1A). Additionally, the relevant biological comparator is lacking again, because BMP-treated hESCs would not be expected to correspond to $Eomes^+$ anterior primitive streak but rather posterior mesoderm and extraembryonic mesoderm.

Faced with these uncertainties about the identity of BMP-induced progeny of hESCs, we have sought to define more complex criteria as an evidence for a bona fide TB phenotype (Bernardo *et al*. 2011).

i) In one approach, antibodies to a constellation of HLA epitopes were used to assess cell surface properties of BMP-induced hESC progeny, based on their presence in villous and/or extravillous

human placental TB (Apps *et al*. 2009, Bernardo *et al*. 2011). Reportedly, BMP-induced hESC progeny stains positively for extravillous TB marker HLA-G (Amita *et al*. 2013). However, even when cells were differentiated on Matrigel and in KSR-containing medium as described by Amita *et al*. (2013), we were unable to detect HLA-G expression in quantitative flow cytometry assays (Bernardo *et al*. 2011). Moreover, the TB specificity of HLA-G immunostaining (Amita *et al*. 2013) remains uncertain. For example, amniotic epithelial cells also express HLA-G (Houlihan *et al*. 1995) and they (along with extraembryonic mesoderm lineages) similarly originate from a region of the mammalian embryo that is strongly influenced by BMP signaling (Lawson *et al*. 1991, Bosman *et al*. 2006).

ii) Migratory behavior is another complex property that could potentially be informative of TB phenotype. BMP-induced hESCs display migratory properties *in vitro* (Amita *et al*. 2013, Telugu *et al*. 2013; thought to be evocative of invasive TB, Roberts *et al*., (this Debate)). Yet dissected primitive streak/nascent mesoderm similarly displays migratory capacities upon explant culture (Burdsal *et al*. 1993). This is likely a feature shared with all populations in the early embryo engaged in morphogenetic movements.

iii) The 'epigenetic' status of key genes involved in placental TB development is another 'complex' criterion for TB identity. In this regard, the promoter of *ELF5* remains highly methylated in most (if not all) hESC-derived candidate TB (Hemberger *et al*. 2010, Bernardo *et al*. 2011), reflecting general transcriptional inactivity at both the mRNA and chromatin levels. This approach to assessing chromatin status could be readily extended to other key TB-associated genes using bisulfite sequencing and histone ChIP-seq methods. Even if additional TB-associated genes were shown to be unmethylated and expressed, however, such *in vitro* evidence for TB identity is purely circumstantial, as it does not demonstrate that the cells could function as bona fide TB during normal placental development.

Researchers have been faced with a similar quandary in delineating the fate of the embryo's own cells and of the stem cells derived from them. The developmental competence of the mouse embryo's native pluripotent cells has been studied extensively in classical studies using heterotopic grafting (Grobstein 1951, 1952) and *in situ* labeling (Lawson *et al*. 1991). In those studies, post-implantation embryonic epiblast never developed into TB derivatives. Likewise, late blastocyst stage embryonic epiblast was incapable of TB contribution after injection into recipient blastocysts (Gardner & Rossant 1979), whereas ectoplacental cone and TSCs could contribute to TB in a similar assay (Rossant *et al*. 1978, Tanaka *et al*. 1998). mESCs injected into blastocysts contributed to a very limited extent to the TB lineage (Beddington & Robertson 1989). Despite their limited ability to contribute to the TB lineage in their native state, mESCs can be reprogramed to TB by overexpressing TB transcription factors (Niwa *et al*. 2005) or by relaxing epigenetic restrictions (Ng *et al*. 2008). In recent studies of the developmental competence of EpiSCs grafted into post-implantation mouse embryos *in vitro* (Huang *et al*. 2012, Kojima *et al*. 2013), these integrated into the primitive streak but did not contribute to the TB lineage. The absence of evidence for TB functional identity is a further obstacle to conclude that post implantation-type PSCs (including mEpiSCs, hESCs, and hiPSCs) can be induced to differentiate *in vitro* into bona fide TB (Brons *et al*. 2007).

While it remains formally possible that a previously untested intervention (consisting of extrinsic signaling conditions with or without transcription factor or chromatin perturbations) could respecify hESCs to a phenotype that convincingly models bona fide placental TB, this has not yet been demonstrated. Accordingly, the presently available data do not support the contention of Roberts *et al*. that it is 'unambiguously clear' that hESCs can give rise to TB. In our contributions to this debate, we emphasized the limitations of gene expression for validating *in vitro* differentiation and advocated using more complex, functional criteria to accurately identify TB. In our previously published work, we chose CDM, not for its efficiency of differentiation into cells with a putative TB phenotype, but because it was maximally defined, thereby enabling us to understand the signaling mechanisms responsible for particular cell fate decisions of Bernardo *et al*. (2011). In their contribution, Roberts *et al*. have focused largely on reaffirming their choice of media (DMEM/F12/KOSR) and exogenous factors (the TGFβ inhibitor A-83-01 and the FGFR inhibitor PD173074) that enhance the efficiency of generating cells with a putative TB phenotype, based primarily on marker gene expression. Amita *et al*. (2013) have showed and Roberts *et al*. (current Debate) have reaffirmed that alterations in the conditions used for BMP-induced differentiation of hESCs can increase the incidence of $KRT7^+$ cells. A fraction (4–8%) of $KRT7^+$ cells emerged in our CDM-based culture conditions, but these also expressed mesoderm-associated genes (Bernardo *et al*. 2011). In any case, increasing the frequency of $KRT7^+$cells does not by itself address whether this represents bona fide placental TB.

If hESCs could truly give rise to TB, this would fundamentally revise our view of how developmental competence is relinquished during early embryonic lineage decisions. However, such a conclusion would require unequivocal proof that such cells met more complex *in vitro* criteria than simply expressing

TB-associated markers and would require proof of their ability to function as placental TB. Roger Bacon once wrote 'argument is conclusive, but it does not remove doubt … unless it finds it by the method of experiment' (Bacon, http://en.wikiquote.org/wiki/Roger_Bacon) and likewise only objective evidence will enable us to advance from the present state of affairs and permit a more confident determination of TB identity among the progeny of hESCs. The aspiration for research funding to study this phenomenon is not a compelling argument for its existence.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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