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Journal

Development, 140(19)

ISSN

0950-1991

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Publication Date

2013-10-01

DOI

10.1242/dev.092155

Peer reviewed

Development 140, 3965-3976 (2013) doi:10.1242/dev.092155

BMP4-directed trophoblast differentiation of human embryonic stem cells is mediated through a ΔNp63$^+$ cytotrophoblast stem cell state

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SUMMARY

The placenta is a transient organ that is necessary for proper fetal development. Its main functional component is the trophoblast, which is derived from extra-embryonic ectoderm. Little is known about early trophoblast differentiation in the human embryo, owing to lack of a proper *in vitro* model system. Human embryonic stem cells (hESCs) differentiate into functional trophoblast following BMP4 treatment in the presence of feeder-conditioned media; however, this model has not been widely accepted, in part owing to a lack of proof for a trophoblast progenitor population. We have previously shown that p63, a member of the p53 family of nuclear proteins, is expressed in proliferative cytotrophoblast (CTB), precursors to terminally differentiated syncytiotrophoblast (STB) in chorionic villi and extravillous trophoblast (EVT) at the implantation site. Here, we show that BMP4-treated hESCs differentiate into bona fide CTB by direct comparison with primary human placental tissues and isolated CTB through gene expression profiling. We show that, in primary CTB, p63 levels are reduced as cells differentiate into STB, and that forced expression of p63 maintains cyclin B1 and inhibits STB differentiation. We also establish that, similar to *in vivo* events, hESC differentiation into trophoblast is characterized by a p63$^+$/KRT7$^+$ CTB stem cell state, followed by formation of functional KLF4$^+$ STB and HLA-G$^+$ EVT. Finally, we illustrate that downregulation of p63 by shRNA inhibits differentiation of hESCs into functional trophoblast. Taken together, our results establish that BMP4-treated hESCs are an excellent model of human trophoblast differentiation, closely mimicking the *in vivo* progression from p63$^+$ CTB stem cells to terminally differentiated trophoblast subtypes.

KEY WORDS: ΔNp63, BMP4, Human embryonic stem cells, Placenta, Trophoblast

INTRODUCTION

Survival and development of the embryo *in utero* depends on the placenta, a transient organ composed of extra-embryonic mesoderm (ExM) and extra-embryonic ectoderm (ExE) (John and Hemberger, 2012). The latter is derived from trophectoderm, the outer layer of the blastocyst, and differentiates to give rise to the epithelial portion of the placenta, including villous and extravillous trophoblast (John and Hemberger, 2012; Rossant, 2007; Roberts and Fisher, 2011). Early during human placental development, mononuclear cytotrophoblast (CTB) differentiates into either syncytiotrophoblast (STB) in floating chorionic villi, or into extravillous trophoblast (EVT) in anchoring villi. The former are responsible primarily for gas and nutrient exchange, whereas the latter invade the uterine stroma and spiral arterioles in order to access maternal blood and establish the maternal-fetal interface (Roberts and Fisher, 2011).

Much of what we know about placental development and trophoblast differentiation comes from studies of rodent models (Soares et al., 2012; Cockburn and Rossant, 2010). In particular, this is due to the availability of numerous transgenic mouse models with placental defects combined with the ability to derive and culture trophoblast stem cells (TSCs), thus providing opportunity for both *in vivo* and *in vitro* analysis of trophoblast differentiation and placental function (Cockburn and Rossant, 2010; Rossant and Cross, 2001; Tanaka et al., 1998). Our understanding of human placental development is limited by multiple factors, including limited access to early gestation tissues, primary CTB, which becomes non-proliferative upon isolation, and cell culture models that poorly represent human trophoblast differentiation *in vivo* (Apps et al., 2009; Bilban et al., 2010). Therefore, there is a great need to establish a human 'trophoblast stem cell' model, with the capacity for indefinite self-renewal and the ability to differentiate into both villous and extravillous trophoblast. However, to date, even the defining characteristics of such a human TSC remain controversial. Many genes have been designated, based on rodent studies, as required for establishment of the trophoblast lineage and/or maintenance of TSCs, including *Cdx2*, *Elf5* and *Eomes* (Strumpf et al., 2005; Niwa et al., 2005; Donnison et al., 2005; Ng et al., 2008; Russ et al., 2000; Kidder and Palmer, 2010). Some studies have identified the mRNA for these genes in human trophectoderm or primary trophoblast, and fewer have indisputably confirmed presence of the corresponding proteins in this compartment (Adjaye et al., 2005; Hemberger et al., 2010; Niakan and Eggan, 2013); even fewer studies have shown a definite role for these gene products in human trophoblast proliferation and/or differentiation. More recently, even the site of origin of human trophoblast progenitor cells has been disputed,

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Accepted 12 July 2013

with the non-trophoblastic component of the chorion proposed to contain trophoblast precursor cells (Genbacev et al., 2011); however, the exact identity of these chorion-derived cells remains to be explored.

Over the past decade, multiple studies have identified human pluripotent stem cells (hPSCs), including embryonic (hESCs) and induced pluripotent stem cells (hiPSCs), as useful models for trophoblast differentiation; specifically, in the presence of feeder-conditioned medium (FCM), BMP4 induces this lineage, with production of both human chorionic gonadotrophin (hCG)β-secreting STB and surface HLA-G-expressing EVT (Xu et al., 2002; Das et al., 2007; Wu et al., 2008; Marchand et al., 2011; Bai et al., 2012). These results imply the presence, however transient, of a trophoblast progenitor population; in fact, a recent report indicates that surface aminopeptidase A (APA)-positive cells are progenitors, at least for multinucleated STB (Drukker et al., 2012). The utility of hPSCs for the study of trophoblast differentiation has recently been questioned by findings from a single study, which showed that the generation of a CDX2-positive cell population by BMP4 treatment of hESCs was abrogated by downregulation of brachyury (*T*), an early mesendoderm marker, with the conclusion that these cells are more likely to be mesodermal in origin with aberrant expression of a few trophoblast markers (Bernardo et al., 2011). However, unlike all previous studies, the latter study used BMP4 in the presence, not of FCM, but of chemically defined media, originally formulated for derivation and culture of mouse epiblast stem cells (mEpiSCs). In so doing, these authors assume that early lineage specification is identical in mouse and human, when in fact many differences have been documented (Rossant, 2008; Benirschke and Kaufmann, 2000; Berg et al., 2011; Roode et al., 2012). In fact, a recent study by Amita et al. (Amita et al., 2013) has recently confirmed formation of bona fide trophoblast from hESCs, differentiated in FCM-containing media.

Here, we have employed primary human placental tissues to evaluate CTBs, which, based on previous studies, including our own (Lee et al., 2007), serve as progenitors to all trophoblast lineages. In order to determine whether FCM-BMP4-treated hPSCs can indeed recapitulate the *in vivo* process of trophoblast differentiation, we compared these cells with a series of fetal and placental tissues, as well as isolated CTBs from both early and late gestation placentas. By comparative gene expressing profiling, we find that FCM-BMP4-treated hESCs cluster with placental tissue and isolated CTB, and not with mesoderm-derived placental stroma or amnion. We show that they progress through a CTB-like phenotype, prior to developing into terminally differentiated STB and EVT. We take these findings a step further, based on our previous work identifying ΔNp63α, a *TP63* (also known as *p63*) isoform involved in maintenance and differentiation of stem cells in stratified epithelia (Senoo et al., 2007; Candi et al., 2008), as a proliferative CTB-specific marker in the human placenta (Lee et al., 2007). In the current study, we establish its roles both in maintenance of the CTB phenotype using primary cells isolated from human placentas, and during FCM-BMP4-induced trophoblast differentiation of hESCs. We establish that FCM-BMP4-treated hESCs in fact undergo a $p63^+/KRT7^+$ CTB stem cell state, subsequently differentiating further into hCG-secreting STB and HLA-G-expressing EVT. Moreover, downregulation of ΔNp63α significantly hampers BMP4-directed differentiation into both of these differentiated trophoblast phenotypes. Based on these findings, we conclude that human pluripotent stem cells are in fact a highly useful model for studying trophoblast lineage specification and differentiation as they recapitulate *in vivo* processes during human placental development.

MATERIALS AND METHODS

Human fetal and placental samples, and isolation and culture of primary cytotrophoblast

All fetal and placental tissues were collected under a protocol approved by the Human Research Protections Program Committee of the University of California San Diego (UCSD) Institutional Review Board; all patients gave informed consent for collection and use of these tissues.

First-trimester CTB were isolated from 7- to 12-week gestational age placentas. Chorionic villi were minced, washed in PBS, and subjected to three sequential digestions: Digestion I: 300 U/ml DNase I (Sigma), 150 U/ml collagenase and 50 U/ml hyaluronidase (Stemcell Technologies); Digestions II and III: 0.25% trypsin (Gibco) and 300 U/ml DNase I. Only the second and third digests were used in subsequent Percoll gradients.

Term placentas were obtained and processed within 1 hour after cesarean section delivery. Following washing in PBS with antibiotics, villous tissue was minced, transferred to a cell dissociation sieve and rinsed with PBS. Three enzyme digestion steps were carried out, each using 0.25% trypsin and 300 U/ml DNase I. Only the second and third digests were used in subsequent Percoll gradients.

The pelleted cells from the second and third digests were pooled, resuspended in HBSS, and CTB was separated on a Percoll gradient. Cell purity was determined by KRT7 fluorescence-activated cell sorting (FACS); the majority of preps were >95% KRT7 positive. Term CTB were seeded at a density of 10^6 cells/ml of Iscove's modified Dulbecco's medium (IMDM) with 10% fetal bovine serum (FBS) and antibiotics, and cultured for up to 7 days.

Placental stroma consisted of tissue left over following digestion of villous tissue for isolation of CTB from first trimester placentas; this tissue was evaluated by KRT7 immunostaining to confirm lack of any remaining CTB. Amnion was peeled off from fetal membranes of term placentas.

Human pluripotent stem cell culture and differentiation

The human pluripotent stem cell aspect of the research was performed under a protocol approved by the UCSD Institutional Review Board and Embryonic Stem Cell Research Oversight Committee.

Prior to differentiation, human ESCs (WA09/H9 clone) and induced pluripotent stem cell (iPSC) lines (Laurent et al., 2011) were cultured under feeder-free conditions in StemPro medium (Invitrogen) with 12 ng/ml recombinant basic fibroblast growth factor (bFGF) (Invitrogen) on Geltrex (BD Biosciences)-coated plates. Cells were passaged with StemDS (ScienCell Research Laboratories) every 3 to 5 days.

For trophectoderm differentiation, hPSCs were first treated with a StemPro-based basal medium (Erb et al., 2011) for 2 days, then cultured in feeder-conditioned medium supplemented with 10 ng/ml human BMP4 (StemRD) for the indicated number of days.

Constructs for overexpression and downregulation of p63

Full-length ΔNp63α cDNA was PCR amplified and cloned into pCMV-lentiviral-based vector (kindly provided by Dr Jack Bui, University of California San Diego, CA, USA) by using *Bam*HI/*Eco*RI restriction sites. pLKO.1-based scrambled short hairpin RNA (shRNA) was obtained from Addgene (Cambridge, MA, USA). A set of five Mission shRNA Lentiviral constructs targeting the human *TP63* gene were purchased and packaged for transduction according to the manufacturer's instructions (Sigma). Lentiviral supernatants were concentrated with PEG-it virus precipitation solution (System Biosciences). The concentrated viral particles were then incubated with target cells in the presence of 8 μg/ml polybrene (Sigma). Packaging and infection efficiency were tested using a GFP-expressing lentivirus (same pCMV-lentiviral backbone as used for p63). Overexpression and knockdown efficiency were determined by western blot. Of the five *TP63*-specific shRNA constructs, three worked best, based on protein knockdown in HaCaT cells (data not shown); therefore, subsequent experiments were performed with this subset of shRNAs pooled together.

Immunostaining of cells and tissues

For immunofluorescence on formalin-fixed, paraffin-embedded placental tissues, sections were de-paraffinized and rehydrated, and antigen retrieval was performed using heat and antigen retrieval buffer (Dako) for

20 minutes. Tissues were incubated with mouse anti-p63 (4A4, Sigma), mouse anti-hCGβ (Abcam), rabbit anti-KLF4 (Sigma) and/or rabbit anti-KRT7 (Abcam) primary antibodies, then visualized by Alexa 488- or Alexa 594-conjugated secondary antibodies (Invitrogen).

For immunohistochemistry on formalin-fixed, paraffin-embedded human blastocyst tissue, serial sections were stained with the same antibodies as above, plus mouse anti-inhibin-α or mouse anti-Ki67 antibodies (both from Dako) using a Ventana Discovery Ultra automated immunostainer with standard antigen retrieval and reagents as per the manufacturer's protocol.

For immunofluorescence staining of CTBs and hESCs, cells grown on coated coverslips were fixed with 4% paraformaldehyde, then permeabilized with 0.5% Triton X-100 and incubated with primary antibodies mentioned above, plus mouse anti-HLA-G (4H84 clone, Abcam), rabbit anti-OCT4 (Abcam), rabbit anti-Ki67 (Abcam), rabbit anti-CDX2 (Abcam), goat anti-BRACHYURY (R&D Systems) or mouse anti-PECAM1 (Millipore), and visualized with Alexa Fluor-conjugated secondary antibodies. For nuclear staining, cells were incubated with DAPI (Invitrogen) for 5 minutes.

All stained tissues and cells were imaged using a Leica DM IRE2 inverted fluorescence microscope or a Leica DM2500 transmitted light microscope.

Flow cytometric analysis and FACS

Bromodeoxyuridine (BrdU) cell proliferation assay of primary CTB and fibroblasts was performed using the FITC BrdU Flow Kit (BD Pharmingen), following the manufacturer's protocol.

For immunostaining, cells were fixed with 2% paraformaldehyde (PFA); for intracellular antigens (p63, KRT7, and KRT18) only, cells were permeabilized with 100% ice-cold methanol for 30 minutes. Cells were incubated with antibodies at room temperature for 1 hour, washed and resuspended in an appropriate volume of FACS buffer. FACS analysis was carried out using a BD FACS-Canto Flow Cytometer. Antibodies used for FACS included mouse anti-HLA-G (MEMG9, Abcam), mouse anti-KRT18 (Abcam), PerCP-Cy5.5-conjugated mouse anti-p63 (Santa Cruz), FITC-conjugated mouse anti-KRT7 (EMD Millipore) and APC-conjugated mouse anti-EGFR (BioLegend). The EGFR antibody was also used to sort live cells.

RNA isolation, quantitative real-time PCR (qRT-PCR), and gene expression microarray

Total RNA was extracted with the mirVana RNA Isolation Kit (Ambion) according to the manufacturer's protocol. Purity and concentration of samples were assessed with NanoDrop ND-1000 Spectrophotometer (Thermo Science). RNA was subjected to bioanalysis and only those tissue RNA samples with an RNA integrity number (RIN) >8.0 were subjected to further analysis; all RNA from cell samples had a RIN>9.5.

cDNA was prepared and qRT-PCR performed as described previously (Parast et al., 2009). Relative mRNA expression levels, compared with 18S rRNA, were determined by the $\Delta\Delta C_T$ method. All primer pairs (supplementary material Table S1) were checked for specificity by using BLAST analysis, agarose gel electrophoresis and thermal dissociation curves to ensure amplification of a single product with the appropriate size and melting temperature. Reverse transcriptase PCR for various *TP63* isoforms was performed using the indicated primers (supplementary material Table S2).

Gene expression profiling was performed using HumanHT-12 v4 Expression BeadChips according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Samples were prepared using the TotalPrep Kit (Ambion) according to the manufacturer's instructions. Probes were filtered with a detection *P*-value cut-off of 0.01, and normalized using the LUMI package in R with the RSN (robust spline normalization) method. Microarray data have been deposited in Gene Expression Omnibus (GEO) under accession number GSE49354.

Analysis of gene expression profiling data

The list of probes for cluster analysis was created in Qlucore comparing each fetal sample group against the others, including amnion, placental stroma, and first trimester placental samples and CTBs (variance 0.02, two-group analysis $q \leq 0.05$). The combined probe list was used to perform bi-clustering of FCM-BMP4-treated hESC samples at d4 and d6. Hierarchical clustering of samples was performed by using Cluster 3.0; data were not centered, the distance measure was the Pearson correlation coefficient algorithm, and the linkage criterion was average linkage. Probe clustering was performed by Qlucore using the agglomerative clustering algorithm. Probe lists, with fold change data, are provided in supplementary material Table S3 (for Fig. 1), supplementary material Table S4 (for Fig. 2) and supplementary material Table S5 (for Fig. 7E).

Expression profiles of first (n=6) and third (n=2) trimester CTBs and FCM-BMP4-treated hESC (H9/WA09) at day 2, day 4 and day 6 (n=3 for each time point) were compared with a panel of human pluripotent stem cells, including both hESC (n=26) and hiPSC (n=25) lines (variance 0.02, two-group analysis $q \leq 0.01$). Probes that varied significantly between the PSC panel and the day 0 (undifferentiated) hESC (H9/WA09) were removed in an initial filtering step. A cut-off fold-change of >1.4 between each group and PSC was used. The probe lists obtained for first and third trimester CTBs were separately compared with each time point of FCM-BMP4-treated hESC and proportional Venn diagrams created with Venn Diagram Plotter (http://omics.pnl.gov/software/VennDiagramPlotter.php).

Heatmaps of hESCs and of hESCs transduced with lentiviruses expressing scrambled control and *TP63* shRNAs, treated with FCM-BMP4 were created by using Qlucore. Trophoblast-associated genes were selected based on previously published studies, with trophectoderm (TE)-specific genes inferred specifically from Bai et al. (Bai et al., 2012). Cut-offs were set to variance=0.02 and multi-group comparison $q \leq 0.01$.

Measurement of secreted total and hyperglycosylated hCG

Cell culture supernatants were collected. Levels of total hCG were quantified using hCG ELISA Kit (Calbiotech) according to the manufacturer's protocol. Hyperglycosylated hCG (H-hCG) was quantified by an electrochemiluminescent procedure using specific antibodies at Quest Diagnostics. The results were normalized to DNA content.

Statistical analysis

Unless otherwise stated, data presented are mean ± s.d. of three replicate wells of a single preparation of cells; the results shown are representative of three to five separate experiments each performed on different days and using a different preparation of cells. Student's *t*-test was performed and *P*-values below 0.05 were taken to indicate a statistically significant difference between the populations sampled.

RESULTS

Comparative gene expression profiling of human fetal and placental tissues, isolated CTB and placental stroma, and FCM-BMP4-treated hESCs

To determine the identity of FCM-BMP4-treated hESCs, we subjected these cells, along with a series of fetal and placental tissues and isolated cells, to microarray-based genome-wide gene expression profiling. After filtering for probes that were specifically differentially expressed in each tissue type, hierarchical clustering showed that FCM-BMP4-treated hESCs were most similar to placental tissue and isolated primary CTB, clustering away from placental stroma and amnion (Fig. 1A). Principal component analysis (PCA) also confirmed that these cells were most closely related to first trimester placenta and CTB (Fig. 1B). Samples analyzed at days 0, 2, 4 and 6 of differentiation showed evidence of progressive differentiation, with some early trophoblast progenitor-associated genes, such as *CDX2* and *STRA13* being induced on day 2-4, whereas genes associated with terminally differentiated trophoblast, including *HLA-G*, *CGA* and *CGB1*, were induced later, on day 6 (Fig. 1C,D). Gene expression analysis of a previously published list of human TE-specific transcription factors showed induction of 12 out of 16 TE-specific transcription factors over the first 6 days of differentiation (Fig. 1E) (Bai et al., 2012). These included induction of *TP63* (further discussed below) and *PPARG*; the latter has been identified by our own studies and those of others

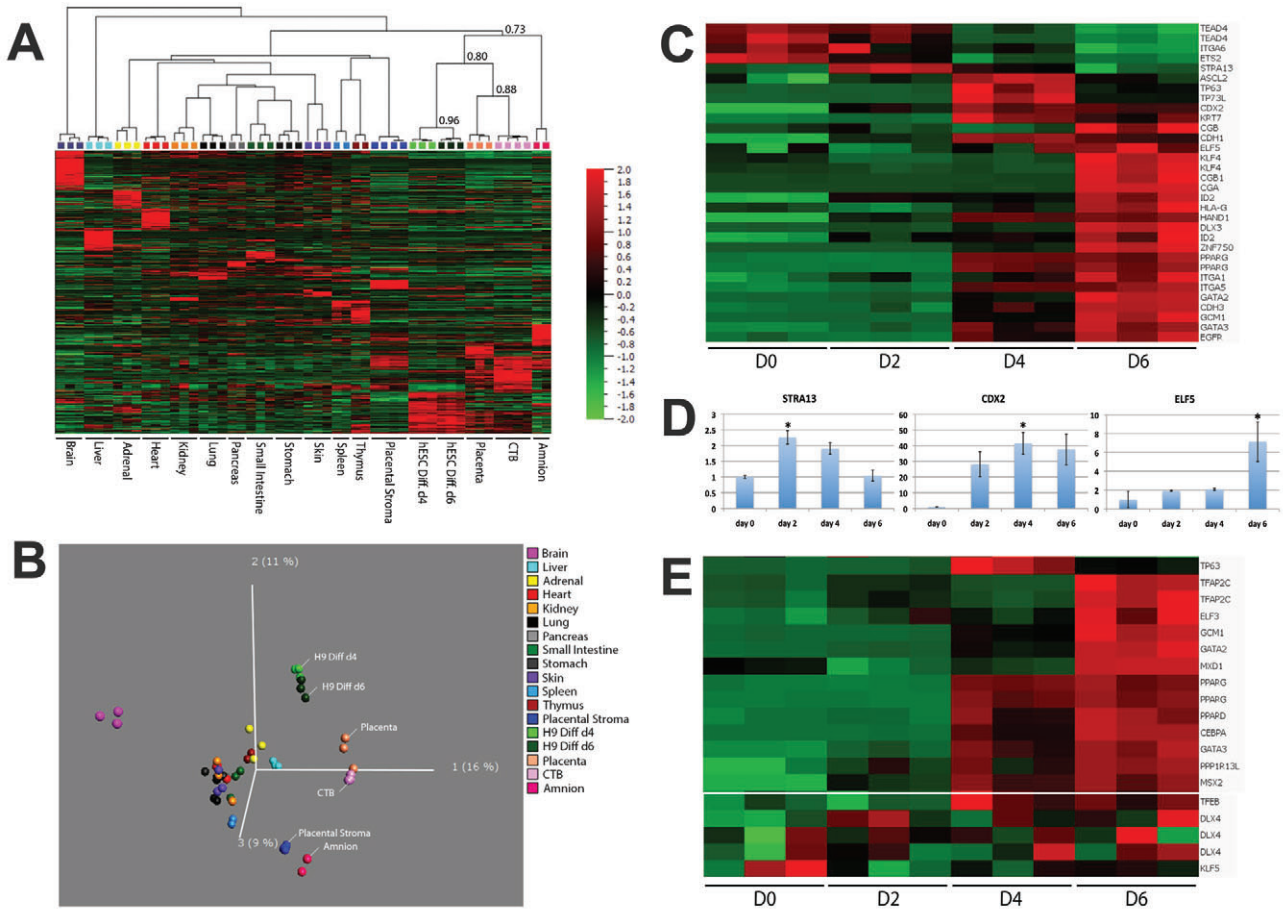

Fig. 1. BMP4-treated hESCs most closely resemble placenta and primary CTB and express trophoblast-associated genes. (**A**) Hierarchical clustering shows that hESCs treated with FCM-BMP4 at days 4 and 6, most closely resemble placental tissues and primary CTB. Pearson correlation coefficients are shown for the FCM-BMP4-day4/day6/placental tissue/CTB cluster. (**B**) PCA shows that the first principal component (horizontal axis) distinctly separates differentiated hESC/placenta/CTB samples from a variety of fetal tissue samples, whereas the second principal component (vertical axis) accounts for the differences among the differentiated hESC/placenta/CTB samples. (**C**) Heatmap of trophoblast-specific genes during the 6-day FCM-BMP4-treatment timecourse (D0-D6) shows progressive differentiation of BMP4-treated hESCs from progenitor to terminally differentiated trophoblast phenotype. (**D**) qRT-PCR for indicated markers, confirming microarray data with peak days of expression indicated by asterisks. Data are expressed as fold change relative to day 0. (**E**) Heatmap of human trophectoderm-specific transcription factors (Bai et al., 2012): out of 16 genes, 12 are induced at one or more time points following FCM-BMP4 treatment of hESCs (top portion of the heatmap); three genes (*TFEB*, *DLX4* and *KLF5*) do not show a consistent pattern of expression during differentiation (bottom portion of the heatmap) and one (*MAFK*) was not expressed at a detectable level in any of the samples (not shown). Some genes are listed twice on the heatmaps, as they are represented by more than one probe on the microarray.

as an upstream regulator of the *GCM1-ERVW1*-directed pathway of syncytiotrophoblast differentiation (Baczyk et al., 2009; Parast et al., 2009).

We next compared the gene expression profile of FCM-BMP4-treated hESCs with those of CTBs isolated from both first trimester and term placental tissues. We used undifferentiated hPSCs (both hESCs and hiPSCs) as the comparison group in order to focus on gene expression changes induced by the differentiation process. We examined the overlap between the gene sets differentially expressed (1) between primary CTBs and undifferentiated hPSCs, and (2) between FCM-BMP4-treated hESCs and undifferentiated hPSCs, demanding a match between both the gene name and the directionality of change in gene expression. This overlap progressively increased over 6 days of differentiation (37% → 58% → 64% for first trimester CTBs; and 32% → 52% → 56% for term CTBs; Fig. 2A). The large majority of the changes in gene expression were maintained over the differentiation timecourse (Fig. 2B, red arrows), as well as between the first and third trimester primary CTBs (Fig. 2B, blue arrows). Taken together, these results suggest that FCM-BMP4-treated hESCs differentiate towards an early CTB-like phenotype.

p63 in cytotrophoblast proliferation and differentiation

TP63 (p63) is a member of the p53 family of nuclear proteins, and has multiple isoforms expressed in a tissue-specific manner (Candi et al., 2008; Crum and McKeon, 2010). The two major isoforms vary at the N-terminus and include the longer, p53-like TA isoform and the truncated ΔN isoform, each of which can be further subdivided into α, β and γ isoforms based on variable C-terminal sequences (Barbieri and Pietenpol, 2006). The best-studied and most abundant isoform is ΔNp63α, which was previously shown to maintain the stem cell state in stratified epithelia (Senoo et al., 2007; Martens et al., 2004). In fact, this was also the most abundant

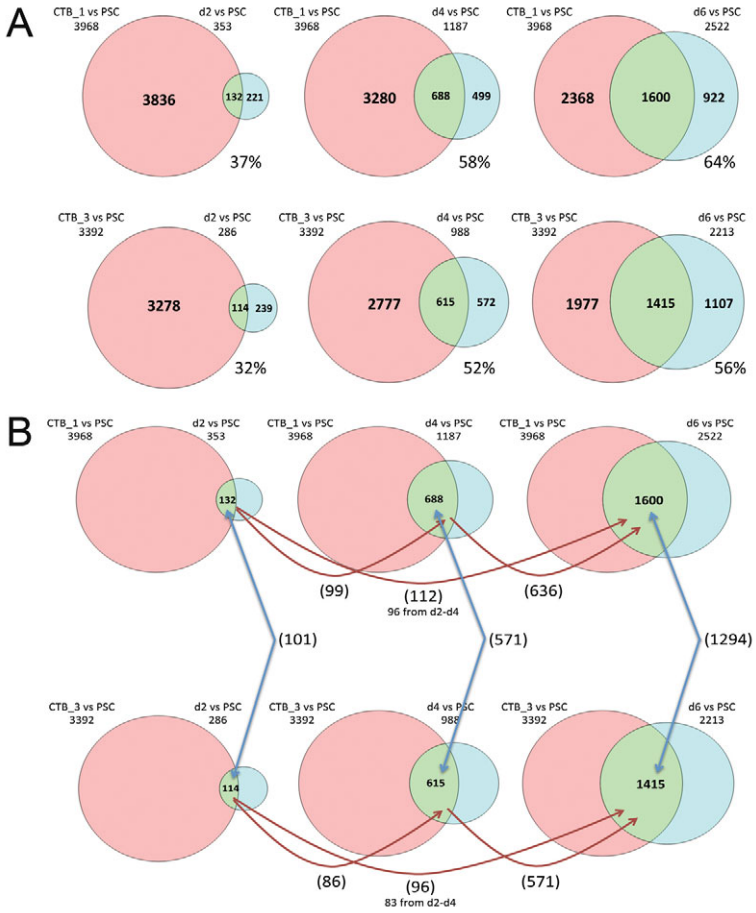

Fig. 2. BMP4-treated hESCs show gene expression changes consistent with differentiation from pluripotency to cytotrophoblast. (**A**) Proportional Venn diagrams, showing overlap (green) of gene expression changes between PSCs-to-CTB (pink) and PSCs-to-FCM-BMP4-treated hESCs (blue). Comparisons including first trimester CTB (top row) and third trimester CTB (bottom row) are shown. Note the progressive increase in the percentage of overlapping genes between the two groups, reaching 64% in the first trimester CTB comparisons, and 56% in the third trimester CTB comparisons. (**B**) The majority of the changes in gene expression were maintained over the differentiation timecourse (red arrows), as well as between the first and third trimester primary CTBs (blue arrows).

isoform in CTB isolated from human placental tissues (supplementary material Fig. S1). Henceforth, the term 'p63' will be used in place of ΔNp63α.

We have previously shown that p63 is expressed in proliferative CTB, and is excluded from terminally differentiated trophoblast, both villous STB and invasive EVT (Lee et al., 2007). Here, we further confirmed that, in first trimester placental tissues, p63 was expressed in all Ki67$^+$ CTB (Fig. 3A). p63 is excluded from placental stroma in both first trimester and term placenta (Fig. 3A,B), and from amnion (Fig. 3B). In fact, dual expression of p63 and cytokeratin 7 (KRT7) in the placenta is confined to CTB (Fig. 3B,C). In addition, *KLF4* has been identified as a gene downstream of p63, involved in terminal differentiation of epidermal cells (Sen et al., 2012). In first trimester placental villous tissue, p63 localized exclusively to KRT7-positive CTB, whereas KLF4 was most abundantly expressed in hCGβ-producing STB (Fig. 3C). In an archived sample of early human blastocyst, immunohistochemistry of serial sections showed p63 co-expressed in Ki67$^+$ and KRT7$^+$ CTB, whereas inhibin and hCGβ were present in the overlying STB layer (Fig. 3D).

We next evaluated the role of p63 in CTB differentiation *in vitro*. CTBs isolated from term placentas quickly lost their proliferative potential (supplementary material Fig. S2). Over time in culture, they lost p63 expression (Fig. 4A-C) and differentiated into KLF4$^+$, hCG-producing STB (Fig. 4D-F).

In order to determine the role of p63 in primary CTB, we generated a lentiviral construct for forced expression of ΔNp63α downstream of the CMV promoter. We optimized and confirmed proper packaging of the construct and infection of the cells using a GFP-expressing lentivirus (data not shown). Transgene expression was high at day 3, but was significantly decreased by day 5 (Fig. 5A); therefore, cells were evaluated only through day 5 (instead of day 6-7, as in Fig. 4). Although forced expression of p63 did not induce primary CTBs to proliferate (supplementary material Fig. S3), it did result in sustained levels of cyclin B1 expression (Fig. 5B), similar to overexpression of another CTB marker, ID2 (Janatpour et al., 2000). Conversely, markers of STB differentiation were markedly reduced based on both ELISA and qPCR (Fig. 5A,C). p63 knockdown in these cells did not further accelerate p63 loss, and as such, did not affect proliferation or differentiation (supplementary material Fig. S3; data not shown).

p63 in hESC-derived trophoblast

We noted *TP63* to peak at day 4 following FCM-BMP4 trophoblastic induction of hESCs based on microarray analysis (Fig. 1C). To confirm this result, we differentiated both hESC-H9 cells and an iPSC line (Laurent et al., 2011) into trophoblast, using FCM-BMP4 over a 10-day period (Xu et al., 2002; Das et al., 2007). The only isoform of *TP63* expressed in BMP4-treated hESC was ΔNp63α (supplementary material Fig. S4). In both H9 and the iPSC line, p63 was initially induced at day 2, peaked at day 4-5, and continued to be expressed at lower levels thereafter (Fig. 6A); *CDX2* expression was induced and peaked one day earlier than p63 (Fig. 6A). Markers of differentiated trophoblast (*CGA*) were induced at day 5-6 and peaked at day 7-8 (Fig. 6A). Differentiation was further confirmed by immunostaining, showing p63$^+$/KRT7$^+$ CTB at day 5, and HLA-G$^+$/KRT7$^+$ EVT and hCG$^+$/KLF4$^+$ STB at day 7 (Fig. 6B). FACS analysis showed almost 90% of cells to be

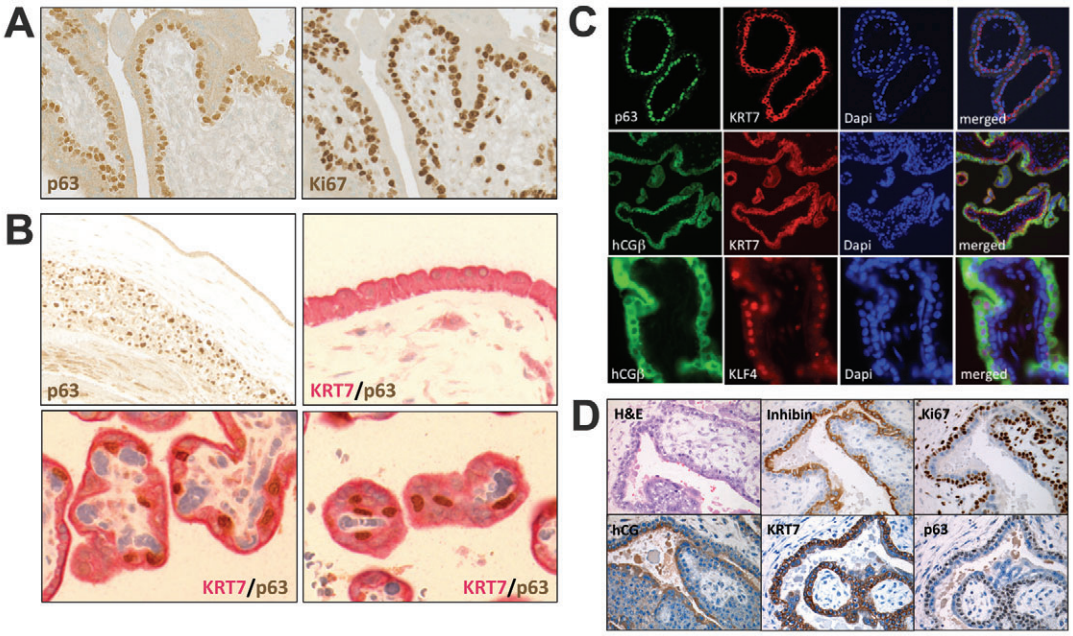

Fig. 3. Markers of CTB and STB in early placental tissues. (**A**) First trimester placental tissue, with consecutive sections stained for p63 (left) and Ki67 (right). (**B**) Term placental tissue: in fetal membranes (top left), p63 stains only chorionic trophoblast and not amnion. Amnion (top right) is KRT7-positive (red), but negative for p63 (brown). In term villous tissue (bottom panels), p63 (brown) highlights CTB whereas KRT7 (red) stains both CTB and STB. (**C**) Immunostaining of first trimester placental villi. Note that p63 highlights CTB, whereas KLF4 and hCGβ highlight STB. KRT7 more prominently stains CTB, but is also expressed in STB. (**D**) Archived human blastocyst sample, early after implantation, shows p63 and KRT7 in the proliferative (Ki67-positive) CTB layer, adjacent to the underlying mesenchyme, whereas inhibin and hCGβ stain the STB layer, adjacent to the maternal vascular space. Brown, immunostaining of indicated marker; blue, hematoxylin-stained nuclei.

double-positive for KRT7 and p63 at day 5 (Fig. 6C), and ~25% of the cells to be HLA-G$^+$ (MEMG9 antibody) at day 7 post-FCM-BMP4 treatment (Fig. 6D). CDX2 and p63 protein expression overlapped in a subset of cells early during differentiation (day 3; Fig. 6E). Functional differentiation was confirmed by secretion of hCG and hyperglycosylated hCG, indicating STB and EVT differentiation, respectively (Fig. 6F) (Guibourdenche et al., 2010). To confirm the identity of the KRT7-p63 double-positive cells as trophoblast and not mesoderm, we sorted EGFR$^+$ cells at day 5 post-FCM-BMP4 treatment, and compared expression of trophoblast- and mesoderm-associated markers in sorted versus non-sorted cells. At this time point, 88% of cells were EGFR$^+$ (data not shown). Compared with non-sorted cells, EGFR$^+$ cells were enriched with the trophoblast markers *p63* and *KRT7* (Fig. 6G); of the two mesoderm-associated markers induced following FCM-BMP4-treatment (*GSC* and *HHEX*), neither was enriched in the EGFR$^+$ cells (Fig. 6G). We also double-stained the FCM-BMP4-treated hESCs for KRT7 and mesoderm markers; KRT7$^+$ cells were negative for both T and PECAM-1 (Fig. 6H).

We next investigated whether p63 is required for FCM-BMP4-based hESC induction towards the trophoblast lineage. We infected H9 cells with lentivirus expressing either an shRNA for *TP63* or a scrambled shRNA, selected for cells expressing the respective shRNA, and differentiated the cells with FCM-BMP4. We performed experiments with three individual *TP63*-specific shRNA clones, all of which showed partial knockdown of p63 in FCM-BMP4-treated hESCs at the RNA level, along with a partial effect on trophoblast markers demonstrated by both qRT-PCR and ELISA (supplementary material Fig. S5A,B). We pooled these shRNAs and showed that they suppressed p63 expression at both RNA (Fig. 7C) and protein (supplementary material Fig. S5C) levels; we therefore used the pooled shRNAs for all remaining experiments.

Morphologically, differentiated p63-shRNA-infected cells showed a similar epithelial phenotype to the scrambled control (supplementary material Fig. S5D); the percentage of KRT7-positive cells was also similar between the two groups (Fig. 7A,B). However, markers of trophoblast differentiation, including both HLA-G and hCG, were undetectable by staining in the differentiated *TP63*-shRNA-infected cells (Fig. 7A) and significantly reduced based on qRT-PCR (Fig. 7C). In addition, secretion of total and hyperglycosylated hCG was also significantly blunted (Fig. 7D). Finally, microarray analysis of samples collected at serial time points during trophoblast differentiation of hESCs, with and without knockdown of *TP63*, showed an overall reduction in expression of trophoblast-associated genes in the absence of p63 (Fig. 7E). We also overexpressed p63 in hESCs grown under pluripotency conditions, to see whether, by itself, p63 is able to induce trophoblast differentiation; however, the cells retained OCT4 (POU5F1 – Human Gene Nomenclature Database) expression and did not induce KRT7 (Fig. 7F; data not shown). Taken together, these results suggest that p63 is required, though not sufficient, for trophoblast differentiation of hESCs.

DISCUSSION

Human trophoblast lineage specification and early differentiation, and mechanisms underlying placental dysfunction, are difficult to study owing to a lack of accurate and relevant model systems. Tissues from early human gestations, including embryos and first trimester placental tissues, are often difficult to obtain. First trimester placentas also yield small numbers of CTB cells, which

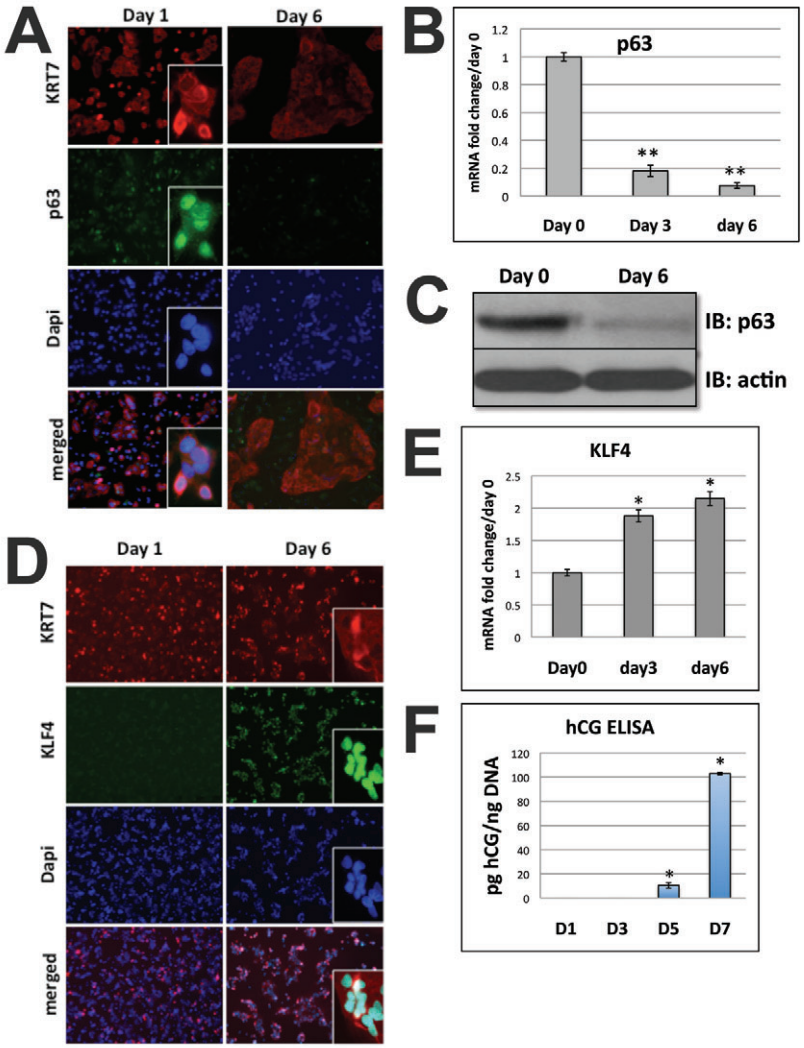

Fig. 4. Primary CTB differentiates from a p63-positive state to KLF4$^+$, hCGβ-secreting STB. (**A**) p63 and KRT7 staining of isolated third trimester CTB, 1 and 6 days after plating. (**B**,**C**) Expression of p63 by qRT-PCR (B) and western blot (C). (**D**) KLF4 and KRT7 staining of isolated third trimester CTB, 1 and 6 days after plating. (**E**,**F**) Expression of KLF4 by qRT-PCR (E) and hCG secretion by ELISA (F). Data are expressed as mean ± s.d. of triplicate samples at the time points shown, and are representative of experiments with eight separate CTB preparations from different placentas. **P*<0.05, ***P*<0.01 in comparison with day 0 or day 1 values.

upon isolation, fail to proliferate, similar to term CTB. Trophoblast stem cells (TSCs) have yet to be derived from human embryos (Roberts and Fisher, 2011), and, although 'trophoblast progenitors' have been cultured from first trimester chorionic mesenchyme (Genbacev et al., 2011), the true nature of these cells remains to be independently confirmed. Established human trophoblast cell lines represent immortalized or transformed versions of previously committed villous or extravillous lineages, and therefore are not good models to study lineage-specific differentiation (Janneau et al., 2002; Borges et al., 2003; Khoo et al., 1998). For these reasons, many researchers focus on mouse models, for which trophectoderm-derived TSCs have been established (Tanaka et al., 1998), and the placental contribution to embryonic phenotype can be easily studied (Rossant, 2007; Cockburn and Rossant, 2010; Rossant and Cross, 2001).

Trophoblast differentiation of hESCs following BMP4 treatment was first reported by Xu et al. (Xu et al., 2002). That BMP signaling is required for human trophoblast induction was later confirmed by Chen et al. (Chen et al., 2008), showing that hESCs lacking GPI-anchored BMP co-receptors were unable to form trophoblast. Although most studies using this model report hCG secretion as a functional test for STB (Xu et al., 2002; Das et al., 2007; Wu et al., 2008; Marchand et al., 2011), presence of HLA-G-positive EVT has also been reported (Das et al., 2007; Amita et al., 2013). In this study, we confirm generation of EVT by both HLA-G expression and secretion of H-hCG (Guibourdenche et al., 2010). For STB, in addition to hCG secretion, we report, for the first time, expression of KLF4 as a nuclear marker in this lineage. Furthermore, we are the first to report identification of CTB in FCM-BMP4-treated hESC cultures, characterized by p63$^+$/KRT7$^+$ cells as seen in bona fide CTBs *in vivo*. We show that, in primary CTB, isolated from human placental tissues, forced expression of p63 maintains the undifferentiated state, whereas in hESC, downregulation of p63 hampers differentiation to the later trophoblast lineages EVT and STB, characterized by decreased HLA-G expression and significantly reduced secretion of hCG and H-hCG.

The use of hPSCs as a model system for trophoblast differentiation has recently come under question (Bernardo et al., 2011). Specifically, based on experiments showing that knockdown of the mesendoderm-specific marker *T* leads to downregulation of *CDX2*, Bernardo et al. claim that the majority of these BMP4-treated cells are likely to be mesodermal in origin (Bernardo et al., 2011). BMP4 is known to induce mesoderm differentiation of hESCs (Zhang et al., 2008; Yu et al., 2011), and it is possible that under the chemically defined culture conditions (CDM) used in the Bernardo et al. study the majority of the differentiated cells are in

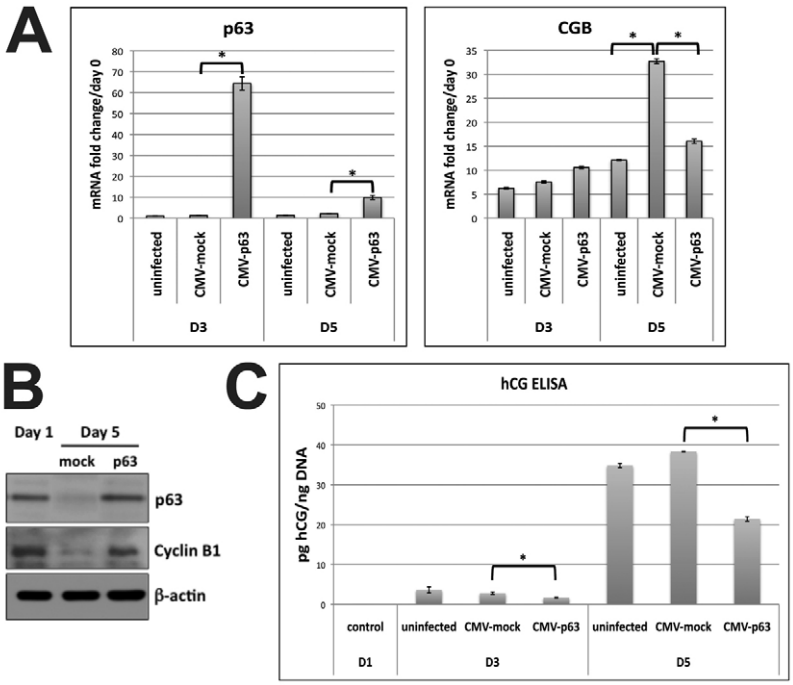

Fig. 5. Overexpression of ΔNp63α in primary CTB inhibits differentiation. (**A**) Expression of ΔNp63α and CGB mRNA in uninfected and mock- and ΔNp63α-infected CTB by qRT-PCR at day 3 (D3) and day 5 (D5) post-infection. (**B**) Western blot for p63 and cyclin B1 in cells infected with lentivirus expressing either ΔNp63α or no transgene ('mock'). (**C**) hCGβ secretion by uninfected and mock- and ΔNp63α-infected CTB. Data shown are from a single CTB preparation and are expressed as mean ± s.d. of triplicate samples; data are representative of experiments with five separate CTB preparations from different placentas. **P*<0.05 in comparison with uninfected or mock-infected values.

fact mesoderm. However, unlike their culture conditions (CDM+BMP4 and activin inhibitor), which at best yield 4-8% KRT7$^+$ cells (Bernardo et al., 2011), we and others use BMP4 in the presence of feeder-conditioned medium (FCM), as per the original report of Xu et al. (Xu et al., 2002), and consistently obtain ~90% KRT7$^+$ cells by day 4-6 after initiation of treatment. We have shown, based on genome-wide expression profiling, that the FCM-BMP4-treated hESCs most closely resemble primary CTB, not mesoderm-derived placental stroma or amnion; we further confirmed lack of mesoderm-associated gene expression in cells sorted based on the CTB-specific surface marker EGFR. More recently, two groups have pointed out that silencing of signaling downstream of FGF2 helps to promote specificity of trophoblast differentiation downstream of BMP4 (Yu et al., 2011; Ezashi et al., 2012). Finally, Amita et al. (Amita et al., 2013) have recently confirmed the importance of culture media in trophoblast induction of hESCs: in a side-by-side comparison with CDM, they confirm that BMP4-induced trophoblast differentiation is significantly more robust in the presence of FCM.

Our study not only confirms the utility of hPSCs in studying trophoblast differentiation, it also establishes, for the first time, a specific transcriptional program that regulates this process, namely one regulated by ΔNp63α. By comparing trophoblast differentiation of hESCs to primary human trophoblast, we make certain that the *in vitro* process generates cells that correspond closely to the *in vivo* cell types, and follow a process that recapitulates normal trophoblast differentiation, with early differentiation markers appearing before markers of the more differentiated cell types. We confirm, based on both marker analysis and secretory function, that true STB and EVT differentiation have taken place. The fact that p63, which in the human placenta is specifically expressed only in CTB, is required for differentiation of hESC into STB and EVT suggests that FCM-BMP4-treated hESC undergo a CTB phenotype prior to terminal differentiation; in fact, we see a relatively uniform p63$^+$/KRT7$^+$ CTB population at day 5 post-FCM-BMP4 treatment, prior to secretion of any hCG or H-hCG.

The study by Bernardo et al. (Bernardo et al., 2011) assumes an identical differentiation potential for hESCs and mEpiSCs. But in fact, early human embryonic development differs from mouse in several ways, including a longer period of time before implantation (Rossant, 2008; Benirschke and Kaufmann, 2000), rapid formation of non-proliferating trophoblast during early implantation (Roberts and Fisher, 2011; Rossant, 2008; Benirschke and Kaufmann, 2000), divergent human *OCT4* promoter sequences which, in the human, lead to co-expression of OCT4 and CDX2 in trophectoderm (Adjaye et al., 2005; Berg et al., 2011), and differences in lineage-specific marker expression and signaling pathways (Roberts and Fisher, 2011; Roode et al., 2012). The inability to apply mouse TSC culture conditions to derive similar cells from human embryos or pluripotent stem cells is additional evidence of these differences in early development (Rossant, 2008; Harun et al., 2006). Our results provide additional proof of this divergence: p63 appears to play a key role in human trophoblast differentiation, whereas p63-null mice survive to term (Mills et al., 1999; Yang et al., 1999; Romano et al., 2012). Although the placenta has never been examined in these strains, it is unlikely that there is a fundamental deficit in the TSC compartment, as such abnormalities are typically associated with very early embryonic lethality (earlier than embryonic day 7.5). In fact, we have been unable to detect p63 protein by western blot or immunostaining in the mouse placenta or TSCs at any developmental stage, whereas the RNA is detectable in undifferentiated TSCs (data not shown). We believe the absence of p63 expression in the mouse placenta is in part due to structural differences between mouse and human placentas; the latter is a more organized stratified epithelium, with STB layered over CTB stem cells, similar to the arrangement of cells in the epidermis (Cross et al., 2003). This directional differentiation is accompanied by a p63-positive cell layer (CTB), adjacent to the underlying basement membrane, similar to the p63-positive stem cells in the epidermis (Yang et al., 1999).

Whether a true 'trophoblast stem cell' (TSC) precedes the differentiation of p63-positive CTB from hESCs remains to be seen.

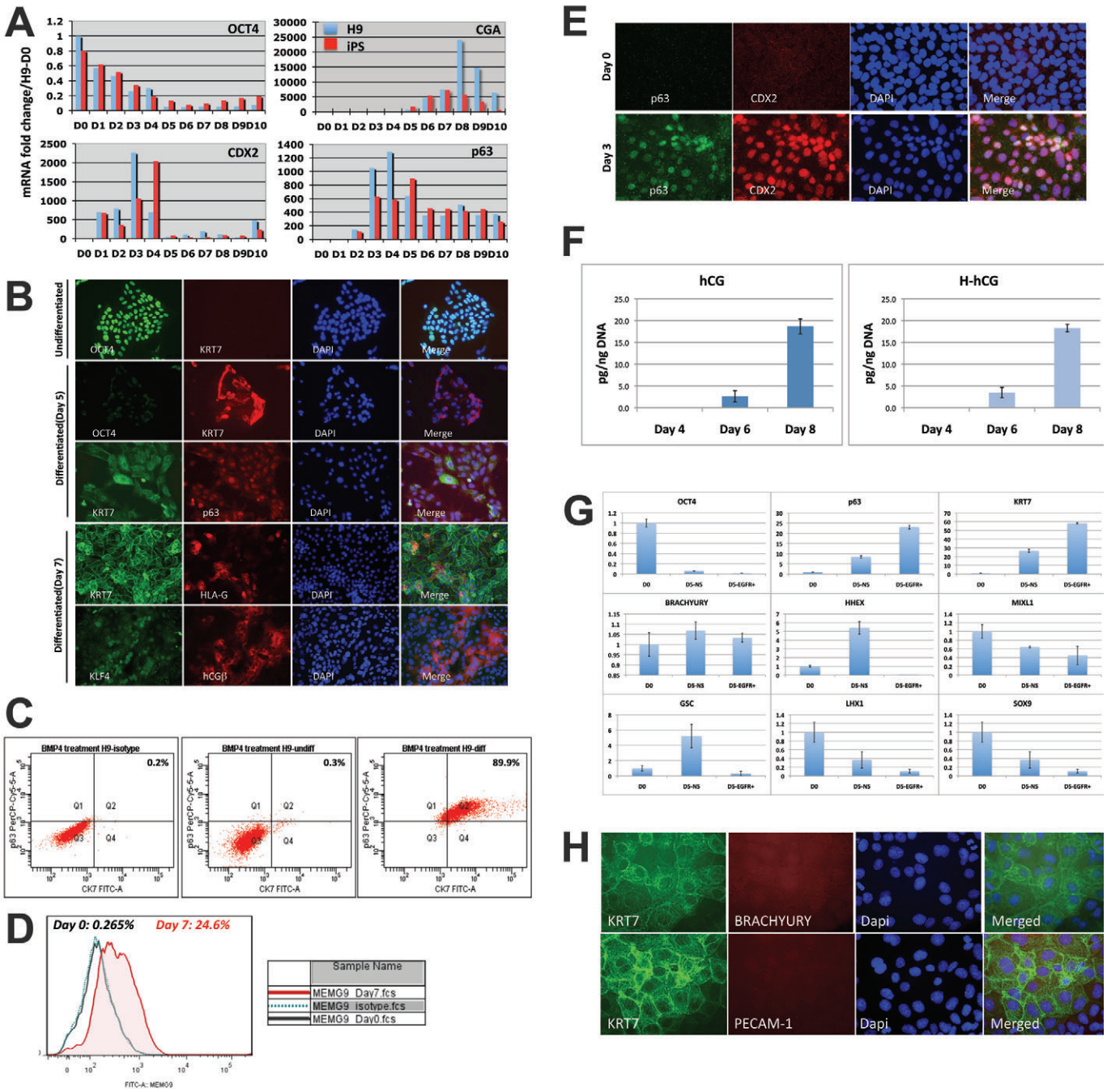

Fig. 6. BMP4 treatment of hPSCs induces a trophoblast phenotype. (**A**) qRT-PCR of various markers in hESCs (H9/WA09) and an hiPSC line differentiated with FCM-BMP4 over 10 days (D1-D10). Data are representative of three independent experiments with each cell line. (**B**) hESCs differentiated with FCM-BMP4 transition through a p63$^+$/KRT7$^+$ cytotrophoblast phenotype at day 5, prior to differentiating into HLA-G$^+$/KRT7$^+$ and KLF4$^+$/hCGβ$^+$ trophoblast, characteristic of EVT and STB, respectively. (**C**) Fluorescent flow cytometry analysis of d0 and d5 hESC9s differentiated in FCM-BMP4. By day 5, almost 90% of cells are p63$^+$/KRT7$^+$ (right-hand panel). (**D**) At day 7, ~25% of cells express surface HLA-G (based on FACS using MEMG9 antibody). (**E**) At day 3, p63$^+$/CDX2$^+$ cells are found among differentiating colonies. (**F**) FCM-BMP4-treated hESCs secrete hCGβ, including a hyperglycosylated form, which is unique to EVT. Data are normalized to DNA content and are expressed as mean ± s.d. of triplicate samples. (**G**) FCM-BMP4-treated cells at day 5 (D5), sorted based on EGFR expression, and subjected to qRT-PCR for the pluripotency marker OCT4, trophoblast-associated markers p63 and KRT7, and a panel of mesoderm markers. NS, non-sorted. Data are expressed as fold change over undifferentiated hESCs (D0); error bars indicate s.d. of technical triplicates. (**H**) FCM-BMP4-treated cells at day 5 are co-stained for KRT7 and the indicated mesoderm markers.

CDX2, a master regulator of trophectoderm establishment and marker of TSCs in the mouse, has been detected at both RNA and protein levels in human trophectoderm (Adjaye et al., 2005; Niakan and Eggan, 2013). We have confirmed expression of *CDX2* and other TSC-associated markers, such as *ELF5*, in FCM-BMP4-treated hESCs, and also noted co-expression of CDX2 and p63 protein in a subset of these cells early in the differentiation process. It is therefore plausible to conclude that FCM-BMP4 treatment allows for a step-wise differentiation from pluripotency to a TSC-like phenotype, followed by differentiation into CTB and eventually

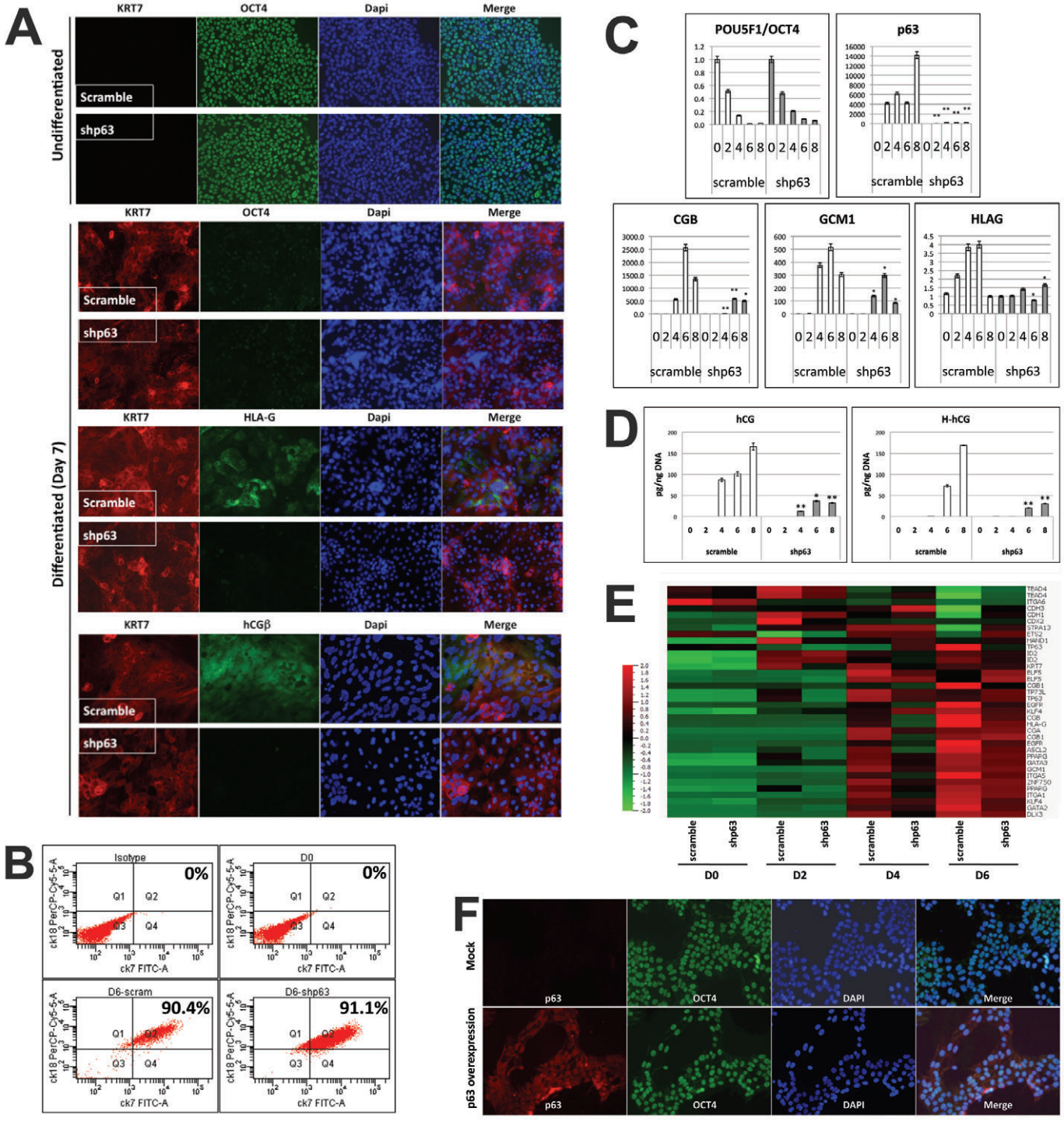

Fig. 7. p63 is required for BMP4-induced trophoblast differentiation of hESCs. (**A**) hESCs expressing either scrambled or p63-specific shRNA (shp63), treated with FCM-BMP4 and stained with a panel of markers. Note lack of HLA-G and hCG expression, but similar expression of KRT7 in p63-shRNA-expressing cells. (**B**) FACS analysis of KRT7 and KRT18 in undifferentiated (D0, upper right) and FCM-BMP4-treated hESCs (day 6) expressing scrambled (D6-scram; bottom left) or p63-shRNA (D6-shp63; bottom right); percentage of double-positive cells is indicated. (**C**) qRT-PCR for markers of pluripotency and trophoblast in scrambled or p63-shRNA-expressing hESCs on days 0-8 following treatment with FCM-BMP4. For all graphs, *y*-axis represents fold change in mRNA relative to scrambled shRNA-infected cells at day 0. Error bars represent s.d. of technical triplicates. (**D**) Secretion of total hCGβ (left) and H-hCG (right) in scrambled or p63-shRNA-expressing hESCs. Data are normalized to DNA content and are expressed as mean ± s.d. of triplicate samples. (**E**) Heatmap of trophoblast-specific genes (same genes as in Fig. 1C) in scrambled- or p63-shRNA-expressing hESCs following treatment with FCM-BMP4. Note an overall decrease in trophoblast-specific gene expression in p63-shRNA versus scrambled-shRNA-expressing hESCs. Data shown are from a single shRNA infection experiment, and are expressed as mean ± s.d. of triplicate samples at the time points shown; data are representative of three independent experiments, with different preparations of hESCs and lentivirus. **P*<0.05, ***P*<0.01, in comparison with scrambled shRNA-infected cells at the same time point. (**F**) ΔNp63α overexpression in hESCs, showing maintenance of the pluripotency marker OCT4.

into STB and EVT. Additional studies are required to determine the exact role of *CDX2* and other early 'TSC'-associated genes in BMP4-induced trophoblast differentiation.

In summary, by detailed comparison to primary human tissues, we have shown that trophoblast differentiation can be modeled using hPSCs, and that this *in vitro* differentiation progresses through a cytotrophoblast stem cell state, mediated by p63. Our data firmly illustrate the importance of going beyond animal models in studying human development, particularly early lineage specification, and the necessity of human cells and tissues for optimizing protocols for hPSC differentiation.

Acknowledgements
We thank Planned Parenthood of the Pacific Southwest for providing samples for this study.

Funding
This work was supported by a California Institute for Regenerative Medicine (CIRM) New Faculty Award [RN2-00931 to M.M.P.]; a grant from the National Institutes of Health (NIH) [R01HD071100 to M.M.P.]; and a grant from Women's Reproductive Health Research (WRHR) [K12 HD001259 to L.C.L.]. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Author contributions
Y.L. designed and performed all experiments and data analysis involving manipulation of p63 gene expression in primary CTB and hPSCs, and contributed to writing the manuscript. M.M.-Z. performed all primary cell isolations and contributed to data analysis and writing of the manuscript. F.S. performed all microarray data analyses and contributed to writing of the manuscript. A.W. performed some experiments with hPSCs and contributed to data analysis and writing of the manuscript. L.W. and S.L.-G. performed collection of human tissue samples and RNA isolation from these tissues. R.P. performed all measurements of hyperglycosylated-hCG secretion. D.P. performed all non-fluorescent tissue staining. L.C. performed some experiments with hPSCs. K.N. and J.F.L. contributed to the performance of gene expression profiling and the subsequent data analysis. C.P.C. contributed archived human placental tissues and helped with manuscript writing and critical review. L.C.L. contributed to performance of gene expression profiling and supervised analysis of all array data. M.M.P. supervised all experimental design, data analysis, and writing of the manuscript, and was responsible for review and approval of the final draft of the manuscript.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.092155/-/DC1

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