Paracrine and Epigenetic Control of Trophectoderm Differentiation from Human Embryonic Stem Cells: The Role of Bone Morphogenic Protein 4 and Histone Deacetylases

Teresa M. Erb,¹ Corinne Schneider,² Sara E. Mucko,² Joseph S. Sanfilippo,¹ Nathan C. Lowry,³ Mukund N. Desai,³ Rami S. Mangoubi,³ Sanford H. Leuba,⁴ and Paul J. Sammak^{4,5}

Our understanding of paracrine and epigenetic control of trophoctoderm (TE) differentiation is limited by available models of preimplantation human development. Simple, defined media for selective TE differentiation of human embryonic stem cells (hESCs) were developed, enabling mechanistic studies of early placental development. Paracrine requirements of preimplantation human development were evaluated with hESCs by measuring lineage-specific transcription factor expression levels in single cells and morphological transformation in response to selected paracrine and epigenetic modulators. Bone morphogenic protein 4 (BMP4) addition to feeder-free pluripotent stem cells on matrigel frequently formed CDX2-positive TE. However, BMP4 or activin A inhibition alone also produced a mix of mesoderm and extraembryonic endoderm under these conditions. Further, BMP4 failed to form TE from adherent hESC maintained in standard feeder-dependent monolayers. Given that the efficiency and selectivity of BMP4-induced TE depended on medium components, we developed a basal medium containing insulin and heparin. In this medium, BMP4 induction of TE was dose dependent and with activin A inhibition by SB431542 (SB), approached 100% of cells. This paracrine stimulation of pluripotent cells transformed colony morphology from a cuboidal to squamous epithelium quantitatively on day 3, and produced significant multinucleated syncytiotrophoblasts by day 8. Addition of trichostatin A, a histone deacetylase (HDAC) inhibitor, reduced HDAC3, histone H3K9 methylation, and slowed differentiation in a dose-dependent manner. Modulators of BMP4- or HDAC-dependent signaling might adversely influence the timing and viability of early blastocyst developed in vitro. Since blastocyst development is synchronized to uterine receptivity, epigenetic regulators of TE differentiation might adversely affect implantation in vivo.

Introduction

TOTIPOTENCY IS A CHARACTERISTIC of the morula stage of embryonic development. This sphere of morphologically indistinguishable totipotent cells is capable of giving rise to all subsequent embryonic and extraembryonic structures. Morula polarization by asymmetric cell division begins the process of differentiation [1–3]. The first lineage commitment is completed at the blastocyst stage, when the inner cell mass (ICM) and the trophoctoderm (TE) are established [4–9]. The ICM, from which embryonic stem cells

(ESCs) are derived, is pluripotent, giving rise to all fetal structures via the 3 germ layers: ectoderm, mesoderm, and endoderm. From TE, the outer epithelial layer of the blastocyst, multipotent trophoctodermal stem cells develop along several lineages [10–18], beginning with placental stem cell progenitors, which ultimately undergo terminal differentiation into multinucleated syncytiotrophoblasts or extravillous trophoblasts. Timing of TE differentiation is critical since the development of this extraembryonic structure must be synchronized with uterine receptivity for successful implantation.

¹Division of Reproductive Endocrinology and Infertility, Department of Obstetrics, Gynecology, and Reproductive Sciences, Magee Women's Hospital of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

²Magee Women's Research Institute, Pittsburgh, Pennsylvania.

³C.S. Draper Laboratory, Cambridge, Massachusetts.

⁴Department of Cell Biology and Physiology, Pittsburgh, Pennsylvania.

⁵McGowan Institute for Regenerative Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

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Given practical and ethical limitations of human blastocyst experimentation, TE differentiation studies have relied extensively on mouse and human ESC culture models. Standard pluripotent hESCs are maintained by growing them on mouse embryonic fibroblasts (MEF) and routinely passaging them to prevent unwanted spontaneous differentiation. A reduction in MEF-secreted pluripotency factors by hESC culture on low-density MEFs encourages spontaneous neurectoderm differentiation [19–22], whereas bone morphogenetic protein 4 (BMP4) supplementation to MEFs at low density [23,24] produces TE from pluripotent hESCs. However, the factors responsible for initiating human TE differentiation from pluripotent hESCs, that is TE induction, are incompletely understood. This is partly due to varied and ill-defined models of preimplantation human development. A complication of in vitro studies of totipotency (TE competent) versus pluripotency (TE incompetent) includes the varied differentiation state of ESCs. While mouse ESCs (mESCs) in standard MEF feeder culture are characterized as ICM-like, hESCs are characterized as epiblast like [25,26]. These developmental states are metastable and can be reprogrammed by transgenic methods [27,28], epigenetic mechanisms [29], or simply by variations in medium composition [28,30] or drug action on intracellular signaling molecules [31]. Therefore, defining conditions for TE induction might also be useful as a functional test of totipotency in vitro.

Studies of hESC differentiation signaling pathways [21,22,24,32–34] suggest a paracrine role of transforming growth factor-beta superfamily members in TE formation. Specifically, BMP4 induces homogenous trophoblast differentiation and further differentiation to syncytiotrophoblast from pluripotent hESCs [21,33], and activin inhibition synergizes with BMP4 to induce trophoblast [24]. Mouse studies confirm a paracrine role of transforming growth factor-beta superfamily members for TE induction, since the first lineage commitment decision is partly regulated by BMP2, BMP4, and BMP6 [35–38]. BMP4 production in mouse TE is maintained by Caudal-like transcription factor CDX2 [39], which is required for TE differentiation [24]. Despite ubiquitous blastomere co-expression of CDX2 with the ICM pluripotency genes, Oct4 and Nanog, CDX2 mRNA and protein levels are heterogeneous at the precommitted 8-cell stage. High-expressing CDX2 blastomeres are allocated to TE, whereas low-expressers contribute to the ICM [3]. Thus, CDX2 becomes progressively upregulated in future TE [40] while repressing Oct4 and Nanog [1,40], setting the stage for morphological transformation and lineage commitment.

Conflicting reports on BMP4 requirement [41] versus dispensability [36] for TE formation suggest that paracrine regulation of embryogenesis is species specific [42]. Transplanted mESCs fail to contribute to the TE in chimeric embryos, suggesting low capacity for TE formation [4]. However, mESC-derived trophoblast can be induced in culture by BMP4 [43] and trophoblast cells [12–14,44] can be maintained in primary culture with activin A and fibroblast growth factor (FGF) 4 in the absence of MEFs or MEF-conditioned medium [45]. Genetic manipulation to artificially increase CDX2 levels in mESCs is sufficient for TE transformation [3], whereas these methods are unsuccessful in human trophoblast cell models [32,46–48]. BMP4 cooperates with LIF to maintain mESC self-renewal [36,37,49], but BMP4 has no role for hESC pluripotency maintenance, which is

FGF2, activin A, and insulin dependent [21–23,30,50,51]. Thus, despite current trophoblast cell biology analysis [3,40,52–54], our understanding of the mechanisms responsible for directing human TE differentiation remains limited by species-specific paracrine regulation and potentially on variations of the developmental stage of pluripotent cells. While detailed molecular studies from primary human placental cell lines circumvent species-specific differences in TE behavior, they too are limited by mixed-cell isolates [41], spontaneous syncytiotrophoblast differentiation [55], and their postimplantation developmental stage. The need remains to clarify regulation of the earliest developmental stages preceding human trophoblast.

In addition to paracrine control of TE differentiation, mouse data suggest epigenetic coordination of whole developmental programs in TE lineage identity and cellular memory [56]. The Oct4 promoter undergoes progressive methylation of specific DNA and histone residues to silence pluripotency genes that are no longer needed upon differentiation [57,58]. These localized, gene-specific epigenetic modifications are followed by higher order chromatin reorganization of multigene loci, which restrict promoter access to transcriptional activators or repressors by physical hindrance [57,59]. CDX2 expression is regulated not only by availability of signal transduction activators and repressors such as BMP4-regulated SMAD 1/5/8 [60,61], but also by chromatin receptivity to activator binding, which is controlled by DNA and histone methylation [62]. Upon hESC differentiation, DNA unneeded for the specified fate is reorganized into transcriptionally inactive heterochromatin. The epigenetic mechanisms of forming heterochromatin are initiated by histone deacetylation by the histone deacetylases (HDACs), subsequent histone and DNA methylation by methyltransferases, and finally chromatin compaction [63–67], although blocking methylation alone does not reverse compaction [68,69]. Deacetylation followed by methylation of H3K9 accompanies mESC differentiation and when blocked by the HDAC inhibitor, trichostatin A (TSA), mESCs remain pluripotent and continue to express Oct4 [56]. Additionally, in TSA-treated trophoblast cells the *Oct4* gene is activated by promoter hypomethylation [58].

Uncertainties in human TE differentiation will remain without a defined in vitro culture that produces TE with high selectivity and efficiency [70]. Protocols using either non-adherent embryoid bodies or adherent hESCs on MEFs produce TE and secrete placental hormones but are heterogeneous [71,72], and thus are not TE selective. For example, short-term BMP4 treatment can also initiate mesoderm induction from hESCs [73]. Further, despite studies showing that hESCs require BMP4 stimulation and activin A inhibition for TE differentiation, MEF culture conditions are ill-defined and complicate mechanistic studies [24,32–34,72]. Medium composition and substrate (including feeder-free systems) have been the focus of attention in attempts to generate TE-directed differentiation. While hESCs have been induced to undergo early extraembryonic differentiation in a chemically defined medium supplemented with BMP4, these cells were still heterogeneous with some cells expressing markers of TE lineages and others expressing markers of primitive endoderm [33,34].

Our objective was to elucidate physiologically relevant paracrine and epigenetic signals in early human TE

differentiation from pluripotent hESCs. To determine specific differentiation stimuli, we took a systems approach to test defined combinations of growth factors and modulators on single cells immunolabeled for lineage-specific transcription factors. Single-cell labeling permitted testing of the TE selectivity of differentiation stimuli. Differentiation conditions were identified with the simplest set of extrinsic stimulators/inhibitors, which facilitated intracellular mechanistic studies [74]. Our objective was met by (1) designing a minimal medium, without unnecessary pluripotency growth factors, that permitted hESCs differentiation in the presence of exogenous paracrine factors; (2) determining the paracrine requirements for TE differentiation with high efficiency (maximize the fraction of TE positive cells) and selectivity (minimize populations of mixed lineage); (3) identifying TE on a single-cell level by transcription factor expression, morphological transformation to epithelium, and terminal differentiation to syncytia; and (4) determining the requirement of histone methylation and HDACs for TE formation from hESCs.

Materials and Methods

Cell culture

Pluripotent hESCs, line H7, were grown on mitomycin-treated MEFs (GlobalStem) at normal density (120,000 cells/6 cm²), maintained in the DSR medium (Supplementary Table S1; Supplementary Data are available online at www.liebertonline.com/scd), and passaged weekly using Collagenase IV (Invitrogen/Gibco) and a Stempro[®]EZPassage™ cutter (Invitrogen) as directed. Cell colonies were briefly spun, resuspended in DSR, and plated onto normal-density MEFs in CM (Supplementary Table S1). For TE-directed differentiation, hESCs were passaged at 1:5 dilution onto low-density MEFs (30,000 cells/6 cm²) using DSR medium minus FGF2 supplemented with BMP4 (Invitrogen; PHC7914) 100 ng/mL. The medium was changed daily.

Feeder-free hESCs were grown on Geltrex (Invitrogen)-coated plates in StemPro (Invitrogen), passaged weekly with Collagenase IV or Accutase (StemCell Technologies) as directed, resuspended in StemPro, and plated onto Geltrex-coated plates. For TE-directed differentiation, pluripotent hESCs maintained in StemPro or in Erb's minimal induction medium (EMIM) were passaged onto feeder-free, Geltrex-coated plates into respective basal medium supplemented with the activin A antagonist, SB431542 (10 μM), and/or BMP4 (100 or 500 ng/mL) as indicated. The medium was changed daily. Terminal syncytiotrophoblast differentiation was accomplished by culturing hESCs in EMIM+SB+100 ng/mL BMP4 for 14 days.

Antigen retrieval and immunocytochemistry

In phosphate-buffered saline (PBS), cell colonies were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma), and nonspecific antibody binding blocked with 10% goat serum. Primary antibodies (Supplementary Table S2) were diluted in 1% goat serum, spun briefly, and incubated overnight at 37°C. After a wash in PBS-Tween 0.05% a species-specific fluorescent secondary antibody (prepared as above) was added for 60 min at 37°C. Cells were washed in PBS-Tween 0.05%, incubated with Hoechst 33342 (1:10,000), and mounted in Gelvatol polyvinyl alcohol-based medium [75].

TE differentiation: induction, selection, and identity

Nuclear labeling (Hoechst 33342) facilitates automated image segmentation for counting cell number. Since pluripotent hESCs can differentiate along multiple lineages, TE differentiation was determined by immunostaining for transcription factors localized to the cell's nucleus or cytoplasm (Supplementary Table S2). Four-color microscopy permits co-labeling of cells with markers of pluripotent hESCs (OCT4), TE cells (CDX2), and alternative markers for early differentiation, including neurectoderm (nestin), mesoderm (brachyury), or extraembryonic endoderm (GATA6). TE induction was determined by 2 criteria: (1) the percentage of all cells that were CDX2-positive and the fold change of CDX2-nuclear intensity over control, unstimulated cells. However, high TE induction (protein level) does not equate to high TE selection (fraction of positive cells). TE selectivity was determined by a significant increase in the percentage of CDX2-positive cells as well as a suppression of the percentage of nestin-, brachyury-, and GATA6-positive cells.

Morphological criteria used to confirm TE identity in TE selective culture conditions were (1) squamous epithelial transformation and (2) subsequent differentiation to multinucleated syncytiotrophoblasts as measured by phase-contrast microscopy and immunofluorescence microscopy of nuclei (Hoechst 33342) and peripheral F-actin (phalloidin) rings to mark cell boundaries. Epithelial transformation was measured by texture analysis (described below). Terminal differentiation to syncytia was determined by bi- or multinucleated cells.

Imaging and analysis

For confocal microscopy, cells were grown on thin, plastic-bottomed multiwell microslides (Ibidi; Integrated Bio-Diagnostics) or 96-well tissue culture-treated plates (Perkin Elmer). Fluorescence images were acquired on a Nikon confocal, Zeiss wide-field, or Thermo Fisher Arrayscan V automated high-content screening reader. Confocal images were acquired using a Nikon TE2000-E inverted microscope with Yokogawa CS10 spinning disk and Photometrics CoolSNAP HQ CCD camera (Photometrics). Images were processed using Nikon NIS-Elements (Nikon, Inc.).

Ninety-six-well microplates were scanned using the 5 or 10× objective on the ArrayScan V, and images were analyzed using the Compartmental Analysis BioApplication using Hoechst for identification of nuclei and various transcription factor antibodies (Supplementary Table S2). The average nuclear-specific fluorescence intensity was determined from ~300 single cells per well from 3 to 6 independent wells. Cell-level measurements were averaged to produce a well-population average nuclear intensity for each measurement. Mean and standard deviation and *t*-tests were calculated from multiple wells. Data are presented as the fold change of the mean intensities that were normalized against matched, unstimulated external controls, usually the pluripotent condition, to divide out channel specific differences due to efficiency of antibody-antigen binding and chromophore quantum yield. The relative transcription factor expression levels were displayed as radar graphs using Microsoft Excel 2003 to facilitate presentation of multiple, independent variables (the induction medium with different

growth factor combinations) plotted around the circumference, and dependent variables (fold change transcription factor levels) displayed as the radial distance from the center. Select conditions are additionally compared in bar graph format to show standard deviations and *t*-tests.

Texture analysis

Colony morphology was quantified by statistical multi-resolution analysis of image texture [76,77]. Image texture is a mathematical measure of the size and sharpness of objects in the image, which is illustrated in 1-dimension by an intensity line profile (Supplementary Fig. S1). The variation in image intensity with position, the spatial frequency, depends on both nuclear and cell size, which changed significantly during differentiation (Supplementary Fig. S1). Therefore, we employed texture analysis as an integrated measure of colony morphology. Texture was measured using wavelet analysis [78], a generalization of Fourier analysis, to decompose colony texture in phase-contrast images (4× objective, Nikon TMS microscope), producing a joint probability density function for the wavelet coefficients from various scales. Different culture conditions were distinguished as belonging to distinct classes. Each class was thus represented by a probability density function, and quantitative comparative features for classifying and statistically distinguishing colony textures were extracted from the density functions. One such feature is the Kullback-Leibler distance [78]. A 2-sample *t*-statistic test conducted at the 0.98 confidence level demonstrates that this feature allowed for successful colony classification. Specifically, the Kullback-Leibler distance between a pair of colonies from the same class was shorter than a pair from different classes.

All images were imported into Adobe Photoshop (Adobe System, Inc.) for final image composition and contrast adjustment. For display, comparable phase-contrast images were adjusted using constant linear contrast and unsharp mask filtering to enhance edges to allow comparison between panels. Quantitation was performed on unenhanced grayscale images of the green channel of an RGB camera (Nikon D40x).

Results

Pluripotent hESCs in feeder-free medium are efficiently but not selectively induced to form TE, unlike hESCs on MEF feeders

TE differentiation from hESCs by addition of BMP4 was disappointing under standard MEF culture conditions and CDX2-positive cells were rarely seen (Fig. 1). Potentially, MEFs maintained pluripotency and inhibited differentiation, so we evaluated reduction of MEF density. Reducing MEF density by half does not maintain pluripotency and enhances neural differentiation, but fails to facilitate BMP4-induced TE from hESCs in monolayers (Supplementary Fig. S2B). Since low CDX2 expression might result from colony size-dependent autocrine factors [46,48], we evaluated whether adherent hESC colony size influenced TE induction after BMP4 supplementation on low density MEFs. However, monolayer colonies from 50 to 1,000 cells rarely expressed CDX2 (Fig. 1C). BMP4 supplementation on low-density MEFs decreased OCT4 expression and transformed cell

morphology (Supplementary Fig. S2B). However, CDX2 was restricted to multilayered colonies (Fig. 1B) and was not seen in monolayers. Thus, BMP4 was insufficient for TE induction from hESC on MEFs, except in compact multicellular bodies.

Inefficient TE induction might result from competing soluble factors secreted from MEFs, such as Activin A, [50,79–81], since TE differentiation by BMP4 was augmented by SB [24,79,80]. To eliminate variable levels of activin A production from MEFs, we evaluated TE induction in a defined, feeder-free, pluripotency medium, StemPro (Supplementary Table S1). Pluripotency in StemPro was confirmed at day 7 with prominent OCT4 staining (Fig. 1D), cuboidal cell morphology, and low differentiation marker expression, including nestin (Supplementary Fig. S2C). Occasional, single hESCs in StemPro expressed high-intensity CDX2 by culture day 2 (Supplementary Fig. S2C) as expected, but early BMP4 supplementation (first 48 h) to hESCs in StemPro produced homogenous, low level CDX2 (Fig. 1E) expression. Quantification of percent CDX2-positive cells revealed higher TE induction from hESCs in StemPro than from hESC on MEFs at low density (Fig. 1F; $P < 0.05$). Despite higher TE induction, BMP4 supplementation to StemPro was not TE selective (Fig. 1G and H), and also induced mesoderm as measured both by average nuclear brachyury intensity (Fig. 1G) and percentage of brachyury-positive cells (Fig. 1H) by day 2 of culture.

The difference in TE induction between pluripotent hESCs grown on MEFs versus StemPro could be explained by inherently different hESC states with different developmental potential when maintained on MEFs versus MEF-free conditions. Alternately, the difference could be explained by the differentiation medium composition since cofactors/inhibitors differ between DSR versus StemPro. We next evaluated medium cofactor and inhibitor requirements for selective TE induction from source cells maintained in StemPro by developing a defined, growth factor-deficient, minimal medium capable of maintaining viable and differentiation-competent hESCs to understand initiating requirements for TE differentiation.

A minimal induction medium maintains viable, differentiation-competent hESCs

We designed a medium to serve as a foundation for differentiation with 2 criteria: high viability and minimal exogenous pluripotency factors that would otherwise compete with differentiating factors. Two candidate starting media from which to devise a minimal basal medium for TE induction studies included StemPro, used primarily for hESC pluripotency maintenance, and RPMI with KSR [73], a TE-supportive medium. RPMI with KSR supported differentiation to TE, but we found that switching basal medium when passaging from the Dulbecco's modified Eagle's medium (DMEM)/F12-based StemPro to an RPMI-based medium was responsible for very low viability (Fig. S3). Thus, to maintain compatibility with StemPro-cultured hESC, we developed a DMEM/F12-based minimal induction medium (MIM, Supplementary Table S1) by testing short-term hESC viability (Supplementary Fig. S4) and OCT4 expression (Fig. 2) after systematically reintroducing cell survival, but not pluripotency, components. Insulin and bovine serum albumin were critical components for cell viability, but while

FIG. 1. Pluripotent hESCs in feeder-free medium are efficiently but not selectively induced to form TE, unlike hESCs on MEF feeders. Pluripotent hESCs grown on normal density MEF feeders (A) or in StemPro (D), a defined pluripotency medium, predominantly stain for the pluripotency marker OCT 4. Pluripotent hESCs grown on low-density MEFs minus fibroblast growth factor 2 and supplemented with BMP4 for 7 days expressed CDX2 in adherent multilayer (B) but not monolayer (C) hESC colonies. In contrast, low-intensity CDX2 expression was observed in monolayer hESCs cultured in the MEF-free, defined medium, StemPro containing BMP4 (E), after only 4 days of culture. TE induction efficiency, as determined by the fraction of cells with CDX2 staining, was significantly lower in monolayer colonies compared to multilayer colonies in DSR/MEF culture, and significantly higher in StemPro, at 3.6% 44.4%, and 93.7%, respectively (F, $P < 0.01$). hESC differentiation in StemPro supplemented with BMP4 is not TE selective, since a dose-dependent, statistically significant increase in brachyury expression is observed with addition of BMP4 to StemPro (G). Further, there is a 6-fold increase in percent brachyury (T)-positive cells after the addition of BMP4 to StemPro (H; $P < 0.005$) for 48 h, suggesting BMP4-induced mesodermal induction by day 2 of culture in StemPro. TE, trophoblast; hESC, human embryonic stem cell; MEF, mouse embryonic fibroblasts; BMP4, bone morphogenetic protein 4. Color images available online at www.liebertonline.com/scd

heparin was nonessential for short-term viability of hESCs in basal medium, it did improve hESC viability once BMP4 was supplemented (not shown). Thus, MIM with heparin (EMIM) became a second-candidate minimal medium for TE induction studies.

One criterion for derivation of an MIM was that hESCs would be viable and continue to express the OCT4 pluripotency marker. Short-term hESC culture in MIM or in EMIM consistently maintained viable, OCT4-expressing hESCs with pluripotent cell morphology (Fig. 2B–D) for up to 4 days when cultured on matrigel or Geltrex. Compared to standard StemPro pluripotency conditions at day 2, expression levels of the differentiation markers, CDX2, brachyury, and GATA6 were not statistically increased (Fig. 2D) in either minimal media. A second criterion for the derivation of an MIM was that hESC would remain competent to differentiate. Spontaneous differentiation in EMIM by day 7 pro-

duced cells with low OCT4 and low CDX2 (Fig. S4). We concluded that MIM and EMIM provided a growth factor-deficient basal medium that maintained hESC competence for lineage-specific induction in the short-term (4 days) as determined by the presence of a pluripotent hESC morphology, continued OCT4 expression (Fig. 2B–D), and the absence of TE, mesoderm, and extraembryonic endoderm induction (Fig. 2D and Supplementary Fig. S5B).

BMP4 produces a dose response increase in TE induction in MIM medium, and is selective when applied with Activin A and heparin in EMIM

Both MIM and EMIM were evaluated as foundation media for evaluating paracrine requirements for selective TE differentiation. To evaluate initiating events, multiple lineage-specific transcription factor levels were quantified after a

FIG. 2. Minimal induction media, MIM and EMIM, maintain viable, differentiation-competent hESCs for 2 days. Pluripotent hESCs maintained in StemPro pluripotency medium were characterized by a cuboidal morphology, closely packed, OCT4 expressing nuclei, and prominent peripheral actin bands shown by phalloidin staining, which is characteristic of tight junctions (A). HESCs maintained in a growth factor-deficient minimal medium without heparin (B, MIM) or with heparin (C, EMIM) were morphologically indistinguishable from pluripotent hESCs in StemPro and continued to express OCT4 at the same level (D). hESCs grown in MIM and in EMIM for 2 days not only continue to express OCT4, but also were not induced to overexpress lineage-specific transcription factors, including CDX2, brachyury (T), and GATA6, when compared to pluripotent hESCs grown in StemPro (D). EMIM, Erb's minimal induction medium.

48-h exposure to presumptive TE-inducing growth factors in StemPro, and growth factor-deficient MIM or EMIM. A systems approach was used to compare multiple independent and dependent variables in radar charts (Fig. 3A, Supplementary Fig. S5E, F) to allow analysis of multiplexed data. The increase in staining of the transcription factors brachyury (T, gray) and CDX2 (black) in the presence of TE-inducing growth factors (SB, low-dose BMP4, and high-dose BMP4) used either alone or in combination in either StemPro or EMIM was plotted as the fold change without stimulant over the basal medium alone (in the pluripotent state). StemPro with all additions showed a tendency for mesoderm induction in StemPro, whereas EMIM showed a preference for TE induction (Fig. 3A).

Statistical analysis specified paracrine requirements for TE induction in these media. Significant CDX2 induction ($P < 0.01$) occurred with combination low-dose BMP4 and SB in StemPro or in EMIM, or when BMP4 was used alone at higher doses (500 ng/mL) in EMIM by day 2 (Fig. 3B). While BMP4 in EMIM was sufficient, dose-dependent, and synergistic with SB for TE differentiation in EMIM, these effects were lost in StemPro except when combined with activin A antagonism (SB). However, selectivity varied with base media. Short-term treatment with SB or with low-dose BMP4 was accompanied by a significant increase in brachyury expression in StemPro, but not in EMIM (Fig. 3C). Thus, activin A inhibition alone or low-dose BMP4 promoted mesoderm induction in StemPro, but not in EMIM, suggesting higher TE selectivity over ME in EMIM compared to StemPro.

Despite the capacity to induce TE in both MIM and EMIM minimal media (Fig. 3D, G), BMP4 was more TE selective in EMIM than MIM (Fig. 3D–G) because the fraction of brachyury and GATA6-positive cells were significantly sup-

pressed in EMIM (Fig. 3G). For example, 100 ng/mL BMP4 induced non-TE lineages in over half of the cell population in MIM (Fig. 3E–G), whereas BMP4 plus SB supplementation to EMIM reduced extraembryonic endoderm from nearly 20% to 8% of cells (Fig. 3F, G; $P < 0.03$) and reduced mesoderm from over 40% to <20% (Fig. 3E, G; $P < 0.024$) producing a population of cells that were predominantly TE (~80% CDX2-positive by day 2). BMP4 selectivity for TE induction was increased by inclusion of both SB and heparin. Interestingly, GATA6 and CDX2 were mutually exclusive at the cellular level, (Supplementary Fig. S5D), demonstrating a mosaic pattern of development, consistent with reports that ICM develops as a mosaic of embryonic and extraembryonic progenitors [82].

TE-selective differentiation is characterized by a quantitative morphological transformation from a cuboidal to a squamous epithelium and subsequent terminal differentiation to multinucleated cells

TE-selective culture, EMIM supplemented with low-dose BMP4 and SB, was evaluated for morphological change to cell colonies. The morphology of pluripotent hESC colonies consisted of small, tightly packed cells that remain tight during colony growth (Fig. 4A). BMP4 (100 ng/mL) with SB in EMIM initiated cell spreading at colony edges by day 2 (Fig. 4B), which progressed to the colony core by day 3. The observed conversion to a squamous epithelium was complete and quantitative before day 4 (Fig. 4B–D). Phase-contrast images of pluripotent hESC and TE-differentiated colonies were compared by texture analysis, which is sensitive to subtle changes in cell and nuclear size, edges, and colony uniformity [76,77]. Texture analysis demonstrated

FIG. 3. BMP4 produces a dose-dependent induction of TE, and is TE selective in EMIM. A radar graph displaying fold change of the transcription factors brachyury (T, gray) and CDX2 (black) expression levels in the presence versus absence of growth factors (Activin antagonist, SB; low-dose BMP4; high-dose BMP4) in either StemPro or EMIM basal medium shows ME induction in StemPro versus TE induction in EMIM (A). CDX2 expression was significantly induced when low-dose BMP4 was used in combination with SB in StemPro or in EMIM or when BMP4 was used alone at higher doses (500 ng/mL) in EMIM (B). In StemPro, but not EMIM, short-term treatment with either SB or low-dose BMP4 alone significantly increased brachyury expression (C). BMP4 supplementation to MIM significantly increased the fold induction of CDX2 over MIM alone, but did not increase brachyury and GATA6 over MIM alone (D). This same pattern of fold increase of transcription factor expression levels was seen with BMP4 and SB addition to EMIM (MIM with heparin, D). Differences between MIM and EMIM best demonstrated by measuring the fraction of CDX2-positive cells rather than the population average CDX2 expression. BMP4 and SB addition to EMIM produced a predominantly CDX2-positive cell population by reducing the percentage of brachyury (E) and GATA6 (F) positive cells, compared to MIM plus BMP4 (G; $P < 0.024$ and $P < 0.03$, respectively).

that pluripotent hESC colony morphology was statistically distinct from TE-differentiated colonies at day 9–10 (Fig. 4C). Further, the kinetics of TE differentiation was quantified by texture analysis (Fig. 4D) of time-lapse images, which revealed that colonies in TE induction media were not significantly different from pluripotent cell colonies on days 1–2 (Fig. 4D), but that colony texture was distinct on day 4–10 with near certainty ($P = 8.6 \times 10^{-7}$). Thus, TE transformation was complete by day 3 in TE-selective culture conditions.

Pluripotent cells with high OCT4 expression had a cuboidal morphology with prominent peripheral F-actin bands suggestive of tight cell–cell junctions, closely packed nuclei and very small cytoplasmic-nuclear ratios (Fig. 4E). Conversely, TE had reduced OCT4 levels, and formed a squamous epithelium with weak peripheral F-actin bands, cytoplasmic stress fibers, large internuclear distance, and increased cytoplasmic-nuclear ratios (Fig. 4F). Finally, TE identity was confirmed by a functional assay of hESC differentiation to binucleated (Fig. 4F, arrows) and multinucleated (Fig. 4H, arrows) syncytium, characteristic of terminally differentiated syncytiotrophoblasts. Time-lapse experiments revealed that 100 ng/mL BMP4 and SB sup-

plementation to EMIM caused a significant ($P < 0.05$) increase in the percentage of binucleated and multinucleated cells by days 4 and 8, respectively. Multinucleated cells were absent from pluripotent hESCs (Fig. 4G) and from neurectoderm culture conditions induced by the BMP4 antagonist, noggin, confirming the selective occurrence of bi- and tri-nucleated cells (Fig. 4H) and of multinucleated syncytium (Fig. 4I, arrows) in TE-selective culture conditions. The morphology of multinucleated cells (Fig. 4I, arrows) on day 8 was typical of syncytiotrophoblast with smaller, darker nuclei and condensed chromatin structures [83,84].

HDAC inhibition with TSA slows BMP4-induced hESC differentiation

Although not TE-selective, BMP4 supplementation to MIM was the most robust protocol for hESC differentiation to multiple lineages. To test whether or not TSA caused lineage-specific inhibition, this protocol was used to assess HDAC inhibition of hESC differentiation to TE, mesoderm, or extraembryonic endoderm. Addition of 2 nM TSA to MIM plus BMP4 for 48 h produced a significant OCT4 level in-

Colony morphology during hESC growth and TE differentiation

FIG. 4. Conversion of hESCs to TE is characterized by a quantitative morphological transformation from a cuboidal to a squamous epithelium and is confirmed by terminal differentiation to syncytiotrophoblast. Pluripotent hESCs in StemPro (**A**) consisted of small, tightly packed cells that remained tight during colony growth by phase-contrast microscopy on day 9. BMP4 (100 ng/mL) in EMIM plus SB initiated colony spreading at day 2 (**B**) that was complete by day 3. Colony texture was quantified by wavelet analysis and displayed with the help of a color scale (**C**, **D**), where blue (black in grayscale image) represents similar (small KL distances) and red (white in grayscale image) represents distinct (large KL distances) colony textures. Colonies were compared just before passaging [5 pluripotent colonies (C1–C5) at day 9 (D9) vs. 4 TE colonies (C6–C9) at day 10 (D10)]. Self-comparisons among pluripotent or among TE colonies showed high uniformity (blue), whereas cross comparisons showed high differences (red, 2-sample *t*-test conducted at the 0.98 confidence level, $P < 1 \times 10^{-6}$). Time-lapse TE differentiation (**B**) was also quantified by texture analysis (**D**). A comparison of each TE colony (C1–C3) showed similarity between all colonies on days 1 and 2 [D1–D2 (blue-green rectangles outlined in white)] and very high similarity between all colonies on days 4 through 10 [D4–D10 (blue rectangles outlined in white)], but that colony textures were different when day 2 and day 4 TE colonies were compared (2-sample *t*-test conducted at the 0.98 confidence level, $P = 8.6 \times 10^{-7}$). Pluripotent cells were further characterized by fluorescence microscopy (**E**), which revealed a cuboidal nuclear morphology, prominent peripheral actin bands, closely packed, OCT4 expressing nuclei and small cytoplasmic-nuclear ratios (**E**). TE cells on day 2 spread out and formed a squamous epithelium with weak peripheral bands, reduced OCT4 levels, and large internuclear distance and cytoplasmic-nuclear ratios (**F**). Frequent cells with 2 nuclei (arrows) were confirmed by peripheral actin bands at cell boundaries. Subsequent to TE induction on day 2, a time-lapse of TE differentiation revealed that bi- and multinuclear trophoblast cells occurred in 10% of cells by day 10 (**G**, bar graph showing mean, SD, and 2 sample *t*-test at $P < 0.05$). Multinucleated cells were absent from hESC cultured in noggin (a BMP4 antagonist) used to promote neural differentiation. Unique to EMIM plus BMP4 plus SB, bi- and tri-nucleated cells (**H**, arrows) were common in trophoblasts and multinucleated syncytium (**I**, arrows) were typical of syncytiotrophoblasts with rounded, smaller, darker cells and condensed chromatin structure (scale bar in **B**, 1 μ m; **F**, 100 μ m; **I**, 10 μ m). KL, Kullback-Leibler. Color images available online at www.liebertonline.com/scd

FIG. 5. HDAC inhibition with TSA slows BMP4-induced hESC differentiation. Compared to BMP4 alone (A, red bars, white in grayscale image) TSA addition to MIM with BMP4 (blue bars, black in grayscale image) significantly increased OCT4 expression and decreased the expression of CDX2, brachyury, and GATA6 (all $P < 0.05$; also shown in D vs. E). TSA also significantly reduced the expression of HDAC3 (B) and methylation of histone H3K9 (C) and redistributed HDAC3 from the plasma membrane (compare HDAC3 in D and E). Net differentiation at 48 h was also significantly reduced with TSA addition to MIM plus BMP as measured by morphological transformation to a squamous epithelium (F and G, $P < 0.05$). TSA, trichostatin A; HDAC, histone deacetylase. Color images available online at www.liebertonline.com/scd

crease with a concomitant decrease in CDX2, brachyury, and GATA6 expression relative to BMP4 addition alone (Fig. 5A; compare panels 5D vs. 5E). Thus, HDAC inhibition delayed BMP4-induced hESC differentiation in the short-term, without altering lineage selection.

HESC colony morphology at 48 h evaluated by texture analysis showed that TSA addition significantly delayed BMP4-induced epithelial transformation (Fig. 5F, G). BMP4 produced heterogeneous morphological transformation at 2 days in MIM. However, addition of 2 nM TSA maintained

tight colonies with small internuclear distance (Fig. 5F) and produced a colony texture that significantly differed from TE (Fig. 5G; $P < 0.05$).

Discussion

Given the literature on TE differentiation from stem cells, we were puzzled by our initial inability to produce TE from BMP4-treated hESCs until we switched from standard MEF cultures to StemPro medium. Several explanations are possible, including inherent differences in cells between culture conditions, or competing medium components that interfered with TE differentiation. Preliminary evidence suggests that not all hESC culture conditions are identical and that StemPro cultures have higher levels of Oct4 expression and histone H3K9 acetylation (manuscript in preparation). Therefore, we cannot exclude differences in source cells used for differentiation as a partial explanation for efficiency of TE derivation from hESCs. Nonetheless, we have identified culture conditions during the initial induction phase of TE differentiation that are useful for selective, synchronous, and quantitative conversion from pluripotent to TE-committed cells after 3 days in culture. Further, we have developed quantitative methods for dynamically measuring morphological transformation during development and suggest that HDACs, especially HDAC3, have a role in regulating the rate of TE induction.

We developed a medium deficient in growth factors that supports pluripotency and that enables high TE induction and selectivity from pluripotent hESCs, line H7. EMIM, comprised of a DMEM/F12 base with insulin, bovine serum albumin, and heparin sulfate, was supplemented with BMP4 and the activin antagonist, SB. Cells differentiated without BMP4 were CDX2 negative, whereas cells differentiated with BMP4 but without the activin antagonist or heparin sulfate produced mixed lineages, including TE, mesoderm, and extraembryonic endoderm. It is likely that while BMP4 is sufficient for extraembryonic lineage formation, activin A inhibition suppresses mesoderm (brachyury) and extraembryonic endoderm (GATA6). Heparin sulfate functions by supporting growth factor stability [60] and cellular delivery during hESC differentiation [85], and in a growth factor-deficient medium, it increased the impact of BMP4 and activin antagonism on TE induction and selection, respectively. EMIM was designed to be compatible with cells cultured in StemPro. It is likely that this protocol for selective TE induction will be suitable for other pluripotent hESC lines in other media. However, cells cultured in mTeSR (StemCell Technologies) efficiently formed TE upon addition of BMP4, but had low viability in EMIM (not shown). Cells cultured in either mTeSR or on MEF feeders could be pretreated with StemPro for 2–4 weeks and then efficiently differentiated to TE.

TE identity after exposure to BMP4 in EMIM plus SB was confirmed by an increase in CDX2 expression in the short-term (48 h), as well as by morphological transformation to a flattened, squamous epithelium, and multinucleated syncytiotrophoblast morphology in the long-term (7–14 days). TE cells are morphologically distinct from pluripotent hESCs. TE cells in the blastocyst express molecules characteristic of a secretory, squamous epithelium, whereas the ICM remains a 3-dimensional, interconnected cuboidal epithelium. These morphological features were utilized to distinguish TE from

pluripotent hESCs and describe a novel, noninvasive measure of TE differentiation, colony texture. Although morphology can distinguish TE from pluripotent hESCs, many other cell types besides TE have an epithelial morphology. Multinucleated cell formation, or syncytia, is a more specific morphological feature and in humans is typically either skeletal muscle or syncytiotrophoblast. Myoblasts form syncytia with normal-sized nuclei and with normal-density chromatin; however, we demonstrated syncytia morphology characteristic of syncytiotrophoblasts, specifically small nuclei with hyper-condensed heterochromatin. Further, the absence of brachyury induction with a concomitant induction of CDX2 in the present protocol suggests that the multinucleated, syncytial cells so formed were of placental type, specifically syncytiotrophoblast, the default pathway of TE cell culture [4,15]. Syncytial formation is not a cell culture artifact since this morphology was never observed in pluripotent cultures, or in neural cultures differentiated with the BMP4 antagonist, noggin (Fig. 4), or in spontaneously differentiating cell culture conditions (not shown). We were unable to confirm secretory function with 2 β -hCG antibodies that failed to stain positive control cells. Thus, evidence of functioning syncytia awaits staining for beta-hCG, or other placenta-specific secretory hormones.

In vivo, activin antagonizes BMP4 signaling through different SMAD factors that compete for the same downstream transcription factor, SMAD 4. Inhibition of activin signaling leads to reduced levels of SMAD 2/3 and in turn increased availability of SMAD 4 for interaction with BMP4-induced SMAD 1/5/8. This is the likely mechanism for augmentation of BMP4-induced TE differentiation by the activin A antagonist, SB, in MEF-monolayer protocols [21,24]. In general, hESC treatment with BMP4 in MEFs [32] or in defined feeder-free conditions after short-term MEF-conditioned medium preconditioning [74] is not TE selective. For example, short-term BMP-4 treatment by these methods also initiates mesoderm induction in hESCs as indicated by brachyury expression. This is consistent with reports that inducing brachyury expression by BMP4 requires FGF2 and activin A [49,85], which are both present in MEFs and in StemPro, but not in EMIM. Also contributing to low TE selectivity in MEFs [9,44] is that TE induction fails except in multilayered adherent colonies where autocrine factors are likely to play a greater role in directing hESC differentiation than soluble growth factors in the medium. Poor TE induction in monolayers is potentially due to exposure to feeder-derived activin A [23,48].

While others have induced early extraembryonic differentiation from hESCs in chemically defined media supplemented with BMP4, these cell products were heterogeneous with some cells expressing markers of TE lineages and others expressing markers of primitive endoderm [33,34]. Therefore, it is useful to evaluate selectivity by single-cell methods when evaluating BMP4 requirements for selective TE induction. The present finding that high-dose BMP4 alone is sufficient for TE induction in a growth factor-deficient (and hence activin A deficient) medium suggests that activin A inhibition is not necessary for TE induction, *per se*. However, at lower BMP4 doses in the growth factor-deficient medium, activin inhibition with SB does augment TE induction, likely by inhibition of the autocrine production of activin A by hESCs [24]. This suggests that BMP4 regulation specifically,

and paracrine regulation generally, of TE differentiation is context dependent.

The foregoing considerations have clinical relevance for the field of assisted reproductive technology (ART). Since proper TE differentiation is a requirement for embryo implantation, paracrine constituent medium differences in human embryo culture systems, which are nonstandardized and ill-defined, could partially account for the observed wide range of embryo implantation rates from 8% to 54% (SART database).

In addition, the present findings suggest that BMP4-induced TE differentiation involves altered epigenetic programs. The HDAC inhibitor, TSA, preferentially reduced HDAC3 levels in parallel with inhibition of TE differentiation and reduced HDAC function by H3K9-trimethylation measures, supporting an HDAC-dependent mechanism for BMP4-induced hESC differentiation to TE. Multiple studies support an epigenetic basis for the developmental plasticity of hESC by showing that tissue-specific genes, though inactive, are not irreversibly silenced [84,86,87]. Despite hESC competence for differentiation toward alternative lineages, TSA slowed hESC differentiation in a dose-dependent manner in response to BMP4, without altering lineage selection. This temporal delay of hESC differentiation by epigenetic modifiers could have clinical consequences. For example, studies on ART have linked epigenetic phenomena to adverse pregnancy outcomes [87–89], including early pregnancy failures. Thus, some ART-associated early pregnancy failures may, in part, reflect errors in embryonic development due to epigenetic defects. Thus, epigenetic modifiers, responsible for a temporal delay in hESC differentiation, could partially account for poor embryo implantation rates if present in nonstandardized embryo culture systems. Since blastocyst development is synchronized to uterine receptivity, delayed TE differentiation from epigenetic modulators might adversely affect implantation, a limiting factor in ART success [90].

Studies of preimplantation human development, mostly from mouse and hESC models, have contributed to our understanding of TE differentiation. However, the varied, ill-defined, and non-TE-selective nature of these models has set the stage for an incomplete understanding of the paracrine and epigenetic transcriptional control of TE induction. The value of a rapid, reproducible, and selective protocol for TE induction is that fundamental questions on the paracrine and epigenetic requirements for TE induction can be tested for an affect thus enabling mechanistic studies of paracrine and environmental factors on human TE development. The TE-selective medium developed should be of considerable value to future studies of placental development. Fundamental questions regarding errors of lineage selection could be asked with this medium, and etiological components identified.

In addition, a defined medium for selective TE induction has practical value. For example, in embryology it could serve as a template for the design of a defined medium to support the blastocyst at the stage where TE is first developed. Similarly, this protocol could serve as a reference standard by which various ART media can be tested for impact on TE development without experimenting on viable embryos. In addition, this protocol for selective TE induction provides a foundation for future studies to explain how

epigenetic modifiers in culture media may adversely affect TE. In toxicology and drug development, this protocol could serve as an assay to identify potential teratogens and/or test developmental toxins that contribute to placental insufficiency and low birth weight. Modifiers present in the media or in the maternal milieu may impact embryo health via TE development. A stable, reproducible in vitro system will advance our mechanistic understanding of agents that affect maternal environment and adversely alter preimplantation embryo health.

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The authors have declared that no competing interests exist.

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Address correspondence to:

Dr. Teresa M. Erb

Division of Reproductive Endocrinology and Infertility
Department of Obstetrics, Gynecology, and Reproductive Sciences,
Magee Women's Hospital of UPMC
University of Pittsburgh School of Medicine
300 Halket Street
Pittsburgh, PA 15213

E-mail: erbtm75@gmail.com

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