Cell Stem Cell

BRACHYURY and CDX2 Mediate BMP-Induced Differentiation of Human and Mouse Pluripotent Stem Cells into Embryonic and Extraembryonic Lineages

Andreia S. Bernardo,[1,9,*] Tiago Faial,[1,4,7] Lucy Gardner,[2,6] Kathy K. Niakan,[1,6,8] Daniel Ortmann,[1,9] Claire E. Senner,[3,6] Elizabeth M. Callery,[1,4] Matthew W. Trotter,[1,9] Myriam Hemberger,[3,6] James C. Smith,[4] Lee Bardwell,[5] Ashley Moffett,[2,6] and Roger A. Pedersen[1,6,9,*]

[1]The Anne McLaren Laboratory for Regenerative Medicine, University of Cambridge, Cambridge CB2 0SZ, UK
[2]Department of Pathology, University of Cambridge, Cambridge CB2 1QP, UK
[3]Laboratory of Developmental Genetics and Imprinting, The Babraham Institute, Cambridge CB22 3AT, UK
[4]MRC National Institute for Medical Research, Mill Hill, London NW7 1AA, UK
[5]Department of Developmental and Cell Biology, University of California, Irvine, CA 92697-2300, USA
[6]Centre for Trophoblast Research, University of Cambridge, Cambridge CB2 3EG, UK
[7]Department of Zoology, University of Cambridge, Cambridge CB2 3EJ, UK
[8]Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge CB2 3EG, UK
[9]Department of Surgery, University of Cambridge, Cambridge CB2 0QQ, UK
*Correspondence: asb63@cam.ac.uk (A.S.B.), ralp2@cam.ac.uk (R.A.P.)
DOI 10.1016/j.stem.2011.06.015

SUMMARY

BMP is thought to induce hESC differentiation toward multiple lineages including mesoderm and trophoblast. The BMP-induced trophoblast phenotype is a long-standing paradox in stem cell biology. Here we readdressed BMP function in hESCs and mouse epiblast-derived cells. We found that BMP4 cooperates with FGF2 (via ERK) to induce mesoderm and to inhibit endoderm differentiation. These conditions induced cells with high levels of BRACHYURY (BRA) that coexpressed CDX2. BRA was necessary for and preceded CDX2 expression; both genes were essential for expression not only of mesodermal genes but also of trophoblast-associated genes. Maximal expression of the latter was seen in the absence of FGF but these cells coexpressed mesodermal genes and moreover they differed in cell surface and epigenetic properties from placental trophoblast. We conclude that BMP induces human and mouse pluripotent stem cells primarily to form mesoderm, rather than trophoblast, acting through BRA and CDX2.

INTRODUCTION

The mesoderm lineage gives rise to the heart, blood, muscle, kidney, and components of most other somatic tissues, plus placental mesenchyme. Cell-based therapy and disease modeling of any of these derivatives thus relies on a thorough understanding of mesodermal origins. Embryonic and extraembryonic mesoderm, together with deÞnitive endoderm, emerge

for the role of BMP4 in the mouse embryo, since homozygous BMP4 mutants have severe defects in embryonic and extraembryonic mesoderm, but none in trophoblast (Winnier et al., 1995).

BMP4, alone or together with Activin, rapidly induces hESCs to express the transcription factor BRACHYURY(BRA) and other genes characteristic of mesendoderm, the common progenitor of mesoderm and endoderm (Zhang et al., 2008; Yang et al., 2008; Vallier et al., 2009). Progressive differentiation of these cells is evident in expression of genes associated with deÞnitive endoderm, such as SOX17 and FOXA2, or with mid/distal and proximal mesoderm, such as TBX6 or FLK1 (Murry and Keller, 2008). Growth factors responsible for mesoderm differentiation of hESCs are not well understood, as BMP does not appear to be the sole driver of mesoderm (Vallier et al., 2009). Fibroblast growth factor (FGF) in particular has been used to promote mesoderm speciÞcation and proliferation (Yang et al., 2008; Yook et al., 2011). Understanding which growth factors distinguish between mesoderm, endoderm, and trophoblast differentiation of hESCs is key to their ultimate use for cell-based therapies and disease modeling.

A recent study used FGF2 to modulate the response of hESCs to BMP4, Þnding that trophoblast-associated genes were

thus appears to be consistent with their effect in gastrulating mouse embryos.

D BRA /CDX2^{+} a BRA /SOX17^{+}
P a E FL B a FL A T a

BRA-positive cells) colocalized with $SOX17^{+}$, and a BRA^{high} population, many of which (45% of the total BRA-positive cells) colocalized with $CDX2^{+}$ (

Figure 2. Distinct BRA ^{high}/CDX2^{+} and BRA^{low}/SOX17^{+} Populations Emerge from FLyA and FLyB Treatments

(A) Flow cytometry histograms of hESCs grown as indicated. Circles represent subpopulations of BRA ^{high} and BRA^{low} cells, which colocalize with CDX2 or SOX17, respectively.

of both mesoderm-associated and trophoblast-associated gene sets.

Characterization of BMP-Treated hESC Differentiated Progeny

To resolve the identity of these cells, we examined BMP-treated hESCs further. We focused on well-characterized epigenetic properties of trophoblast cells, namely on the epigenetic status of their ELF5 promoter (Ng et al., 2008; Hemberger et al., 2010) and on the characteristic repertoire of human leukocyte antigen (HLA) class I molecules expressed by human trophoblast cells in vivo (Apps et al., 2009).

In the mouse and human trophoblast lineages, ELF5 is hypomethylated (≈9%) and highly expressed, but it is hypermethylated and silenced in mEpiSCs and hESCs (Ng et al., 2008; Hemberger et al., 2010). In the absence of FGF, BMP-treated cells expressed some ELF5, albeit at low levels (with a cycle threshold [CT] of 32), and ELF5-positive cells comprised only a small fraction of cells (Figure 4A). Consistent with this, the ELF5 promoter was highly methylated in BMP-treated hESCs and the critical CpG dinucleotides surrounding the transcriptional

Figure 4. Characteristics of BMP-Treated hESCs Distinguish Them from Placental Trophoblast

(Ai) qPCR analysis of ELF5 expression in hESCs

start site (Hemberger et al., 2010) were hypermethylated in all samples. Slightly lower methylation was seen with BMP + Sb treatment (Figure 4B); this correlated with slightly raised ELF5 transcript levels, which were still much lower than in placental trophoblast (Hemberger et al., 2010). Nevertheless, the majority of CpGs remained methylated across the ELF5 promoter.

marker of villous cytotrophoblast; Tavare and Holmes, 1989), and for 1B10-antigen (a marker of villous syncytiotrophoblast; Figure 4D) (Figure 4E). There was no detectable expression of EGFR in the various BMP-treated cultures (data not shown). However, the vast majority (80%Ð90%) of the BMP-treated hESCs expressed the 1B10-antigen, which is also typically expressed in adult Þbroblast tissues of mesodermal origin (Singer et al., 1989). The 1B10 antigen was almost exclusively detected in cells coexpressing HLA class I molecules as detected by W6/32 (Figure 4E). Thus, these data exclude the possibility that an authentic trophoblast population emerges after BMP treatment of hESCs, even in the conditions most favorable to the appearance of such a phenotype.

C E C α, C 1, a 7 R a S a M C

As BMP-induced cells do not fulÞll essential criteria of the trophoblast lineage, it is important to establish their correct lineage identity. All in vivo placental trophoblast subpopulations express KRT7, so we used it as a marker for trophoblast-like cells that resulted from BMP treatment of hESCs. Neither H9 nor HuES9 hESC lines expressed KRT7 when grown in CDM supplemented with Activin + FGF, which maintain pluripotency in chemically deÞ7iny (

Figure 5. Cells Expressing ***HCGα***, ***GCM1***, and ***KRT7*** Represent a Subpopulation of Mesoderm Cells

(A) qPCR analysis of KRT7^{+} and KRT7^{-} cells sorted from hESCs grown as indicated.

(B) Representative ßuorescent images of hESCs grown as indicated. Samples were immunostained for KRT7, ISL1, and the nuclear marker DAPI.

(C) Flow cytometry histograms showing FLK1 (upper panels), VCAM-1 (lower panels), and KRT7 coexpression in hESCs grown as indicated.

(D) qPCR analysis of mouse late epiblast explants grown in pluripotency (A, ActivinA, 10 ng/ml; F, FGF2, 20 ng/ml) or differentiation conditions as indicated. *$p \leq 0.05$; $^{+}p \leq 0.01$; t test.

(Ei) Representative ßuorescent image of human placental mesenchyme plated for a week in serum-containing medium. Samples were immunostained for KRT7 and with DAPI.

(Eii) qPCR analysis of human placental mesenchyme before plating cells to generate outgrowths.

(F) Representative light and ßuorescent images of hESCs differentiated as indicated. White arrowheads point to multinucleated cells.

(G) Flow cytometry histograms showing KRT7 expression, class 1 HLA epitopes (W6/32 antibody), class I A and B HLA epitopes (Tu155 antibody), and the epitope detected by 1B10-Þbroblast (1B10-Fib) in hESCs grown as indicated. See also Figure S5.

on BRA. Hence, we sought to deÞne culture conditions that would distinguish between these possibilities.

Complementary to the recent report of Yu et al. (2011), we Þnd that BMP-induced hESCs express mesoderm-associated genes (ISL1, NKX2.5, CD31, LMO2, and CDX2) in the presence of FGF, whereas they express trophoblast-associated genes (KRT7, GCM1, and HCGa) only in the absence of FGF. Importantly, we show that hESC-derived cells induced by BMP to express the trophoblast-associated gene KRT7 coexpressed mesoderm- and trophoblast-associated genes in single-cell assays (immunostaining and ßow cytometry). The BMP4 mutant mouse phenotype adds further support to the hypothesis of a mesodermal identity for BMP-induced hESCs expressing those genes, as it

H a E a M I a a , C

Placental tissue was obtained from elective terminations of normal pregnancies between 6 and 12 weeks gestation. Ethical approval for the use of these tissues was obtained from the Cambridge Local Research Ethics Committee. Human placental mesenchyme for culture was isolated by negative selection (for details see the

Goldman, D.C., Bailey, A.S., Pfafße, D.L., Al Masri, A., Christian, J.L., and Fleming, W.H. (2009). BMP4 regulates the hematopoietic stem cell niche. Blood 114, 4393Ð4401.

Gurtner, G.C., Davis, V., Li, H., McCoy, M.J., Sharpe, A., and Cybulsky, M.I. (1995). Targeted disruption of the murine VCAM1 gene: essential role of VCAM-1 in chorioallantoic fusion and placentation. Genes Dev. 9, 1Ð14.

Hayashi, Y., Furue, M.K., Tanaka, S., Hirose, M., Wakisaka, N., Danno, H., Ohnuma, K., Oeda, S., Aihara, Y., Shiota, K., et al. (2010). BMP4 induction of trophoblast from mouse embryonic stem cells in deÞned culture conditions on laminin. In Vitro Cell. Dev. Biol. Anim. 46, 416Ð430.

He, S., Pant, D., Schiffmacher, A., Meece, A., and Keefer, C.L. (2008). Lymphoid enhancer factor 1-mediated Wnt signaling promotes the initiation of trophoblast lineage differentiation in mouse embryonic stem cells. Stem Cells 26, 842Ð849.

Hemberger, M., Udayashankar, R., Tesar, P., Moore, H., and Burton, G.J. (2010). ELF5-enforced transcriptional networks deÞne an epigenetically regulated trophoblast stem cell compartment in the human placenta. Hum. Mol. Genet. 19, 2456Ð2467.

Inman, K.E., and Downs, K.M. (2006). Localization of Brachyury (T) in embryonic and extraembryonic tissues during mouse gastrulation. Gene Expr. Patterns 6, 783Ð793.

Kwon, G.S., Viotti, M., and Hadjantonakis, A.K. (2008). The endoderm of the mouse embryo arises by dynamic widespread intercalation of embryonic and extraembryonic lineages. Dev. Cell 15, 509Ð520.

Lawson, K.A., Meneses, J.J., and Pedersen, R.A. (1991). Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. Development 113, 891Ð911.

McLean, A.B., DÕAmour, K.A., Jones, K.L., Krishnamoorthy, M., Kulik, M.J., Reynolds, D.M., Sheppard, A.M., Liu, H., Xu, Y., Baetge, E.E., and Dalton, S. (2007). Activin a efÞciently speciÞes deÞnitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed. Stem Cells 25, 29Ð38.

Murry, C.E., and Keller, G. (2008). Differentiation of embryonic stem cells to