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Optimizing bone morphogenic protein 4-mediated human embryonic stem cell differentiation into trophoblast-like cells using fibroblast growth factor 2 and transforming growth factor-β/activin/nodal signalling inhibition

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KEY MESSAGE

FGF2 inhibition accelerates and TGF-β/activin/nodal inhibition decreases BMP4 mediated differentiation of syncytiotrophoblasts from hESC. Furthermore, inhibition of FGF2 is a major trigger for the production of hyperglycosylated HCG. This knowledge enables us to find an optimal model for studying the early development of human trophoblasts in normal and complicated pregnancy.

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A B S T R A C T

Several studies have demonstrated that human embryonic stem cells (hESC) can be differentiated into trophoblast-like cells if exposed to bone morphogenic protein 4 (BMP4) and/or inhibitors of fibroblast growth factor 2 (FGF2) and the transforming growth factor beta (TGF-β)/activin/nodal signalling pathways. The goal of this study was to investigate how the inhibitors of these pathways improve the efficiency of hESC differentiation when compared with basic BMP4 treatment. RNA sequencing was used to analyse the effects of all possible inhibitor combinations on the differentiation of hESC into trophoblast-like cells over 12 days. Genes differentially expressed compared with untreated cells were identified at seven time points. Additionally, expression of total human chorionic gonadotrophin (HCG) and its hyperglycosylated form (HCG-H) were determined by immunoassay from cell culture media. We showed that FGF2 inhibition with BMP4 activation up-regulates syncytiotrophoblast-specific genes (*CGA*, *CGB* and *LGALS16*), induces several molecular pathways involved in embryo implantation and triggers HCG-H production. In contrast, inhibition of the TGF-β/activin/nodal pathway decreases the ability of hESC to form trophoblast-like cells. Information about the conditions needed for hESC differentiation toward trophoblast-like cells helps us to find an optimal model for studying the early development of human trophoblasts in normal and in complicated pregnancy.

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Introduction

The first cell fate decision in mammalian development is the segregation of the trophectoderm and the inner cell mass that leads to blastocyst formation. During human embryo implantation, the outer trophectoderm layer attaches to the endometrial epithelial cells and differentiates into villous cyto- and syncytiotrophoblasts which, in combination with extravillous trophoblast (EVT) cells, are required for the formation of a functional placenta. Several studies have demonstrated that human embryonic stem cells (hESC) can be directed to differentiate into trophoblast cells if exposed to the transforming growth factor **beta** (TGF-β) superfamily member bone morphogenetic protein 4 (BMP4) (Amita et al., 2013; Chen et al., 2013; Das et al., 2007; Erb et al., 2011; Li et al., 2013; Marchand et al., 2011; Sudheer et al., 2012; Telugu et al., 2013; Xu et al., 2002; Yabe et al., 2016; Yang et al., 2015). However, it is known that bone morphogenic proteins (BMP) are able to induce differentiation not only into trophoblast cells, but also into embryonic lineages (mesoderm and endoderm) (Teo et al., 2012; Zhang et al., 2008). To prevent the formation of other cell lineages and to produce a more homogenous trophoblast cell population, the inhibition of fibroblast growth factor 2 (FGF2) and TGF-β/activin/nodal signalling pathways in combination with BMP4 treatment have recently been utilized (Amita et al., 2013; Lee et al., 2015; Sudheer et al., 2012).

The TGF-β/activin/nodal and FGF2 signalling pathways are major components that support hESC self-renewal (Vallier et al., 2005). TGF-β/activin/nodal ligands belong to the TGF-β superfamily that also includes members of the BMP family. These two branches of the TGF-β/BMP signalling pathways, BMP and TGF-β/activin/nodal signalling, normally antagonize each other, partly because they need to compete for the common factor SMAD4, which is required for the activation of both branches (**Figure 1A**). FGF2 has been shown to inhibit the BMP4-directed differentiation of hESC (Das et al., 2007), or even switch it toward mesendodermal differentiation, characterized by a uniform expression of T (brachyury) and other primitive streak markers (Yu et al., 2011). FGF2 signal inhibition inversely directs BMP4-mediated differentiation of hESC to human chorionic gonadotrophin (HCG)-producing syncytiotrophoblasts (Sudheer et al., 2012; Yu et al., 2011).

Several independent studies have previously utilized BMP4 with or without FGF2 and/or TGF-β/activin/nodal signalling inhibitors to differentiate hESC into trophoblastic cells (Amita et al., 2013; Chen et al., 2013; Das et al., 2007; Erb et al., 2011; Li et al., 2013, p. 4; Lichtner et al., 2013; Marchand et al., 2011; Shirley et al., 2012; Sudheer et al., 2012; Telugu et al., 2013; Xu et al., 2002). However, the individual molecular effect of BMP4 induction and FGF2 and/or TGF-β/activin/nodal inhibition is still not known. Therefore, the aim of the current study was to investigate the individual effects and potential co-action between FGF2 and TGF-β/activin/nodal pathway inhibition on trophoblast differentiation. To obtain a deeper insight into the biological effects of the aforementioned inhibitors, full transcriptome analysis by RNA sequencing (RNA-seq) was used to describe the changes in gene expression during BMP4-activated differentiation.

Materials and methods

Cell culture

The pluripotent hESC line H9 (WiCell Research Institute, Madison, WI, USA) was cultured on a growth factor-reduced Matrigel® coating (BD Biosciences, Bedford, UK) in StemPro medium (Life Technologies, Carlsbad, CA, USA). For trophoblast-directed differentiation, hESC were split at a 1:5 ratio using 0.02% EDTA (Sigma-Aldrich, St. Louis, MO, USA) and mechanical scraping. After splitting, cells were cultured in StemPro medium for 24 h to let them attach to the plate. The next day (day 0), StemPro was replaced with N2B27 medium after gentle washing twice with Dulbecco's phosphate-buffered saline (DPBS; Life Technologies). N2B27 medium consists of DMEM/F12 (1:1) supplemented with 1 × N2 supplement, 1 × B27 supplement, 0.1 mmol/l β-mercaptoethanol, 1% non-essential amino acids solution (NEAA) and 0.5 mg/ml bovine serum albumin (all from Life Technologies). For trophoblast differentiation, four different conditions were used (**Figure 1A**): (i) BMP4, induction by 10 ng/ml BMP4 (PeproTech, NJ, USA) without inhibitors (marked in yellow throughout the study); (ii) 'iFGF2 + BMP4', induction by 10 ng/ml BMP4 and inhibition by 0.1 μmol/l fibroblast growth factor and vascular endothelial growth factor (FGF/VEGF) receptor tyrosine kinase inhibitor PD173074 (Santa Cruz Biotechnology, CA, USA) (marked in blue); (iii) 'iTGFb + BMP4', induction by 10 ng/ml BMP4 and inhibition by 1 μmol/l TGF-β type I receptors (ALK4/5/7) inhibitor A83–01 (Abcam, UK) (marked in green); and (iv) 'iFGF2 + iTGFb + BMP4', induction by 10 ng/ml BMP4 and co-inhibition by 0.1 μmol/l FGF/VEGF receptor inhibitor PD173074 and 1 μmol/l ALK4/5/7 inhibitor A83–01 (marked in red). Untreated hESC were marked in violet throughout the study. Media and supplements were replaced every 24 h. Every other day, for a period of 12 days (**Figure 1B**), two 35-mm plates of cells were dissociated in TRIzol

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