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All-*trans* retinoic acid and basic fibroblast growth factor synergistically direct pluripotent human embryonic stem cells to extraembryonic lineages

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Abstract Human embryonic stem cells (hESCs) can be used to model the cellular and molecular mechanisms that underlie embryonic development. Understanding the cellular mechanisms and pathways involved in extraembryonic (ExE) differentiation is of great interest because of the important role of this process in maternal health and fertility. Fibroblast growth factor 2 (FGF-2) is widely used to maintain the self-renewal of hESCs and induced pluripotent stem cells, while all *trans* retinoic acid (RA) is used to facilitate the directed differentiation of hESCs. Here, we monitored the RA induced differentiation of hESCs to the ExE lineage with and without FGF-2 over a 7-day period via global transcriptional profiling. The stemness markers *POU5F1*, *NANOG* and *TDGF1* were markedly downregulated, whereas an upregulation of the ExE markers *KRT7*, *CGA*, *DDAH2* and *IGFBP3* was observed. Many of the differentially expressed genes were involved in WNT and TGF-β signaling. RA inactivated WNT signaling even in the presence of exogenous FGF-2, which that promotes the maintenance of the pluripotent state. We also show that BMP4 was upregulated and that RA was able to modulate the TGF-β signaling pathway and direct hESCs toward the ExE lineage. In addition, an epigenetic study revealed hypermethylation of the *DDAH2*, *TDGF1* and *GATA3* gene promoters, suggesting a role for epigenetic regulation during ExE differentiation. These data reveals that the effect of RA prevails in the presence of exogenous FGF-2 thus resulting in the direction of hESCs toward the ExE lineage.

Introduction

Human embryonic stem cells (hESCs) derived from the inner cell mass of blastocysts are pluripotent and self-renewing cells that are capable of differentiating into all organ-specific cell types (Greber et al., 2007). hESCs were initially grown in serum-containing medium on feeder layers of inactivated mouse embryonic fibroblasts (MEFs), thus, allowing them to maintain their ability to self-renewing or to differentiate depending on the cell culture conditions (Thomson et al., 1998). Defined media such as StemPro and mTeSR are often used to culture hESCs in the absence of the supportive feeder cells (Ludwig et al., 2006). Growth factor supplements commonly used to promote self-renewal in hESC cultures include fibroblast growth factor-2 (FGF-2)

Abbreviations: hESCs, human embryonic stem cells; RA, all-*trans* retinoic acid; ExE, extraembryonic; FGF-2, basic fibroblast growth factor; MEF, mouse embryonic fibroblasts; TGF-β, transforming growth factor beta.

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(Dvorak et al., 2005; Levenstein et al., 2006), transforming growth factor beta1 (TGF-β1) (Amit et al., 2004), activin A (Vallier et al., 2005), and Wnt1 (Cai et al., 2007; Dravid et al., 2005). FGFs are secreted polypeptide ligands that are essential for the initiation, growth and maturation of many tissues and organs during embryonic, fetal and postnatal vertebrate development (Goldfarb, 1996). The external addition of FGF-2 activates FGF receptors and their downstream targets, such as mitogen-activated protein kinase (MAPK) and participates in maintaining the undifferentiated growth of hESCs by increasing self-renewal, cell survival and adhesion (Eiselleova et al., 2009). In addition, FGF-2 has been found in the embryonic ectoderm, muscle cells and limbs from the initiation of budding through differentiation (Dono et al., 1998; Savage et al., 1993; Storey et al., 1998). Furthermore, bone morphogenetic protein (BMP) signaling sustains the differentiation of hESCs into various lineages, including the extraembryonic (ExE) lineage (Pera et al., 2004; Xu et al., 2002). A combination of FGF, activin/nodal and BMP signaling coordinates the early lineage segregation of hESCs (Erlebacher et al., 2004; Wu et al., 2008). BMPs belong to the TGF-β superfamily which includes activins and inhibins. TGF-β signaling has a vital role in stem cell development, organogenesis (Kitisin et al., 2007) and the maintenance of pluripotency (James et al., 2005). The synergism of BMP-4 and FGF-2 signaling is imperative to direct trophoblast differentiation (Das et al., 2007; Golos et al., 2006; Xu et al., 2002).

All-*trans* retinoic acid (RA) belongs to the retinoid family, a group of analogs of vitamin A (retinols), and regulates embryonic growth and patterning decisions. RA acts as a ligand for inactive transcription factors and enables them to bind to nuclear retinoic acid receptor (RAR), thus switching the transcription factors to transcriptional activators (for review see (Rhinn and Dolle, 2012). RA, which is used for the treatment of cystic acne and other chronic skin disorders, is considered to be teratogenic in humans when taken during early pregnancy (Klug et al., 1989), to be embryotoxic following exposure prior to and during early organogenesis in the cynomolgus monkey (Hendrickx and Hummler, 1992) and to cause limb deformities in mice (Collins et al., 2006). RA is a critical signaling molecule during embryogenesis and is responsible for proximodistal patterning, limb development and regeneration, neural differentiation and axon outgrowth (Maden, 2007; Yashiro et al., 2004). A deficiency of RA during development results in a spectrum of malformations that includes defects of the lungs, cardiovascular system and urogenital system (MorrissKay and Sokolova, 1996). RA is regularly used to direct the in vitro differentiation of ESCs toward the pancreatic cell fate, epithelial, neural and smooth muscle cell fates (Dhara and Stice, 2008; Metallo et al., 2008; Potta et al., 2009; Shim et al., 2007).

Previously, RA and FGF-2 have been used together to induce neuronal differentiation. FGF-2 has been introduced not only during the early stages of neurogenesis but also to regulate processes that occur in the later stages, such as the caudalization of neuronal tissues, motor neuron specification and the enhancement of the proliferation of neuronal progenitor cells (Baharvand et al., 2007). The external addition of RA to trophoblast stem cells augments the formation of giant cells and causes a reduction in spongiotrophoblasts via the expression of RARβ in trophoblast cell fate specification during placentation (Yan et al., 2001). Homogeneous expression of FGF and its receptors was observed in bovine trophoblast giant cells, and the external addition of growth factors in the presence of extra cellular matrix on blastocysts synergistically promotes trophoblast outgrowth and could influence implantation (Haimovici and Anderson, 1993; Pfarrer et al., 2006). The ExE lineage which is essential for the survival of the embryo in the uterine environment arises from trophectoderm and primitive endoderm. These two types of cell lineage largely contribute to the ExE membranes, such as the placenta and the yolk sac. A number of signaling pathways and transcription factors are implicated in the perpetuation of these lineages (Rossant, 1995). During pre-implantation and conceptus development, bovine trophoblast cell migration, reorganization and morphogenesis are influenced by the FGF-2 and FGF-10. The interaction between FGF and RA has been successfully utilized previously for anterior–posterior neural patterning, endoderm patterning and the initiation of differentiation in vertebrate body axis formation in extending limbs using different in vitro methods (Johannesson et al., 2009; Olivera-Martinez and Storey, 2007; Shiotsugu et al., 2004). We hypothesized that the synergistic effect of two mutually opposing morphogens in early embryonic development in hESCs may at least partially elucidate the mechanism of ExE lineage development. To elucidate the combinatorial effect of RA and FGF-2 on the lineage decisions of hESCs and to identify mechanisms by which RA might bypass the self-renewal signaling pathway induced by FGF-2 in hESCs to thereby promote ExE lineage differentiation, we investigated the effect of RA in the presence and absence of FGF-2 using global expression microarrays.

Methods

Media and cell culture

H9 hESCs (WiCell, Madison, WI, USA) at passages 38 to 45 were expanded on irradiated MEFs in knockout (KO)-DMEM-F12, 20% KO serum replacement, 1% non-essential amino acids, penicillin (100 units/ml)/streptomycin (100 mg/ml) and 0.1 mM β-mercaptoethanol supplemented with 4 ng/ml FGF-2. For experiments, cells were cultured on plates coated with Matrigel (BD Scientific) in conditioned medium (CM). CM was prepared by conditioning unconditioned medium on fibroblast (CF1 feeders) overnight and then supplementing with FGF-2 (final concentration 8 ng/ml). RA treatment was performed at a 5 μM concentration, which is not cytotoxic to ESCs (Zhuang and Gudas, 2008). For treatments, RA was added on day 0 in the presence or absence of FGF-2 in the medium, and the vehicle was added to the controls. Experiments were performed with three biological replicates. Cells were harvested on day 7, lysed in TRIzol and processed for RNA isolation (see below).

Cell proliferation assay

The cell proliferation assay was performed as previously described (Jagtap et al., 2011). Briefly, hESC colonies were dissociated with StemPro Accutase (Invitrogen, Carlsbad,