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Fosl1 overexpression directly activates trophoblast-specific gene expression programs in embryonic stem cells

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ABSTRACT

During early development in placental mammals, proper trophoblast lineage development is essential for implantation and placentation. Defects in this lineage can cause early pregnancy failures and other pregnancy disorders. However, transcription factors controlling trophoblast development remain poorly understood. Here, we utilize Fosl1, previously implicated in trophoblast giant cell development as a member of the AP-1 complex, to trans-differentiate embryonic stem (ES) cells to trophoblast lineage-like cells. We first show that the ectopic expression of Fosl1 is sufficient to induce trophoblast-specific gene expression programs in ES cells. Surprisingly, we find that this transcriptional reprogramming occurs independently of changes in levels of ES cell core factors during the cell fate change. This suggests that Fosl1 acts in a novel way to orchestrate the ES to trophoblast cell fate conversion compared to previously known reprogramming factors. Mapping of Fosl1 targets reveals that Fosl1 directly activates TE lineage-specific genes as a pioneer factor. Our work suggests Fosl1 may be used to reprogram ES cells into differentiated cell types in trophoblast lineage, which not only enhances our knowledge of global trophoblast gene regulation but also may provide a future therapeutic tool for generating induced trophoblast cells from patient-derived pluripotent stem cells.

1. Introduction

During early embryo development, cells in inner cell mass (ICM) responsible for fetal development do not contribute to the trophectoderm (TE) or trophoblast lineages engendering placenta. Surprisingly, it has been shown that multiple TE lineage-specific transcription factors (TFs), such as Cdx2, Gata3, Hand1, and Tfap2c, are significantly up-regulated upon spontaneous differentiation of embryonic stem (ES) cells, an *in vitro* model for ICM (Hailesellasse Sene et al., 2007). Knockout (KO) or knockdown (KD) of a key pluripotency factor Oct4 (Pou5f1) in ES cells also induces multiple TE-specific marker genes (Niwa et al., 2000, 2005). Moreover, overexpression (OE) of individual TE-specific TFs, such as Cdx2 and Gata3 in ES cells, up-regulates TE lineage marker genes (Niwa et al., 2005; Ralston et al., 2010), revealing that trans-differentiation of ES cells towards trophoblast stem (TS)-like cells by modulating a single regulator or TF is feasible. More recent works have additionally showed that Arid3a, a previously known B-cell regulator, reprograms ES cells to TS-like cells upon OE (Rhee et al., 2017a, 2014). These Arid3a-OE cells can successfully be incorporated into the TE of developing embryos *ex vivo*. Subsequent study on the reprogramming mechanisms of ES cells to TS-like cell fate conversion further revealed that this process is achieved through a specific series of sequential epigenetic and transcriptional events. First, an initial suppression of the ES cell core pluripotency factors was observed, followed by a dramatic activation of TE lineage-specific genes (Rhee et al., 2014, 2017b). These findings demonstrate that ectopic expression of a single TE-specific transcription factor is sufficient to overcome the barrier between ES and TS cell identity. This implies that TE lineage-specific genes may exist in a poised configuration in terms of their proximal chromatin landscape, or that there exist additional factors sequestered in ES cells that may be liberated to activate the TE-specific transcriptional program. Therefore, ES cells can serve as a reliable model system to study important factors responsible for TE lineage development (Murry and Keller, 2008; Niwa, 2010).

Fosl1 (also known as Fra1) is a component of activator-protein 1 complex (AP-1), which comprises a heterodimer of Fos-Jun family proteins. The Fos family includes cFos, FosB, Fosl1, and Fosl2, whereas the JunB family comprises cJun, JunB, and JunD. The exact configuration of the heterodimer determines the cell-specific role of the AP-1 complex. For example, an AP-1 complex composed of cFos and JunB regulates cell proliferation and differentiation (Shaulian and Karin, 2002). Meanwhile, another AP-1 complex composed of Fosl1 and JunB is implicated

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in endocrine and invasive trophoblast differentiation (Kubota et al., 2015; Renaud et al., 2014). Fosl1 has numerous biological roles, highlighting its importance as a versatile transcription factor. Fosl1 can contribute significantly to tumorigenesis, cell invasion (Verde et al., 2007), bone development (Wagner, 2002), and somatic cell reprogramming processes (Chronis et al., 2017). Although Fosl1 null mice die due to placental defects at approximately E10.5 (Schreiber et al., 2000), the mechanisms through which Fosl1 regulates TE lineages have not been fully understood, and furthermore, whether the Fosl1 alone can induce TE lineage-specific gene expression programs in ES cells has not been tested.

In the current study, we tested the potential of Fosl1 in trans-differentiation of mouse ES cells to TS or TE lineage-like cells. We found that OE of Fosl1 in ES cells induces TE-specific gene expression programs, especially genes active in the later stage of TE lineage development or differentiated TS cells. Surprisingly, unlike Arid3a, Cdx2, and Gata3, OE of Fosl1 does not significantly repress core pluripotency factors. Rather, Fosl1 activates the genes involved in TE lineage development, in particular genes associated with terminal TE differentiation. This suggests that Fosl1-mediated reprogramming may be used in the future as a tool to directly establish patient-specific specialized cells in the TE lineage, such as trophoblast giant cells.

2. Materials and methods

2.1. Cell culture

Mouse ES cell lines (J1 and E14) were maintained on 0.1% gelatin-coated plates in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 18% fetal bovine serum (FBS), 50 U/ml penicillin/streptomycin (Gibco), 2 mM L-glutamine (Gibco), 100 μM MEM nonessential amino acids (Gibco), nucleosides (Millipore), 100 μM β-mercaptoethanol (Sigma) and 1000 U/ml recombinant leukemia inhibitory factor (LIF, Millipore). Mouse TS cells were maintained at a ratio of 3:7 of TS medium to MEF-conditioned TS medium with 25 ng/ml Fgf4 and 1 μg/ml heparin. The TS medium consisted of RPMI 1640 (Gibco) supplemented with 20% FBS, 100 μM β-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 mg/ml streptomycin. To make MEF-conditioned medium, mitomycin-treated MEF cells were cultured in TS medium for 3 days and the medium was collected every 3 days three times. To differentiate TS cells, we cultured the cells without Fgf4 and heparin.

2.2. Stable cell line generation

Full length Fosl1 cDNA was cloned into pEF1a-FLBIO (FB) vector (Kim et al., 2009, 2010). Primer sequences used for cloning are listed in Supplemental Table 1. Fosl1-containing vector (FB-Fosl1) was electroporated into BirA-expressing ES cells. Cells grew under puromycin and G418 selection for 9 days before picking colonies. OE of Fosl1 was confirmed by RT-qPCR and Western blotting. Fosl1 OE cells were maintained under ES cell culture conditions.

2.3. Generation of inducible cell lines

Lenti-X Tet-on 3G inducible expression system containing pLVX-Tet3G and pLVX-TRE3G-ZsGreen1 vectors was used following manufacturer's instruction (Clontech). Fosl1 cDNA was prepared by PCR using primers listed in Supplemental Table 1 and cloned into the pLVX-TRE3G-ZsGreen1. The Tet3G or TRE3G-Fosl1 expression vectors were transfected with pCMV-Δ8.9 and VSV-G helper plasmids into 293T cells using Fugene (Promega), according to the manufacturer's instruction. After 24 h, the 293T medium was replaced with ES medium. The supernatants containing virus particles were collected 48 h post transfection and filtered through 0.45 μm pore-size cellulose acetate filters (Pall). E14 ES cells were plated at ~2.5×10^5 cells per one well of 24-well plate with Tet3G virus-containing supernatant. After 24 h, the cells were selected with G418 for 2 days and re-infected with the TRE3G-Fosl1 virus. The co-infected E14 ES cells were placed under G418 and puromycin selection for 2 days. Fosl1 was induced by 500 ng/ml of doxycycline in ES cell culture media.

2.4. Western blotting

Cells were washed with PBS and lysed in 2× Laemmli sample buffer (Bio-Rad). Cell lysates were boiled at 100 °C for 15 min and centrifuged prior to loading. Proteins were separated on 4–20% gradient acrylamide gels (Bio-Rad) and transferred onto PVDF membranes using Trans-Blot® Turbo™ Transfer Starter System (Bio-Rad). After transfer, membranes were blocked with 5% BSA (Sigma) in TBST (20 mM Tris-HCl, pH 7.6, 13 mM NaCl, and 0.1% Tween-20) for an hour and incubated with primary antibody (or streptavidin-HRP) at 4 °C overnight. Membranes were then washed with TBST and incubated with secondary antibody for 1 h at room temperature. Antigens were detected using ECL reagents (GE Healthcare Amersham ECL prime) with Bio-Rad Molecular Imager® ChemiDoc™ XRS+ system. The antibodies used are streptavidin-HRP (1:2000, RPN1231V, GE Healthcare Life Sciences), anti-Fosl1 (1:1000, sc-183, Santa Cruz Biotechnology), and anti-β-actin (1:20,000, ab20272, Abcam).

2.5. Alkaline phosphatase (AP) staining

Alkaline phosphatase (AP) staining was performed according to manufacturer's protocol using Alkaline Phosphatase Detection Kit (Millipore).

2.6. Real time-quantitative PCR (RT-qPCR)

Total RNA was isolated using RNeasy plus Mini Kit (Qiagen). 500 ng of total RNA was used for cDNA synthesis with ReadyScript® cDNA Synthesis Mix (Sigma). cDNA generated was diluted (20×) and RT-qPCRs were performed using 2 μl of diluted cDNA and PerfeCTa SYBR® Green FastMix, Low ROX™ (Quanta). RT-qPCR primers were designed to amplify exon junctions. Primer sequences are listed in Supplemental Table 1 and Gapdh was used as an internal control.

2.7. bioChIP-seq

bioChIP assays were performed as previously described (Beck et al., 2014; Lee et al., 2015). Briefly, cells were cross-linked in 1% formaldehyde for 7 min at room temperature. The reaction was quenched for 5 min with 125 mM glycine followed by washing with PBS. Cells were centrifuged and the pellets were used immediately for experiments or stored at −80 °C. Cells were resuspended in ChIP buffer (1% TritonX-100, 2 mM EDTA, 20 mM TrisCl, pH 8.1, 150 mM NaCl, 0.1% SDS and protease inhibitor), sonicated for 30 min (30 s on/1 min off) and centrifuged at maximum speed for 10 min. The supernatant was pre-cleared with Protein A beads for 4 h, rotating in 4 °C. Samples were then centrifuged and the supernatant was incubated in 10 μg streptavidin beads (Roche) overnight. Beads were washed for 8 min, twice with 2% SDS, once with high salt buffer (0.1% Deoxycholate, 1% Triton X-100, 1 mM EDTA, 50 mM HEPES (pH 7.5), and 500 mM NaCl), once with LiCl wash buffer (250 mM LiCl, 0.5% NP40, 0.5% Deoxycholate, 1 mM EDTA, and10mM TrisCl pH 8.1), and twice with TE buffer. Samples were eluted by incubating the beads in SDS Elution buffer (1% SDS, 10 mM EDTA and 50 mM TrisCl pH 8.1) overnight at 65 °C. 200 μl of TE buffer with 1 μg RNase A was added and incubated for 30 min at 37 °C. 1 μg of Proteinase K was added and incubated for 2 h at 37 °C. ChIP-seq library prep kits (New England BioLabs) were used to generate ChIP-seq libraries and the libraries were sequenced using an Illumina HiSeq 2500 machine.