Biochemical and Biophysical Research Communications xxx (2017) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Inhibition of Wnt/β-catenin signaling by IWR1 induces expression of Foxd3 to promote mouse epiblast stem cell self-renewal

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ARTICLE INFO

Article history:
Received 8 June 2017
Accepted 15 June 2017
Available online xxx

Keywords:
Epiblast stem cells
Self-renewal
IWR1
Foxd3

ABSTRACT

Inhibition of Wnt/β-catenin signaling facilitates the derivation of mouse epiblast stem cells (EpiSCs), as well as dramatically promotes EpiSC self-renewal. The specific mechanism, however, is still unclear. Here, we showed that IWR1, a Wnt/β-catenin signaling inhibitor, allowed long-term self-renewal of EpiSCs in serum medium in combination with ROCK inhibitor Y27632. Through transcriptome data analysis, we arrived at a set of candidate transcription factors induced by IWR1. Among these, Forkhead box D3 (Foxd3) was most abundant. Forced expression of *Foxd3* could recapitulate the self-renewal-promoting effect of IWR1 in EpiSCs. Conversely, knockdown of *Foxd3* profoundly compromised responsiveness to IWR1, causing extinction of pluripotency markers and emergence of differentiation phenotype. Foxd3 thus is necessary and sufficient to mediate self-renewal downstream of Wnt/β-catenin signaling inhibitor. These findings highlight an important role for Foxd3 in regulating EpiSCs and will expand current understanding of the primed pluripotency.

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1. Introduction

Epiblast stem cells (EpiSCs) are pluripotent stem cells derived from the epiblasts of early post-implantation mouse embryos [1,2]. EpiSCs can proliferate indefinitely in culture and differentiate into derivatives of all three germ layers in vitro. EpiSCs are similar to human embryonic stem cells (ESCs) in growth requirements, morphology, clonogenicity and gene expression pattern [3,4]. Notably, EpiSCs have little or no ability to give rise to chimeras when injected into blastocysts [1,2], and thus represent a 'primed' pluripotent state, in contrast to 'naïve'-type stem cells such as mouse and rat ESCs, characterized by the expression of pluripotency markers (e.g. *Rex1* and *Nr0b1*) and the ability to generate germline-competent chimeric offspring [5–8]. Since the original derivation of mouse EpiSCs in 2007 [1,2], culture conditions for sustaining pluripotency in vitro have been progressively refined. The cytokines Activin A and basic fibroblast growth factor (bFGF) potently promotes EpiSC self-renewal and is routinely used in the derivation and culture of mouse EpiSCs [1,2]. However, EpiSCs cultured in Activin A/bFGF appear to be in various heterogeneous states, further addition of an inhibitor of Wnt/β-catenin signaling can enable robust EpiSC propagation, and they display rather uniform marker expression [9–12]. However, understanding how these intracellular signaling pathways engage with the core circuitry to maintain primed pluripotent state remains elusive.

The canonical Wnt/β-catenin signaling pathway plays pivotal roles not only in early embryogenesis but also in stem cell maintenance [13]. In the absence of Wnt ligand, β-catenin is phosphorylated by glycogen synthase kinase 3 (GSK3) GSK3)/Axin/adenomatouspolyposis coli (APC) complex, leading to ubiquitin-mediated degradation of β-catenin. When Wnt ligand binds to its receptor complex, the canonical Wnt/β-catenin pathway is activated, leading to the stabilization of β-catenin. Stabilized β-catenin then translocates to the nucleus, where it interacts with T-cell factors (TCFs) to regulate gene expression [13]. Wnt signaling has been described as a positive regulator of self-renewal in mouse ESCs, while induces EpiSCs differentiation [10,14]. Therefore, inhibition of Wnt/β-catenin signaling favors the growth of EpiSCs. Blocking the secretion of all WNT proteins by deleting the *Porcupine* gene or retention of β-catenin in the cytoplasm through stabilization of AXIN proteins can promote propagation of EpiSCs in the undifferentiated state [9–11]. But it is unclear whether inhibition of Wnt/β-catenin signaling acts via a master downstream targets.

In this study, we devised a simple and robust approach to maintain homogeneous properties of the undifferentiated EpiSCs

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http://dx.doi.org/10.1016/j.bbrc.2017.06.086
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Please cite this article in press as: K. Liu, et al., Inhibition of Wnt/β-catenin signaling by IWR1 induces expression of Foxd3 to promote mouse epiblast stem cell self-renewal, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.06.086

using the small molecule Wnt-inhibitor IWR1 combined with ROCK inhibitor Y27632. IWR1-mediated inhibition of tankyrase stabilizes AXIN, leading to the formation of the β-catenin destruction complex, and consequently pharmacologically inhibits downstream Wnt signaling [15]. We then integrated available transcriptome profiles from our previous study [9] and identified Foxd3 as a key target of IWR1, that when overexpressed, can reproduce the self-renewal-promoting effect of IWR1.

2. Material and methods

2.1. Small-molecule inhibitors and cytokines

The following small-molecule inhibitors and cytokines were used at the indicated final concentrations: CHIR99021 (Sigma, 3 μM), IWR-1 (Sigma, 4 μM), Y27632 (Tocris, 0.5 μM), recombinant human bFGF (PeproTech, 10 ng/ml), and recombinant human Activin A (PeproTech, 10 ng/ml).

2.2. Culture media for mouse EpiSCs

The basal medium for EpiSC culture is the conventional mouse ESC medium, which consists of GMEM (Sigma) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 1% nonessential amino acids (Invitrogen) and 0.1 mM β-mercaptoethanol. EpiSCs were maintained in the basal medium supplemented with IWR1/Y27632 with or without bFGF/Activin A.

2.3. Plasmid construction

The coding regions of the mouse *Foxd3* and *Otx2* genes were inserted into PiggyBac transposon vectors and then were introduced into EpiSCs with the LTX reagent. For RNA interference in EpiSCs, short hairpin RNA (shRNA) constructs were designed to target 21-base specific regions of *Foxd3* and were then cloned into the pLKO.1-TRC plasmid. The targeted sequences were as follows:

Foxd3 shRNA #1: GCCTGCAGCTACAGCTCAACA;

Foxd3 shRNA #2: GCATCTGCGAGTTCATCAGCA.

2.4. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted with a *TransZol* Up plus RNA Kit (ER501-01, TRANSGEN BIOTECH, China). cDNA was synthesized from 1 μg of total RNA using TransScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal) (AT341-02, TRANSGEN BIOTECH, China) according to the manufacturer's instructions. qRT-PCR was performed with TransStart Top Green qPCR SuperMix (AQ131-03, TRANSGEN BIOTECH, China) in a PikoReal Real-time PCR machine (Themo Scientific). Gene expression was normalized to mouse β-Actin expression. The primers used are listed in Table S1.

2.5. Western blotting

Cells were lysed in ice-cold RIPA cell buffer (P0013B, Beyotime Biotechnology, China) supplemented with Protease Inhibitor Cocktail (DI111-02, TRANSGEN BIOTECH, China). Proteins were separated with a 10% PAGE gel and electrotransferred to a PVDF membrane. Probing was performed with specific primary antibodies and HRP-conjugated secondary antibodies. The primary antibodies used were HA (3724S, Cell Signaling Technology, 1:1000), Oct4 (SC-5279, Santa Cruz, 1:500), Nanog (ab808692, Abcam, 1:200) and α-tubulin (SC-8035, Santa Cruz, 1:2000).

2.6. Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde for 20 min at room temperature, washed in PBS, blocked for 1 h at 37 °C in blocking buffer (PBS containing 5% BSA and 0.2% Triton X-100), and incubated overnight at 4 °C with the primary antibodies: Oct4 (SC-5279, Santa Cruz, 1:200), Gata4 (SC-25310, Santa Cruz, 1:100), Tuj1 (MAB1195, R&D Systems, 1:200) or Troponin T (ab8295, Abcam, 1:100). Alexa Fluor 488 or 594 (Invitrogen, 1:1000) conjugated secondary antibody was used at 1:1000. Nuclei were stained with Hoechst (Invitrogen, 1:5000).

2.7. Embryoid body (EB) formation

Mouse EpiSCs were dissociated with CTK [9] and cultured in suspension on ultralow adhesion plates in mouse ESC basal media. Cells were allowed to grow in suspension for 8 days, and the resulting EBs were digested and then plated onto 0.1% gelatin coated dishes, in serum medium. The expression of markers for the three germ layers was examined by immunostaining at day 15.

2.8. Statistical analysis

All data are reported as the mean ± s.d. Student's t-test was used to determine the significance of differences in comparisons. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. IWR1 maintains EpiSC self-renewal combined with Y27632

Mouse E3 EpiSCs growing on 0.1% gelatin-coated dish and cultured in serum medium supplemented with Activin A/bFGF ('Ab' hereafter) rapidly differentiated (Fig. 1A), indicating that other than Activin A/bFGF might be involved in regulating EpiSC self-renewal. Previously, we and other groups showed that small-molecule inhibition of Wnt signaling is able to reduce the spontaneous differentiation of EpiSCs [9]. We next added IWR1, a small-molecule inhibitor of Tankyrase, and ROCK inhibitor Y27632 [10,12,16], which also can greatly increase the surviving ability of EpiSCs and human ESCs, into Ab condition. Culture in the presence of Ab, IWR1 and Y27632 generated a number of undifferentiated colonies (Fig. 1A). To refine the culture condition, we withdraw Ab. Surprisingly, dual administration of IWR1 and Y27632 ('IY' hereafter) allowed long-term maintenance of the undifferentiated EpiSCs in serum condition without exogenous growth factors or cytokines (Fig. 1A). Immunostaining and western blotting showed that IY-treated cells expressed higher levels of *Oct4* and *Nanog*, the key pluripotency genes (Fig. 1B and C), while exhibited lower expression of differentiation genes, such as *Cdx2*, *Gata4* and *Mixl1*, when compared with no treatment (NT) and Ab-treated cells (Fig. 1C). To determine whether the cells maintained in the IY condition retain an EpiSC identity, we examined their molecular profile and tested their differentiation potential. Quantitative real-time PCR (qRT-PCR) showed that they expressed pluripotency markers (*Oct4* and *Sox2*), and *Fgf5*, a post-implantation epiblast specific marker [1,2]. Their expression of *Rex1*, *Tfcp2l1*, *Nr0b1* and *Stella*, markers for the pre-implantation epiblast [4,17], was significantly lower than that of 46C mouse ESCs (Fig. 1E). Moreover, EpiSCs readily formed embryoid bodies (EBs) in suspension culture upon withdrawal of IY and could differentiate into TUJ1-positive ectoderm cells, TROPONIN T-positive mesoderm cells and GATA4-positive primitive endoderm cells in vitro (Fig. 1F), suggesting that IY-treated EpiSCs remain pluripotent. These results collectively indicate that EpiSCs in IY exhibit key EpiSC features.

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