Stem Cell Rev and Rep (2014) 10:16–30
DOI 10.1007/s12015-013-9473-0

Inhibition of TGFβ Signaling Promotes Ground State Pluripotency

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Published online: 15 September 2013

Abstract Embryonic stem (ES) cells are considered to exist in a ground state if shielded from differentiation triggers. Here we show that FGF4 and TGFβ signaling pathway inhibitors, designated R2i, not only provide the ground state pluripotency in production and maintenance of naïve ES cells from blastocysts of different mouse strains, but also maintain ES cells with higher genomic integrity following long-term cultivation compared with the chemical inhibition of the FGF4 and GSK3 pathways, known as 2i. Global transcriptome analysis of the ES cells highlights augmented BMP4 signaling pathway. The crucial role of the BMP4 pathway in maintaining the R2i ground state pluripotency is demonstrated by BMP4 receptor suppression, resulting in differentiation and cell death. In conclusion, by inhibiting TGFβ and FGF signaling pathways, we introduce a novel defined approach to efficiently establish the ground state pluripotency.

Keywords Embryonic stem cells · ERK pathway · Ground state pluripotency · TGF-β pathway

Introduction

Pluripotent mouse embryonic stem (ES) cells were initially generated by culturing 3.5-day blastocysts on mitotically-inactivated mouse embryonic fibroblast (MEF) feeder cells in the presence of fetal calf serum (FCS) [1, 2]. The feeder cells and FCS were later substituted by leukemia inhibitory

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Electronic supplementary material The online version of this article (doi:10.1007/s12015-013-9473-0) contains supplementary material, which is available to authorized users.

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factor (LIF) [3] and bone morphogenetic protein 4 (BMP4) [4], respectively. LIF by activating signal transducer and activator of transcription 3 (STAT3) transcription factor [5, 6] and BMP4 by inducing inhibitor of differentiation (Id) proteins [4] could sustain long-term self-renewal. Subsequently, STAT3 or Id has been observed to primarily perpetuate pluripotency by restricting multi-lineage differentiation rather than by directly affecting the transcriptional core of pluripotency [7]. Therefore, the ground state hypothesis of pluripotency proposes and Smith and colleagues showed that the chemical inhibition of endogenous differentiation signals, fibroblast growth factor 4 (FGF4) and glycogen synthase kinase 3 (GSK3), by PD0325901 and CHIR99021, respectively, known as 2i, can replace the need for exogenous signals in ES cell culture [8]. However, despite the remarkable features, the exact mechanisms of 2i in governing pluripotency have been debated primarily owing to the complexity of GSK3 signaling. Although, the activation of the nuclear receptor Esrrb has been recently shown as the main outcome of GSK3 inhibition in 2i-grown mouse ES cells [9], GSK3 affects numerous other intracellular signaling pathways by targeting more than 40 known substrates [10]. Additionally, suppression of GSK3 compromises the genomic integrity of cultured cells [11, 12].

To circumvent these issues, we have shown that dual inhibition of mitogen-activated protein kinase (MAPK) kinase (also known as MEK) and transforming growth factor β (TGFβ) type I receptors (also known as activin receptor–like kinase [ALK]−4, −5, and −7) by PD0325901 and SB431542, leads to the derivation of mouse ES cells [13, 14]. However, these lines were established in non-defined culture condition in the presence of canonical serum, MEFs and LIF. Moreover, the homogeneity (i.e., no cell-to-cell nonconformity in expression of pluripotency genes such as Nanog and Dppa3), genomic integrity, ground state pluripotency, and whole gene expression of ES cells under MEK and TGFβ inhibition was not determined.

In this study, we find highly efficient establishment of mouse ES cells from different ES cell-refractory strains using chemical inhibitors of the MEK and TGFβ signaling pathways, with R2i designated here for two such small molecule inhibitors in a chemically defined culture condition even in absence of LIF. We provide evidence that TGFβ inhibition leads to the efficient generation of homogenous and naïve ES cells through augmenting BMP4 signaling pathway, as evidenced by the global transcriptome analysis and the detrimental effect of BMP4 suppression on pluripotency. In addition, R2i offers a 'superior' environment for mouse ES cell derivation and maintenance compared with the well-known 2i condition in terms of maintaining ES cells with higher genomic integrity following long-term cultivation; and providing a less complex condition for investigating the molecular mechanisms of ground state pluripotency, in contrast to inhibition of the multifunctional GSK3 molecule in 2i.

Materials and Methods

Animals and Embryos

Mice strains used in this study were maintained on a 12-h light/dark regimen. All mouse embryos were recovered by flushing blastocysts from the uteri through superovulation of the animals by a standard protocol. We obtained blastocysts at E3.5 either from the cross of C57BL/6 (Oct4ΔPE: GFP+ or OG2) and CD-1 or from the inbred mice C57BL/6, BALB/c and DBA/2 strains.

Derivation of R2i Mouse ES Cells from 3.5-Day Blastocysts

Derivation of mouse ES cells at the blastocyst stage was performed by plating the zona-free embryonic day (E)3.5 embryos on 0.1 % gelatin-coated plates (Sigma-Aldrich) that contained N2B27 defined medium plus R2i (R2i was composed of 1 μM PD0325901 [Stemgent] and 10 μM SB431542 [Sigma-Aldrich] either with or without LIF [ESGRO, Millipore]). Alternatively, immunosurgery was performed to establish ES cell lines. Five to seven days after blastocyst or ICM plating, the ICM cells were disaggregated by using 0.05 % trypsin (Invitrogen). Trypsin was neutralized by mouse ES cell medium (or "serum" medium) containing FBS. The dissociated cell solution was centrifuged to remove the serum and harvested cells were transferred to bacterial dishes with N2B27+R2i±LIF for cultivation as a cell suspension. Alternatively, an adequate number of dissociated cells (1:3–1:6) were plated and passaged on gelatin-coated plates containing N2B27+R2i±LIF for cultivation as an adherent cell layer, with fresh defined medium added after removing the remnant serum after cell adhesion (usually 2–3 h after plating). N2B27 medium consisted of DMEM/F12 (Invitrogen) and neurobasal (Invitrogen) at a 1:1 ratio, 1 % N2 supplement (Invitrogen), 1 % B27 supplement (Invitrogen), 2 mM L-glutamine (Invitrogen), 1 % nonessential amino acids (Invitrogen), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), and 5 mg/mL BSA (Sigma-Aldrich). Mouse ES (serum) medium consisted of knockout Dulbecco's modified Eagle's medium (Invitrogen), 15 % fetal bovine serum (FBS, HyClone), 2 mM L-glutamine, 1 % nonessential amino acids, 100 U/ml penicillin and 100 mg/ml streptomycin, 0.1 mM β-mercaptoethanol, and 1,000 U/ml mouse LIF.

Culture of Mouse ES Cells

Mouse ES cells were cultured without MEF, on gelatin coated plates. R2i cells routinely passaged every 2 days as the route mentioned earlier. Usually 1:3 ratio of cells passaged in every culture. The concentrations of other TGFβ chemical inhibitors used in this study were 0.5 μM and 1 μM for A83-

01(Stemgent) and Alk5i (Stemgent), respectively. For transferring R2i cells in 2i or serum, N2B27+2i or mouse ES (serum) medium, respectively, were used with or without LIF. 2i include 1 μM PD0325901 and 3 μM CHIR99021 (Stemgent).

In Vitro and In Vivo Differentiation of ES Cells

In vitro differentiation was performed via spontaneous differentiation by EB formation or by induced differentiation. For EB formation, ES cells were dissociated by trypsin and cultured in bacterial dishes in mouse ES cell medium in the absence of LIF. Usually, 7-day EBs were plated onto gelatinized plates and 7 days later were assayed for expression of pluripotency or lineage- specific genes. For induced differentiation into neuronal lineages, 3-day EBs were transferred in medium containing 2 μM retinoic acid for 1 week then plated onto gelatinized plates and assayed 3–4 days later. For cardiomyocyte differentiation, ES cells were trypsinized and cultured at a density of 800 cells/20 μl hanging drops for 2 days in serum medium without LIF supplemented with 0.1 μM ascorbic acid. Subsequently, cells were transferred to a bacterial dish for an additional 3 days, and then rinsed onto gelatinized plates. Beating cells were usually observed 1–5 days after plating. For endoderm lineage differentiation, EB formation was performed in N2B27 medium for 3–4 days, after which 50 nM Activin A was added for 5 days followed by plating onto gelatinized cell culture dishes and assaying 72 h later. For teratoma formation, $3–5\times10^6$ ES cells were resuspended in matrigel and injected subcutaneously into nude or syngeneic mice and teratoma growth was monitored. Paraffin sections of tumor masses were stained with hematoxylin and eosin (H&E) for all histological determinations. Chimeric mice were generated as standard protocol. Chimerism was determined by coat color. To test for germline transmission, the chimeras were mated to mice from the injected strain of ES cells.

Karyotype

For karyotype analysis, ES cells were treated with thymidine (0.66 mM, Sigma-Aldrich) for 12 h at 37 °C, washed with fresh medium, and 4 h later treated with colcemid (0.15 mg/ml, Invitrogen) for 30 min. ES cells were trypsinized, swelled with KCl (75 mM) for 15 min, fixed in ice-cold methanol and acetic acid (3:1), and dropped onto chilled slides. Chromosomes were visualized using standard G-band staining. For each sample, 50 metaphase spreads were screened and analyzed for numerical and structural aberrations.

Alkaline Phosphatase Activity and Immunofluorescence Staining

Alkaline phosphatase (ALP) activity was detected by a manufacturer's kit (Sigma-Aldrich, 86R). Immunofluorescence was performed after fixation of the cultured cells in 4 % paraformaldehyde for 20 min, permeabilization with 0.2 % Triton X-100 for 30 min, and blocking in PBS supplemented with 10 % goat serum for 1 h. Cells were incubated overnight with the primary antibodies at 4 °C, washed, and incubated with secondary antibodies (Supplemental Table 1). Nuclei were counterstained with 1 μg/ml DAPI (Sigma-Aldrich). Cells were visualized using an Olympus fluorescent microscope (Olympus, Japan).

Reverse Transcription and Quantitative-Real Time PCR

ES cells were collected and preserved at −80 °C until RNA extraction. Total RNA was isolated using the RNeasy Mini kit with on-column DNase I digestion (QIAGEN). Integrity and quality of RNA samples was checked using a Nanodrop (ND-1000) spectrophotometer and RNA 2100 Bioanalyzer (Agilent). Reverse transcription was carried out using the High Capacity cDNA Reverse Transcription Kit (Life Science) and Random Hexamer primers according to the manufacturer's instructions. Transcript levels were determined using the SYBR Green master mix and 7900HT Sequence Detection System (Life Science). Gene expression level was normalized to the mouse *Ppib*, *B2m*, *Pgk*, *Actb*, and *Gapdh* housekeeping genes. Relative quantification of gene expression was calculated using the $\triangle\triangle$Ct method. Primer sequences for RT-PCR and real-time qRT–PCR are listed in Supplemental Table 2–3.

Flow Cytometry

Trypsinized ES cells were fixed with ice cold methanol, blocked by 2 % normal goat serum for 60 min, washed, incubated with primary antibody (Oct4, Nanog and Dppa3, Supplemental Table 1) for 1 h at 37 °C, washed again, and incubated with secondary antibody (Supplemental Table 1) for 30 min at 37 °C. After the final washing, flow cytometric analysis was performed using a FACSCalibur Flow Cytometer (BD Biosciences). As a negative control, the cells were stained with the appropriate isotype-matched control. The acquired data was analyzed using BD CellQuest™ Pro software.

Western Blot Analysis

Cells were lysed with commercial lysis buffer (Qproteome Mammalian Protein Prep Kit, QIAGEN). Cell lysates (50 μg) were separated on 10 % SDS-polyacrylamide gel and then transferred to PVDF membranes (Bio-Rad). The blots were blocked with TBST (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.1 % Tween-20) that contained 5 % BSA, and then incubated overnight at 4 °C with primary antibody solution. After washing with TBST, the membranes were

Table 1 Derivation efficiency of mouse ES cell lines under chemically defined medium supplemented with R2i

Strain	# of Blastocysts	# of attached embryos	# of derived lines	Efficiency of derivation (%)
N2B27+R2i				
F1[a]	4	2	2	100
BALB/c	8	6	3	50
DBA/2	7	5	3	60
N2B27+R2i+LIF				
F1[a]	8	6	6	100
C57BL/6	20	20	20	100
DBA/2	15	13	13	100
BALB/c	52	50	50	100

[a] C57BL/6 (Oct4-EGFP) × CD-1 hybrid

incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Signals were detected with ECL substrate (GE) using Hyperfilm (GE). Supplemental Table 1 shows the first and secondary antibodies used.

siRNA Analysis

For siRNA transfection, 20×10^4 cells of mouse ES cells, Royan B20, were seeded on gelatin coated 6-well plates that contained 1 ml N2B27+LIF medium. Three different siRNAs that targeted different regions of each gene were used. The sequences of siRNAs are presented in Supplemental Table 4. The medium was changed 24 h later, and 48 h after transfection the cells were collected for analysis.

Microarray Analysis

Total RNA of two mouse ES cell lines, Royan B18 and Royan B20, which were cultivated in 2i or R2i for 10 passages, was extracted using the RNeasy Mini Kit (QIAGEN). cRNA samples for microarray gene expression profiling were prepared with the Illumina TotalPrep RNA Amplification Kit (Life Science). Purified and labeled cRNA was then hybridized for 17 h onto BeadChip Array MouseRef-8 v2.0 (Illumina, San Diego, CA, USA). After washing, chips were stained with streptavidin-Cy3 (GE Healthcare, Germany) and scanned using the iScan reader (Illumina, San Diego, CA, USA) as recommended by the manufacturer. The raw signal intensities of regular and control probes were extracted from Illumina® GenomeStudio software and used for normexp [15] background correction followed by quantile normalization using the limma package [16]. For most of the bioinformatics analyses, we developed several scripts in R statistical language [17], which are available upon request. Functional pathway analysis was performed using DAVID software and the Kyoto Encyclopedia of Genes and Genomes (KEGG).

Statistical Evaluation

Statistical analyses of all graphs were performed by one-way ANOVA to determine the statistical significance of the data, using the SPSS/PC + statistics 11.0 software (SPSS). All data are presented as mean ± s.d. values in the graphs from a minimum of three independent experiments.

Accession Numbers

The raw and analyzed microarray data are deposited in NCBI Gene Expression Omnibus and accessible through GEO series accession number GSE43682.

Results

Efficient Derivation of Continuous Mouse ES Cell Lines with R2i

To determine the effect of R2i on ES cell derivation and maintenance in a chemically defined medium, we cultured 3.5-day Oct4-GFP (C57BL/6 [Oct4 ΔPE:GFP$^+$ or OG2] × CD-1) blastocysts lacking the zona pellucida on gelatin-coated 96-well plates with N2B27 culture medium supplemented with either R2i or serum plus LIF (serum+LIF). Oct4-GFP expression decreased gradually in inner cell mass (ICM) cells and disappeared by day 6 under the serum+LIF condition, with numerous trophectodermal and other differentiated cell types detectable around the outgrowth (Fig. 1a). In striking contrast, in the presence of R2i, the outgrowth maintained transgenic Oct4-GFP expression even after 6 days, with only very few trophectodermal cells. The R2i outgrowths were dissociated enzymatically into single cells and replated on gelatin-coated plates containing R2i. Six days later, colonies typical for ES cells appeared that could be passaged continuously and that maintained Oct4-GFP expression (Fig. 1a). This protocol could be successfully applied to derive ES cells from DBA/2 and BALB/c strains, which are

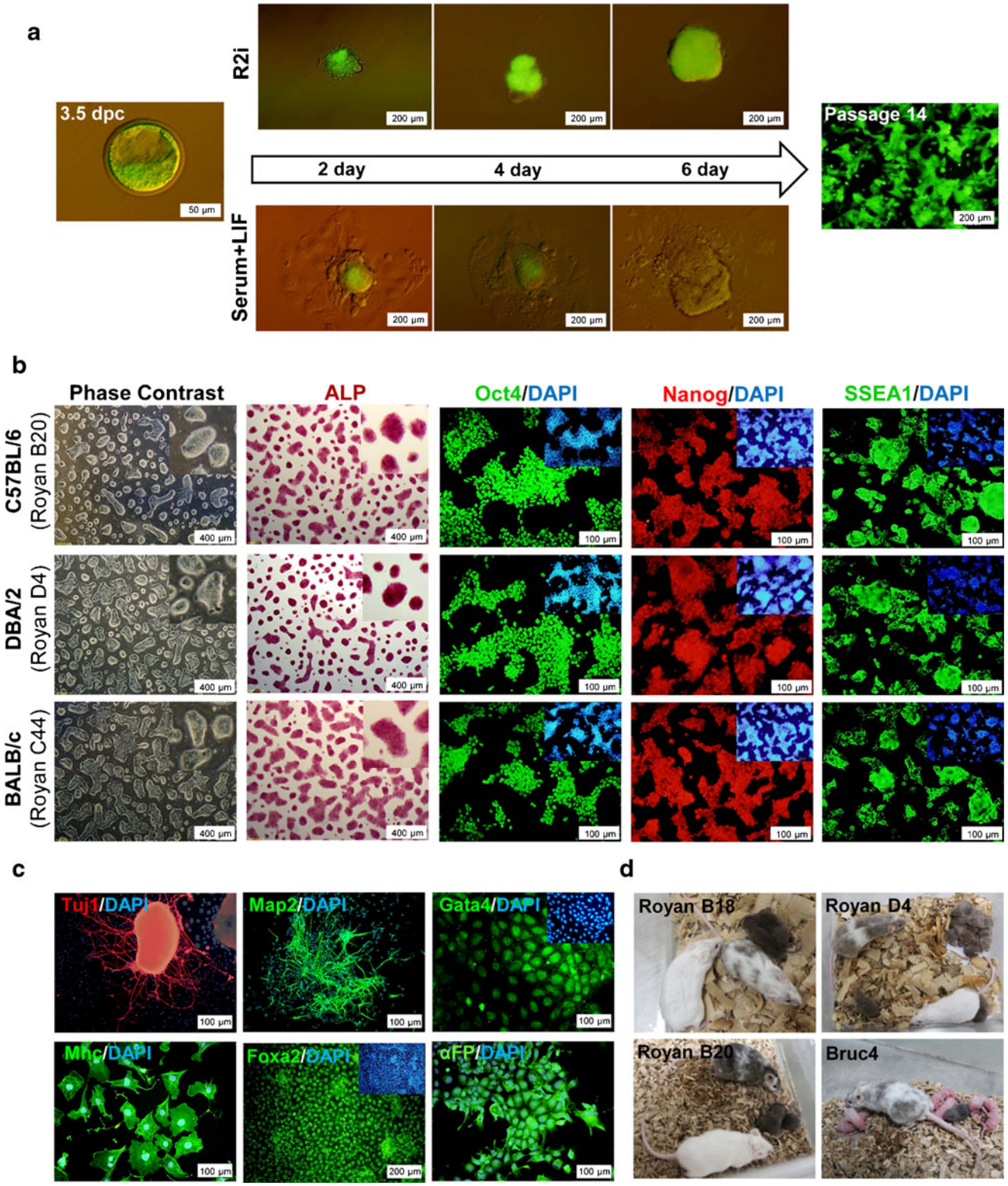


Fig. 1 R2i preserves expression of pluripotency markers in mouse inner cell mass primary outgrowths and extended ES cell lines. **a** R2i maintains Oct4 expression in the outgrowth of Oct4-EGFP blastocysts in N2B27 defined medium. **b** Characteristics of the mouse ES cells derived and cultivated in R2i. Phase contrast, alkaline phosphatase (ALP) staining, and immunofluorescence labeling for Oct4, Nanog, and SSEA-1, counterstained for DAPI, are shown. Data are from cells after > 20 passages. **c** Immunostaining for the derivatives of three embryonic germ layers of an R2i cell line, Royan B20, after directed differentiation; Tuj1 and Map2 are neuronal markers; Gata4 is a mesendodermal marker, Mhc is a cardiomyocyte marker, and αFP and Foxa2 are endodermal markers. **d** Generation of chimeric mice with germline transmission after blastocyst injection of different R2i lines (Royan B18, Royan B20, and Royan D4) or serum ES cells (Bruc4) passaged 10 times in R2i

refractory to ES cell line generation under conventional conditions [18, 19]. However, under the R2i condition, some replated cells of the ICM of DBA/2 and BALB/c strains did not adequately propagate, with efficiency for ES generation at 50 %–60 % (Table 1). As LIF has been reported to be an important factor for clonogenicity in 2i culture [20], we added LIF to R2i during the procedure to further improve the derivation efficiency. In the presence of R2i+LIF, ES cell lines were derived from ICM cells from all the different mouse strains tested, including Oct4-GFP, C57BL/6, DBA2, and BALB/c (Table 1).

All derived cell lines propagated continuously in R2i (R2i cells) to produce colonies of packed cells with a high nucleus to cytoplasm ratio and obvious nucleoli-two characteristics typical for stable ES cell lines. R2i cells could be maintained with or without LIF for more than 50 passages, with no detectable changes in morphology. To study the pluripotency identity of R2i cells, we examined the expression of stem cell markers in at least two randomly selected lines per strain. All assessed lines demonstrated positive staining for alkaline phosphatase (ALP) and expressed Oct4, Nanog, and SSEA1 (Fig. 1b and Supplemental Fig. 1A). The developmental potential of selected R2i cells was also evaluated by in vitro and in vivo differentiation assays (Fig. 1c, d and Supplemental Fig. 1B-D). The differentiated cells formed embryoid bodies (EBs) that expressed markers of three embryonic germ layers as assessed by RT-PCR (Supplemental Fig. 1B). In addition, R2i cells are exhibited lineage-specific differentiation, as they were capable of forming neuronal lineage ($Tuj1^+$ and $Map2^+$), mesendoderm ($Gata4^+$), cardiomyocytes (Mhc^+), and endoderm ($Foxa2^+$ and αFP^+), as examined by immunocytochemistry (Fig. 1c). R2i cells were further assessed for chimera formation. Colored C57BL/6- and DBA2-derived R2i cells indicated high contribution to chimeras and also germline transmission (Fig. 1d, Supplemental Fig. 1C and Supplemental Table 5).

To assess whether the serum culture condition could be adapted to R2i, we transferred serum+LIF–derived ES cell lines (serum cells) to the R2i condition. Within two or three passages, cultures had adapted morphological characteristics of the new condition. Additionally, the potential for teratoma formation (Supplemental Fig. 1D) and chimera and germline contribution (Fig. 1d and Supplemental Table 5) was ensconced after long-term passages under the R2i condition. Consequently, the data obtained show that R2i induces the generation of ES cells from different mouse strains, with maintenance of self-renewal and pluripotency after several passages.

R2i Maintains Ground State of ES Cell Pluripotency

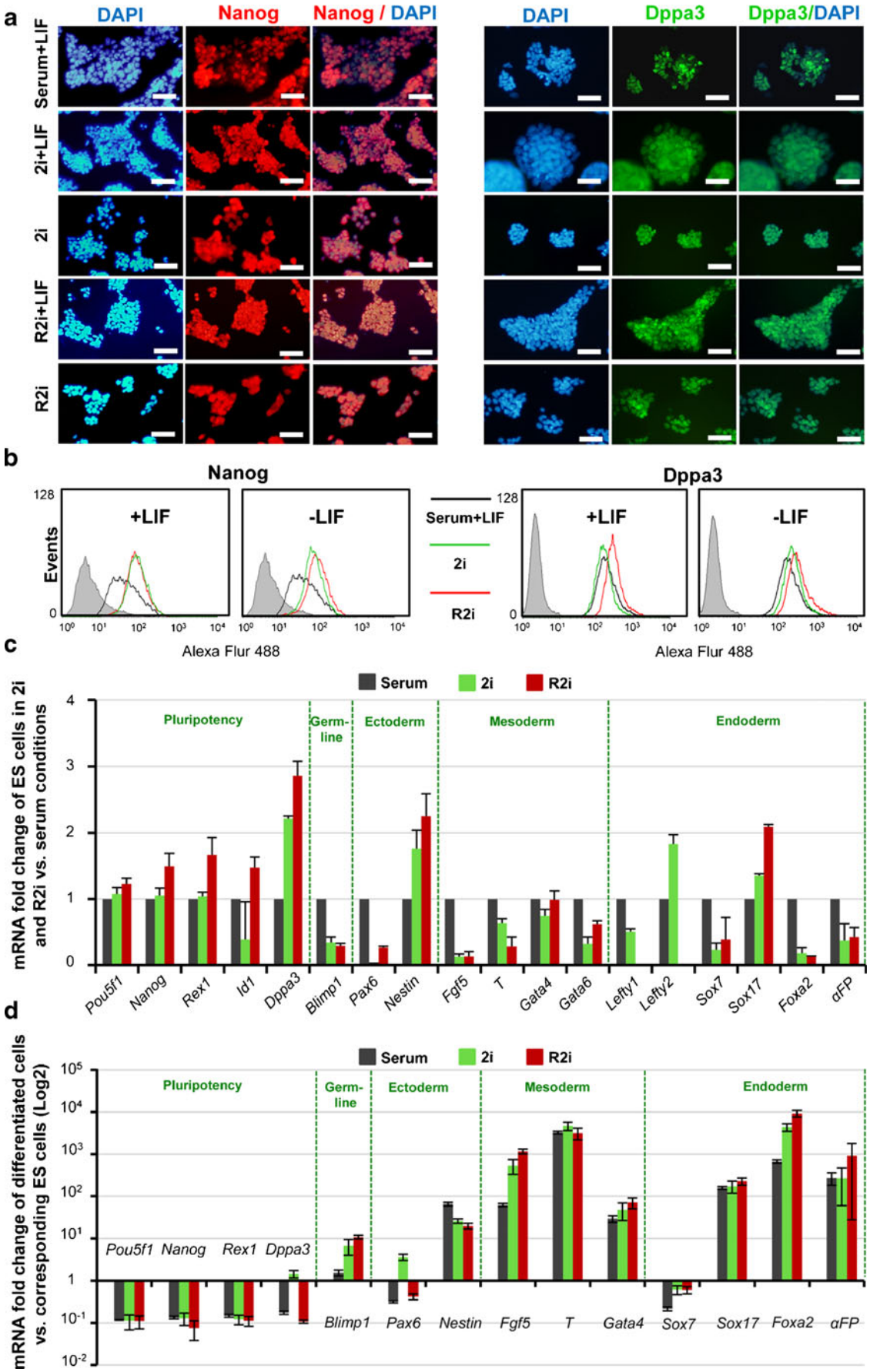
With the introduction of 2i, the ability for ES cell generation was bestowed upon ES-recalcitrant rodent strains [7, 8]. ES cells show more homogeneity in 2i compared with serum, with no cell-to-cell nonconformity in Nanog expression in 2i [7].

Fig. 2 R2i maintains ground state pluripotency. **a**, **b** Mouse ES cells are homogeneous in R2i, **a** Immunostaining for Nanog and Dppa3 (Stella) counterstained by DAPI in an R2i cell line, Royan B20, after cultivation for seven passages in the indicated medium. Scale bar, 50 μm; **b** Flow cytometric analyses for Nanog and Dppa3 expression profile in another R2i cell line, Royan D4, after cultivation for seven passages in the indicated medium. **c** qRT-PCR of genes associated with pluripotency and the various germ layers. Royan B20 ES cell line was cultivated for seven passages in serum, 2i, and R2i (all treatments plus LIF). Serum cells were considered as the control. Values are mean ± s.d. ($n=3$). **d** Embryoid body (EB) differentiation potential (7d EB and 7d plating) of Royan B20 that had been passaged seven times concurrently in serum, 2i and R2i (all treatments plus LIF) before EB formation. Expression levels were determined by qRT-PCR. Undifferentiated states in each condition were considered as the controls. Relative expression levels normalized to the housekeeping gene *Gapdh*. Values are mean ± s.d. ($n=3$)

Like 2i, R2i supports the derivation of ES cell lines from various mouse strains (Fig. 1, Supplemental Fig. 1 and Table 1). To evaluate ES cell homogeneity in R2i, we cultivated two R2i cell lines concurrently in serum+LIF, 2i±LIF, and R2i ±LIF for seven passages. We used immunostaining and flow cytometry to examine the cells' protein profiles for Nanog and Dppa3 (Stella). Although ES cells under different culture conditions showed homogeneous expression of Oct4 (Supplemental Fig. 2), expression of Nanog and Dppa3 proteins was more uniform in 2i and R2i compared with serum (Fig. 2a, b).

The transcriptional profile of ES cells has recently been shown not to exhibit a "fixed" state, as it could be interconvertible following transfer of 2i ES cells into serum, and vice versa [21]. Therefore, we assessed expression of some pluripotency and early lineage differentiation markers in an R2i cell line that was grown for seven passages in serum, 2i, and R2i, all three in the presence of LIF (Fig. 2c). Results showed high expression of pluripotency-affiliated genes and low or no expression of most lineage-associated genes, suggesting that R2i could support a stable "ground state" in ES cells [21]. R2i significantly reduced the expression of various developmental genes, including *Blimp1*, *Pax6*, *Fgf5*, *T (Brachyury)*, *Sox7*, *Foxa2,* and *αFP* (Fig. 2c). In addition, direct target genes of TGFβ signaling, such as *Lefty1* and *Lefty2* [22], were undetectable, which provided evidence for the inhibition of this pathway. In contrast, some genes related to ectoderm or to endoderm, such as *Nestin* or *Sox17*, respectively, are expressed in R2i cells at higher level than serum and 2i. These observed upregulation of early lineage marker genes in R2i cells, similar to upregulation of *Lefty2* and *Sox17* in 2i cells in our study and as reported associated genes with the germline and endoderm in Marks et al. report [21] indicates that the expression of all early differentiation genes is not inhibited in the ground state of pluripotency.

As remarkable downregulation of some lineage-specific genes was observed in R2i cells compared with serum- or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mRNA fold change of ES cells in 2i and R2i vs. serum conditions
Pluripotency
Germ-line
Ectoderm
Mesoderm
Endoderm
Pou5f1
Nanog
Rex1
Id1
Dppa3
Blimp1
Pax6
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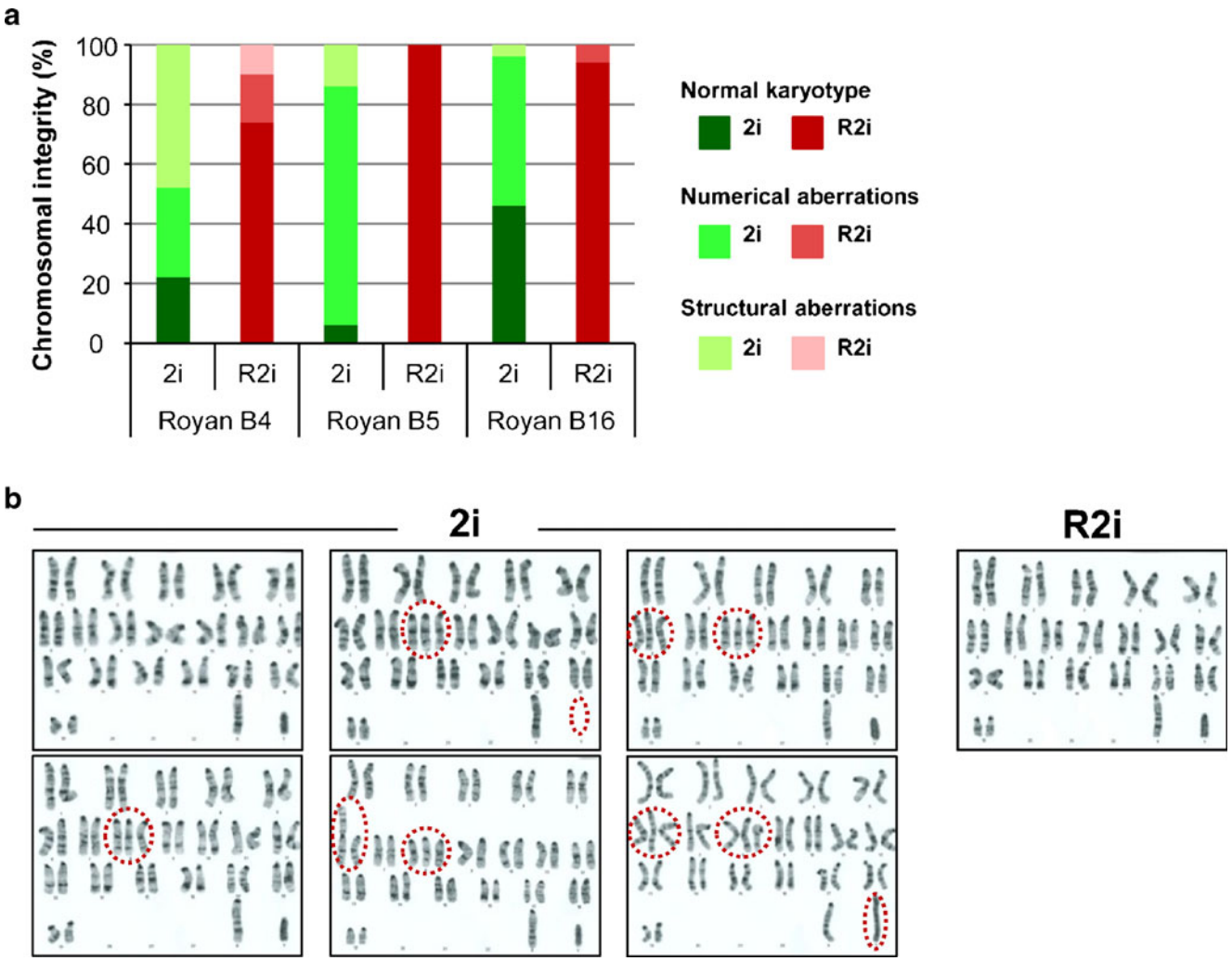


Fig. 3 R2i preserves genomic integrity after long-term maintenance. **a** Karyotypic status of three ES cell lines after ≥ 20 passages in 2i and R2i cultures. Royan B4 and Royan B5 are serum-grown ES cell lines, derived as previously described [13]. **b** Representative images from the metaphase spread of one ES cell line (Royan B5) after long-term expansion in 2i or R2i

2i-grown cells, we had to ascertain whether this status influence the differentiation potential of R2i cells. We compared the ability of serum-, 2i-, and R2i-grown cells to differentiate into different lineages by EB-induced differentiation. qRT-PCR analysis of the differentiated cells showed similar expression patterns between R2i, serum-, and 2i-grown cells in pluripotency, germline, and lineage-specific markers, indicating that the differentiation events occur properly after R2i removal (Fig. 2d). Taken together, our results suggest that R2i cells exhibit a bona fide ground state of pluripotency, with the ability to differentiate into different embryonic lineages.

R2i Asserts Genomic Integrity After Long-Term Cultivation

A basic requirement of any ES cell culture medium is the maintenance of a stable karyotype with the continuous passaging of ES cells [23]. Although the use of small molecule inhibitors in defined media has enhanced the generation of mouse pluripotent stem cells, concerns abound that these chemical perturbations may compromise the genomic integrity of the cells [24]. To evaluate the effects of the 2i and R2i culture conditions on the genomic stability of ES cells, we assessed the karyotype of three ES cell lines after simultaneous long-term passaging (≥20 passages) in both media. Our data showed that while 2i-grown cells exhibited a 6 %–46 % normal karyotype, R2i-grown cells showed 74 %–100 % normal karyotype (Fig. 3). These results suggest that R2i preserves a more stable karyotype during long-term ES cell cultivation compared with 2i, and presents a reliable alternative to 2i for the cultivation of naïve mouse ES cells.

TGFβ Inhibition Sustains the Pluripotency of Mouse ES Cells

There are contradictory arguments regarding the role of TGFβ signaling in pluripotency. Although TGFβ signaling has well-known function in human ES cells [25], the role of this

pathway in mouse ES cells faces with opposing views [26–28]. In this study, we have demonstrated that inhibition of TGFβ signaling supports the derivation and maintenance of mouse ES cells. The TGFβ signaling pathway exerts its function by phosphorylating regulatory Smad2/3 proteins via activated receptors [29] (Supplemental Fig. 3). Thus, we analyzed the activation of endogenously activated Smad2 using an antibody that specifically detected phosphorylated Smad2 (pSmad2). We found markedly diminished Smad2 phosphorylation in R2i cells compared with serum-grown cells (Fig. 4a and Supplemental Fig. 4). Consistent with this observation, we showed that inhibition of TGFβ signaling in R2i cells led to undetectable expression levels of transcriptional targets of Smad2 in ES cells, *Lefty1* and *Lefty2* (Fig. 2c).

To assess the effects of TGFβ inhibition on pluripotency, we evaluated different TGFβ receptor inhibitors including SB431542, A83-01, and the ALK5 inhibitor (ALK5i) in ES cell culture in the absence of PD0325901. Our results demonstrated that ES cells were readily cultivated only when LIF was added to each of these inhibitors and only if the ES cells were cultured in bulk (Fig. 4b, c). Additionally, knockdown of *Smad2* and *Smad3* by the use of siRNA in ES cells had relatively similar effects as the aforementioned chemicals in the expression of pluripotency markers (Fig. 4c, Supplemental Fig. 5). However, when ES cells were plated as single cells for assessing clonogenicity-an uncompromising assay for the acceptability of an ES cell culture condition [7]-TGFβ inhibitor plus LIF was unable to support colony formation. This capability, however, was maintained in R2i (Fig. 4d and Supplemental Fig. 6). Of note, TGFβ inhibitor plus LIF led to ES cell line derivation from F1, with low efficiency, but not from inbred strains (Supplemental Table 6). Therefore, TGFβ inhibitors alone did not provide an optimal culture condition for the derivation of mouse pluripotent stem cells. Instead, the use of PD0325901 with different TGFβ inhibitors manifested the prominent features of pluripotency. Mouse ES cells that were passaged for at least seven times in R2i containing various TGFβ inhibitors exhibited the main signatures of pluripotency (Fig. 4e). Furthermore, qRT-PCR analysis confirmed the same results in regard to the expression of pluripotency and lineage-specific marker genes (Fig. 4f).

TGF-β/Nodal/Activin signaling is counted among the important signaling in modulating proliferation of mouse ES cells [27]. Then, we assessed the growth rate of R2i cells, as one of the major controversial issues of TGFβ signaling in mouse ES cells [26, 28]. The cell number for two R2i cell lines that were concurrently passaged seven times in serum+LIF, 2i±LIF, and R2i±LIF was counted at 24, 48, and 72 h after cell seeding to determine the growth rate. Our results demonstrated that the proliferation rate of the cells was not significantly affected in R2i compared with serum or 2i. LIF was indispensable for optimal cell growth, both in 2i and R2i (Fig. 4g). Taken together, in contrast to numerous reports, our findings firmly indicate that the inhibition of TGFβ signaling, in combination with MEK suppression, could support all aspects of pluripotency in mouse ES cells.

R2i Enhances Expression of BMP Signaling Mediators Compared with 2i

To elucidate the molecular basis by which R2i maintains pluripotency, a detailed Microarray Analysis was performed. In this analysis, the transcriptomes of two R2i cell lines were compared after passaging them in parallel for ten times in 2i and R2i, both in the presence of LIF (Fig. 5). These cell lines have shown their original full pluripotency potential by high-contribution chimera formation and germline transmission. Microarray analysis was performed to examine expression levels of 25,697 probes under each condition, for both cell lines (Supplemental Fig. 7). Of 18,138 unique genes, 10,055 genes were expressed in at least one condition ($p < 0.02$), which emphasized the lack of global transcriptional hyperactivity by undifferentiated ES cells [21]. We observed similar expression levels of most of the transcripts, including pluripotency factors, in 2i and R2i (Fig. 5a). However, a greater than twofold difference in expression levels for 122 transcripts was observed between the two conditions. Of these, 43 genes, including *Dppa3* and BMP-related signaling mediators (*Bmp4*, *Id1*, *Id2*, and *Ctgf)*, were upregulated and 79 genes, such as *Lefy1*, *Lefty2*, *Pitx2*, *Krt17*, *Fgf10*, and *Thr*, were downregulated in R2i compared to 2i (Fig. 5b). qRT-PCR analysis of a selected set of genes also validated the microarray data results (Fig. 5c).

On the other hand, functional pathway analysis showed signaling cross talk between BMP4 and TGFβ pathways in R2i cells (Supplemental Fig. 8). The significant elevation of BMP4-associated genes and the signaling cross talk between BMP4 and TGFβ pathways in R2i cells prompted us to clarify the role of BMP4 in this context. To this end, we added noggin (500 nM) or noggin (250 nM) plus dorsomorphin (5 μM), two potent BMP signaling inhibitors, to the 2i and R2i cultures. We observed no significant changes in the morphology of ES cells and Oct4 expression in 2i-grown cells in the presence of the BMP4 signaling inhibitors, even after several passages. However, the self-renewal capability of R2i cells was strongly and adversely affected over a brief time period (Fig. 6a and Supplemental Fig. 9). Overall, microarray analysis showed that R2i maintains the expression of most transcripts, including pluripotency-associated genes, similarly to 2i. However, the expression of early lineage-specific genes is maintained at lower levels in R2i compared with 2i. Notably, BMP4 signaling appears to have a crucial role in the self-renewal ability of R2i cells.

Discussion

In this study, we introduced R2i inhibitors, PD0325901 and SB431542, which selectively target MEK and the TGFβ receptor, respectively, and resulting in a novel culture condition for the efficient and reproducible establishment ES cell lines from all mouse strains tested. R2i preserves various features of the ground state of pluripotency in ES cells, including the homogeneous expression of pluripotency markers such as Nanog and Dppa3 (Stella) proteins and minimal leakage of lineage-specific genes. Efficient clonal propagation of single ES cells and, above all, the integrity of genome during serial passaging are among the prominent advantages of the R2i culture.

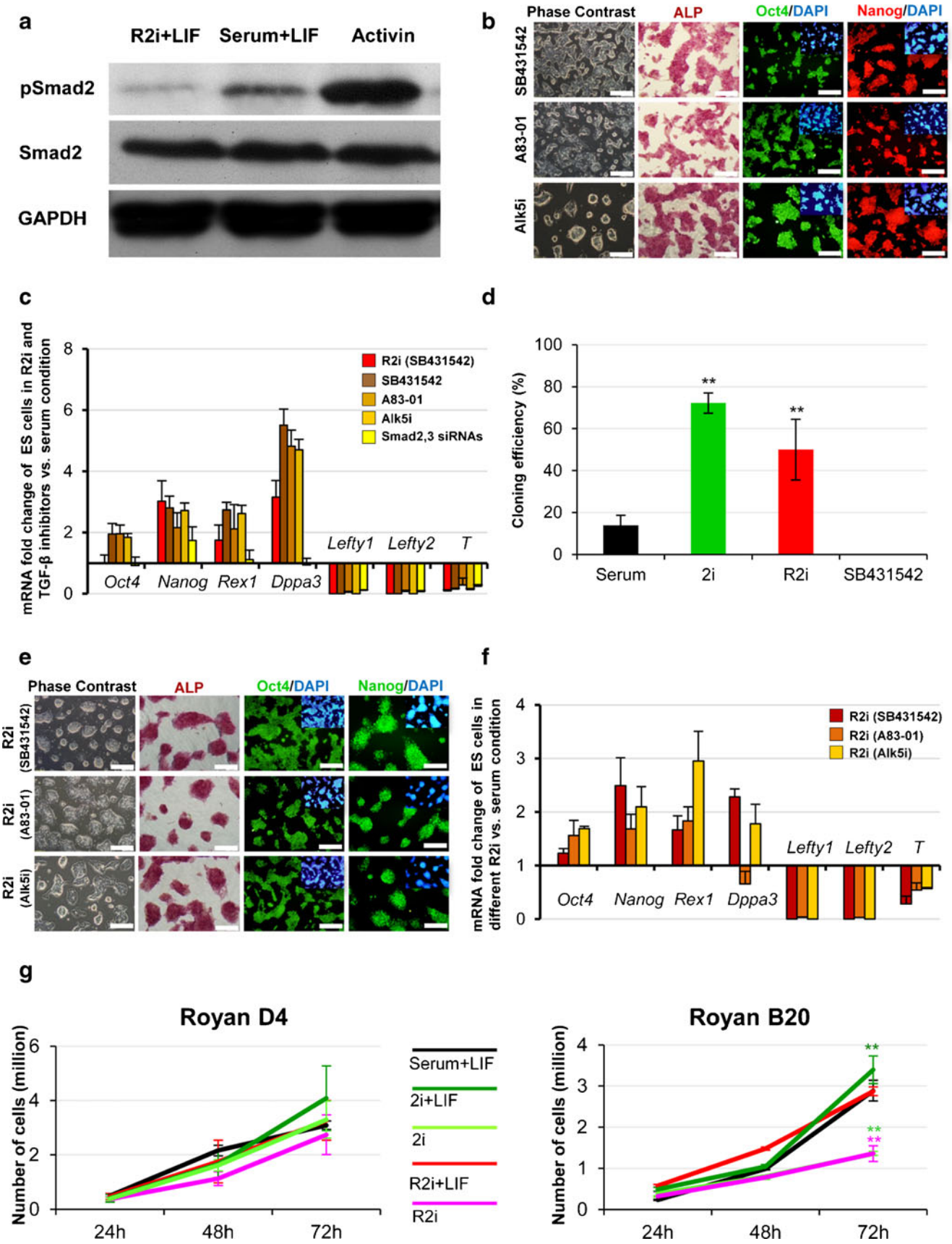
TGFβ-Smad2/3 signaling plays crucial roles in various lineage specification pathways [30, 31]; however, the mode of action of this signaling pathway in the self-renewal and pluripotency of mouse ES cells remains controversial. The prevalent presumptions underscore the important roles for the TGFβ signaling pathway in the pluripotency of mouse ES cells [27, 28, 32, 33]. Nevertheless, other evidence indicates this pathway is dispensable for ES cell self-renewal and pluripotency [13, 26, 34]. Interestingly, inhibition of this pathway has improved the efficacy of generating induced pluripotent stem (iPS) cells from mouse fibroblasts [35, 36]. By efficient derivation of ES cells from diverse mouse strain and more importantly establishment of the ground state of pluripotency in culture, our present study is also supportive of the beneficent effects of TGFβ inhibition on pluripotent mouse ES cells.

FGF signaling is a well-known pro-differentiation pathway in mouse ES cells. Its inhibition under both the 2i and R2i conditions suggests that the nature of pluripotency obtained by the two approaches may be, to a large extent, similar. However the difference in culture conditions (GSK3 vs. TGFβ inhibition in 2i vs. R2i, respectively) may lead to a distinct molecular signature in the resultant pluripotent cells. Consistently, our genome-wide analysis of 2i- and R2i-grown cells has indicated that the expression of most of the transcripts, including pluripotency-associated genes, remained unchanged under these two conditions. Of approximately 18,000 genes, only 122 were significantly different ($p<0.02$, and twofold change in expression) between 2i and R2i cells. Interestingly, of these genes, some of the lineage-affiliated genes, including *Lefty1* and *Lefty2*, were downregulated and BMP4-signaling mediators were upregulated in R2i cells compared with 2i cells. To our surprise, although the BMP4 pathway produced negligible effects in 2i cells, its blockade in R2i cells led to the potent inhibition of the cells' self-renewal capability. Several studies have demonstrated that BMP4 signaling through Smad1/5/8 suppresses developmental regulators such as neuroectodermal-associated genes and FGF signaling [4, 37]. BMP4 signaling may thus confer additional robustness to the pluripotent state in R2i cells even though this pathway seems to be dispensable in 2i.

Fig. 4 TGFB inhibition supports pluripotency. **a** Western blot analysis of Smad2 phosphorylation (pSmad2) in an R2i cell line, Royan B20, cultured in R2i medium and for three passages in serum-containing medium. As a positive control, serum-grown cells were treated with the TGFβ ligand activin (50 nM) for 4 h to induce Smad2 phosphorylation. **b** Characteristics of Royan B20 after cultivation for seven passages in different TGFβ inhibitors (SB431542, A83-01, and ALK5i) plus LIF. Phase contrast, ALP staining, and immunostaining for Oct4 and Nanog expression, counterstained by DAPI, are shown. Scale bar, 100 μm. **c** qRT-PCR of pluripotency and differentiation-specific genes in Royan B20 after cultivation for seven passages in different TGFβ inhibitors (SB431542, A83-01, and ALK5i), *Smad2,3* siRNAs, and serum as control (all plus LIF). Relative expression levels normalized to the housekeeping gene *Gapdh*. Values are mean ± s.d. ($n=3$). **d** Cloning efficiency of single mouse ES cells (Royan $OG_2$5). Single cells of Oct4-EGFP ES cell line were cultivated for five passages in the indicated medium (all plus LIF), then transferred into laminin-coated 96-well plates (single cell/well) for evaluation of clonal growth (ANOVA $^{**}p<0.01$, shows significance relative to serum). Values are mean ± s.d. ($n=3$). **e** The characteristics of Royan B20 after cultivation for seven passages in different R2i (PD0325901 + different TGFβ inhibitors: SB431542, A83-01, and ALK5i) plus LIF. Phase contrast, ALP staining, and immunostaining for Oct4 and Nanog expression, counterstained by DAPI, are shown. Scale bar, 100 μm. **f** qRT-PCR of pluripotency and differentiation-specific genes in Royan B20 after seven passages in different R2i (PD0325901 + different TGFβ inhibitors: SB431542, A83-01, and ALK5i) and serum as control (all plus LIF). Relative expression levels normalized to the housekeeping gene *Gapdh*. Values are mean ± s.d. ($n=3$). **g** Growth curves of mouse ES cell lines (Royan B20 and Royan D4) were determined after seven passages in the mentioned media. For counting, 20×10^4 cells per well were seeded on 6-well plates at passage seven (ANOVA $^{**}p<0.01$, shows significance relative to serum). Values are mean ± s.d. ($n=3$)

For an ES cell culture condition to be optimal, it must preserve the cells' genomic integrity. We observed a more stable karyotype in R2i cells compared with 2i cells in various mouse ES cell lines. This observation might be attributable to the application of the small molecule CHIR99021 in 2i culture, which is known to increase the number of chromosomal aberrations [11, 12].

Conflicting opinions abound about the nature of pluripotency in ES cells. Pluripotency may be a precarious condition with an unstable pluripotent state established by extrinsic signals [38] or a stable self-sustaining "ground state" independent of any extrinsic stimulation [8]. The ground state theory of pluripotency has been challenged by the finding that GSK3 inhibition inevitably activates the Wnt/β-catenin pathway-a signal pivotal for the maintenance of ES cells [38]. The results of the present study are supportive of a stable ground state pluripotency in which the activation of the endogenous signaling pathways (Wnt pathway in 2i and BMP4 pathway in R2i cells) is an integral part of the basal state of pluripotency. However, in contrast to activation of the pluripotency-associated genes and Wnt/β-catenin pathway in 2i, the R2i approach appears to exert its effects by shielding cells


a
R2i+LIF
Serum+LIF
Activin
pSmad2
Smad2
GAPDH
b
Phase Contrast
ALP
Oct4/DAPI
Nanog/DAPI
SB431542
A83-01
Alk5i
c
mRNA fold change of ES cells in R2i and TGF-β inhibitors vs. serum condition
R2i (SB431542)
SB431542
A83-01
Alk5i
Smad2,3 siRNAs
Oct4
Nanog
Rex1
Dppa3
Lefty1
Lefty2
T
d
Cloning efficiency (%)
Serum
2i
R2i
SB431542
e
Phase Contrast
ALP
Oct4/DAPI
Nanog/DAPI
R2i (SB431542)
R2i (A83-01)
R2i (Alk5i)
f
mRNA fold change of ES cells in different R2i vs. serum condition
R2i (SB431542)
R2i (A83-01)
R2i (Alk5i)
Oct4
Nanog
Rex1
Dppa3
Lefty1
Lefty2
T
g
Royan D4
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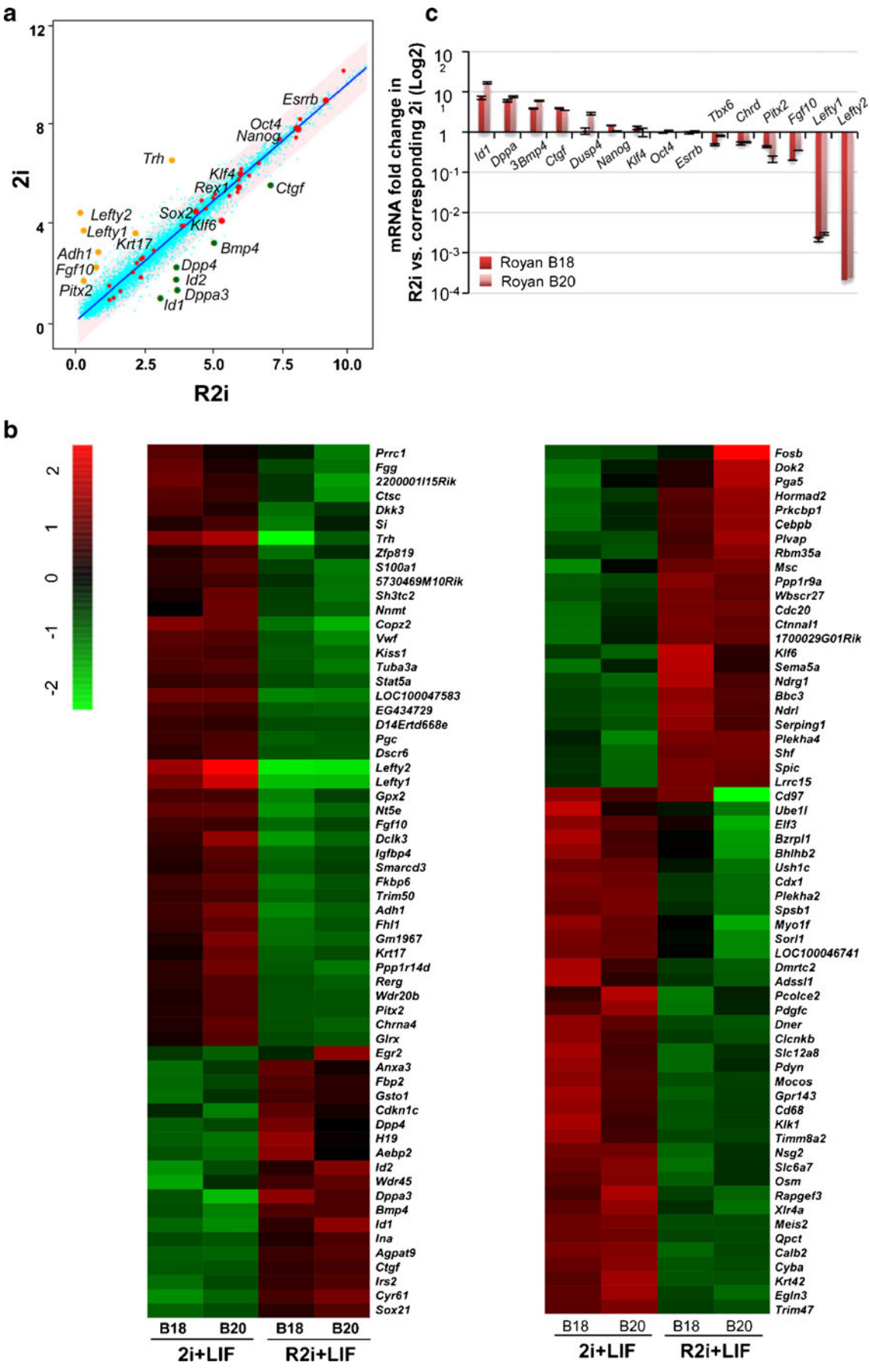
R2i
24h
48h
72h


a
2i
R2i
Esrrb
Oct4
Nanog
Trh
Klf4
Rex1
Ctgf
Lefty2
Sox2
Lefty1
Klf6
Krt17
Bmp4
Adh1
Dpp4
Fgf10
Id2
Pitx2
Dppa3
Id1
c
mRNA fold change in R2i vs. corresponding 2i (Log2)
Royan B18
Royan B20
Id1
Dppa
3Bmp4
Ctgf
Dusp4
Nanog
Klf4
Oct4
Esrrb
Tbx6
Chrd
Pitx2
Fgf10
Lefty1
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b
Prrc1
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2200001I15Rik
Ctsc
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Si
Trh
Zfp819
S100a1
5730469M10Rik
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Fkbp6
Trim50
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Fhl1
Gm1967
Krt17
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Rerg
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Chrna4
Glrx
Egr2
Anxa3
Fbp2
Gsto1
Cdkn1c
Dpp4
H19
Aebp2
Id2
Wdr45
Dppa3
Bmp4
Id1
Ina
Agpat9
Ctgf
Irs2
Cyr61
Sox21
Fosb
Dok2
Pga5
Hormad2
Prkcbp1
Cebpb
Plvap
Rbm35a
Msc
Ppp1r9a
Wbscr27
Cdc20
Ctnnal1
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Klf6
Sema5a
Ndrg1
Bbc3
Ndrl
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Plekha4
Shf
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Lrrc15
Cd97
Ube1l
Elf3
Bzrpl1
Bhlhb2
Ush1c
Cdx1
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Clcnkb
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Pdyn
Mocos
Gpr143
Cd68
Klk1
Timm8a2
Nsg2
Slc6a7
Osm
Rapgef3
Xlr4a
Meis2
Qpct
Calb2
Cyba
Krt42
Egln3
Trim47
B18
B20
2i+LIF
R2i+LIF

◀ **Fig. 5** Global gene expression of ES cells in R2i vs. 2i conditions. **a** Global gene expression patterns were compared between R2i- and 2i-grown cells (log2). The pink ribbon indicates less than twofold differential expression between the R2i and 2i-grown cells. Red dots represent key pluripotency factors; green and orange dots indicate significantly overrepresented genes in R2i and 2i, respectively. **b** Heatmap showing microarray analysis of genes with at least twofold differential expression between R2i and 2i media. Transcript levels are expressed as mean-centered in $\log_2$ fold change. **c** qRT-PCR validation of some genes identified in the microarray of R2i cells compared with the corresponding 2i cells. Ppib was used as a housekeeping control. Values are mean ± s.d. ($n=3$)

from differentiation signals via activation of the 'differentiation-inhibiting' BMP4 signaling. We thus argue that R2i supports ground state pluripotency by suppressing differentiation-related pathways rather than directly influencing self-renewal (Fig. 6b).

In conclusion, R2i may provide a superior environment for mouse ES cells compared with 2i for preservation of a more stable karyotype, and for investigation of the molecular mechanisms of ground state pluripotency thanks to the modulation of the less-complex pathways. With regard to the similarity

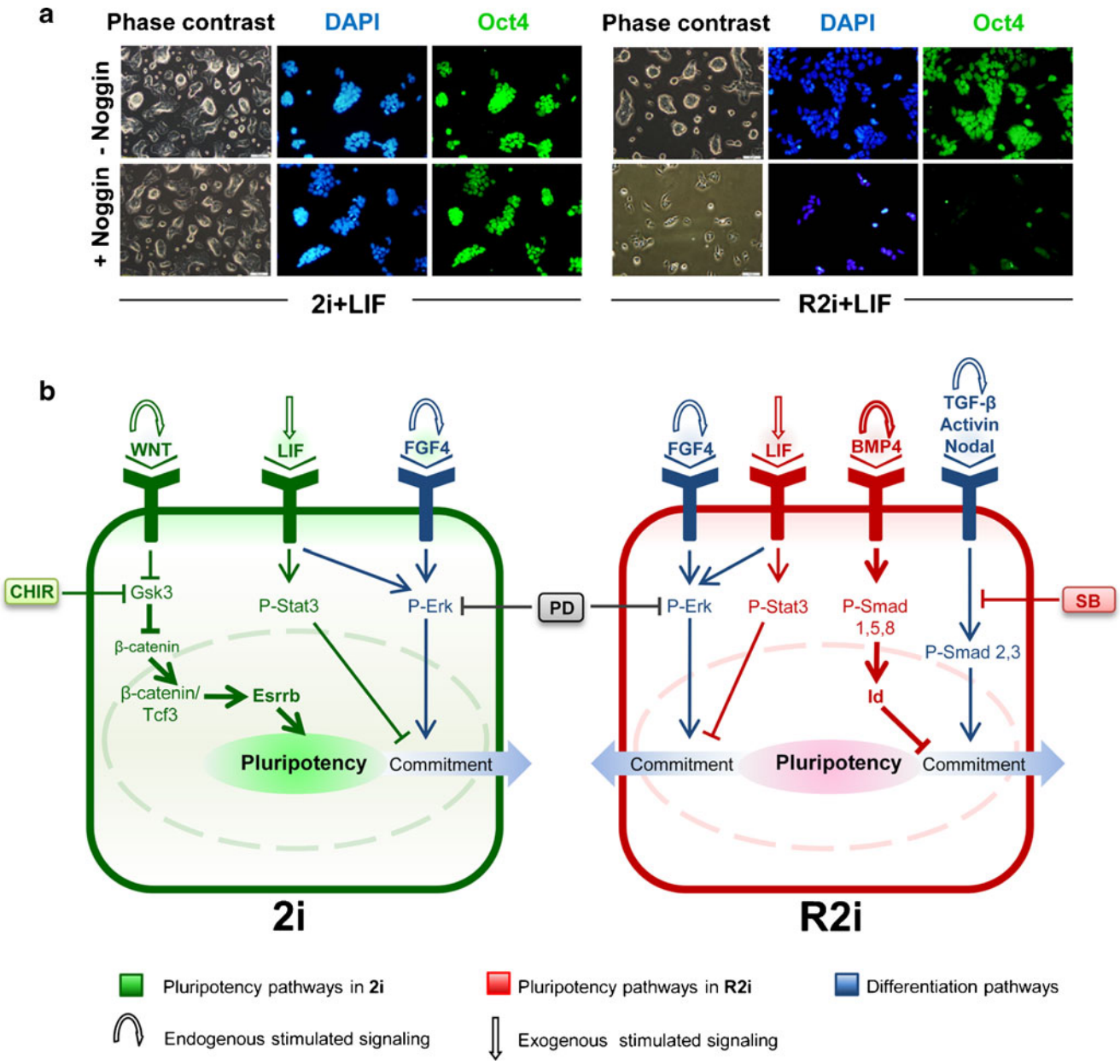

Fig. 6 Stable ground state pluripotency maintained by activated endogenous signaling pathways. **a** Pluripotency in R2i cells depends on endogenously activated BMP4 signaling. Treatment of R2i- and 2i-grown cells with Noggin (500 nM, 24 h). Phase contrast image, Oct4 immunostaining, and DAPI counterstaining are shown. **b** Ground state pluripotency under 2i and R2i conditions. Although in 2i cells the endogenously-activated Wnt/β-catenin signaling directly potentiates the pluripotency regulatory circuitry, pluripotency of R2i cells is stabilized by the activation of endogenous BMP4 signaling, which is a well-known differentiation-inhibiting pathway in mouse ES cells

(PD0325901) and difference (SB431542 vs. CHIR99021) between the R2i and 2i approaches and the two rather distinct routes to naïve pluripotency, it would be informative to investigate the molecular mechanisms underlying the derivation of mouse ES cells using these small-molecule–based strategies. However, given that R2i modulates less-complex pathways, dissecting the molecular mechanism by this approach is likely to provide better insight into the ground state of pluripotency.

Acknowledgments We thank the members of the Department of Stem Cells and Developmental Biology labs for their helpful suggestions and critical reading of the manuscript. We thank Behrouz Asgari for chimera formation and germline transmission. This study was funded by grants provided from Royan Institute and Iranian Council of Stem Cell Technology and the Iran National Science Foundation (INSF).

Author Contributions S. H., M. T., H. R. S. and H. B. designed all experiments and wrote the manuscript. S. H. and S. M. performed cell culture. M. T. and A. S. performed real-time PCR analysis. A. F. and M. P. operated in vivo experiments. N. M. and H. G. performed karyotype analysis. S. M. and G. S. H. performed western blot analysis. M. T., A. S., M. S., B. G. and M. J. A. designed and interpreted microarray analysis. G. H. S., S. M. and D. S. contributed to the overall design and writing of the article.

Conflict of Interest The authors indicate no potential conflicts of interest.

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