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Hesx1 enhances pluripotency by working downstream of multiple pluripotency-associated signaling pathways

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ABSTRACT

Hesx1, a homeobox gene expressed in embryonic stem cells (ESCs), has been implicated in the core transcription factors governing the pluripotent state. However, data about the underlying mechanism of how Hesx1 is involved in maintaining pluripotency is still scarce. In this study, we find Hesx1 responds to multiple pluripotency-related pathway inhibitors as well as LIF stimulation. Particularly, the expression of Hesx1 can be readily induced by dual inhibition (2i) of glycogen synthase kinase 3 and mitogen-activated protein kinase. Forced expression of Hesx1 can partially compensate for the withdrawal of either LIF or each component of 2i. We also demonstrate that LIF and each inhibitor of 2i can induce Hesx1 independent of one another. We tentatively put forward that Hesx1 is a common downstream target of LIF- and 2i-mediated self-renewal signaling pathways and plays an important role in maintaining ESC identity. Our study extends the methods of identifying the missing crucial factors in establishing ESC pluripotency.

1. Introduction

Pluripotent stem cells in early embryos are transient cell populations that exist in the epiblast of the peri-implantation embryos, but can be captured in vitro as embryonic stem cells [1]. Embryonic stem cells (ESCs) self-renew indefinitely while holding significant potential of differentiation [2,3]. Maintenance of self-renewal in ESCs relies on complex transcriptional regulatory network, and continuous inputs from extrinsic stimuli are required to sustain an intrinsic transcription factor circuitry [4].

Conventionally, activation of leukaemia inhibitory factor (LIF)-Stat3 and bone morphogenetic proteins (BMP)-Smad by exogenous growth factors are required for mouse ESCs (mESCs) to retain a pluripotent state [5]. Hence, LIF and BMP have been considered to be indispensable extrinsic factors together to enhance the self-renewal and pluripotency of mouse ESCs. However, recent studies have shown that chemical pathway inhibitors also own an extraordinary ability to maintain self-renewal and pluripotency of mESCs independent of LIF and BMP [6,7].

In 2006, a chemical inhibitor named pluripotin/SC1 was identified to be capable of deliberating mESCs from requirements for exogenous factors without compromise to developmental potency [8]. SC1 can maintain long-term self-renewal of mESCs under feeder-, serum- and LIF-free conditions. Biochemical and cellular experiments showed that SC1 specifically targeted two exogenously expressed differentiation-triggered proteins: RasGAP and extracellular signal-regulated kinase-1 (ERK1). SB431542 (SB), a potent and specific transforming growth factor-beta receptor kinase inhibitor, has been proved to establish the ground state pluripotency when used together with PD0325901 (PD) [9]. Consistently, SB431542 was reported to greatly improve the efficiency of generation of induced pluripotent stem cells [10]. More and more signaling pathway inhibitors that can sustain the pluripotent state of ESCs were later identified. The most striking discovery was development of “2i” inhibitor-based culture system

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[11,12]. Dual inhibition of glycogen synthase kinase 3 (GSK3) and mitogen-activated protein kinase (MEK), regulated by CHIR99021 (CHIR) and PD respectively, termed "2i," was reported to sustain the pluripotency of mESCs in the absence of exogenous cytokines. These findings provided a conceptually new view on the understanding of ESCs self-renewal: ESCs could act internally to maintain pluripotency through separate signaling pathways as long as differentiation activity is balanced out.

The remarkable role of signaling pathway inhibitors in maintaining ESCs pluripotency has also drawn our attention to investigation of intrinsic signaling pathways of ESCs. The way how the signaling pathways modulate the pluripotent state of ESCs depends on their downstream targets, and each signaling pathway ends up with multiple targets. It seems intriguing to assume that a common target of different pluripotency-related signaling pathways plays a significant role in stabilizing the pluripotency of ESCs. Unfortunately, little effort has been made towards this goal.

Hesx1 is a transcription factor that belongs to the paired class of homeobox genes. Hesx1 is expressed in the rostral region of the developing vertebrate embryo, but expression has not been detected in any adult tissues or established cell lines, with the exception of ESCs [13,14]. Previous reports have shown that Hesx1 is crucial for normal forebrain and pituitary gland formation in mammals [15,16]. In mESCs, Hesx1 sustains a robust level of expression, and previous report implies that Hesx1 is a member of the core ESCs transcriptional circuit [17]. To date, little research has been conducted with respect to the detailed connection between Hesx1 and mESCs pluripotency.

2. Materials and methods

2.1. Cell culture

J1 mESCs were cultured on 0.1% gelatin-coated dishes, at 37 °C in 5% CO2. Medium for routine maintenance was GMEM (Sigma, G5414) supplemented with 10% FCS (HyClone), 1% MEM non-essential amino acids (Invitrogen), 2 mM GlutaMax (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen), and 1000 units/ml LIF (Merck Millipore). For serum-free culture, mESCs were maintained in N2B27 supplemented with 3 μM CHIR and 1 μM PD (Sigma).

2.2. Generation of inducible Hesx1

Hesx1 was cloned from J1 mESC cDNA with LA Taq DNA Polymerase (Takara) and inserted into the SalI and NotI sites of the p2Lox vector. J1 mESCs were seeded onto gelatin in a 3.5-cm dish and transfected with 2 μg p2Lox-Hesx1 plasmid using the Lipofectamine 2000 Transfection Reagent (Invitorgen) according to the manufacturer's instructions. Selection was initiated the next day by adding 300 μg/ml of G418 (Gibco) and maintained for 3 weeks. Surviving colonies were isolated and replated onto gelatin for expansion.

2.3. Overexpression and knockdown plasmid construction

The coding regions of Hesx1 was cloned from J1 mESC cDNA with LA Taq DNA Polymerase and inserted into the pCDH-CMV-MCS-EF1-Puro vector (SBI). For RNA interference in J1 mESCs, shRNA constructs were designed to target 21 base-pair gene-specific regions of Hesx1 and were then cloned into Tet-pLKO-puro vector (purchased from addgene). The targeted sequences are as follows: Hesx1 RNAi 1: GATTGTACAA- CGTCAGTAAGA; Hesx1 RNAi 2: GAAGAAAGGGCTCCGAAATAT. J1 cells engineered for inducible expression of shRNA were generated by puromycin selection.

2.4. Cell transfection and virus production

For gene overexpression, J1 mESCs were transfected with 2 μg of pCDH-CMV-MCS-EF1-Puro inserted with Hesx1 using Lipofectamine 2000 Transfection Reagent according to the manufacturer's instructions. Selection was begun the next day by adding puromycin to a final concentration of 2 μg/ml and continued for 1 week.

2.5. qRT–PCR

Total RNA was isolated from J1 mESCs using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed using a SYBR PrimeScript™ RT-PCR Kit (Takara). Real-time quantification of mRNA was performed with an ABI StepOnePlus PCR system (Applied Biosystems) using SYBR Premix ExTaq II kits (Takara). Target gene expression was normalized to GAPDH expression. The primer sequences used will be available upon requested.

2.6. Western blot analysis

Cells were lysed in ice-cold RIPA cell buffer (prepared in-house) supplemented with protease inhibitors (Thermo Scientific). The proteins were separated with 12% acrylamide gels and transferred to PVDF membranes (Millipore). Probing was performed with specific primary antibodies and HRP-conjugated secondary antibodies (Beyotime technology). The primary antibodies used were Hesx1 (ab187172; Abcam, 1:500), α-tubulin (ab125267; Abcam, 1:2000).

2.7. Alkaline phosphatase activity assay

The alkaline phosphatase activity of J1 mESCs cultured on gelatin-coated plates was detected using the Alkaline Phosphatase Kit (Beyotime technology)

2.8. Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde/PBS for 20 min, permeabilized with 0.2% Triton X-10 0/PBS for 10 min, rinsed in PBS and then blocked in blocking buffer (Beyotime technology). Primary antibodies were diluted in blocking buffer. Cells were incubated in the presence of primary antibodies at 4 °C overnight and then washed three times in PBS. Cells were then incubated with Alexa Fluor 555 (Beyotime technology, 1:1000) secondary antibody for 1 h at 37 °C. Nuclei were stained with DAPI (Sigma, 1:10 000). The primary antibody and dilution was Oct4 (ab18976; Abcam, 1:200).

2.9. Statistical analysis

Data are reported as the mean ± standard deviation (SD) and analyzed using the Student's t -test. A value of $p < 0.05$ was considered significant.

3. Results

Inhibition of ERK, TGF-beta, and MEK and GSK3 pathway individually showed potent ability to sustain the pluripotent state of mESCs. We hypothesized that common downstream targets of these pathways played a comprehensive and significant role in maintaining mESCs pluripotency. We performed DNA microarray analysis to identify genes differentially expressed in J1 mESCs treated with or without SC1, SB, PD, CHIR and etinoic acid (RA) for 12 h. We found Hesx1 was upregulated by all the four pathway

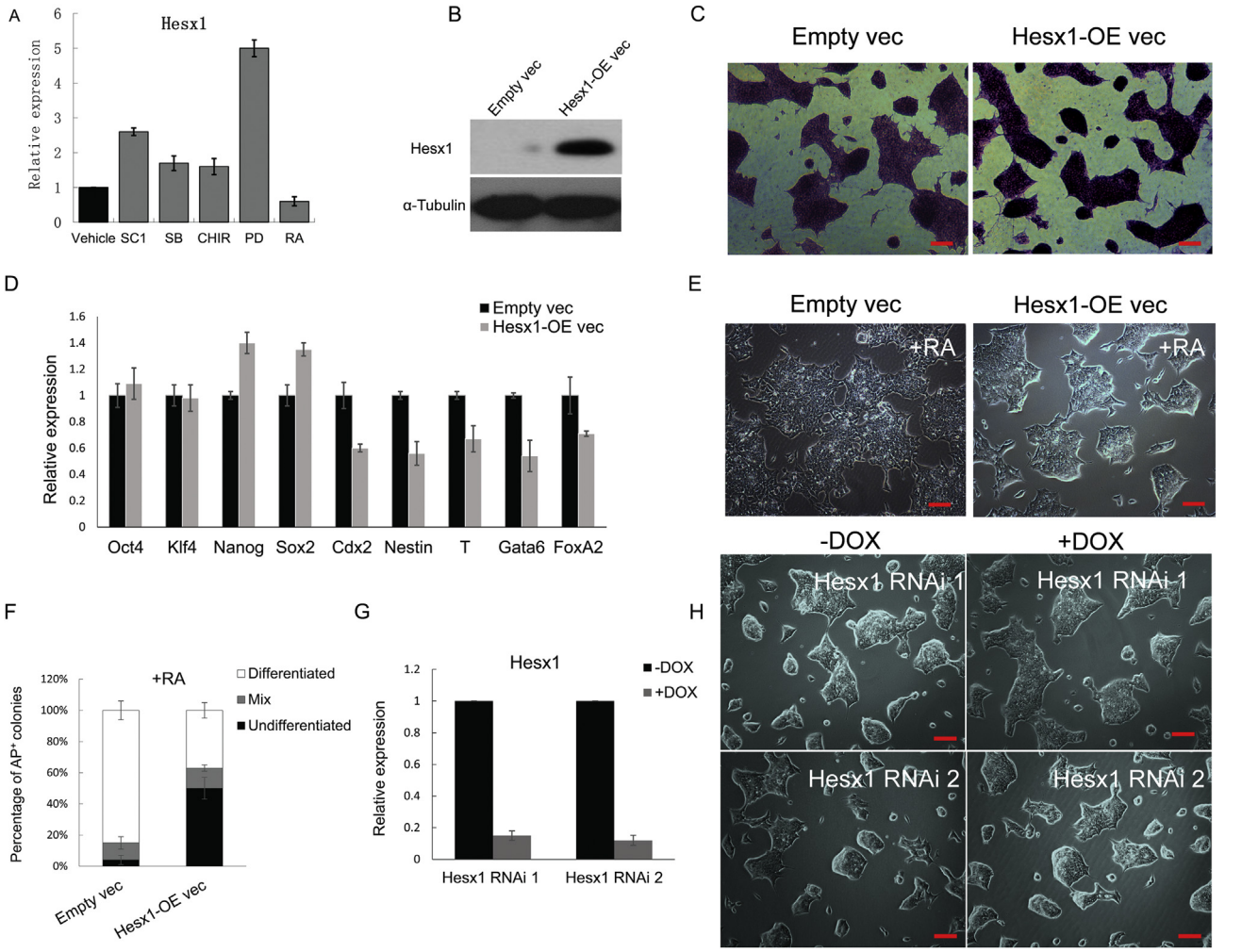

Fig. 1. Impact of Hesx1 on mESC pluripotency (A): qRT-PCR analysis of Hesx1expression in J1 mESCs cultured in the presence of serum/LIF or serum/LIF/inhibitors for 12 h. Error bars are the s.d. of three biological replicates. (B): Western blot analysis of Hesx1 in J1 mESCs cultured in serum/LIF. α-Tubulin is a loading control. (C): Alkaline phosphatase staining of colonies arising from transfectants cultured in serum/LIF for two passages. Scale bar, 50 μm. (D): qRT-PCR analysis of Oct4, Klf4, Nanog, Sox2, Cdx2, Nestin, T, Gata6 and FoxA2 expressed in J1 mESCs cultured in serum/LIF with or without exogenous Hesx1. Data represent mean ± s.d. of three biological replicates. (E): J1 mESCs transfectants cultured in serum/LIF plus RA for six days. (F): Quantification of alkaline phosphatase-positive colonies. Data represent mean ± s.d. from triplicate experiments. (G): qRT-PCR analysis of Hesx1 expression in shRNA knockdown cells. The transcript level was normalized against vector control. Data represent mean ± s.d. of three biological replicates. (H): Morphology of J1 mESCs harboring inducible shRNA expression construct cultured in serum/LIF with or without DOX. Vehicle, DMSO; vec, vector; OE, overexpression, DOX, DOXycycline.

inhibitors, whereas downregulated by RA. The miroarray data was confirmed by Quantitative real-time PCR (qRT-PCR) (Fig. 1A). We then overexpressed Hesx1 in J1 mESCs (Fig. 1B), and they showed a higher alkaline phosphatase (AP) activity compared with cells transfected with empty vector (Fig. 1C). AP is a typical pluripotency marker, and any change of its activity signals the fluctuation of pluripotent status. To gain insight into the molecular alteration induced by Hesx1, the expression of pluripotency genes and differentiation-associated genes were evaluated. The qRT-PCR analysis revealed that the expression of Sox2 and Nanog were elevated slightly but significantly, whereas expression of OCT4 and Klf4 remained undisturbed (Fig. 1D). Notably, differentiation-related genes tested were all downregulated by overexpression of Hesx1 (Fig. 1D). Hence, Hesx1 may contribute to enhance the pluripotency mainly by repressing the differentiation activity. To further characterize this finding, we treated the J1 mESCs with RA to induce differentiation while overexpressed Hesx1. We observed impaired differentiation in Hesx1-overexpressed J1 mESCs. ES cell-like colonies were present in the culture with exogenous Hesx1 after six days of RA stimulation, whereas vector control J1 mESCs lost most colonies within six days (Fig. 1E and F). This line of evidence further suggested that Hesx1 could maintain the pluripotency state of J1 mESCs probably by inhibiting differentiation.

To determine whether Hesx1 was required in maintaining mESC pluripotency, we depleted endogenous Hesx1 by RNAi. Two short hairpin RNA constructs targeting different regions of Hesx1 coding sequence were used to ensure that the effects were specific. Both RNAi constructs were effective in reducing the transcript level of Hesx1 (Fig. 1G). The repression of Hesx1 had no obvious morphological effect on the J1 mESCs (Fig. 1H). There were no differences in the expression of the pluripotency markers Oct4, Sox2, Nanog and Klf4 (data not shown). Markers of differentiation showed small increases in expression in the Hesx1-knockdown cells (data not shown), these same markers were slightly repressed in the Hesx1 overexpression cells. To further examine the role of Hesx1 as a regulator of ESC pluripotency, we tested our Hesx1-knockdown cells to see whether suppression of Hesx1 rendered cells more responsive to differentiation induction. The Hesx1-knockdown cells were treated with RA to induce differentiation. Over a 6-day time course, Hesx1-knockdown cells were observed to be

morphologically differentiated to greater extent than control cells (data not shown). These data establish that mESC with knocked down expression of Hesx1 have enhanced responsiveness to differentiate, which is opposite to the effect we observed when Hesx1 was overexpressed. Together, the knockdown and overexpression data indicate that Hesx1 plays a role in maintenance of mESC pluripotency.

Hesx1 had a strong response to either CHIR or PD stimulation (Fig. 1A and B). To determine whether Hesx1 could compensate for the withdrawal of either CHIR or PD in N2B27/2i medium to maintain mESC self-renewal, we generated a mESC line harboring a DOXycycline (DOX)-inducible Hesx1 transgene (Hesx1 mESCs). Western blot analysis showed that Hesx1 expression in these cells was efficiently induced by DOX treatment (Fig. 2A). Hesx1 mouse ESCs as well as control mESCs could be sustained in the N2B27/2i medium with or without DOX. Withdrawal of 2i immediately caused cell differentiation within four days even in the presence of DOX (data not shown), indicating that Hesx1 was far from enough to function as the combination of CHIR and PD. Hesx1 mESCs could be cultured for at least five passages without obvious differentiation in N2B27/PD plus DOX (Fig. 2B and C). The qRT-PCR analysis revealed a robust expression level of pluripotency marker genes (data not shown), and immunofluorescence staining showed positive expression of Oct4 (Fig. 2C). In contrast, Hesx1 mESCs cultured in N2B27/PD without DOX quickly lost the typical mouse ESC morphology and became flattened and fibroblast-like. In N2B27/CHIR plus DOX, Hesx1 mESCs also exhibited a considerable resistance to differentiation, although they proliferated more slowly (Fig. 2D and E). Our findings suggested that the artificial expression of Hesx1 could partially recapitulate the effect of either CHIR or PD in boosting J1 mESCs pluripotency in serum-free condition.

Previous studies have demonstrated that LIF can work in concert with either CHIR or PD to sufficiently prompt the self-renewal of mESCs in serum-free condition, which suggested LIF and 2i might converge on the same downstream targets. To investigate whether Hesx1 was a downstream target of LIF signaling, mESCs were cultured in serum-based medium without LIF for twelve hours, and then supplemented with LIF for twelve more hours. The expression of Hesx1 as well as the Stat3 target Klf4 was analyzed by qRT-PCR. The expression level of both Hesx1 and Klf4 was significantly downregulated after twelve hours of LIF starving, and restored after LIF was supplemented (Fig. 3A). Following withdrawal of LIF, Hesx1 mESCs remained undifferentiated for at least five passages in the presence of DOX, though they proliferated more and more slowly (Fig. 3B and C). Thus Hesx1 might serve as a downstream effector of LIF signaling and its continued expression could partly compensate for the deprivation of LIF.

Binding of LIF to the LIF receptor/gp130 complex can trigger three signaling pathways: janus kinase (JAK)/Stat3, phosphoinositide 3-kinase (PI3K)/AKT, and mitogen-activated protein kinase (MAPK). To determine which pathway is responsible for the induction of Hesx1 by LIF, we treated J1 mESCs with inhibitors specific for JAK, PI3K, and MAPK, and evaluated the induction of Hesx1 by LIF. As expected, the induction of the direct target of Stat3 to LIF stimulation was fully blocked by JAK inhibitor, while PI3K and

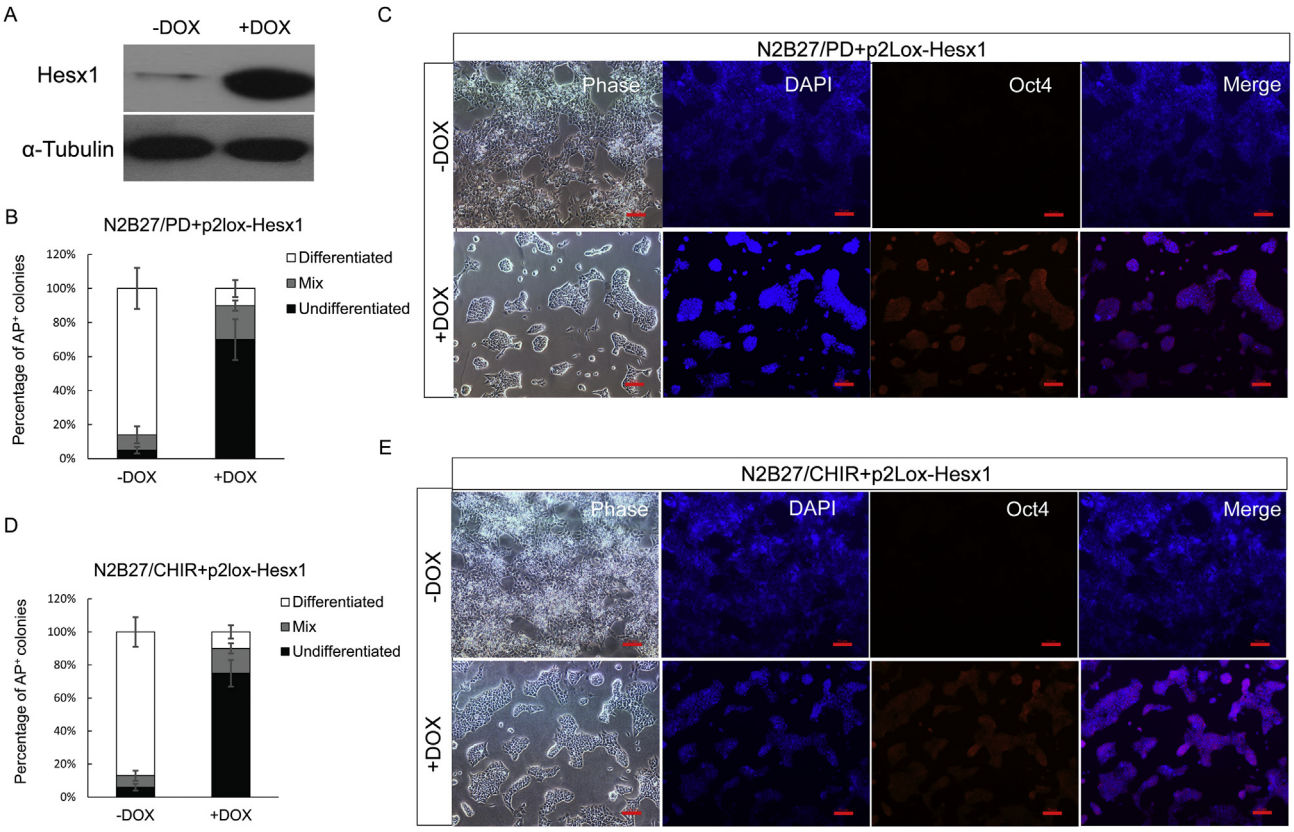

Fig. 2. Artificial expression of Hesx1 can partially substitute CHIR or PD for the maintenance of mESC. (A): Western blot analysis of Hesx1 expression in Hesx1 J1 mESCs cultured in the presence or absence of 0.2 μg/ml DOX for 12 h (B): Quantification of alkaline phosphatase-positive colonies maintained in N2B27/PD. Data represent mean ± s.d. from triplicate experiments. (C): Immunostaining of Hesx1 J1 mESCs cultured in N2B27/PD with or without DOX for five passages. Scale bars, 50 μm. (D): Quantification of alkaline phosphatase-positive colonies maintained in N2B27/CHIR. Data represent mean ± s.d. from triplicate experiments. (E): Immunostaining of Hesx1 J1 mESCs cultured in N2B27/PD with or without DOX for five passages. Scale bars, 50 μm.

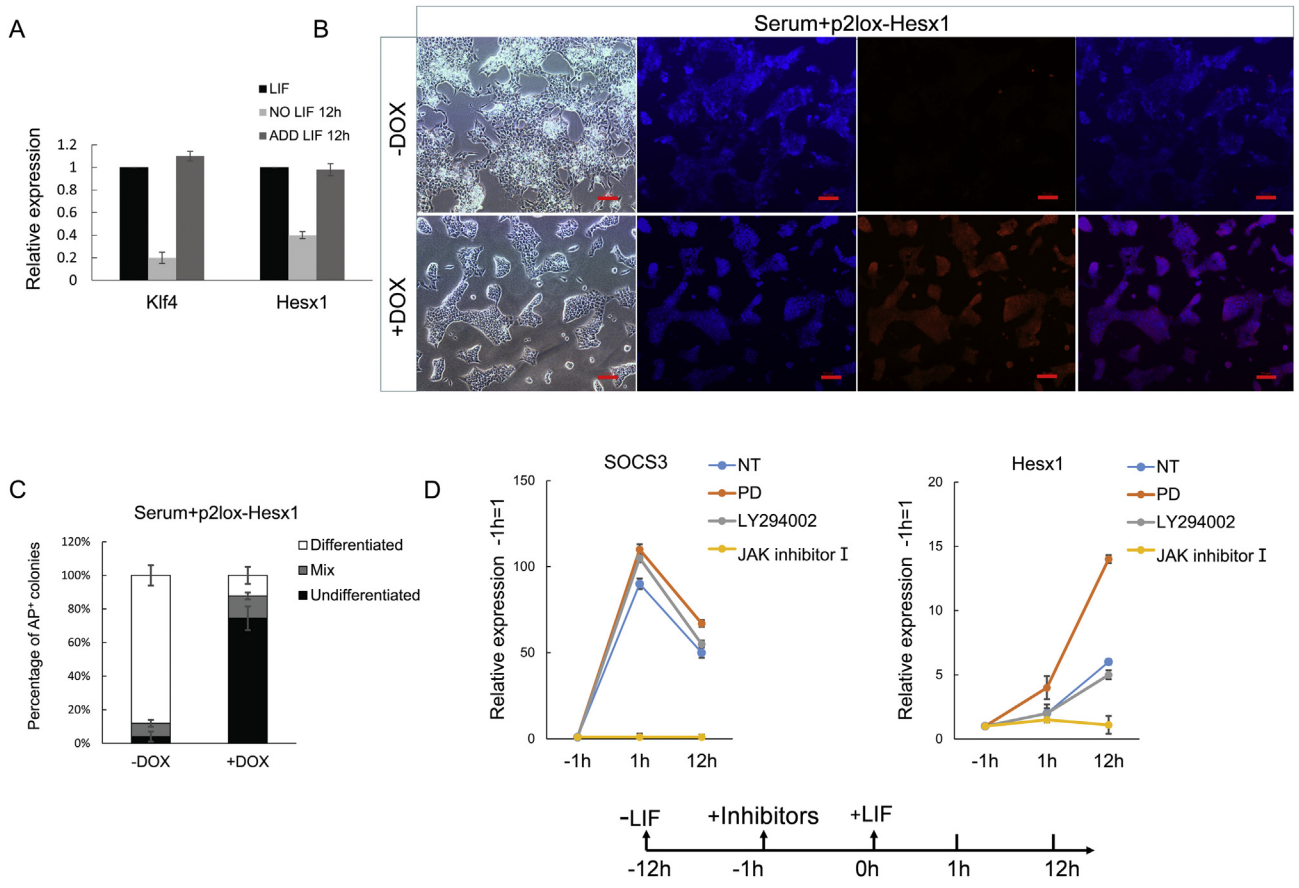

Fig. 3. Enforced Hesx1 can partially recapitulate the effect of LIF on sustainning mESC pluripotency (A): qRT-PCR analysis of Klf4 and Hesx1 expression in J1 mESCs that are deprived of LIF for 12 h and then supplemented with LIF for 12 h. Data represent mean ± s.d. of three biological replicates. (B): Immunostaining of Hesx1 J1 mESCs cultured in serum-based medium with or without DOX for five passages following withdrawal of LIF. Scale bars, 50 μm. (C): Quantification of alkaline phosphatase-positive colonies maintained in serum-based medium. Data represent mean ± s.d. from triplicate experiments. (D): J1 mESCs were deprived of LIF for 12 h, and then treated with indicated kinase inhibitors for 1 h before LIF stimulation for 12 h, as indicated on timeline (lower). Socs3 and Hesx1 transcripts were evaluated at −1, 1, and 12 h by qRT-PCR. Data represent mean ± s.d. of three biological replicates.

MAPK inhibitors did not show any blockade (Fig. 3D). Expression of Hesx1 was not sensitive to LIF stimulation when JAK inhibitor was present. The presence of PI3K did not alter the sensitivity of Hesx1 expression to LIF stimulation, and treatment of PD significantly enhanced the expression level of Hesx1 (Fig. 3D). Altogether, our results indicated that expression of Hesx1 was upregulated by LIF through JAK.

CHIR, PD, and LIF are all capable of inducing Hesx1 expression, which prompted us to explore whether there is any interplay between the signaling pathways downstream of CHIR, PD, and LIF. We pre-treated mESCs with JAK inhibitor (Stat3 signaling inhibitor), 53AH (Wnt signaling inhibitor), or bFGF (activator of FGF/MEK signaling pathway) for one hour before adding LIF, CHIR, or PD. Twelve hours after the addition of LIF, CHIR, or PD, Hesx1 expression was significantly induced following treatment with LIF, CHIR, PD, even in the presence of irrelevant signaling pathway inhibitors/activator (Fig. 4A–C). Egr1, a target of the FGF/MEK signaling pathway, was induced by bFGF but repressed by MEK inhibitor PD (Fig. 4A). Axin2, a target of the Wnt signaling pathway, was induced by CHIR but repressed by 53AH (Fig. 4B); and Socs3, a target of the LIF/Stat3 signaling pathway, was induced by LIF but repressed by JAK inhibitor I (Fig. 4C). Taken together, our observations implied that CHIR, LIF, and PD didn't rely on one another to exert their upregulative effect on Hesx1.

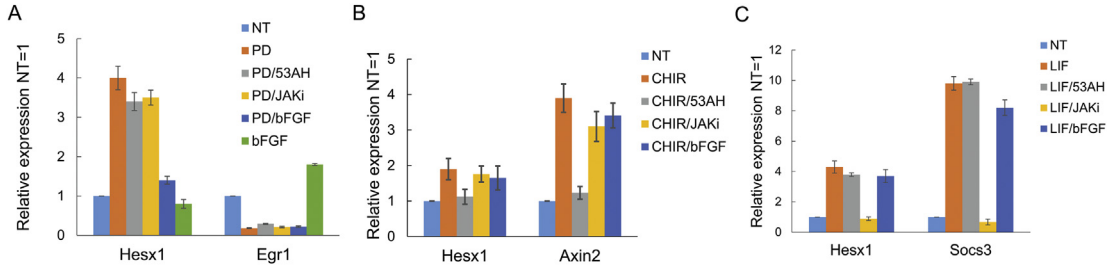

Fig. 4. PD, CHIR, and LIF induce Hesx1 independently. (A–C): J1 mESCs were deprived of LIF for 12 h, and then treated with 1 μM 53AH, 10 μM JAK inhibitor I, or 20 ng/ml bFGF for 1 h before LIF, CHIR, or PD stimulation. Egr1, Axin2, Socs3, and Hesx1 transcript levels were evaluated 12 h later by qRT-PCR. Data represent mean ± s.d. of three biological replicates.

4. Discussion

The maintenance and culture of ES cells in the artificial system of cell culture requires a fine balance of the signaling pathways maintaining pluripotency and inhibiting differentiation, and this has been fully documented by recent breakthrough in identifying signaling pathway inhibitors that can modulate the fate of stem cells. Strikingly, the discovery of 2i renders mESCs completely independent from activation of the JAK-Stat and BMP-Smad pathways that have been conventionally regarded to be essential. Other signaling pathway inhibitors such as SC1 and SB, also have been proved to be extraordinary manipulators in the maintenance of pluripotency. These chemical compounds modulate the pluripotency of mESCs through seemingly independent pathways, however, these signaling pathways may converge on same downstream targets.

Gene expression profiles of mESCs treated with pathway inhibitors revealed that each inhibitor could boost the expression of numerous pluripotency-related genes in J1 mESCs. We noticed that mRNA abundance of Hesx1 is exceptionally augmented by all fours pathway inhibitors SC1, SB, CHIR and PD, and we also found Hesx1 was a downstream target of JAK-Stat pathway, which may confer Hesx1 an important position in establishing the pluripotency-maintaining network. Given that CHIR, PD and LIF show greater power in maintaining pluripotency, our research focuses on studying the connection between Hesx1 and these three mediators.

Forced expression of Hesx1 can't fully recapitulate the effect of CHIR, PD and LIF, but greatly postpone the differentiation process induced by withdrawal any of the three mediators. Similarly, artificial expression of Hesx1 can't render J1 mESCs immune to RA stimulation, but the initiation of differentiation seems to be delayed. Likewise, knockdown of Hesx1 was not sufficient to induce differentiation. But Hesx1-overexpression and -knockdown cells showed an altered response to RA-induced differentiation. Moreover, ESC with reduced levels of Hesx1 were significantly more inclined toward differentiation upon RA treatment. Given that Hesx1 negatively correlates with differentiation-related genes, we can conclude that Hesx1 prevents ESC from differentiation mainly through negative regulation of lineage markers.

The established pluripotency circuitry of mESCs encompasses a small number of potent transcription regulators, such as Oct4, Nanog, STAT3, Esrrb, Tfcp2l1 and Klf4. Notably, all these key factors are transcriptional activators [18]. How transcriptional repressors highly expressed in ESCs influence pluripotency have been barely investigated. There is evidence indicating that Hesx1 can function as a transcriptional repressor in vitro and in vivo [19,20]. Hesx1 contains two repressor domains, one located in the N-terminus (eh1 and HRPW motifs) and the other in the homeodomain. The N-terminal repressing domain binds N-CoR, whereas the homeodomain interacts with Tle1, a mammalian ortholog of Groucho [20]. Tle1 has been defined as an early stage marker gene of successful reprogramming of human B-lymphocytes [21], and N-coR was reported to control the undifferentiated state of mouse neural stem cells [22]. These lines of evidences imply that Hesx1 may guard the steady state of pluripotency in cooperation with Tle1 and N-coR. Another research has demonstrated Hesx1 interacts with DNA methyltransferase 1 [23], suggesting that Hesx1 may induce gene silencing by specific methylation of CpG residues of its target genes.

Genome-wide studies have shown that many repressors are associated with actively transcribed loci and they are believed to provide transcriptional find-tuning by modulation of expression level of target genes. Transcriptional repressors can co-work with chromatin modifiers and co-repressor complexes as multifaceted regulators of gene expression [24]. Zfp281, for example, a transcription repressor, has been reported to not only repress but activate its target genes in mESCs. Further analysis implies Zfp281 can fine-tunes the expression level of Nanog to facilitate ESC pluripotency [25]. The multifaceted property of transcription repressor may explain why Hesx1 responds to multiple dominant pluripotency-related signaling pathways.

To date, Tfcp2l1 and Nanog are the only two transcription factors identified as common downstream targets of CHIR, PD and LIF that possess the capacity for sustaining mESC self-renewal [26]. Our data shows that Hesx1 owns a limited capacity for prompting mESC self-renewal. Nevertheless, Hesx1 shows considerable ability to delay the differentiation process. The fact that constitutive expression of Hesx1 can't stop the differentiation implies that more factors are needed. Overall, Hesx1 may sustain the pluripotent state mainly by suppressing differentiated-associated genes and further consolidates the pluripotency-maintaining transcription factor network.

Acknowledgments

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Transparency document

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