Dichotomous role of Shp2 for naïve and primed pluripotency maintenance in embryonic stem cells

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Abstract

The requirement of the Mek1 inhibitor (iMek1) during naïve pluripotency maintenance results from the activation of the Mek1-Erk1/2 (Mek/Erk) signaling pathway upon leukemia inhibitory factor (LIF) stimulation. Through a meta-analysis of previous genome-wide screening for negative regulators of naïve pluripotency, *Ptpn11* (encoding the Shp2 protein, which serves both as a tyrosine phosphatase and putative adapter), was predicted as one of the key factors for the negative modulation of naïve pluripotency through LIF-dependent Jak/Stat3 signaling. Using an isogenic pair of naïve and primed mouse embryonic stem cells (mESCs), we demonstrated the differential role of Shp2 in naïve and primed pluripotency. Loss of Shp2 increased naïve pluripotency by promoting Jak/Stat3 signaling and disturbed *in vivo* differentiation potential. In sharp contrast, Shp2 depletion significantly impeded the self-renewal of ESCs under primed culture conditions, which was concurrent with a reduction in Mek/Erk signaling. Similarly, upon treatment with an allosteric Shp2 inhibitor (iShp2), the cells sustained Stat3 phosphorylation and decoupled Mek/Erk signaling, thus replacing the use of iMek1 not only for maintenance but also for the establishment of naïve ESCs through reprogramming. Taken together, our findings highlight the differential roles of Shp2 in naïve and primed pluripotency and propose the usage of iShp2 instead of iMek1 for the efficient maintenance and establishment of naïve pluripotency.

Introduction

Although mouse embryonic stem cells (mESCs) have been widely used to characterize the early embryonic development of mammals, these cells exhibit clear differences from human ESCs at the cellular (e.g., colony morphology and surface markers) [1] and molecular levels, such as different epigenetic states, X-chromosome inactivation, chimera formation, major signaling pathways [2, 3], and glucose metabolism [4], among others [5]. Based on recent advances in our understanding of pluripotency, two discrete pluripotent states, naïve (or ground) and primed, which represent pre- and post-implanted epiblast stem cells (Epi-SCs) respectively [6], account for most of the differences between mouse and human ESCs [7] when naïve hESCs are established by conversion from primed hESCs [8].

A constant supply of leukemia inhibitory factor (LIF) along with two chemical inhibitors for Mek1 (iMek1) and Gsk3 β (iGsk3 β) (hereinafter referred to as LIF + 2i) is indispensable [9] not only for maintenance but also for the establishment of naïve ESCs, thus mimicking the characteristics of naïve pluripotency of pre-implantation embryos *in vitro*. LIF-mediated propagation and maintenance of naïve ESCs depend on the Jak/Stat3 pathway [10], which is also conserved in embryo development and implantation [11]. Two inhibitors are required for maintaining naïve pluripotency, which is likely attributed to the innate mechanism of Gsk3 α/β inhibition in the inner cell mass (ICM) of blastocysts [12] coupled with a lower basal activity of Erk1/2 (hereinafter Erk) in early blastocysts [13], the latter of which is possibly due to the expression of Erk-specific dual-specificity phosphatases (DUSPs) [14, 15].

Src homology region 2 domain-containing phosphatase 2 (Shp2), which is encoded by protein tyrosine phosphatase non-receptor type 11 (*Ptpn11*), transmits the receptor signaling to Erk [16] and negatively

modulates Jak/Stat3 signaling [17]. Phosphorylation of Shp2 upon cytokine stimulation by interleukin-6 (IL-6) or LIF, leads to intramolecular conformational changes to relieve autoinhibition of tyrosine phosphatase activity [18] and dephosphorylates the Jak/Stat3 pathway [18, 19]. In parallel, the recruitment of phosphorylated Shp2 to gp130 forms a protein complex with Gab1 to transduce signals from the receptor to Ras/Raf/Mek/Erk [20]. Considering the positive role of Shp2 in Ras-dependent Erk activation (Ras-to-Erk), as well as in oncogenic Ras-to-Erk [21, 22] and target therapy resistance[23], small molecules to inhibit the bivalent roles of Shp2 have been developed as novel anti-cancer therapeutic agents[24].

Shp2 deficiencies promote self-renewal capacity and suppress mESC differentiation through increased sensitivity to LIF on Jak/Stat3 and attenuation of Ras-to-Erk [17]. Similarly, failure to recruit Shp2 [25] and Shp1 [2] to the site of Stat3 activation sensitizes mESCs to LIF in the Jak/Stat3 signaling pathway, thus favoring self-renewal. In contrast, the molecular mechanisms that control the pluripotency and differentiation of hESCs via SHP2 depletion are different from those of mESCs [26], which suggests the dichotomous role of Shp2 in naïve and primed ESCs.

Here, we demonstrated that transient activation of Shp2 by LIF negatively modulated Jak/Stat3 signaling and transmitted the signal to Ras-to-Erk, which consequently resulted in an attenuation of naïve pluripotency. Accordingly, depletion of Shp2 or iShp2 (a chemical inhibitor of Shp2) treatment favors naïve pluripotency by sensitizing the Jak/Stat3 signaling pathway to LIF stimulation and decoupling Ras-to-Erk signaling, whereas primed ESCs were intolerant to Shp2 perturbation. The dichotomous effect of Shp2 inhibition thus favored the enrichment of naïve ESCs. Additionally, iShp2 could efficiently substitute iMek1 for the maintenance and establishment of naïve pluripotency.

Results

Transient LIF-dependent response

Constant iMek1 and LIF supplementation would be necessary to inhibit Erk activation on Klf4 and Nanog destabilization [27,28]. Upon LIF stimulation, we also observed that Erk activation occurred along with Stat3 phosphorylation (Fig. 1A). To determine the occurrence of naïve pluripotency, we took advantage of OG2 mESCs, which express naïve specific green fluorescent protein (GFP) under the control of the Oct4 promoter due to the lack of a proximal enhancer [29]. Consistent with previous reports [6], a lack of 2i resulted in the loss of the dome shape that characterizes naïve specific colony morphology (Fig. 1B, Movie S1) and significantly attenuated GFP signals even under LIF stimulation (Fig. 1C). Due to negative feedback mechanism(s) toward Stat3[30], triggered just after LIF stimulation, Stat3 phosphorylation (Fig. 1A) and its transcriptional activity (Fig. 1D) were attenuated along with Erk activation through a series of protein interactions [31]. Therefore, the putative negative feedback mechanism(s) and Erk activation after LIF stimulation would be responsible for the constant supply of LIF+2i for maintaining naïve pluripotency.

Shp2 activation by LIF negatively affects naïve pluripotency

In a previous study, genome-wide CRISPR screening identified a set of negative and positive regulators of naïve pluripotency [32]. A total of 156 genes (Fig. S1A and Table S1), whose perturbation significantly affected naïve pluripotency [32], were examined via gene ontology (GO) and KEGG pathway analysis. As expected based on the significance of LIF-mediated signaling on naïve pluripotency described in other studies, these 156 genes were highly enriched in several associated pathways including ‘Interleukin-6 signaling,’ ‘Jak/Stat3 signaling,’ and ‘PluriNetWork’ (Fig. 2A). Particularly, a few genes among a set of 27 positive regulators (shown in red) and 128 negative regulators (shown in blue) were associated with the ‘Naïve pluripotency signaling’ (Fig. S1B), ‘JAK/STAT signaling’ (Fig. S1C), and ‘Ras signaling’ (Fig. S1D) pathways. Among 27 putative negative regulators, three genes including *Grb2*, *Ptpn2*, and *Ptpn11* belonged to the “IL-6/JAK/STAT signaling” pathway according to MSigDB Hallmark 2020 (<https://maayanlab.cloud/Enrichr/>) [33] (Fig. 2B). We next focused on *Ptpn11*, which encodes the Shp2 protein that dephosphorylates Stat3, thus acting as an important negative regulator of IL-6 stimulation [34], and transduces signals toward Ras-to-Erk possibly through Grb2 [35] (Fig. S1D). Considering the close similarity between LIF and IL-6[36], we presumed that the activation of Shp2 by LIF would act as a negative regulator on naïve pluripotency by Stat3 dephosphorylation and Erk1/2 activation. As predicted, active phosphorylation of Shp2 (Y542) occurred promptly after LIF stimulation in parallel with signal transduction to Ras-to-Erk, thus relieving its auto-inhibitory regulation on phosphatase activity [18] (Fig. 2C). In parallel with Shp2 active phosphorylation (Fig. 2C), the phosphatase activity of Shp2 [inhibited by Shp2 inhibitor (iShp2) treatment], was also clearly induced by LIF stimulation (Fig. 2D). These data suggest that Shp2 activation by LIF simultaneously controls both Jak/Stat3 and Ras-to-Erk signaling to affect naïve pluripotency (Fig. 2E).

Role of Shp2 in naïve pluripotency

Although the roles of Shp2 were well-characterized in mESCs with knockdown (KD) or knockout (KO) models [17], we noticed that the effect of Shp2 in human ESCs (hESCs) was less evident than that of mESCs [26]. Given that hESCs share the molecular and cellular characteristics of primed ESCs[7], we hypothesized that the effect of Shp2 in naïve pluripotency would differ from that of primed pluripotency. To evaluate this hypothesis, we utilized primed ESCs (P-OG2) converted from naïve ESCs (OG2). As previously described [37], naïve ESCs with a ‘colonial dome shape’ exhibit GFP signals unlike primed ESCs, which exhibit a ‘flat disc shape’ (Fig. 3A), expressing typical marker genes of the naïve and primed state (Figs. S2A and B). Stable knockdown of *Ptpn11* was performed using a pair of naïve and primed ESCs. As expected based on previous studies performed in mESCs, naïve ESCs with clear *Ptpn11* knockdown (hereinafter referred to as Shp2 KD or KD naïve ESCs) (Fig. S2C) exhibited a clear ‘colonial dome shape’ with an increased GFP signal (Fig. 3B). Consistently, naïve cell-specific marker genes were also significantly enhanced in KD Naïve ESCs (Fig. 3C), whereas core pluripotency genes only exhibited marginal changes (Fig. S2D). Interestingly, KD Naïve ESCs maintained a stable GFP signal even under LIF treatment without 2i supplement (LIF only), which significantly affected the aforementioned ‘colonial dome shape’ morphology (Fig. S2E) as well as the GFP signal of wild-type (WT) cells (Fig. 3D). Based on the drastic increase in active phosphorylation (Fig. 2C) and phosphatase activity (Fig. 2D) of Shp2 by LIF, the prolonged naïve characteristics of KD naïve ESCs under the LIF-only condition resulted from

sustained Stat3 phosphorylation via stable (Fig. 3E) and transient (Fig. 3F) Shp2 depletion. These results were also supported by the gene set enrichment analysis (GSEA) [38] of FPKM values from the WT and KD transcriptomes. Consistently, the gene set for 'Hallmark IL6 JAK STAT3 signaling', 'KEGG JAK STAT3 signaling pathway,' and 'LIF signaling 1 UP' were significantly enriched in the KD cells compared to their WT counterparts (Fig. 3G). However, we still could not fully account for the marginal effect of 2i withdrawal on GFP signals (Fig. 3D and Movie S2), as well as the morphological changes illustrated in Figure S2E. Therefore, the transcriptomes of WT and KD naïve ESCs after depletion of iMek1 or iGsk3b were analyzed next. Compared to the transcriptome of LIF+2i, the WT transcriptome lacking 2i (WT LIF only) exhibited the largest number of DEGs, thus manifesting the most severe alterations. Therefore, to compare the effect of depletion of each inhibitor, 'WT LIF only' DEGs were designated as 'gene sets for 2i' for comparison (Table S1). Within the 'gene sets for 2i', KD naïve ESCs lacking iMek1 [KD (-) iMek1] were altered the least (Fig. 3H) compared to the other cells, suggesting that KD naïve ESCs would be more tolerant to iMek1 depletion.

As predicted, unlike WT cells, the 'colonial dome shape' morphology of KD naïve ESCs remained unaltered without iMek1 supplementation but was quickly lost after iGsk3b withdrawal (Fig. 3I). Given that Shp2 has another role as a signal transducer to Ras-to-Erk in addition to tyrosine phosphatase, a lack of Shp2 may decouple the signal transduction to Erk upon LIF stimulation. Consistent with the results in Figure 3G and H, phosphorylated Mek1 and Erk (Fig. S2F) was attenuated in KD naïve ESCs, whereas no clear alteration in GSK3b phosphorylation was observed (Fig. S2G). These data were also supported by GSEA, showing that the gene signatures of "Hallmark KRAS signaling DN and UP" were more enriched in KD naïve ESCs when iMek1 was depleted (Fig. S2H).

Role of Shp2 in primed pluripotency

Unlike naïve ESCs, primed ESCs were likely intolerant to the absence of Shp2. Despite multiple trials, the establishment of primed ESCs with stable Shp2 depletion was unsuccessful due to the severe growth retardation of primed ESCs after Shp2 knockdown (Fig. S3). Alternatively, KD naïve ESCs were subjected to primed culture conditions (with bFGF/Activin) to establish KD primed-like ESCs. Although KD naïve ESCs propagated well in naïve culture conditions, they did not successfully grow in primed culture conditions (Fig. 4A and Movie S3). This dichotomous effect of Shp2 depletion on primed ESCs compared to naïve ESCs was further highlighted by clonogenic assays (Fig. 4B), which account for prompt culling of KD ESCs (labeled with GFP only) from the WT ESCs (labeled with RF/GFP) mixture under primed conditions unlike naïve conditions (Fig. 4C and Movie S4). As similar as that of naïve ESCs, signaling to Erk upon bFGF/Activin stimulation was markedly attenuated in KD ESCs (Fig. 4D). These results indicate that the self-renewal of primed ESCs depends on Shp2-dependent signaling unlike that of naïve ESCs.

Defects in the differentiation of naïve ESCs by Shp2 depletion

Shp2 null mice failed to develop as a result of peri-implantation lethality, which is caused by trophoblast failure [39]. Similar to the high bFGF signaling dependence described above, trophoblast stem cells (TS) and epiblast stem cells (EpiSCs), which share a close similarity to primed ESCs, rely on bFGF

signaling [40]. As previously proposed [39], embryo development failure in Shp2 null mice may result from defects in the transition from naïve to primed ESCs in accordance with the TS impediment. Naïve ESCs after Shp2 depletion tended not only to maintain naïve pluripotency (Fig. 3) but also to hinder the progression to primed ESCs, and therefore the differentiation potential of KD naïve ESCs would be disturbed. Thus, WT and KD naïve ESCs were subjected to spontaneous differentiation via LIF/2i withdrawal from embryoid body (EB) formation (Fig. 5A). During spontaneous differentiation, which initiates with EB formation, typical marker genes of naïve pluripotency were clearly upregulated in the embryoid bodies of KD naïve ESCs (Fig. 5A), remaining marginally high one day after differentiation induction and becoming sharply suppressed when core pluripotency marker genes were similarly repressed (Fig. 5C). Thus, spontaneous differentiation was enforced with the serum-induced exit from naïve pluripotency regardless of Shp2. However, unlike *in vitro* differentiation, the formation of teratoma from KD naïve ESCs was significantly impaired in multiple sites compared to those from WT (Fig. 5D). One teratoma-like mass that was formed out of a total of 13 injections of KD naïve ESCs (Fig. 5E) only exhibited a few ectoderm and endoderm tissue structures without clear mesoderm tissue formation, unlike the well-developed teratoma from WT (Fig. 5F). It is also worth noting that Shp2 is required for proper gastrulation and mesoderm patterning in mouse [41] and *Xenopus* [42] development.

Shp2 chemical inhibitor as an iMek1 replacement

There is emerging evidence that Shp2 contributes to chemoresistance and cancer development [21,22,43], and therefore Shp2 allosteric inhibitors that interfere in both phosphatase and signal transduction have been developed as novel anti-cancer therapeutic agents [21]. We first examined whether an allosteric Shp2 inhibitor (Fig. 6A, RMC-4550: iShp2), which was initially developed to decouple the oncogenic Ras-to-Erk signaling in human cancers [21], inhibits LIF-dependent Erk activation. At an iShp2 concentration known to decrease the basal level of Erk2 phosphorylation (Fig. 6B), Stat3 phosphorylation was significantly sustained after LIF stimulation in naïve ESCs (Fig. 6C), which was associated with higher Stat3 reporter activity in the presence of iShp2 24 hours after the first LIF stimulus (Fig. 6D). Additionally, iShp2 treatment also preserved Stat3 phosphorylation even at a 1/100-fold LIF concentration (Fig. 6E) and markedly rescued naïve ESCs from cell death at a low LIF concentration (Fig. 6F). Similar to our observations in KD naïve ESCs (Fig. 3H), iShp2 treatment could likely replace the effects of iMek1 (but not iGSK3b) on the ‘colonial dome shape’ morphology of naïve ESCs (Fig. 6G), which may result from the clear decoupling of LIF mediated Ras-to-Erk signaling by iShp2 treatment (Fig. 6C). Similarly, typical naïve marker genes (Fig. 6H) and the GFP signal (Fig. 6I) indicated that iShp2 treatment compensated for the loss of iMek1 in naïve ESCs. Next to validate the dichotomous effect of iShp2 in naïve and primed ESCs, we took advantage of mESCs expressing GFP and/or RFP due to distinct enhancer activity of Pou5f1 (encoding Oct4) in naïve (or ICM) [under control of distal enhancer (DE)] and primed (or epiblast) [under control of proximal enhancer (PE)] ESCs [29] (Fig. 6J). As illustrated in Figure 6J, while ESCs of intermediate status expressing both GFP and RFP proliferate under LIF only condition (Fig. 6J), naïve (e.g., GFP+ only) and primed (e.g., RFP+ only) ESCs would exclusively expand under LIF+2i and bFGF/Activin culture condition respectively (Fig. 6J). As expected, both GFP and RFP signal from intermediate ESCs was gradually increased under ‘LIF only’ condition (Fig. S4A). To contrast, GFP but not

RFP signal became readily dominant under LIF+2i while RFP signal was only marginally affected by bFGF/Activin culture (Figs. S4B and C). As conversion from the intermediate status to primed ESCs (expressing only RFP+) requires multiple passaging as described previously [29], RFP as well as GFP signal just barely maintained by bFGF/Activin. Of note, iShp2 treatment was likely to interfere in the increase of RFP rather than GFP signal under LIF+2i (vs LIF+2i') and bFGF/Activin (vs F.A+iShp2) (Fig. 6K), implying that Shp2 inhibition would be unfavorable for ESCs that are under control PE of Pou5f1.

Reprogramming of cells to naïve pluripotency using an Shp2 chemical inhibitor

To reprogram cells to naïve pluripotency, additional 2i supplementation is required after the induction of the four Yamanaka factors (4F: Oct4, Sox2, Klf4, and c-Myc, hereinafter referred to as OSKM) with LIF [44]. To examine whether iShp2 could replace iMek1 during naïve reprogramming, we used mouse embryonic fibroblasts obtained from inducible-OSKM (iOSKM-MEFs) mice [45], which readily achieves OSKM induction via doxycycline (Dox) treatment. These iOSKM-MEFs were subjected to naïve reprogramming with each different condition (Fig. 7A). As expected, multiple colonies with 'colonial dome shape' that were positive to alkaline phosphatase (AP) activity were obtained via LIF+2i supplementation but not the absence of 2i nor iMek1 (Figs. 7B and C). In this condition, iShp2 treatment was likely to reverse the effect of iMek1 depletion for naïve reprogramming (Figs. 7B and C). Notably, the marker expression of naïve (Fig. 7D) and core pluripotency (Fig. 7E) indicated that a new combination of LIF with iGSK3b and iShp2 (instead of iMek1) could enrich the reprogrammed iPSCs more efficiently than the conventional 2i supplementation.

Discussion

The disrupted activity of Shp1 [2] and 2 [25] in mESCs due to a lack of Zap70, a protein that acts as a non-receptor tyrosine kinase upon LIF stimulation, increases Jak/Stat3 signaling and self-renewal. However, although we observed that the effect of Zap70 depletion on hESCs was only marginal unlike mESCs (data not shown), we simply assumed that these discrepancies were largely attributable to species-specific factors and other inherent differences [1]. However, recent advances in the characterization of naïve and primed pluripotency have now revealed that such dissimilarities between mouse and human ESCs result from the unique characteristics of naïve and primed pluripotent cells.

Unlike in primed ESCs, the simultaneous chemical inhibition of Mek1 and Gsk3 β is critical for the maintenance of naïve pluripotency [9], thus highlighting the unique cellular signaling in the ICM of blastocysts [12, 46]. Particularly, the normal development of Erk2 null embryos until pre-implantation [47] and low basal Erk activity in ICM [13] suggest that Erk activity is dispensable during the pre-implantation state, which is consistent with the characteristics of naïve pluripotency. Similarly, Shp2 null embryo lethality also resulted from trophoblast failure [39] and possible epiblast cell death with primed characteristics.

The requirement of DUSPs, Erk1/2 specific phosphatases, in mESCs also demonstrates that Erk activity needs to be maintained at a lower level to hold naïve pluripotency [14, 15]. Nevertheless, the constant

requirement of the Mek1 inhibitor during *in vitro* culture of naïve ESCs implies that constant Erk activation occurs due to LIF stimulation, which eventually impedes naïve pluripotency via Erk-dependent phosphorylation [27, 28].

Meta-analysis of previous datasets from a genome-wide study [32] revealed that Shp2 was predicted to serve as a negative regulator for naïve pluripotency through its involvement in both LIF-Jak/Stat3 and Ras-to-Erk signaling (Fig. 2). Notably, Shp2, whose catalytic activity was induced by LIF along with tyrosyl phosphorylation[18](Fig. 2), serves not only as a negative regulator for Stat3 (Fig. 3) but also as a positive regulator for Ras-to-Erk (Fig. 3). This is why the decoupling of 'Ras-to-Erk' signaling occurred through Shp2 inhibition, thus liberating the demand of iMek1 for naïve pluripotency (Fig. 6) as well as enhancing naïve pluripotency (Fig. 3). In sharp contrast, Shp2 appeared to be indispensable for primed ESCs, whose pluripotency relies on bFGF/Activin stimuli. Perturbation of Shp2 significantly delayed the self-renewal of ESCs in primed conditions (Figs. 4 and 6J) with concurrent decoupling of Erk signaling (Fig. 4D). Additionally, Shp2 suppression can successfully substitute the usage of iMek1 not only for maintenance of naïve ESCs (Fig. 6) but also during reprogramming (Fig. 7), suggesting that iShp2 can be used as an alternative to improve iPSC formation. Therefore, the interfering dual roles of Shp2 with the allosteric inhibitor of Shp2 [21] used in this study prolonged Stat3 and attenuated Erk phosphorylation, which mimicked the effect of Shp2 depletion on naïve pluripotency (Fig. 6) and naïve reprogramming (Fig. 7).

Conclusion And Perspectives

Shp2 serves as a negative regulator for naïve pluripotency due to its dual roles in Jak/Stat3 and Ras-to-Erk signaling, whereas the pluripotency of primed cells depended on bFGF/Activin, which could also be modulated by Shp2-dependent signaling. Allosteric inhibition of Shp2 with an inhibitor to disrupt these dual functions can therefore be used to improve naïve pluripotency and replace the use of iMek1. Considering the complex protocol for human naïve pluripotent stem cell conversion [48], iShp2 treatment would be a promising strategy for the efficient establishment of human naïve PSCs, which will be examined in future studies.

Materials And Methods

Reagents

The primary antibodies against α -tubulin (#sc- 8035), b-actin (#sc-47778), Stat3 (#sc-8019), Erk2 (#sc-154) were purchased from Santa Cruz Biotechnology, Inc. Antibodies against phospho-Shp2 (Tyr542) (#3151), phospho-Stat3 (#9145s), phospho-Erk (#9101) were purchased from Cell Signaling Technology. Antibody against Shp2 (#VL3159027A) was purchased from Invitrogen. siRNAs targeting Negative Control (#SN-1003) and the others (listed at supplement table) were obtained from Bioneer. Expression vectors of 6X Stat3-luciferase was kindly gifted by Prof. Hyewon Youn from Seoul National University.

Cell culture

Naïve mouse ESCs were cultured on 0.5% porcine gelatin-coated dish in Naïve mESC culture media - DMEM high glucose (Gibco) supplemented with 15% FBS (Gibco), 1% Glutamax (Gibco), 1% MEM-nonessential amino acids (Gibco), 0.1% Gentamycin (Gibco), 0.1 mM β -mercaptoethanol (Gibco), 1,000 U/ml mouse leukemia inhibitory factor (mLIF) (Millipore, Merk), 1 mM PD0325901 (Peprotech) and 3 mM CHIR99021- at 37°C and 5% CO₂ incubating condition. Cells were passaged 1:10 ratio every 3 days using 0.25% Trypsin/EDTA (Wellgene) as a dissociation reagent. Primed mouse ESCs were cultured on Matrigel (Corning# 354277)-coated dish in EpiSC culture media -DMEM/F12 (Gibco) supplemented with 15% KnockOut SR (Gibco), 1% GlutaMAX (Gibco), 1% MEM-nonessential amino acids (Gibco), 0.1% Gentamycin (Gibco), 10 ng/ml murine bFgf (Peprotech), 20 ng/ml murine Activin (Peprotech)- at 37°C and 5% CO₂ condition. Primed mESCs were passaged every 3-4 days using Dispase (Gibco) as a colony detachment reagent. Detached colony clumps were transferred 1:15-1:20 ratio on Matrigel (Corning# 354277) coated dishes. Culture media was changed every day for all cell lines. Oct4- Δ PE-GFP/ Δ DE-RFP ESCs were cultured on mitogen-inactivated C57BL/6 feeder cells on 0.15% porcine gelatin-coated dish in culture media - DMEM Low (Gibco) supplemented with 15% FBS (Gibco), 1% P.S.G (Gibco), 1% MEM-nonessential amino acids (Gibco), 0.1 mM β -mercaptoethanol (Gibco) and 1,000 U/ml mouse leukemia inhibitory factor (mLIF) (Millipore, Merk)- at 37°C and 5% CO₂ incubating condition.

Teratoma formation assay

5x10⁵ cells of each OG2 and OG2Shp2KD mESCs were injected into the testes of 5-weeks-old male BALB/C nude mice (n=3), and subcutaneously injected in the neck (n=5) and abdomen (n=5) of 5-weeks-old male BALB/C nude mice. Mice were euthanized 6 weeks after injection. These animal experiments were conducted under the permission of Seoul National University Institutional Animal Care and Use Committee (Permission number: SNU-190716-5-2).

RNA-sequencing analysis

Total RNA was isolated from cells by using Easy-BLUE™ RNA isolation kit (iNtRON Biotechnology). 1mg of total RNA was processed for preparing mRNA sequencing library using MGI Easy RNA Directional Library Prep Kit (MGI) following manufacturer's instruction. The mRNA is fragmented into small pieces under elevated temperature. The cleaved RNA fragments are copied into first strand cDNA using reverse transcriptase and random primers. Strand specificity is achieved in the RT directional buffer and second strand cDNA synthesis was done. Single 'A' base was added to the cDNA fragments, followed by ligation of the adapter. The products are purified and enriched by PCR procedure to acquire the final cDNA library. The cDNA library is quantified using QuantiFluor ONE dsDNA System (Promega). Using DNA nanoball (DNB) enzyme, the library is incubated at 30°C for 25min to make DNB. Finally, sequencing of the prepared DNB was performed using MGISEQ system (MGI) with 100bp paired-end reads.

Gene Set Enrichment Analysis (GSEA)

FPKM values of RNA sequencing data were formatted and loaded to run Gene Set Enrichment Analysis. GSEA software and the Gene Sets (Hallmark_IL6_JAK_STAT3 signaling, KEGG_JAK_STAT3 signaling, ABBUD_LIF signaling_up, Hallmark_KRAS_signaling_DN, Hallmark_KRAS_signaling_UP) were downloaded from Molecular Signatures Database v7.4 (<https://www.gsea-msigdb.org>)

Alkaline phosphatase assay

After reprogramming of iOSKM MEF, Alkaline phosphatase staining assay was performed by using AP staining kit (Cat# 86R-1KT; Sigma-Aldrich) to visualize the reprogrammed cells. Overall procedure was followed by the manufacturer's instructions.

Statistical analysis

The quantitative data are presented as the mean values $\pm$ standard deviation (SD). Unpaired two-tailed *t*-tests or one-way ANOVA following Dunnett multiple comparison, was performed to analyze the statistical significance of each response variable. *p*-values less than 0.05 were considered statistically significant (* < 0.05, ** <0.001, ***<0.0001, ****<0.0001 and n.s. for not significant).

Declarations

Supplementary data

Online supplementary data are available.

Data availability

Source data are available from the corresponding authors upon request.

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Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors Contributions

HJ.C and KT.K conceived the overall study design and led the experiments. SM.K mainly conducted the experiments, data analysis, and critical discussion of the results. EJ.K performed RNAseq data analysis, YJ.K, YH.K, JY.O, S.P and JT.D produced mutant lines and provided key cell line systems.

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Figures

Figure 1

Transient LIF dependent response. (A) Immunoblotting at indicative time after LIF stimulation, Erk2 and β -actin were used as loading controls. (B) Fluorescent microscopic images at indicated time under LIF+2i, LIF only and LIF deprivation (LIF-) media respectively. (C) Flow cytometry of GFP after 60hr stimulated by LIF+2i, LIF only, LIF- media respectively (left panel), Graphical presentation of GFP positive population (right panel) (D) Luciferase reporter activity of Stat3 in OG2 cells at indicated time after LIF stimulation (*, $p < 0.05$, **, $p < 0.001$, ***, $p < 0.0001$, n.s. for not significant)

Figure 2

Shp2 activation by LIF negatively affects naïve pluripotency (A) Top enriched categories of GO from BioPlanet 2019 (top), Wiki Pathways 2019 Mouse (middle) and MSigDB Hallmark 2020 (bottom) from DEG, ranked by $-\log(p\text{-value})$ (B) List of 27 genes that were predicted as putative negative regulators of naïve pluripotency (top), gene list involved in 'IL-6/JAK/STAT3 Signaling Pathway' (bottom) (C) Immunoblotting for indicative proteins at 10 and 30 min after LIF stimulation, α -tubulin was used as a

loading control. (D) Graphical presentation of phosphatase activity of OG2 ESCs at 10 min after LIF stimulation with or without iShp2 (10μM) for 1 hour (n=4) (E) Graphical illustration of Shp2 in LIF mediated signaling of naïve ESCs, (*, $p < 0.05$, **, $p < 0.001$, ***, $p < 0.0001$, n.s. for not significant)

Figure 3

Role of Shp2 in naïve pluripotency (A) Graphical illustration of isogenic pair of naïve (OG2) and primed (POG2) ESCs (left), brightfield and fluorescent microscopic images of OG2 and POG2 ESCs (scale bars,

500µm) (right panel). (B) Representative light microscopic images of WT and KD ESCs (scale bars, 200µm) (left panel) and fluorescent microscopic images of WT and KD ESCs (scale bars, 500µm) (right panel) (C) Fold mRNA expression of typical naïve pluripotency marker genes in WT and KD ESCs (**, $p < 0.001$ and ***, $p < 0.0001$, $n=3$) (D) Fluorescent microscopic images of WT and KD ESCs at indicated time under LIF+2i and LIF only culture condition (scale bars : 500µm) (E and F) Immunoblotting for pStat3, Stat3 and Erk2 in WT and KD ESCs at indicated time after LIF stimulation, Erk2 was used as a loading control. (F) Immunoblotting of OG2 ESCs with control (siNC) or Ptpn11 siRNA (siPtpn11) at indicated time after LIF stimulation, Erk2 was used as a loading control (G) The normalized enrichment score by gene set enrichment analysis (GSEA) of WT and KD ESCs for Hallmark_IL6_JAK_STAT3 signaling (left panel), KEGG_JAK_STAT3 signaling (middle panel), and ABBUD_LIF signaling up (right panel). (H) DEGs of WT LIF+2i vs WT (-)2i were assigned as 'gene sets for 2i'. The number of Up-DEGs and Down-DEGs within the 'gene sets for 2i' group are presented. (I) Light microscopic images of WT and KD 24 hours after indicated culture condition (scale bars: 200µm)

Figure 4

Role of Shp2 in primed pluripotency (A) Light microscopic images of WT and KD ESCs at indicated times under LIF+2i or bFGF/Activin culture condition (scale bars : 500 µm) (left), Graphical presentation of cell number at indicated time after seeding of 1x10⁴ ESCs (right). (B) Images of clonogenic assay of WT and

KD ESCs at indicated time after culture in LIF+2i or bFGF/Activin (left), Graphical presentation of area ratio (A.U.) of clones at day 2 and day 4 determined by ImageJ (right). (C) Graphical illustration of competition assay with WT or KD ESCs, labeled with red fluorescence (RF)/GFP or GFP only respectively (top), Fluorescence microscopic images of WT or KD ESCs at 2 days after indicated culture condition, arrowheads indicate KD ESCs (labeled with GFP only) (scale bars: 500 μ m) (D) Immunoblotting analysis of WT and KD ESCs grown under bFGF/Activin for Shp2, pStat3, pErk1/2, Erk2 and β -actin. β -actin was used as a loading control.

Figure 5

Defects in the differentiation of naïve ESCs by Shp2 depletion (A) Light microscopic images of embryonic body (EB) cultured for 3 days of WT or KD ESCs (scale bars, 500µm) (left), Graphical presentation of relative mRNA expression of typical naïve pluripotency and core pluripotency markers (right) of WT (blue) and KD (red) (B) Light microscopic images of WT and KD before (LIF+2i) or 48 hours after differentiation [(-)LIF+2i] (C) Relative mRNA expressions of naïve pluripotency (left) and core pluripotency (right) markers at indicative time after differentiation in WT (blue) and KD (red) (D) Representative images of teratomas formed in mouse testes at 6 weeks after injection of WT (top) and KD (bottom) ESCs (E) Graphical presentation of teratoma volume [$V = a \times b \times c \times \pi / 6$ (a = length, b = width, and c = depth)] of total 13 teratoma from WT or KD ESCs (left), formed three different areas (right), Volumes of normal testis after teratoma injection were considered as 0. (F) Hematoxylin & eosin (H&E) (for ectoderm and endoderm) and Masson's trichrome (for mesoderm) staining of teratoma section from WT and KD, Uncharacterized tissue from teratoma of KD were shown in the box.

Figure 6

Shp2 chemical inhibitor as an iMek1 replacement (A) Chemical structure of Shp2 inhibitor (iShp2: RMC-4550) (B) Immunoblotting analysis of WT ESCs at 30 min after indicative dose of iShp2 treatment, α -tubulin was used as a loading control. (C) Immunoblotting analysis of WT ESCs at indicted time after 1000 units of LIF stimulation, pretreated with either DMSO (Mock) or 5 μ M of iShp2 (+ iShp2) for 1 hour (L/2i: LIF+2i control) (D) Graphical presentation of luciferase reporter activity of Stat3 at 8 hours after LIF

deprivation with either DMSO (Mock) or 5 μ M of iShp2 (+ iShp2) for 1 hour compared to prior to LIF deprivation as a control (LIF) ***, $p < 0.0001$). (E and F) Immunoblotting analysis for pStat3 at 30 min (E) and light microscopic images (scale bars, 500 μ m) (F) after indicative dose of LIF stimulation in WT ESCs pretreated with either DMSO (Mock) or 5 μ M of iShp2 (+iShp2) for 1 hour (G) Light microscopic images of WT ESCs under control (LIF+2i), deprivation of iMek1 [(-) iMek1], iGsk3 β [(-)iGsk3 β] or iMek1/iGsk3 β [(-2i)] with DMSO (Mock) or 5 μ M of iShp2 (+iShp2) treatment for 2 days (scale bars: 200 μ m) (H) Relative gene expression of Rex1 (left) and Essrb (right) at indicated culture condition with DMSO (Mock: blue) or 5 μ M of iShp2 (+iShp2: red) for 2 days (*, $p < 0.05$, **, $p < 0.001$, ***, $p < 0.0001$, n.s. for not significant) (I) Flow cytometry of GFP intensity of WT ESCs with either LIF+2i control (LIF+2i) or iMek1 depletion [(-)iMek1] for 2 days, pretreated with DMSO (Mock) or 5 μ M of iShp2 (+iShp2) (****, $p < 0.00001$, $n = 6$) (J) Graphical illustration of endogenous Oct4 with distal enhancer (DE) and proximal enhancer (PE)(top), Oct4-PE-GFP (middle) and Oct4-DE-RFP (bottom), activation of DE and PE at naïve and inner cell mass (ICM) and at primed and epiblast stem cells (EpiSCs) respectively (left), ESCs with GFP+/RFP- (Green), GFP+/RFP+ (Yellow) and GFP-/RFP+ (red) at naïve, intermediate and primed status respectively (top), expected growth of GFP+ or RFP+ ESCs under each indicative condition was shown (bottom). (K) Cell growth ratio at indicated time of GFP+ or RFP+ ESCs under LIF+2i / LIF+2i' (iShp2 instead of iMek1) and bFGF/Activin with DMSO (F.A) or iShp2 (F.A+iShp2) (top panels), Table of slope and p value of linear regression of GFP+ or RFP+ at indicated culture condition, effect of iShp2 on slope was highlighted in red.

Figure 7

Reprogramming of cells to naïve pluripotency using an Shp2 chemical inhibitor (A) Graphical illustration for naïve reprogramming of mouse embryonic fibroblast introducing doxycycline inducible reprogramming factors (iOSKM MEF) under indicated culture condition (B) Light microscopic images (Bright Field) and alkaline phosphatase staining (AP) of iOSKM MEF reprogrammed for 15 days with indicated culture condition (C) Representative images of AP staining of iOSKM MEF under indicated culture condition (left) (scale bars: 3 mm) and graphical quantification of AP positive area (arbitrary unit: A.U) at indicated condition (**, $p < 0.001$, $n = 3$) (D) Relative mRNA expressions of naïve pluripotency markers (left) and core pluripotency marker of reprogrammed iOSKM MEF at indicated condition (*, $p < 0.05$ and ****, $p < 0.00001$, $n = 3$).

Supplementary Files

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