

Gbx2, a LIF/Stat3 target, promotes reprogramming to and retention of the pluripotent ground state

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Summary

Activation of signal transducer and activator of transcription 3 (Stat3) by leukemia inhibitory factor (LIF) maintains mouse embryonic stem cell (mESC) self-renewal and also facilitates reprogramming to ground state pluripotency. Exactly how LIF/Stat3 signaling exerts these effects, however, remains elusive. We identified gastrulation brain homeobox 2 (Gbx2) as a LIF/Stat3 downstream target that, when overexpressed, allows long-term expansion of undifferentiated mESCs in the absence of LIF/Stat3 signaling. Elevated Gbx2 expression also enhanced reprogramming of mouse embryonic fibroblasts to induced pluripotent stem cells. Moreover, overexpression of Gbx2 was sufficient to reprogram epiblast stem cells to ground state ESCs. Our results reveal a novel function of Gbx2 in mESC reprogramming and LIF/Stat3-mediated self-renewal.

Key words: Embryonic stem cell, Pluripotency, Self-renewal, LIF, Stat3, Gbx2, Reprogramming

Introduction

Embryonic stem cells (ESCs) are derived from the inner cell mass of the preimplantation blastocyst. ESCs can be maintained indefinitely as self-renewing populations while retaining the ability to differentiate into any type of cell in the body. Extrinsic signals and intrinsic transcriptional circuitries govern ESC fate decisions. Notably, exogenous provision of leukemia inhibitory factor (LIF) can maintain mouse ESC (mESC) self-renewal (Smith et al., 1988; Williams et al., 1988) by activating signal transducer and activator of transcription 3 (Stat3) (Niwa et al., 1998). Inactivation of Stat3 abrogates LIF-dependent mESC propagation (Niwa et al., 1998). In addition, artificial, hormone-dependent activation of Stat3 can maintain mESCs in an undifferentiated state (Matsuda et al., 1999). These findings implicate Stat3 as a key component in LIF-mediated self-renewal signaling.

Stat3 forms a homodimer upon induction by LIF and subsequently translocates into the nucleus, where it regulates transcription of its downstream targets to maintain ESC identity. Several downstream targets of Stat3, such as Krüppel-like factor 4 (Klf4) and Klf5, have been shown to promote mESC self-renewal when overexpressed (Li et al., 2005; Ema et al., 2008; Bourillot et al., 2009). Nevertheless, overexpression of neither Klf4 nor Klf5 can fully recapitulate the self-renewal effect mediated by LIF (Hall et al., 2009). Moreover, self-renewal in the presence of LIF is unaffected by the knock down of Klf4 or Klf5 expression (Jiang et al., 2008). These lines of evidence suggest that LIF/Stat3 might trigger multiple downstream targets to maintain mESCs in an undifferentiated state.

Previous attempts to identify LIF/Stat3 downstream targets responsible for mediating mESC self-renewal have been hampered because LIF activates not only Stat3 but also many other pathways, including the mitogen-activated protein kinase

(MEK)/extracellular signal-regulated kinase (ERK) pathway, which negatively regulates mESC self-renewal (Burdon et al., 1999). In order to identify novel self-renewal-associated genes induced by LIF, we focused on direct downstream targets of Stat3 by stimulating the LIF/Stat3 pathway while blocking activation of the MEK/ERK pathway, and screened for factors crucial for mESC self-renewal. Here, we identified gastrulation brain homeobox 2 (Gbx2) as a downstream target of Stat3, that when overexpressed was sufficient to sustain mESC self-renewal in the absence of added LIF. We also found that Gbx2 expression distinguishes naïve state ESCs from primed-state epiblast stem cells (EpiSCs), and promotes the conversion of EpiSCs to ESCs.

Results and Discussion

Identification of direct downstream targets of Stat3 in mESCs

To facilitate the identification of Stat3 targets that contribute to mESC self-renewal, we exploited the GRgp-Y118F chimeric receptor, which can be stimulated with granulocyte colony-stimulating factor (GCSF) to activate Janus kinase (Jak)/Stat3 signaling (Burdon et al., 1999). Substitution of phenylalanine for tyrosine 118 in the gp130 segment of the receptor prevents it from inducing MEK/ERK signaling, thereby enabling a high-fidelity examination of Stat3-specific effects. We introduced GRgp-Y118F into B6 ESCs derived from the C57BL/6 mouse strain. As expected, treatment of B6-Y118F ESCs with GCSF strongly activated Stat3 without affecting ERK1/2 phosphorylation (supplementary material Fig. S1). To examine transcriptional changes associated with Stat3 activation, we starved B6-Y118F ESCs in serum-free medium for 6 hours and then treated them with GCSF for 1 hour to stimulate transcription of primary Stat3 targets but limit indirect downstream transcription. We treated a separate culture with LIF and the MEK inhibitor PD0325901, as we

reasoned this condition would mimic the effect of GCSF stimulation and thereby corroborate results obtained with GCSF (supplementary material Fig. S2). RNA from the treated cells was extracted and analyzed by microarray. Genes upregulated in both GCSF and LIF/PD0325901 treatments were considered candidate primary Stat3 targets. Based on the fold change values from microarray analysis and the functional role of each gene as reported in the relevant scientific literature, 19 genes were selected and their expression was confirmed by quantitative real-time PCR (qRT-PCR) (supplementary material Table S1). The microarray data are accessible through GEO series accession number GSE38719.

Overexpression of *Gbx2* maintains mESC self-renewal in the absence of LIF

To screen for genes that can promote mESC self-renewal, we artificially expressed each of the 19 candidate genes in 46C ESCs and cultured them in the absence of LIF. 46C ESCs are feeder-independent ESCs derived from the 129 mouse strain (Ying et al., 2003). Among the 19 genes screened, *Gbx2* was found capable of supporting mESC self-renewal when overexpressed. Specifically, 46C ESCs transduced to overexpress a *Gbx2* transgene formed colonies exhibiting typical undifferentiated morphology in the absence of LIF (Fig. 1A,B). To further evaluate their status, we fixed the cells and stained them for alkaline phosphatase (AP) and classified the colonies as differentiated, undifferentiated or mixed. Approximately 25% of the *Gbx2* transfectant colonies were undifferentiated and 50% were mixed while 90% of the EGFP transfectant colonies were fully differentiated (Fig. 1C). Overexpression of *Gbx2* supported long-term maintenance of undifferentiated 46C ESCs in the absence of added LIF

(Fig. 1D,E). The results were validated in another mESC line, R1 (supplementary material Fig. S3A–C). shRNA knock down of *Gbx2* in *Gbx2*-overexpressing R1 ESCs (supplementary material Fig. S4A) rendered them unable to retain an ESC identity in the absence of LIF (supplementary material Fig. S4B,C), confirming the observed self-renewal-promoting effect is indeed attributed to *Gbx2* overexpression.

To determine whether ESCs maintained by *Gbx2* overexpression retain pluripotency, we exploited a *loxP*-based excisable vector containing the *Gbx2* transgene. Transient expression of Cre recombinase results in excision of the *Gbx2* transgene and simultaneously brings GFP under the CAG promoter. ESCs transfected with the excisable vector were clonally selected and then maintained in serum without added LIF for 20 days. The cells were then transiently transduced to express Cre. *Gbx2* excision transfectants enriched by FACS sorting reacquired LIF dependence (supplementary material Fig. S4D). The revertant cells were injected into 12 mouse blastocysts and gave rise to three chimeric fetuses with widespread GFP expression (Fig. 1F), suggesting that *Gbx2* overexpressing ESCs retain their pluripotency.

Gbx2 is a direct downstream target of the LIF/Stat3 pathway

Jak1-mediated phosphorylation of Stat3 results in homodimerization and activation of Stat3 (Stahl et al., 1995). To further confirm that *Gbx2* is downstream of the LIF/Stat3 pathway, we evaluated *Gbx2* gene induction in 46C cells treated with a Jak1 inhibitor. In the absence of the inhibitor, *Gbx2* transcription showed a prompt induction within one hour of continuous LIF treatment, whereas the induction was abolished in the presence of the inhibitor (Fig. 2A). Moreover, overexpression

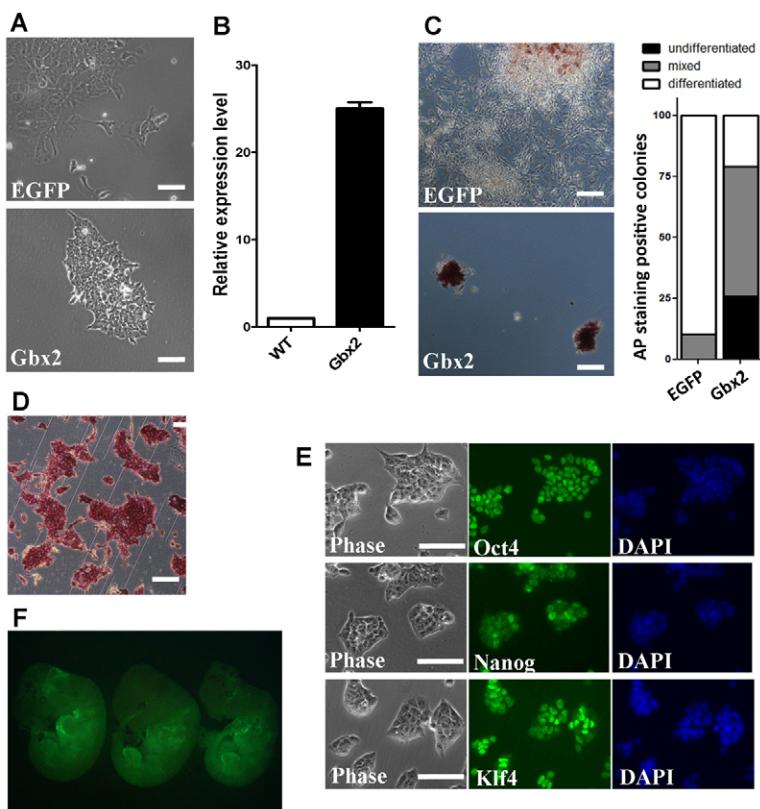


Fig. 1. Overexpressing *Gbx2* sustains mESC self-renewal.
(A) Phase-contrast images of 46C ESCs overexpressing EGFP or *Gbx2* after culture for 5 days in the absence of LIF. **(B)** qRT-PCR analysis of *Gbx2* mRNA expression in 46C ESCs transduced to express EGFP and *Gbx2*. **(C)** AP staining and quantification of colonies of 46C ESCs overexpressing EGFP or *Gbx2* after 7 days without LIF supplementation. **(D)** AP staining of 46C ESCs overexpressing *Gbx2*, cultured for more than 40 days (passage >9) in the absence of LIF supplementation. **(E)** Immunofluorescence staining of Oct4, Nanog and Klf4 in 46C ESCs overexpressing *Gbx2*, cultured more than 40 days (passage >9) in the absence of LIF supplementation. Scale bars: 50 µm (A,C–E). **(F)** The three chimeric embryos (E11.5) with widespread GFP, generated from *Gbx2* transfectants subjected to Cre-mediated excision of *Gbx2*.

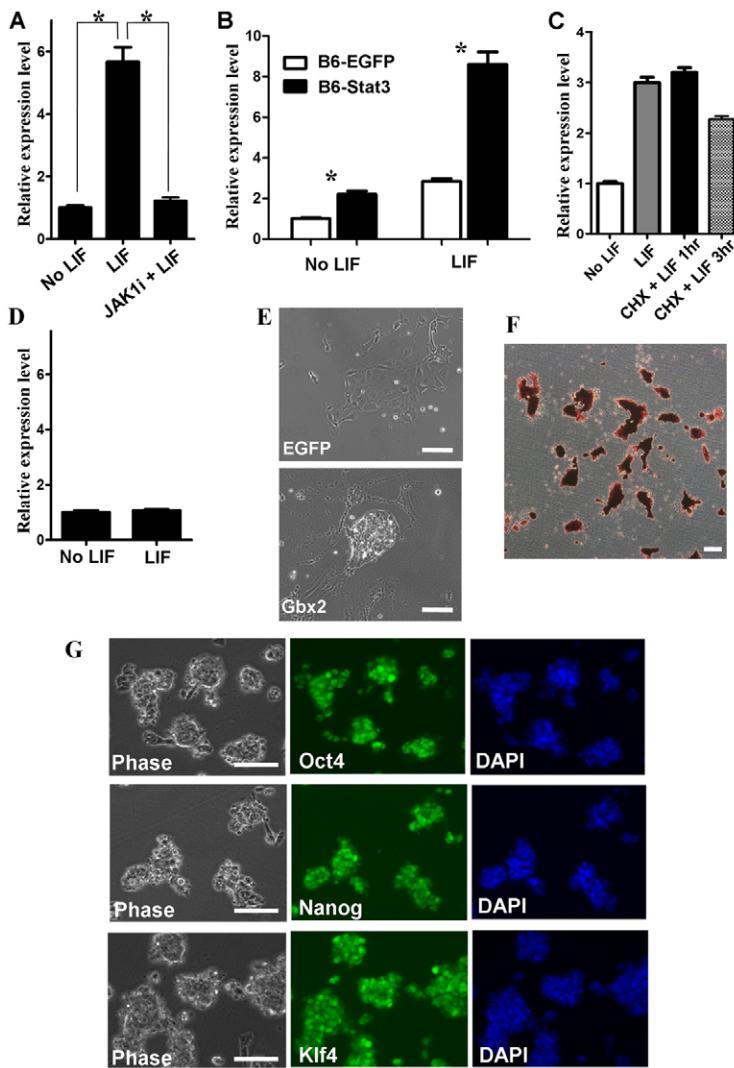


Fig. 2. Gbx2 is directly regulated by LIF/Stat3 and its overexpression is sufficient to sustain *Stat3*^{-/-} ESC self-renewal. (A) qRT-PCR analysis of *Gbx2* mRNA expression in 46C ESCs after LIF treatment for 1 hour. 46C ESCs were starved for 6 hours in serum-free medium before LIF stimulation. Jak1 inhibitor (Calbiochem; 10 µM) was administered 1 hour before LIF. (B) qRT-PCR analysis of *Gbx2* mRNA expression in B6 ESCs overexpressing EGFP or Stat3 after LIF treatment for 1 hour. These cells were starved for 6 hours in serum-free medium before LIF stimulation. (C) qRT-PCR analysis of *Gbx2* mRNA expression in 46C ESCs after LIF stimulation in the presence or absence of cycloheximide (CHX; 50 µg/ml) for 1 or 3 hours. (D) qRT-PCR analysis of *Gbx2* mRNA expression in *Stat3*^{-/-} ESCs after LIF treatment for 1 hour. (E) Colony morphology of *Stat3*^{-/-} ESCs overexpressing Gbx2, cultured for 6 days in serum-only medium. (F) AP staining of *Stat3*^{-/-} ESCs overexpressing Gbx2 cultured for more than 3 weeks (passage >6). (G) Immunofluorescence staining of Oct4, Nanog and Klf4 in *Stat3*^{-/-} ESCs overexpressing Gbx2, cultured for more than 3 weeks (passage >6). In A–D error bars represent the s.d. ($n=4$); * $P<0.01$. Scale bars: 50 µm (E–G).

of Stat3 in mESCs was associated with higher basal and LIF-induced expression of *Gbx2* than was detected in control mESCs expressing Stat3 at the endogenous level (Fig. 2B), indicating that the expression level of Stat3 is correlated with the expression level of *Gbx2*. The induction of *Gbx2* expression by LIF was similar with or without cycloheximide (Fig. 2C), an inhibitor of protein biosynthesis, which excludes the possible regulation of *Gbx2* by other LIF-induced downstream targets of Stat3. We next exploited *Stat3* null (*Stat3*^{-/-}) ESCs to further test the requirement for Stat3 in *Gbx2* induction. *Stat3*^{-/-} ESCs are routinely maintained in the 2i condition containing Gsk3 inhibitor CHIR99021 and MEK/ERK inhibitor PD0325901 (Ying et al., 2008). Expression of *Socs3*, a direct downstream target of Stat3, was upregulated in 46C ESCs upon 1 hour of LIF treatment, but not in equivalently treated *Stat3*^{-/-} ESCs (supplementary material Fig. S5). Likewise, LIF failed to induce *Gbx2* expression in *Stat3*^{-/-} ESCs (Fig. 2D). A chromatin immunoprecipitation (ChIP) assay performed on mESCs by using antibodies against Stat3 indicated an association between Stat3 and *Gbx2* loci (Zhou et al., 2007; Chen et al., 2008). Taken together, these data convincingly demonstrate that *Gbx2* is a direct target of the LIF/Stat3 signaling pathway.

Overexpressing Gbx2 sustains *Stat3*^{-/-} ESCs in an undifferentiated state

Having established a role for Gbx2 as a downstream agent of LIF/Stat3-mediated self-renewal, we next sought to determine whether overexpression of Gbx2 would suffice to maintain the undifferentiated state in *Stat3*^{-/-} ESCs. In serum medium without LIF or 2i, *Stat3*^{-/-} ESCs transduced to overexpress Gbx2 could be continually propagated and passaged while retaining typical ESC morphology and AP activity and expression of Oct4, Nanog and Klf4 (Fig. 2E–G). In contrast, control *Stat3*^{-/-} ESCs transduced to express EGFP died or differentiated within three days in the same condition (Fig. 2E). These results imply that overexpression of Gbx2 can compensate for the absence of Stat3 to maintain the self-renewal phenotype.

Gbx2 exhibits a redundant role in LIF-mediated self-renewal

To ascertain whether Gbx2 is essential for LIF/Stat3-mediated ESC self-renewal, we infected 46C ESCs with shRNAs designed to knock down endogenous *Gbx2* expression, and collected RNA samples 5 days later. Expression of *Gbx2* was 70% to 80% reduced as measured by qRT-PCR (Fig. 3A). In the presence of

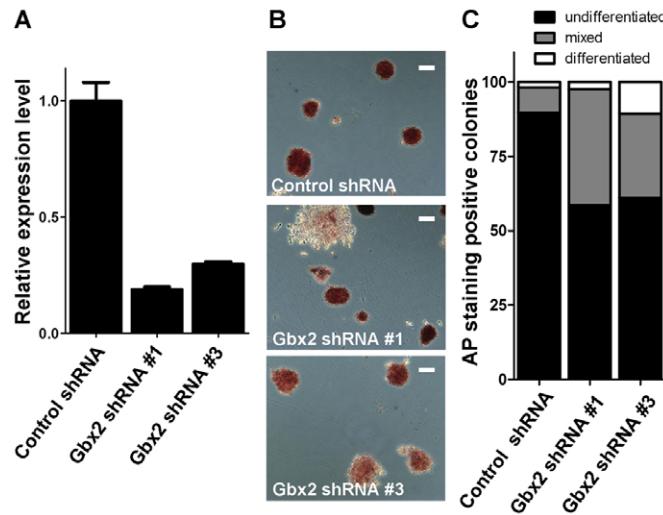


Fig. 3. *Gbx2* is redundant in LIF-mediated self-renewal. (A) qRT-PCR analysis of endogenous *Gbx2* mRNA expression in 46C ESCs transduced to express control or *Gbx2* shRNAs. Error bars represent the s.d. ($n=4$). * $P<0.01$. (B) AP staining of 46C ESCs transduced to express control and *Gbx2* shRNAs. Scale bars: 50 μ m. (C) Quantitative analysis of AP staining in 46C ESCs transduced to express control and *Gbx2* shRNAs.

LIF, a greater percentage of partially differentiated colonies was observed for *Gbx2* knockdown 46C ESCs than cells infected with scramble control shRNA (Fig. 3B). However, knockdown of *Gbx2* was incapable of totally impairing ESC self-renewal: More than 50% of *Gbx2* knockdown colonies remained undifferentiated, and only 5–15% of colonies fully differentiated (Fig. 3C). These results suggest that while *Gbx2* plays a role in promoting mESC self-renewal, its expression is not essential for LIF/Stat3-mediated self-renewal. This is consistent with the finding that knockout of *Gbx2* results in no morphological abnormalities in the blastocyst that would indicate a defect in self-renewal (Wassarman et al., 1997). Moreover, knockdown of two other Stat3 targets, *Klf4* and *Klf5*, does not lead to mESC differentiation in the presence of LIF (Jiang et al., 2008). Collectively, these results imply that LIF/Stat3 supports mESC self-renewal through redundant mechanisms involving multiple factors downstream of Stat3, such as *Gbx2*, *Klf4* and *Klf5*.

***Gbx2* is specifically expressed in naïve ESCs and promotes reprogramming of MEFs and EpiSCs to a state of naïve pluripotency**

We next examined the expression of *Gbx2* in undifferentiated and differentiating 46C ESCs. *Gbx2* mRNA expression diminished

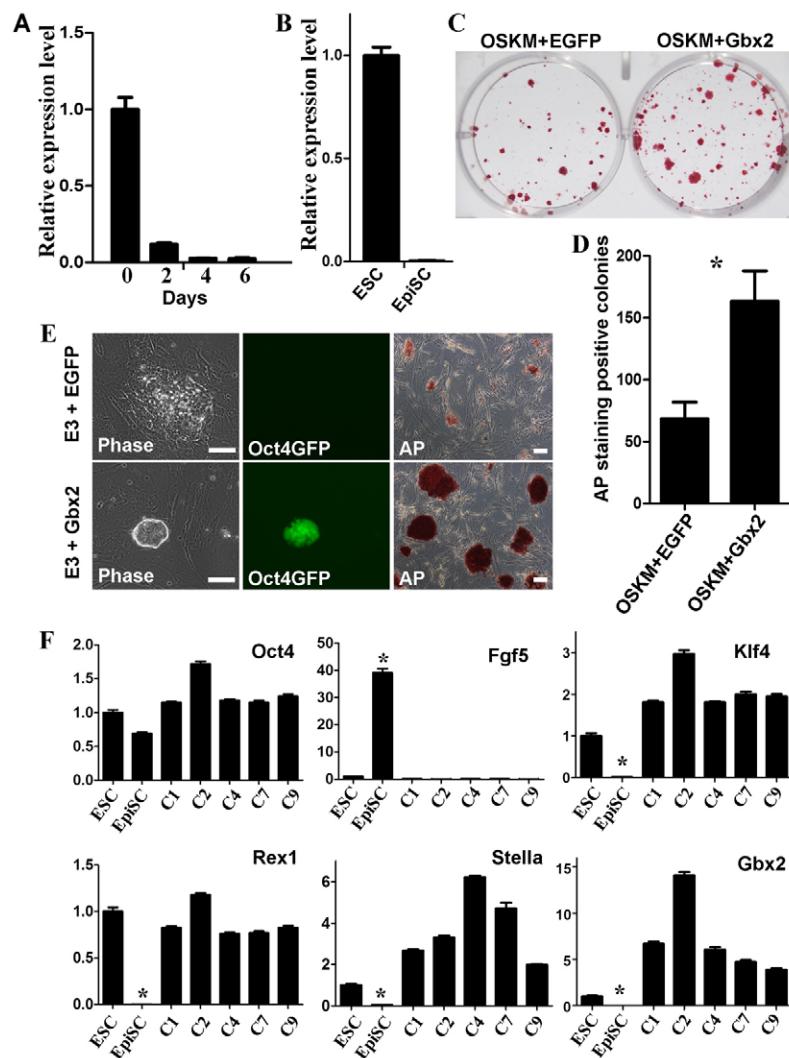


Fig. 4. *Gbx2* promotes reprogramming to the pluripotent ground state. (A) qRT-PCR analysis of *Gbx2* mRNA expression at different time points of embryoid body differentiation. (B) qRT-PCR analysis of *Gbx2* mRNA expression in 46C ESCs and EpiSCs. (C) AP staining conducted on MEFs on day 20 of OSKM reprogramming with EGFP or *Gbx2*. O, Oct4; S, Sox2; K, *Klf4*; M, c-Myc. (D) Quantitative analysis of colony formation of OSKM+EGFP- and OSKM+*Gbx2*-expressing cells. Error bars represent the s.d. ($n=2$). * $P<0.05$. (E) Oct4 expression and AP activity in E3 EpiSCs transduced to express EGFP or *Gbx2*. The images were taken 5 days after transfer to LIF plus 2i-supplemented medium. Scale bars: 50 μ m. (F) qRT-PCR analysis of expression of key markers in ESCs, EpiSCs and ESC-like cells. C1, C2, C4, C7 and C9 are the individual colonies of ESC-like cells. The y-axis is the relative expression normalized to that of ESCs. (A,B,D,F) Error bars represent the s.d. ($n=4$). * $P<0.01$.

rapidly during embryoid body formation (Fig. 4A), and was only negligibly detectable in epiblast stem cells (EpiSCs) derived from 46C ESCs (Fig. 4B; supplementary material Fig. S6). These results indicate that loss of the ESC state coincides with downregulation of *Gbx2* following removal of the LIF/Stat3 signal.

Because *Gbx2* is a transcription factor and is specifically expressed in the ESCs, we asked whether it could function as a reprogramming factor analogous to Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006; Yu et al., 2007). We transduced mouse embryonic fibroblasts (MEFs) to express all five of these factors and found that they exhibited higher reprogramming efficiency than controls expressing Oct4, Sox2, Klf4, c-Myc and EGFP, as indicated by the number of induced pluripotent stem cell (iPSC) colonies that formed (Fig. 4C,D). This result implies that *Gbx2* synergistically cooperates with the other four factors in the reprogramming process.

Recent reports have established that primed-state EpiSCs can be converted to naïve-state ESCs by activation of Stat3 signaling and induced expression of its downstream targets, such as Klf4 and Nanog (Guo et al., 2009; Yang et al., 2010; Theunissen et al., 2011). To test whether *Gbx2* is sufficient to mediate reprogramming of EpiSCs to a naïve pluripotent state, we used the lentiviral system to introduce the *Gbx2* transgene into 129 mouse strain-derived E3 EpiSCs carrying an *Oct4-GFP* transgene (Greber et al., 2010). After selection, the cells were cultured atop feeders in LIF plus 2i and within 5 days formed compact, dome-shaped colonies exhibiting strong AP activity, while wild-type E3 EpiSCs differentiated or died in the same condition (Fig. 4E). qRT-PCR analysis of the *Gbx2* transfectants showed *Oct4* expression and a typical ESC marker profile, with upregulation of *Rex1*, *Stella* and *Klf4*. Conversely, the expression of *Fgf5*, a marker for EpiSCs, was lost. The mRNA expression level of *Gbx2* in the transfectants was 4- to 14-fold higher than in ESCs (Fig. 4F). These data demonstrate that forced expression of *Gbx2* can direct EpiSCs back to naïve pluripotency.

Conclusions

Our approach in this study provides a platform to identify genuine LIF/Stat3 direct targets in mESCs. Some of the targets we detected, such as Klf4, Klf5 and Pim3, have been previously identified as direct Stat3 targets that have roles in self-renewal (Aksoy et al., 2007; Hall et al., 2009). Among other downstream targets identified in our study, *Gbx2* showed a unique expression pattern in mESCs and sufficiency to sustain expansion of mESCs in the absence of LIF over multiple passages. Overexpression of *Gbx2* can not only mimic the self-renewal-sustaining effect of activated LIF/Stat3, but also overcome the differentiation barrier to convert EpiSCs to naïve pluripotency.

Materials and Methods

ESC and EpiSC culture

46C ESCs were routinely maintained on gelatin-coated plates in GMEM (Sigma) containing 10% FBS (HyClone), 1 mM sodium pyruvate (Gibco), 100 μM non-essential amino acids (Gibco), 2 mM GLUTAMAX (Gibco), 1 μM β-Mercaptoethanol, and 1000 U/ml LIF (Stemgent), a formulation hereinafter referred to as ‘mESC medium’. R1 ESCs were maintained in the same condition but with feeders. *Stat3*^{-/-} ESCs were maintained with feeders in serum-free N2B27 medium supplemented with 3 μM CHIR99021 (Stemgent) and 1 μM PD0325901 (Stemgent) (Ying et al., 2008). EpiSC derivation and culture were conducted as described previously (Guo et al., 2009).

Microarray analysis

B6-Y118F ESCs starved in serum-free medium for 6 hours were treated with 30 ng/ml GCSF (Preprotech) or 1000 U/ml LIF plus 1 μM PD0325901 for 1 hour.

Total RNA was then extracted by TRIzol (Invitrogen) and submitted to the University of California, Los Angeles DNA Microarray core facility for analysis. The samples were hybridized to GeneChip Mouse Gene 1.0 ST Array. The data analysis was performed by using the Microarray Σ US program provided by the USC Norris Medical Library.

Gene transfection

The identified genes from microarray analysis were cloned individually into the pSIN-EF2-PURO lentiviral vector (Addgene). The primers used for cloning each gene are shown in supplementary material Table S2. 293T cells were cultured in DMEM containing 10% FBS. mESCs 80–90% confluent on 10 cm plates were transfected with 5 μg pSPAX2 (Addgene), 3 μg pVSVG (Addgene) and 8 μg of each pSIN-EF2 construct via LTX (Invitrogen). Six hours after transfection, the medium was replaced with mESC culture medium. The viruses were then collected and filtered after incubation for 48 hours.

Self-renewal assay

For lentiviral infection, mESCs were seeded 10⁵ cells per well in a 24-well plate, in 600 μl of medium composed of 300 μl filtered virus medium and 300 μl mESC medium with 4 μg/ml Polybrene (Millipore). After 12–18 hours, the cells were trypsinized and replated onto a six-well plate and cultured in the presence of 1 μg/ml puromycin for 48 hours. After selection, transduced ESCs were plated 10⁴ cells per 6 cm dish and subsequently cultured in mESC medium without LIF. Undifferentiated ESC colonies were isolated and propagated for further characterization.

Gbx2 reversion and chimera production

46C ESCs were electroporated with linearized pPyCAG-loxP-Gbx2-IRES-pac-STOP-loxP-eGFP. After electroporation, the cells were plated onto 6 cm culture dishes, in serum without LIF and selected in 1 μg/ml puromycin. Self-renewing colonies were isolated and then maintained for at least 20 days in the absence of added LIF. The Gbx2-IRES-pac-STOP cassette was then excised by transfecting the cells with a Cre recombinase expression plasmid. After 48 hours, GFP-positive cells were enriched by FACS sorting. Complete excision of the Gbx2 cassette was determined by the reacquisition of LIF dependence. The GFP-positive colonies were expanded and then microinjected into C57BL/6 blastocysts. Microinjected blastocysts were transferred to pseudo-pregnant CD1 mice.

qRT-PCR

Total RNA was extracted with TRIzol (Invitrogen). cDNA was synthesized with 1 μg of total RNA, using the QuantiTech Rev. Transcription Kit (Qiagen). qRT-PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. Signals were detected with an ABI7900HT real-time PCR System (Applied Biosystems). The relative expression level was determined by the 2-ΔCT method and normalized against *Gapdh*. The primers used for qRT-PCR are listed in supplementary material Table S2.

Immunostaining and AP staining

Immunostaining was performed according to a standard protocol. Primary antibodies used were the following: Oct4 (C-10, Santa Cruz, 1:200), SSEA-1 (480, Santa Cruz, 1:200), Klf4 (R&D Systems, 1:200) and Nanog (R&D Systems, 1:200). Alexa Fluor fluorescent secondary antibodies (Invitrogen) were used at a 1:2000 dilution. Nuclei were visualized with DAPI. AP staining was performed with an alkaline phosphatase kit (Sigma) according to the manufacturer’s instructions.

Gbx2 knockdown

shRNA-expressing plasmids were generated according to Addgene PLKO.1 protocol. The target-specific shRNA sequences used in this study are as follows: Control shRNA: 5'-AATTCTCGAACGTGTACGT-3'; Gbx2 shRNA#1: 5'-GGTTCGCTATTCGAAGTCATT-3'; Gbx2 shRNA#3: 5'-GAGAGCGATGTG-GATTACA-3'. After lentiviral infection, the cells were incubated with 1 μg/ml puromycin, 10 μg/ml Blasticidin S deaminase, or 100 μg/ml hygromycin for 48–72 hours. The drug-resistant cells were then replated onto a six-well plate, 5000 cells/well, and incubated for 5–7 days before AP staining.

Reprogramming

MEFs were transfected with the four factors using the piggyBac transposon system and LTX. The MEFs expressing the four factors were selected in 1 μg/ml puromycin for 2 days and then treated with Gbx2 or EGFP virus-containing medium for 18 hours before the medium was replaced. After incubation for 10 days in mESC medium supplemented with LIF, the cells were stained for AP and the number of positive colonies was tallied. EpiSCs expressing EGFP or Gbx2 following lentiviral transduction were selected with 1 μg/ml puromycin for 2 days and then plated at 3×10⁴ cells/well onto a six-well plate, in mESC medium supplemented with LIF plus 2i.

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Author contributions

C.I.T. performed all the experiments. C.I.T. and Q.L.Y. conceived and designed the study and wrote the manuscript.

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