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Increased efficiency in the generation of induced pluripotent stem cells by Fbxw7 ablation

(Short title: Promoted iPSC formation by Fbxw7 loss)

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Abbreviations: ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; UPS, ubiquitin-proteasome system; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; ATRA, all-*trans* retinoic acid; HSP, Heat shock protein

ABSTRACT

Induced pluripotent stem cells (iPSCs) share many biological properties with embryonic stem cells (ESCs), and are generated from somatic cells by expression of some transcriptional factors such as Oct3/4, Sox2, Klf4, and c-Myc. Among these factors, the abundance of c-Myc is strictly regulated by Fbxw7, a subunit of SCF-type ubiquitin ligase. We have now shown that the expression of Fbxw7 was increased as ESCs differentiated. To investigate the role of Fbxw7 in the ESCs/iPSCs, we examined the impact of Fbxw7 ablation in the efficiency in iPSC generation. The frequency of iPSC generation from mouse embryonic fibroblasts (MEFs) lacking Fbxw7 was markedly greater than that from control MEFs. Depletion of Fbxw7 also resulted in promotion of iPSC generation. Morphology of iPSC clonies from Fbxw7-depleted MEFs appeared more undifferentiated than that from MEFs overexpressing c-Myc. Additional depletion of c-Myc did not abrogate the effect of Fbxw7 depletion, suggesting that c-Myc accumulation is not necessarily required for the increased efficiency in iPSC generation by Fbxw7 ablation. Substrates of Fbxw7 other than c-Myc might therefore play a key role in iPSC generation. These results suggest that transient inhibition of Fbxw7 would be a more promising approach to efficient generation of iPSCs than c-Myc overexpression.

Key words: Fbxw7; c-Myc; induced pluripotent stem cell; reprogramming; ubiquitin proteasome system

Introduction

Embryonic stem cell (ESC)-like cells designated as induced pluripotent stem cells (iPSCs) are generated from somatic cells by introduction of the four transcription factors: Oct3/4 (also known as Pou5f1), Sox2, Klf4, and c-Myc (Takahashi & Yamanaka 2006; Maherali *et al.* 2007; Meissner *et al.* 2007; Okita *et al.* 2007; Wernig *et al.* 2007). Human iPSCs could be used to study pathogenesis of diseases, and potentially to treat patients suffering from incurable diseases by transplanting the regenerated grafts derived from their own cells. However, many iPSC-derived animals develop tumors as a result of the reactivation of the retrovirus encoding c-Myc (Okita *et al.* 2007), and this would represent a major safety concern if this approach is translated to humans. Although iPSCs can be generated without introduction of c-Myc (Nakagawa *et al.* 2008; Wernig *et al.* 2008), the efficiency of reprogramming process is markedly reduced without c-Myc. To solve this problem, considerable effort has been devoted to finding other factors or small molecules that facilitate the generation of iPSCs (Yoshida *et al.* 2009; Esteban *et al.* 2010; Nakagawa *et al.* 2010; Li *et al.* 2011; Maekawa *et al.* 2011).

c-Myc is a basic helix-loop-helix/leucine zipper (bHLH/Zip)-type transcription factor and plays a pivotal role in cellular proliferation. Expression of c-Myc and N-Myc is strictly regulated by the ubiquitin-proteasome system (UPS). In the UPS, ubiquitin polypeptides are covalently attached to lysine residues of the target protein by the concerted action of at least three enzymes: the ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). When a polyubiquitin chain is formed on the substrate protein, it is targeted for rapid proteolysis by the 26S proteasome (Nakayama

& Nakayama 2006). Fbxw7 (also known as Fbw7, SEL-10, hCdc4, or hAgo) is the F-box protein subunit of an Skp1-Cul1-F-box protein (SCF)-type E3 ubiquitin ligase complex and plays a central role in the degradation of members of the Myc family: c-Myc and N-Myc are ubiquitylated by SCF^{Fbxw7} in a manner dependent on phosphorylation of the Myc box 1 (MB1) domain (Welcker *et al.* 2004, Otto *et al*. 2009, Yada *et al.* 2004). L-Myc also contains the MB1 domain, suggesting that L-Myc is also a target of Fbxw7-mediated proteolysis (Adhikary & Eilers 2005).

In addition to transcriptional regulations, post-transcriptional controls such as microRNAs can also induce iPSCs (Miyoshi *et al.* 2011). These observations led us to the hypothesis that the UPS might play an important role in iPSC generation. We have now shown that downregulation of Fbxw7 promotes the reprogramming process. Surprisingly, this effect appears to be independent of c-Myc accumulation. These results thus suggest that transient inhibition of Fbxw7 is a useful and efficient method to generate iPSCs.

Results

Increased expression of Fbxw7 in the process of ESC differentiation

To examine the possible correlation between Fbxw7 expression and pluripotency of ESCs/iPSCs, we first compared the amount of Fbxw7 mRNA in undifferentiated ESCs and those treated with 2 μM ATRA to induce differentiation (Fig. 1A). The ESCs treated with ATRA showed a typical spindle-like morphology, indicative of neuronal differentiation. RT and real-time PCR analysis revealed that the abundance of Fbxw7 mRNA in differentiated ESCs was greater than that in undifferentiated ESCs, whereas undifferentiation markers Oct3/4 and Nanog were markedly downregulated as ESCs differentiated (Fig. 1B). These results thus suggested that Fbxw7 expression is increased during the process of ESC differentiation. This led us to the speculation that the abundance of Fbxw7 remains low to achieve high expression of c-Myc that maintains the stemness of undifferentiated ESCs, and that downregulation of Fbxw7 might recapitulate this situation to facilitate the reprogramming process during iPSC generation.

Fbxw7 depletion increases efficiency in iPSC generation

To test our hypothesis that the downregulation of Fbxw7 contributes to the reprogramming process, we compared the frequency of iPSC generation from *Fbxw7*$^{F/F}$ or *CAG-CreER*^{T2}*;Fbxw7*$^{F/F}$ MEFs that were exposed to 1 μM tamoxifen before OSK infection (Fig. 2A). Genomic PCR analysis revealed that the efficiency in tamoxifen-induced deletion of floxed *Fbxw7* alleles was almost complete (Fig. 2B). The number of colonies that were positive for alkaline phosphatase activity was counted: The efficiency of iPSC generation

from Fbxw7-ablated MEFs was more than twice as many as that from control MEFs (Fig. 2C,D). Furthermore, RT and real-time PCR analysis revealed that the iPSC colonies induced from Fbxw7-deficient MEFs expressed Nanog and Oct3/4 to a similar extent in ESCs (Fig. 2E), suggesting that the iPSCs formed from Fbxw7-deficient MEFs were indeed undifferentiated. It is also possible that the increase in the number of the iPSC colonies from Fbxw7-null cells might be attributable to faster iPSC formation.

We also examined the effect of RNAi-mediated Fbxw7 depletion on iPSC generation. To this end, Fbxw7 was depleted in wild-type MEFs by either of two independent shRNA vectors (shFbxw7-2 and shFbxw7-8) targeting different Fbxw7 sequences. Depletion efficiency was slightly greater in cells induced with shFbxw7-8 than those with shFbxw7-2 (28% vs. 38% of control, respectively, $P < 0.001$) (Fig. 3A). Similar to the results from Fbxw7 gene ablation by the Cre-loxP system, MEFs depleted of Fbxw7 by shFbxw7-2 or shFbxw7-8 also resulted in a marked increase in the frequency of iPSC generation compared with control cells (Fig. 3B). The levels of Nanog and Oct3/4 expression in iPSCs induced from Fbxw7-depleted MEFs were similar to those in ESCs or iPSCs induced by regular OSK infection (Fig. 3C). Collectively, these results suggested that downregulation of Fbxw7 greatly promotes the generation of iPSCs.

We next measured the abundance of the substrates that have been shown to be targeted by Fbxw7 including c-Myc, Notch1, mTOR, and c-Jun (Fig. 3D). Consistent with a previous study (Li *et al.* 2009) showing that OSK expression represses *Ink4/ARF* locus resulting in promotion of cell cycle progression, induction of OSK promoted expression of c-Myc, Notch1 and c-Jun. Depletion of Fbxw7 by shFbxw7-8 resulted in an increase in the abundance of c-Myc and c-Jun, whereas such effect was not evident in cells depleted with

shFbxw7-2.

Fbxw7-depleted iPSCs appear more undifferentiated than those overexpressing c-Myc

Given that c-Myc accumulated in Fbxw7-depleted iPSCs, we compared the characteristics in iPSCs formed from Fbxw7-depleted MEFs with those in iPSCs induced by OSKM infection. Immunoblot analysis revealed that the level of c-Myc expression was much smaller in Fbxw7-depleted iPSCs than in iPSCs induced by OSKM infection (Fig. 4A). Nevertheless, the number of iPSCs transfected with shFbxw7-8 was increased to a similar extent that was achieved in c-Myc-overexpressing MEFs (Fig. 4B). However, the morphology of iPSCs formed by OSK infection with Fbxw7 depletion was similar to that by OSK infection alone, whereas most iPSCs generated from c-Myc-overexpressing MEFs did not form colonies with a clear boundary, suggesting that c-Myc overexpression induces partially reprogrammed cells (Fig. 4C). Alkaline phosphatase staining revealed that the number of undifferentiated colonies was much less in c-Myc overexpressing iPSCs than that in Fbxw7-depleted iPSCs (Fig. 4D). We thus concluded that iPSCs formed by Fbxw7 downregulation quantitatively and qualitatively differ from those formed by c-Myc overexpression, and that Fbxw7 depletion is more advantageous for efficient generation of undifferentiated iPSCs than simple overexpression of c-Myc.

c-Myc accumulation is not necessary for increased generation of iPSCs by Fbxw7 depletion

Given that Fbxw7 targets not only c-Myc but also many other proteins that contribute to cellular proliferation for degradation, we examined the effect of additional c-Myc depletion

in MEFs that had been depleted of Fbxw7 on the efficiency in iPSC generation. c-Myc was depleted in MEFs by either of two independent shRNA vectors (shc-Myc-3 and shc-Myc-5) targeting different c-Myc sequences. RT and real-time PCR analysis revealed that the depletion efficiency was greater in cells induced with shc-Myc-3 than those with shc-Myc-5 (Fig. 5A), which was consistent with the results with immunoblot analysis (Fig. 5B). However, the number of iPSC colonies formed with OSK expression and Fbxw7 depletion did not differ among cells infected with vectors encoding control shRNA, shc-Myc-3 and shc-Myc-5 (Fig. 5C). Collectively, these results suggested that accumulation of the Fbxw7 substrates other than c-Myc is sufficient for increased generation of iPSCs.

Discussion

Our present study demonstrates that *Fbxw7* ablation markedly increases the efficiency in the generation of iPSCs similar to c-Myc overexpression. Unexpectedly, however, c-Myc depletion did not abrogate the effect of Fbxw7 depletion. In addition, the formed colony appears more undifferentiated when Fbxw7 is inhibited than when c-Myc is overexpressed. Fbxw7 inhibition is therefore a promising approach to the efficient generation of iPSCs.

Fbxw7 functions as the substrate-recognition subunit of an SCF-type ubiquitin ligase complex and targets for degradation various mammalian oncoproteins that promote cell cycle progression (Nakayama & Nakayama 2006; Welcker & Clurman 2008). These targets of Fbxw7 include cyclin E (Koepp *et al.* 2001; Moberg *et al.* 2001; Strohmaier *et al.* 2001), c-Myc (Welcker *et al.* 2004; Yada *et al.* 2004), Notch (Hubbard *et al.* 1997; Gupta-Rossi *et al.* 2001; Oberg *et al.* 2001; Wu *et al.* 2001), c-Jun (Nateri *et al.* 2004), mTOR (Mao *et al.* 2008), SREBP (Sundqvist *et al.* 2005), PGC-1α (Olson *et al.* 2008), Mcl-1 (Inuzuka *et al.* 2011; Wertz *et al.* 2011) and NF-κB2 (Busino *et al.* 2012; Fukushima *et al*. 2012). Given that most of these substrates promote cellular proliferation, Fbxw7 is thought to be a negative regulator of the cell cycle. Unlike our expectation that the effect of Fbxw7 depletion is mainly attributable to c-Myc accumulation, additional depletion of c-Myc in Fbxw7-depleted MEFs did not affect the promoted generation of iPSCs. These results thus suggest that accumulation of other substrates in Fbxw7-depleted cells might play a key role in iPSC generation (Fig. 6).

Regulation of the cell cycle is thought to play a key role in the maintenance and function of stem cells. One of the substantial differences between embryonic and adult tissue

stem cells is their dependency on quiescence: Maintenance of quiescence is thus thought to be more important for adult stem cells than for embryonic stem cells, the latter of which rapidly proliferate while maintaining their stemness. Fbxw7 was shown to be essential for the maintenance of adult hematopoietic stem cells (HSCs), and disruption of *Fbxw7* impairs the quiescence as well as stemness in the HSCs. Mutant mice with deletions in other genes for various cell cycle regulators also manifest defects in HSC maintenance and function (Kozar *et al.* 2004; Malumbres *et al.* 2004; Kalaszczynska *et al.* 2009).

Our observations implicate that *Fbxw7* ablation increases the stemness of ESCs/iPSCs. This effect of *Fbxw7* ablation on iPSCs is thus opposite to that on adult HSCs. Although the reason why Fbxw7 behaves differently in embryonic and adult stem cells remains largely unknown, several previous studies have suggested that Fbxw7 functions differently in distinct cell types. We and others have shown that T cell–specific ablation of Fbxw7 leads to overproliferation of immature T cells as a result of c-Myc accumulation (Onoyama *et al.* 2007), whereas Fbxw7 deficiency in bone marrow cells leads to apoptosis of hematopoietic stem cells, also as a result of c-Myc activation (Matsuoka *et al.* 2008; Thompson *et al.* 2008); mice with brain-specific deletion of *Fbxw7* exhibit enhanced astrogenesis in association with the accumulation of Notch1 and Notch3 (Matsumoto *et al.* 2011); ablation of Fbxw7 in the intestine leads to intestinal and colonic polyposis mediated by elevated Notch1, Notch3, and c-Jun (Babaei-Jadidi *et al.* 2011); and loss of Fbxw7 in the liver results in development of nonalcoholic steatohepatitis–like disease associated with the accumulation of SREBP1 (Onoyama *et al.* 2011). Given the variety of phenotypes induced by Fbxw7 ablation in different tissues, it is not necessarily surprising that the substrates targeted for degradation by Fbxw7 and the phenotypes of Fbxw7 deficiency differ between embryonic

and adult stem cells. The phenotypic differences between Fbxw7-deficient embryonic and adult stem cells are thus likely attributable to differences in the accumulating Fbxw7 substrates, corresponding kinases, or cellular responses to the accumulation of the substrates such as c-Myc. The details of the molecular mechanisms underlying these differences remain to be examined.

Although our study suggests that inhibition of Fbxw7 might represent a new approach to efficient iPSC generation, the application of Fbxw7 inhibition to human iPSC generation warrants careful consideration. Given that Fbxw7 has been regarded as an oncosuppressor protein because it targets many proto-oncoproteins, growth promoters, and antiapoptotic molecules (Nakayama & Nakayama 2006; Welcker & Clurman 2008), sustained suppression of Fbxw7 might induce carcinogenesis or promote cancer growth. Indeed, mice lacking Fbxw7 in hematopoietic cells or T cells exhibit ALL and T cell lymphoma, respectively (Onoyama *et al.* 2007; Thompson *et al.* 2007; Matsuoka *et al.* 2008). Moreover, in humans, mutations in Fbxw7 have been detected in individuals with ovarian cancer, breast cancer, lymphoma, or colorectal cancer (Nakayama & Nakayama 2006; Welcker & Clurman 2008). To minimize such risk, the inhibition of Fbxw7 should be limited for the transient use. Procedures such as siRNA-mediated depletion or a small compound that inhibits Fbxw7 function will be a promising approach to achieve such transient inhibition of Fbxw7 function.

Experimental Procedures

Mouse iPSC generation

Generation of iPSCs with the use of retroviruses was performed as described (Hong *et al.* 2009). Wild-type (*Fbxw7*$^{+/+}$), *Fbxw7*$^{F/F}$ or *CAG-CreER*^{T2}*;Fbxw7*$^{F/F}$ mouse embryonic fibroblasts (MEFs) were prepared as described previously (Nakayama *et al.* 1996). "F" means the allele flanked by loxP sites, and "CreER^{T2}" is a mutant estrogen receptor fused with Cre recombinase. For *Fbxw7* deletion, *CAG-CreER*^{T2}*;Fbxw7*$^{F/F}$ MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 1 μg/ml tamoxifen at 37°C for 48 h until a day before infection of retroviruses encoding Oct3/4, Sox2, and Klf4 (OSK).

ESC differentiation

ESCs were incubated in DMEM containing 15% FCS and 2 μM all-*trans* retinoic acid (ATRA) for 48 h from day 1 to day 3 after plating. The cells were harvested at the indicated days.

RT and real-time PCR analysis

Total RNA (1μg) isolated from cultured cells with use of ISOGEN (Nippon Gene) was subjected to reverse transcription (RT) with a QuantiTect Reverse Transcription kit (Qiagen). The resulting cDNA was subjected to polymerase chain reaction (PCR) with a StepOne Real Time PCR System (Applied Biosystems) and SYBR Premix Ex Taq (Takara). Data were analyzed according to the relative standard curve method and were normalized relative to the

amount of acidic ribosomal phosphoprotein (ARBP) mRNA. The primer sequences for RT-PCR analysis are listed in Table 1.

RNAi

The pMX-puro II vector was constructed by deletion of the U3 portion of the 3′ long terminal repeat of pMX-puro. The mouse U6 gene promoter, followed by DNA corresponding to an shRNA sequence, was subcloned into the NotI and XhoI sites of pMX-puro II, yielding pMX-puro II-U6/siRNA. The DNA for each shRNA encoded a 21-nucleotide hairpin sequence specific to the mRNA target, with a loop sequence (-TTCAAGAGA-) separating the two complementary domains, and it also contained a tract of six T nucleotides to terminate transcription. The resulting vectors were used to transfect Plat E cells and thereby to generate recombinant retroviruses. The sequences targeting mouse Fbxw7 mRNA are 5'-GGTTCTGAAGTTCGTTCCTTT-3' (shFbxw7-2) and 5'-GGATCTCTTGATACATCAATC-3' (shFbxw7-8), and those targeting mouse c-Myc mRNA are 5'-GCGACGAGGAAGAGAATTTCT-3' (shc-Myc-3) and 5'-GCTCTGCTCTCCATCCTATGT-3' (shc-Myc-5). A vector encoding shRNA targeting enhanced green fluorescent protein (EGFP) mRNA was used as a control.

Immunoblot analysis

Cells were lysed by incubation for 20 min at 4°C with lysis buffer (50 mM Tis-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 400 μM Na_3VO_4, 400 μM EDTA, 10 mM NaF, 10 mM Na pyrophosphate, 10 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride). The lysates were centrifuged at 20,400 × *g* for 20 min at 4°C,

and 20 μg amounts of protein from the supernatants were subjected to immunoblot analysis. Antibodies to cleaved Notch1 (Val^{1744}), to c-Jun, or to mammalian target of rapamycin (7C10) were obtained from Cell Signaling Technology, Inc. Antibodies to c-Myc and Heat shock protein (HSP) 90 were from Epitomics, Inc. and BD Biosciences, respectively.

Statistical analysis

Quantitative data are presented as means ± standard deviations (SD) and were analysed by one-way ANOVA, which was followed by multiple comparisons with the Tukey-Kramer test. A p value of <0.05 was considered statistically significant.

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Figure Legends

Fig. 1. Expression of Fbxw7 during the differentiation of mouse embryonic stem cells (ESCs). (**A**) Experimental strategy for differentiation of ESCs by treatment with all-*trans* retinoic acid (ATRA). (**B**) Quantitative RT-PCR analysis of Nanog, Oct3/4 and Fbxw7 mRNAs in undiffentiated ESCs (no treatment) or those exposed to ATRA at day 4 or day 8. Data are means ± SD from three independent experiments. $^{**}P < 0.01$.

Fig. 2. Increased efficiency in iPSC generation by Fbxw7 deletion. (**A**) Experimental strategy for iPSC generation from Fbxw7-deficient MEFs. (**B**) PCR analysis of genomic DNA from MEFs of the indicated genotypes. The positions of amplified fragments corresponding to floxed (*Flox*), and ΔE5 (exon 5-deleted) alleles are indicated. (**C**) Alkaline phosphatase staining of iPSCs in the 100-mm dishes 20 days after retroviral transduction. (**D**) Number of iPSC colonies positive for alkaline phosphatase from control and Fbxw7-deficient MEFs at 20 days after retroviral transduction. Data are from three independent experiments. $^{***}P < 0.001$. (**E**) RT and real-time PCR analysis of Nanog and Oct3/4 mRNA in ESCs, iPSCs and MEFs. Data are means ± SD from three independent experiments.

Fig. 3. Increased efficiency in iPSC generation by Fbxw7 depletion. (**A**) Quantitative RT-PCR analysis of Fbxw7 mRNA in MEFs infected with a retroviral vector encoding control (EGFP) or Fbxw7 shRNAs. Data are means ± SD from three independent experiments. $^{***}P < 0.001$. (**B**) Number of iPSC colonies from control and Fbxw7-depleted MEFs in a 100-mm dish at 19 days after retroviral infection. Data are from three independent experiments. $^{*}P <$

0.05, ***$P < 0.001$. (**C**) RT and real-time PCR analysis of Nanog and Oct3/4 mRNA in ESCs, iPSCs and MEFs. Data are means ± SD from three independent experiments. (**D**) Immunoblot (IB) analysis of Fbxw7 substrates and HSP90 (loading control) in extracts from MEFs at 4 days after the retroviral infection. mTOR, mammalian target of rapamycin.

Fig. 4. Comparison of Fbxw7-depleted iPSCs with c-Myc-overexpressing iPSCs. (**A**) Immunoblot (IB) analysis of c-Myc and HSP90 (loading control) in extracts from MEFs at 4 days after the retroviral infection. (**B**) Number of iPSC colonies from MEFs infected with indicated retroviruses in a 100-mm dish at 17 days after retroviral infection. Data are from three independent experiments. (**C**) Appearance of iPSCs infected with the indicated retroviruses at 17 days after retroviral infection. Scale bars: 500 μm. (**D**) Number of iPSC colonies positive for alkaline phosphatase from control and Fbxw7-depleted MEFs at 17 days after retroviral transduction. Data are from three independent experiments.

Fig. 5. Effect of additional depletion of c-Myc in Fbxw7-depleted iPSCs. (**A**) RT and real-time PCR analysis of c-Myc mRNA in MEFs infected with a retroviral vector encoding control (EGFP) or c-Myc shRNAs. Data are means ± SD from three independent experiments. ***$P < 0.001$. (**B**) Immunoblot (IB) analysis of c-Myc and HSP90 (loading control) in extracts from MEFs infected with a retroviral vector encoding control (EGFP) or c-Myc shRNAs. (**C**) Number of iPSC colonies from MEFs infected with indicated retroviruses in a 100-mm dish at 17 days after retroviral infection. Data are from three independent experiments.

Fig. 6. Model of the role of Fbxw7 in iPSC formation.

Figure 1

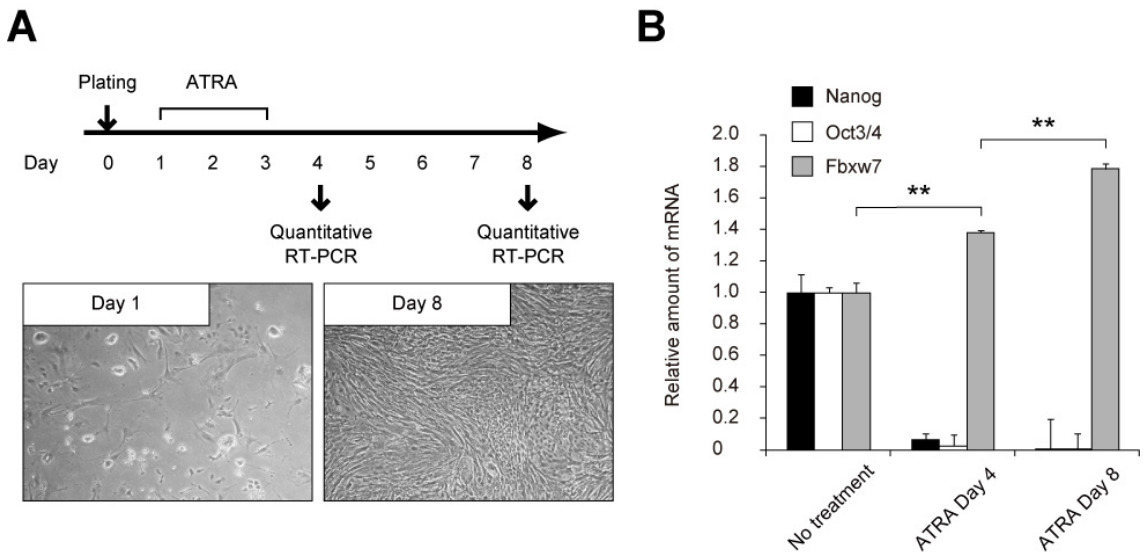
A
Plating
ATRA
Day 0 1 2 3 4 5 6 7 8
Quantitative RT-PCR
Quantitative RT-PCR
Day 1
Day 8
B
Nanog
Oct3/4
Fbxw7
Relative amount of mRNA
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
**
No treatment
ATRA Day 4
ATRA Day 8

Figure 2

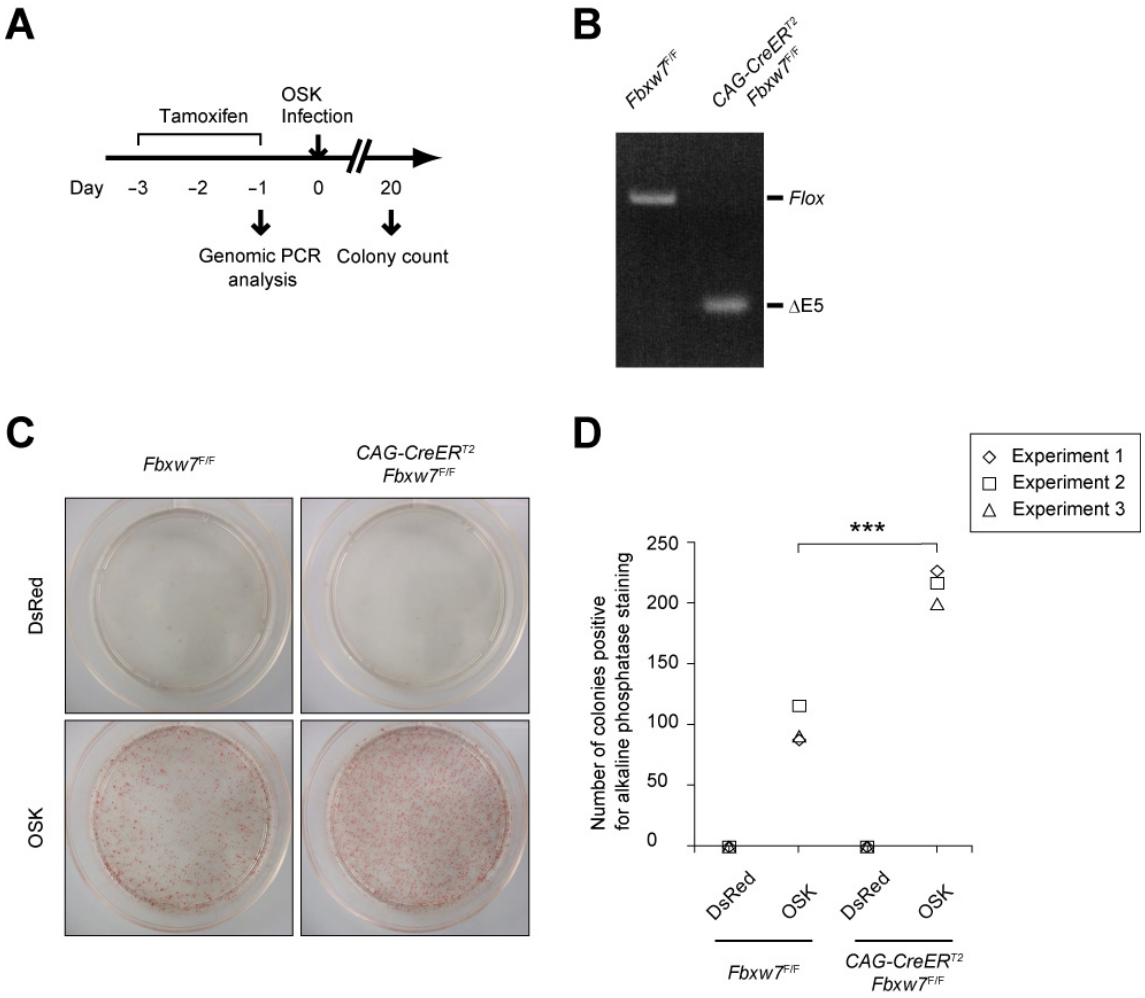
A
Tamoxifen
OSK Infection
Day −3 −2 −1 0 20
Genomic PCR analysis
Colony count
B
Fbxw7F/F
CAG-CreERT2 Fbxw7F/F
Flox
ΔE5
C
Fbxw7F/F
CAG-CreERT2 Fbxw7F/F
DsRed
OSK
D
Experiment 1
Experiment 2
Experiment 3
***
Number of colonies positive for alkaline phosphatase staining
250
200
150
100
50
0
DsRed
OSK
DsRed
OSK
Fbxw7F/F
CAG-CreERT2 Fbxw7F/F
E
Nanog
Oct3/4
Relative amount of mRNA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
ESC
CAG-CreERT2 Fbxw7F/F iPSC
MEF

Figure 3

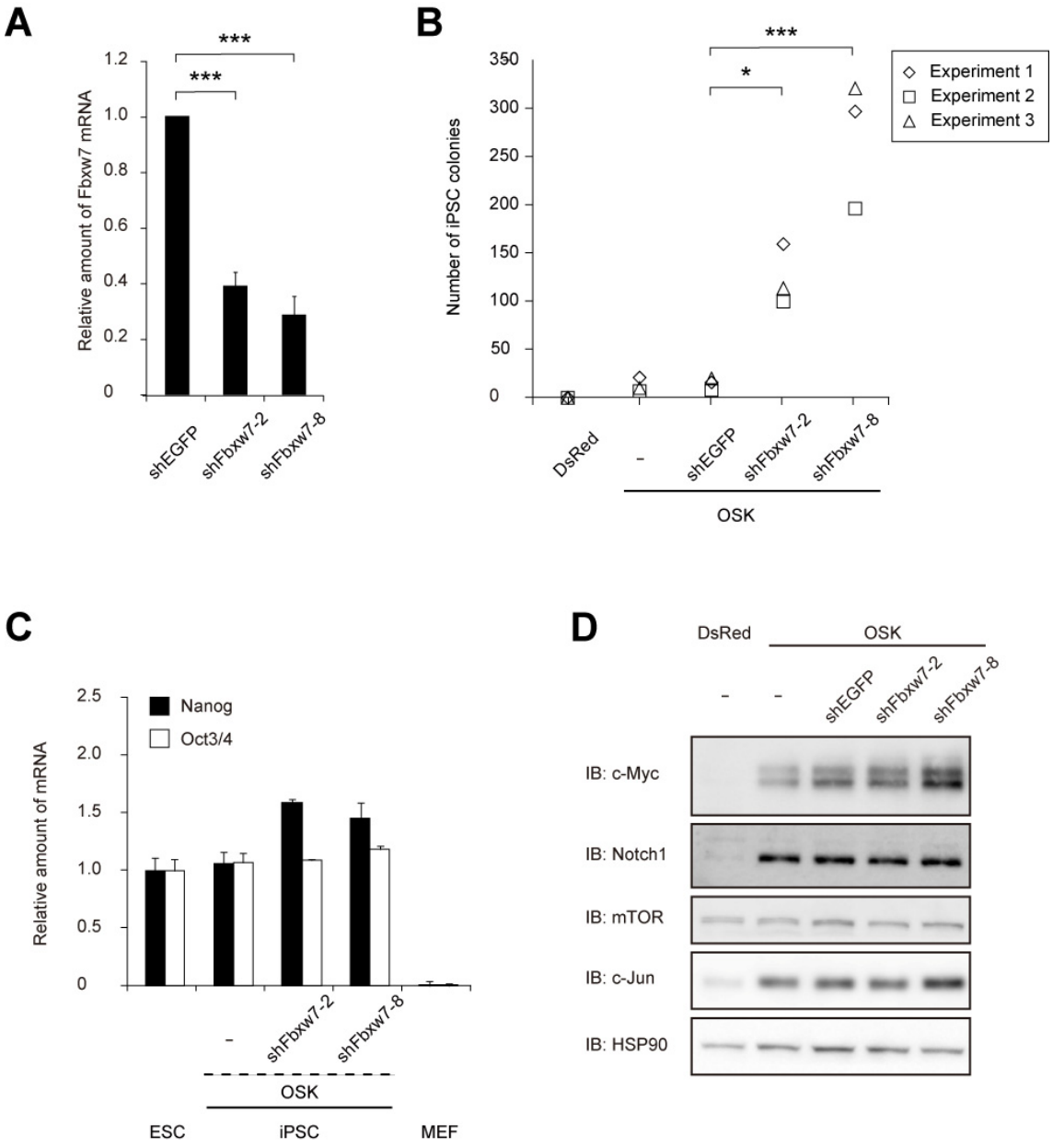
A
***
***
Relative amount of Fbxw7 mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0
shEGFP
shFbxw7-2
shFbxw7-8
B
***
*
Number of iPSC colonies
350
300
250
200
150
100
50
0
Experiment 1
Experiment 2
Experiment 3
DsRed
-
shEGFP
shFbxw7-2
shFbxw7-8
OSK
C
Nanog
Oct3/4
Relative amount of mRNA
2.5
2.0
1.5
1.0
0.5
0
-
shFbxw7-2
shFbxw7-8
OSK
ESC
iPSC
MEF
D
DsRed
OSK
-
-
shEGFP
shFbxw7-2
shFbxw7-8
IB: c-Myc
IB: Notch1
IB: mTOR
IB: c-Jun
IB: HSP90

Figure 4

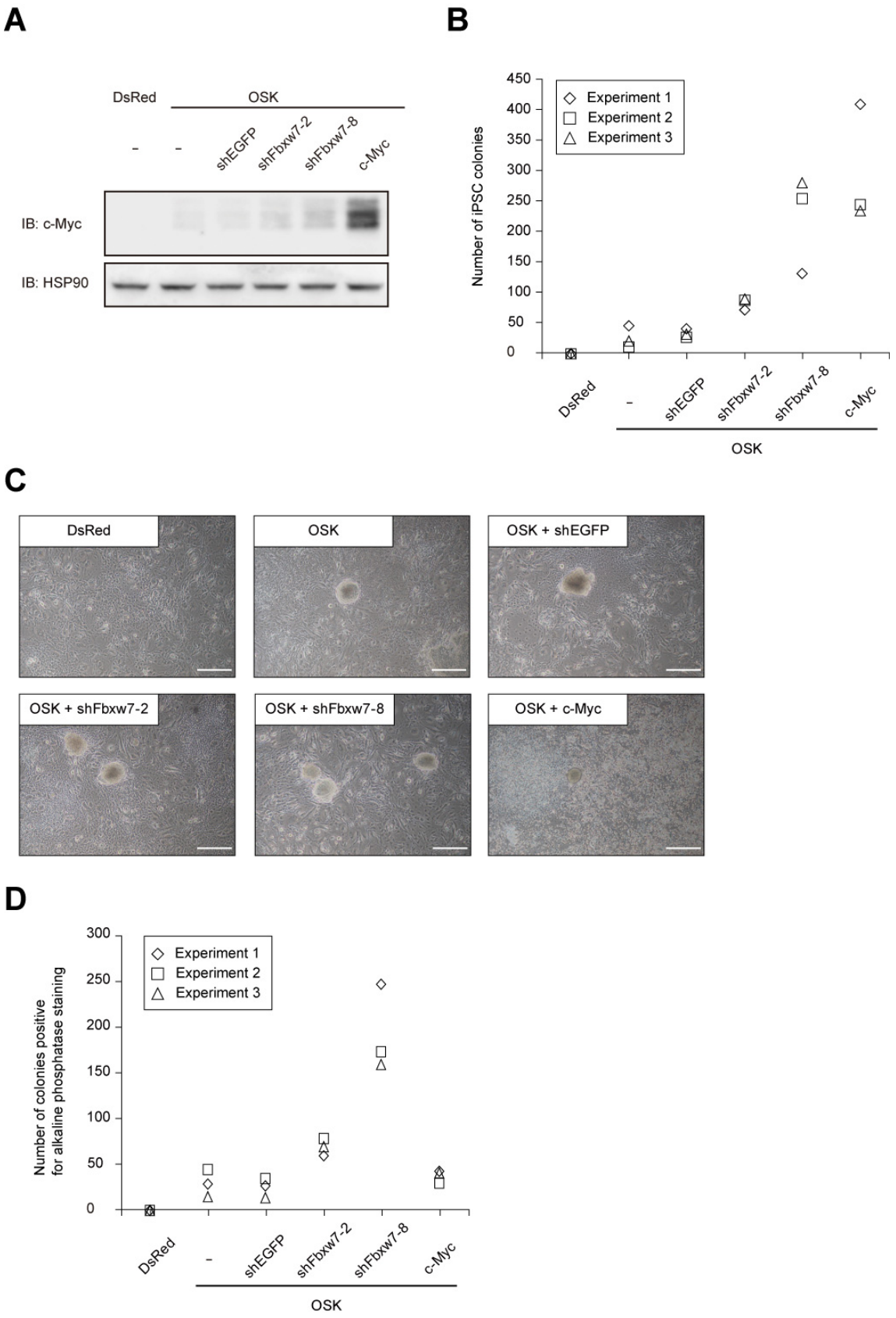
A
DsRed
OSK
-
-
shEGFP
shFbxw7-2
shFbxw7-8
c-Myc
IB: c-Myc
IB: HSP90
B
Number of iPSC colonies
Experiment 1
Experiment 2
Experiment 3
DsRed
-
shEGFP
shFbxw7-2
shFbxw7-8
c-Myc
OSK
C
DsRed
OSK
OSK + shEGFP
OSK + shFbxw7-2
OSK + shFbxw7-8
OSK + c-Myc
D
Number of colonies positive for alkaline phosphatase staining
Experiment 1
Experiment 2
Experiment 3
DsRed
-
shEGFP
shFbxw7-2
shFbxw7-8
c-Myc
OSK

Figure 5

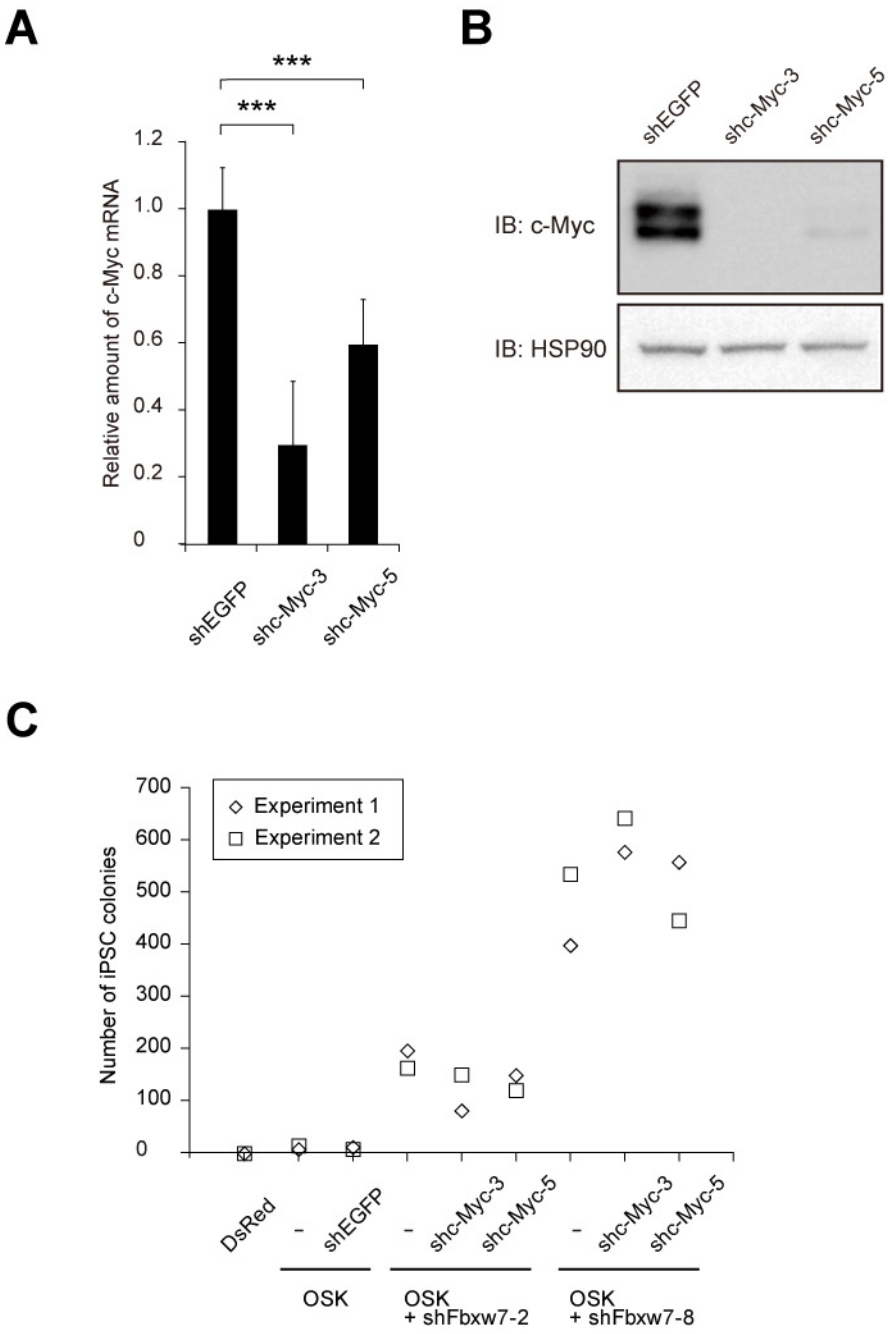
A
***
***
Relative amount of c-Myc mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0
shEGFP
shc-Myc-3
shc-Myc-5
B
shEGFP
shc-Myc-3
shc-Myc-5
IB: c-Myc
IB: HSP90
C
Number of iPSC colonies
700
600
500
400
300
200
100
0
Experiment 1
Experiment 2
DsRed
-
shEGFP
-
shc-Myc-3
shc-Myc-5
-
shc-Myc-3
shc-Myc-5
OSK
OSK
+ shFbxw7-2
OSK
+ shFbxw7-8

Figure 6

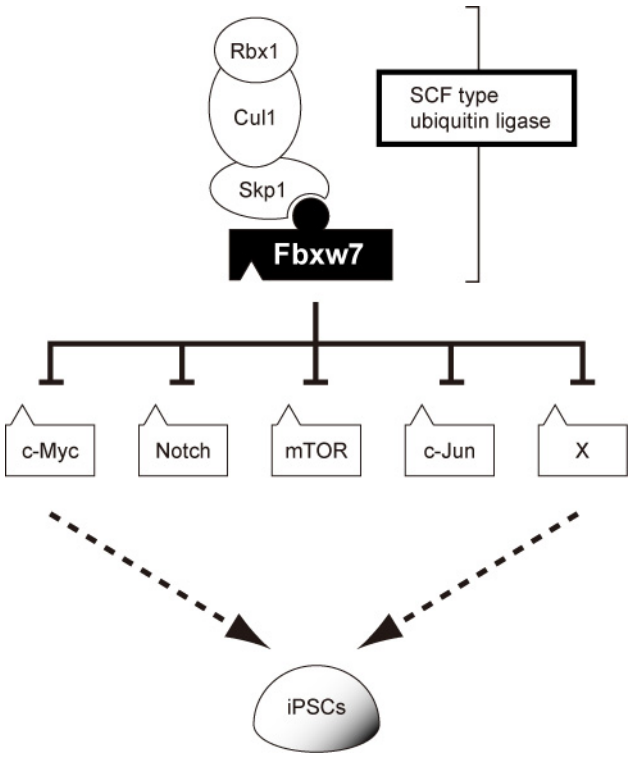
Rbx1
Cul1
Skp1
Fbxw7
SCF type
ubiquitin ligase
c-Myc
Notch
mTOR
c-Jun
X
iPSCs

Table 1 Primer sequences (5′→3′) for RT-PCR analysis.

Gene	Forward primer	Reverse primer
Nanog	AGGGTCTGCTACTGAGATGCTCTG	CAACCACTGGTTTTTCTGCCACCG
Oct3/4	CTGAGGGCCAGGCAGGAGCACGAG	CTGTAGGGAGGGCTTCGGGCACTT
Fbxw7	TGCAAAGTCTCAGATTATACC	ACTTCTCTGGTCCGCTCCAGC
c-Myc	ATGCCCCTCAACGTGAACTT	AATTCTCTTCCTCGTCGCAGAT
ARBP	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG