Regenerative Therapy 24 (2023) 443–450

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

Cbx7 promotes the generation of induced pluripotent stem cells

Chie-Hong Wang [a, b, c], Yi-Fang Huang [d, e, f], Woei-Cherng Shyu [b, c, 1, ***], Long-Bin Jeng [a, g, 1, **], Shih-Ping Liu [b, c, h, 1, *]

[a] Cell Therapy Center, China Medical University Hospital, Taichung 411, Taiwan
[b] Neuroscience and Brain Disease Center, College of Medicine, China Medical University, Taichung 411, Taiwan
[c] Translational Medicine Research Center, China Medical University Hospital, Taichung 411, Taiwan
[d] Department of General Dentistry, Linkou Chang Gung Memorial Hospital, No. 5, Fuxing Street, Gueishan Dist, Taoyuan City 333423, Taiwan
[e] School of Dentistry, College of Oral Medicine, Taipei Medical University, Taipei 110301, Taiwan
[f] Graduate Institute of Dental and Craniofacial Science, College of Medicine, Chang Gung University, Taoyuan City 333323, Taiwan
[g] Organ Transplantation Center, China Medical University Hospital, Taichung 404, Taiwan
[h] Program for Aging, College of Medicine, China Medical University, Taichung 404, Taiwan

ARTICLE INFO

Article history:
Received 16 May 2023
Received in revised form
22 August 2023
Accepted 7 September 2023

Keywords:
Cbx7
Induced pluripotent stem cells
Yamanaka factors

ABSTRACT

The iPS cells were discovered in 2006. With their ability to differentiate into cells of all three germ layers, iPS cells have great potential for clinical applications. Oct4, Sox2, c-Myc, and Klf4 were identified as the most effective factors for generating iPS cells. Despite this, iPS cells manufactured with these factors would still be inefficient. As a member of the chromobox family, chromobox protein homolog 7 (Cbx7) binds to PRC1 and PRC2 to inhibit genes involved in differentiation. A decrease in the expression of Cbx7 is observed during embryonic stem cell differentiation. Currently, no report discusses the role of Cbx7 in the production of iPS cells. In this study, we hypothesized that Cbx7 could increase iPS cell generation. We confirmed that Cbx7 is highly expressed in pluripotent stem cells (including ES and iPS cells). In addition, transfecting Cbx7 into fibroblasts increased Oct4, Sox2, c-Myc, and Klf4 expression. Moreover, we describe a novel approach to producing iPS cells using Cbx7 in combination with Oct4, Sox2, c-Myc, and Klf4. In summary, we have demonstrated that Cbx7 enhances the reprogramming of iPS cells and characterized the stemness and pluripotency of iPS cells.

1. Introduction

Because of the ability to differentiate into three-germ layer cells, pluripotent stem cells have long been considered to be one of the most powerful stem cells. Induced pluripotent stem cells (iPSCs), one kind of pluripotent stem cells, were first generated by Dr. Shinya Yamanaka's team at Kyoto University, Japan, in 2006 [1]. Several key factors required for pluripotency are also expressed in stem cells but not in somatic cells. Twenty-four genes previously identified by Yamanaka's team as critical factors in embryonic stem cells (ESCs) were delivered to mouse fibroblasts through retroviruses and removed one factor from twenty-four genes at a time to eventually identify Oct4, Sox2, cMyc, and Klf4 were necessary to generate iPSCs. Unfortunately, two of the four genes (c-Myc and Klf4) are oncogenic, and 20% of the chimeric mice developed cancer. In another study, Yamanaka reported that iPSCs were produced without c-Myc and the chimeras did not acquire cancer. Recently studies also indicated that iPSCs generation only uses Oct4 and Sox2 without oncogenes involvement [2,3]. Further, Bmi1 can replace Sox2, Klf4, and C-Myc during reprogramming, but Bmi1 was identified as a proto-oncogene that cooperates with c-Myc to promote the formation of B- and T-cell lymphomas [4]. It has been demonstrated that the Oct4 factor alone can reprogram human neural progenitor cells to pluripotency without the assistance of

*Corresponding author. Program for Aging, College of Medicine, China Medical University, Taichung 404, Taiwan.
** Corresponding author. Cell Therapy Center, China Medical University Hospital, Taichung 411, Taiwan.
*** Corresponding author. Translational Medicine Research Center, China Medical University Hospital, Taichung 411, Taiwan.
E-mail addresses: shyu9423@gmail.com (W.-C. Shyu), longbin.cmuh@gmail.com (L.-B. Jeng), spliu@mail.cmu.edu.tw (S.-P. Liu).
Peer review under responsibility of the Japanese Society for Regenerative Medicine.
[1] all authors contributed equally.

https://doi.org/10.1016/j.reth.2023.09.008

additional factors [5]. However, the iPS generation efficiency using only Oct4 was low [6].

Oct4 is an epigenetic regulator that plays critical roles in stem cell pluripotency, somatic cell reprogramming, and early development. There are several epigenetic regulatory pathways that Oct4 encompasses, including Polycomb repressive complex 1 (PRC1). A previous study also showed that Kdm2b promotes Oct4-induced somatic reprogramming through the recruitment of a variant PRC1 to CpG islands (CGIs) [7]. PRC1 belongs to polycomb group protein complexes and is a key regulator of chromatin structures that is required for segmental identity in the developing embryo [8]. The PRC1 and PRC2 maintained embryonic stem cell (ESC) pluripotency by silencing lineage-specifying developmental regulator genes [9]. PRC1 complex acts via chromatin remodeling and modification of histones. Histone modifiers inducing H3K27me3 and H3K4me3 have a key function in maintaining pluripotency is also regulated by PRC1 [10]. As a result, PRC1 components may be one of the key factors for the generation of iPS cells.

Cbx7 is the main CBX protein and a component of PRC1 that is expressed in embryonic stem cells (ESCs) and is highly expressed in other multipotent cells, such as hematopoietic stem cells [11–13]. Previously, it has been reported that Cbx7 is downregulated during ESC differentiation, and overexpression of Cbx7 increases self-renewal through its interaction with H3K27me3 to repress progenitor-specific genes in hematopoietic stem cells [11]. Conversely, Cbx7 knockdown induces ES cell differentiation and derepresses lineage-specific markers [13]. ESCs have shown that PRC1 and PRC2 localize to the promoters of a subset of genes encoding pluripotency-associated transcription factors (TFs) such as Oct4, Sox2, Nanog, and Sall4 [13]. Cbx7 involves in those pluripotency-associated TFs expression through promoter regulation is required to confirm.

However, there isn't real research or a discussion about the possibility of using Cbx7 as iPS cells generated factor. Herein, we first examined the hypothesis that Cbx7 could use to increase the iPS cell generation efficiency and might possess the reprogramming potential for turning somatic cells into iPS cells.

2. Materials and methods

2.1. Cell culture

We isolated MEFs from the uteri of pregnant mice (E13.5). Embryos were washed with phosphate-buffered saline (PBS) and the heads, hearts, and spinal cords were dissected. The remainder of the embryo was minced using scissors and forceps and digested with trypsin/EDTA solution (GIBCO BRL, Grand Island, NY, USA) at 37 °C for 3 min. After incubation, the sample was gently pipetted up and down several times to produce a single-cell suspension. Cells from each embryo were cultured in a T75 flask with fresh medium (Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, N.Y., USA) containing 10% fetal bovine serum (HyClone, Logan, Utah, USA), 2 mmol/l L-glutamine (GIBCO BRL), 100 μM non-essential amino acids (Invitrogen), and penicillin/streptomycin (Invitrogen)) at 37 °C and in a 5% CO2 incubator. MEFs collected within the first three passages were used. iPS cells were generated from our laboratory and published previously [14]. iPS cells were seeded on MEF feeder cell layers mitotically inactivated with 10 μg/ml mitomycin C (Sigma-Aldrich, St. Louis, Mo., USA). iPS cells were cultured in DMEM; containing 15% fetal bovine serum, 2 mmol/l L-glutamine, 100 μM non-essential amino acids, 100 μM 2-mercaptoethanol (Sigma-Aldrich), 10^3 units/ml leukemia-inhibiting factor (EMD Millipore Corporation, Temecula, California, USA), and penicillin/streptomycin.

2.2. Construction of plasmids expressing Oct4, Sox2, c-myc, Klf4 and Cbx7

The cDNA sequences of mouse Oct4, Sox2, c-Myc, Klf4, and Cbx7 were obtained from commercial clones (Thermo Fisher Scientific, Waltham, MA, USA). Plasmids containing the Oct4, Sox2, c-Myc, Klf4, and Cbx7 genes were digested by restriction enzymes to obtain cDNA fragments and then ligated into pcDNA 3.1 vector. The resulting pcDNA3.1-Oct4, pcDNA3.1-Sox2, pcDNA3.1-c-Myc, pcDNA3.1-Klf4, and pcDNA3.1-Cbx7 plasmids were used in the transfection experiments to generate the iPS cells.

2.3. Transfection

pcDNA3.1-Oct4, pcDNA3.1-Sox2, pcDNA3.1-c-Myc, pcDNA3.1-Klf4, and pcDNA3.1-Cbx7 plasmids were purified using Qiagen Megaprep Kit (Qiagen, Hilden, Germany). MEF cells were transfected with four plasmids (pcDNA3.1-Oct4, pcDNA3.1-Sox2, pcDNA3.1-c-Myc, and pcDNA3.1-Klf4) or five plasmids (pcDNA3.1-Oct4, pcDNA3.1-Sox2, pcDNA3.1-c-Myc, pcDNA3.1-Klf4, and pcDNA3.1-Cbx7) by using TransIT-X2® Dynamic Delivery System transfection reagent (Mirus Bio LLC, Madison, WI, USA). To explore the effects of Cbx7 on regulating Oct4, Klf4, c-Myc, and Sox2 expression, MEFs were transfected with pcDNA3.1-Cbx7. Real-time polymerase chain reaction analysis was performed 24 h after transfection (Applied Biosystems, Waltham, MA, USA) to detect Oct4, Klf4, c-Myc, and Sox2 expression levels.

2.4. iPS cells generation

As shown in Fig. 2A, we co-transfected pcDNA3.1-Oct4, pcDNA3.1-Sox2, pcDNA3.1-c-Myc, pcDNA3.1-Klf4, and pcDNA3.1-Cbx7 into MEF once every 2 days (four times in total). On day 9, the cells were gently dissociated and transferred onto mitomycin C-treated feeder-coated culture dishes and cultured until ES-like colonies appeared. As soon as the cells were seeded, their medium was changed to an iPS cell culture medium containing non-essential amino acids (0.1 mM), L-glutamine (2 mM), 2-Me (0.1 mM), and leukemia inhibitory factor (10^3 U/mL). In all subsequent experiments, we used the passaged cells for ES cell-like clones obtained on day 30 (21 days after passing on feeder cells).

2.5. Reverse transcription-PCR and real-time PCR

Total RNA was extracted using the TRIzol reagent (Thermo Fisher Scientific) and used to synthesize cDNA using an RT premix (Thermo Fisher Scientific) and oligo-dT (Thermo Fisher Scientific), according to the manufacturer's instructions. PCR was performed with ExTaq (Takara Bio, Japan) to detect the expression levels of the Nanog, Ecat1, Eras, Sox2, Oct4, Zfp, Dax1, Rex1, Gdf3, and Nat1. Real-time PCR was performed to determine the gene expression levels of Oct4, Klf4, c-Myc, and Sox2 with Fast SYBR™ Green Master Mix (Applied Biosystems, MA, USA) and StepOnePlus™ Real-Time PCR Environment (Applied Biosystems). Gapdh was also determined as an internal control. Primer sequences used for reverse transcription-PCR and real-time PCR are listed in Table 1.

2.6. Alkaline phosphatase (AP) staining and immunofluorescence analysis

AP staining was performed using the Alkaline Phosphatase Detection Kit (Millipore) according to the manufacturer's instructions. The cultured cells were fixed for 30 min at 37 °C with fixing solution I [4% paraformaldehyde plus 400 mM sucrose in phosphate-buffered saline (PBS)]. After fixing the slides with fixing

Table 1
RT-PCR primer sequences.

Reverse transcription PCR		
Gene	Forward Primer	Reverse Primer
Nanog	GAGCTATAAGCAGGTTAAGACC	TGCTGAGCCCTTCTGAAT
Ecat1	TGTGGGGCCCTGAAAGGCGAGCTGAGAT	ATGGGCCGCCATACGACGACGCTCAACT
Eras	ACTGCCCCTCATCAGACTGCTACT	CACTGCCTTGTACTCGGGTAGCTG
Sox2	TAGAGCTAGACTCCGGGCGATGA	TTGCCTTAAACAAGACCACGAAA
Oct4	TCTTTCCACCAGGCCCCCGGCTC	TGCGGGCGGACATGGGGAGATCC
Zfp	CCATTAGGGGCCATCATCGCTTTC	CACTGCTCACTGGAGGGGGCTTGC
Dax1	TGCTGCGGTCCAGGCCATCAAGAG	GGGCACTGTTCAGTTCAGCGGATC
Rex1	ACGAGTGGCAGTTTCTTCTTGGGA	TATGACTCACTTCCAGGGGGCACT
Gdf3	GTTCCAACCTGTGCCTCGCGTCTT	AGCGAGGCATGGAGAGAGCGGAGCAG
Nat1	ATTCTTCGTTGTCAAGCCGCCAAAGTGGAG	AGTTGTTTGCTGCGGAGTTGTCATCTCGTC
Real-time PCR		
Gene	Forward Primer	Reverse Primer
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Oct4	GCATTGAGAACCGTGTGA	GATTGGCGATGTGAGTGAT
Klf4	CCTTTCAGTGCCAGAAGT	ACTACGTGGGATTTAAAAGTGC
C-Myc	CATTCAAGCAGACGAGCA	CGAGTTAGGTCAGTTTATGCAG
Sox2	AGGGCTGGACTGCGAACT	TTTGCACCCCTCCCAATTC

solution II, they were held at room temperature for 15 min in a solution containing 0.5% Triton X-100. As soon as the slides were washed with PBS, they were treated with blocking buffer (0.5% BSA in PBS) at room temperature for 1 h and then washed three times with PBS before being incubated with various primary antibodies at 1:100 dilutions, either overnight at 4 °C or for 1 h at 37 °C. The antibodies used were anti-Cbx7 (Invitrogen, PA5-114951), anti-SSEA1 (Merck Millipore, MAB4301), anti-Oct4 (Genetex, GTX101497), and anti-Sox2 (Genetex, GTX101507). Following five rounds of cold PBS washing, slides were treated with either FITC-conjugated anti-mouse IgG (Sigma-Aldrich) or TRITC-conjugated antirabbit IgG (Sigma-Aldrich). After four more washes with cold PBS, slides were mounted and observed using a confocal fluorescence microscope (TCS-NT). DNA was stained to identify nuclei using DAPI (Sigma-Aldrich).

2.7. In vitro differentiation of iPS cells to three germ layers

Embryoid body (EB) formation was conducted by transferring iPS clumps to low attachment plates (Corning, NY, USA). After 2 days of culture, the medium was replaced by DMEM/F12 containing 20% knockout serum replacement (Thermo Fisher Scientific), 1× nonessential amino acids, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol and 1× Pen/Strep. At day 7, the EBs were plated on 0.5 μg/cm^2 vitronectin (Thermo Fisher Scientific) coated dishes and cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 1× Pen/Strep for up to three weeks. The following antibodies were used for ICC analyses on EBs fixed in 4% PFA for 15 min: anti-Gata4 (Genetex, CTX113194), anti-alpha-SMA (Merck Millipore, CBL171), and anti- Tuj-1 (Genetex, GTX631863).

2.8. Teratoma formation

iPS cells (10^6 cells/per mouse) were injected under the subcutaneous of Balb/c nude mice. The mice were sacrificed 4 weeks later and the teratomas were harvested. For histological analysis, tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The Institutional Animal Care and Use Committee at China Medical University approved all experimental protocols (CMUIACUC-2020-017).

2.9. Statistical analysis

Data were obtained from at least three independent experiments and compared for statistical significance using the Prism software (version 5.01, San Diego, CA, USA) at a significance level of $p < 0.05$ (*$p < 0.05$, and ***$p < 0.001$). All quantitative data were expressed as mean ± standard deviation (SD) and analyzed by Student's t-test.

3. Results

3.1. Cbx7 is highly expressed in pluripotent stem cells

We examined the expression levels of Cbx7 in MEF, iPSC, and ESC using immunofluorescence staining. As shown in Fig. 1A, Cbx7 was highly expressed in ESC and iPS cells as well as SSEA1, which is a surface marker of mouse ES and iPS cells. The average expression levels of Cbx7 in ES and iPS cells were 5–7 times higher than those in MEF, according to quantitative analysis. Further, SSEA1 expression was 13–30 times higher in ES and iPS cells than in MEF cells. (Fig. 1B).

3.2. Cbx7 promotes the generation of iPS cells

For the reprogramming of MEFs into iPS, plasmids containing Oct4, Sox2, c-Myc, Klf4, or Cbx7 genes were transduced into the MEFs (Fig. 2A) to generate two iPS clones, iPS-OKMS and iPS-OKMSC. Introducing four factors (Oct4, Sox2, c-Myc, Klf4) and five factors (Cbx7, Oct4, Sox2, c-Myc, Klf4) on day 30 (21 days post-seeding to feeder layers) resulted in a significant difference in iPS generation efficiency. Under the five-factors group (OKMSC), the number of ES-like colonies obtained was 1.6 times higher than under the four factors group (OKMS) (Fig. 2B). The results demonstrated that the expression of Cbx7 increases the generation of iPS cells.

3.3. Regulation of Yamanaka factors by Cbx7

The aforementioned data indicated that Cbx7 enhances the reprogramming efficiency of iPS cells with Yamanaka factors. To investigate whether Cbx7 might enhance Yamanaka factor

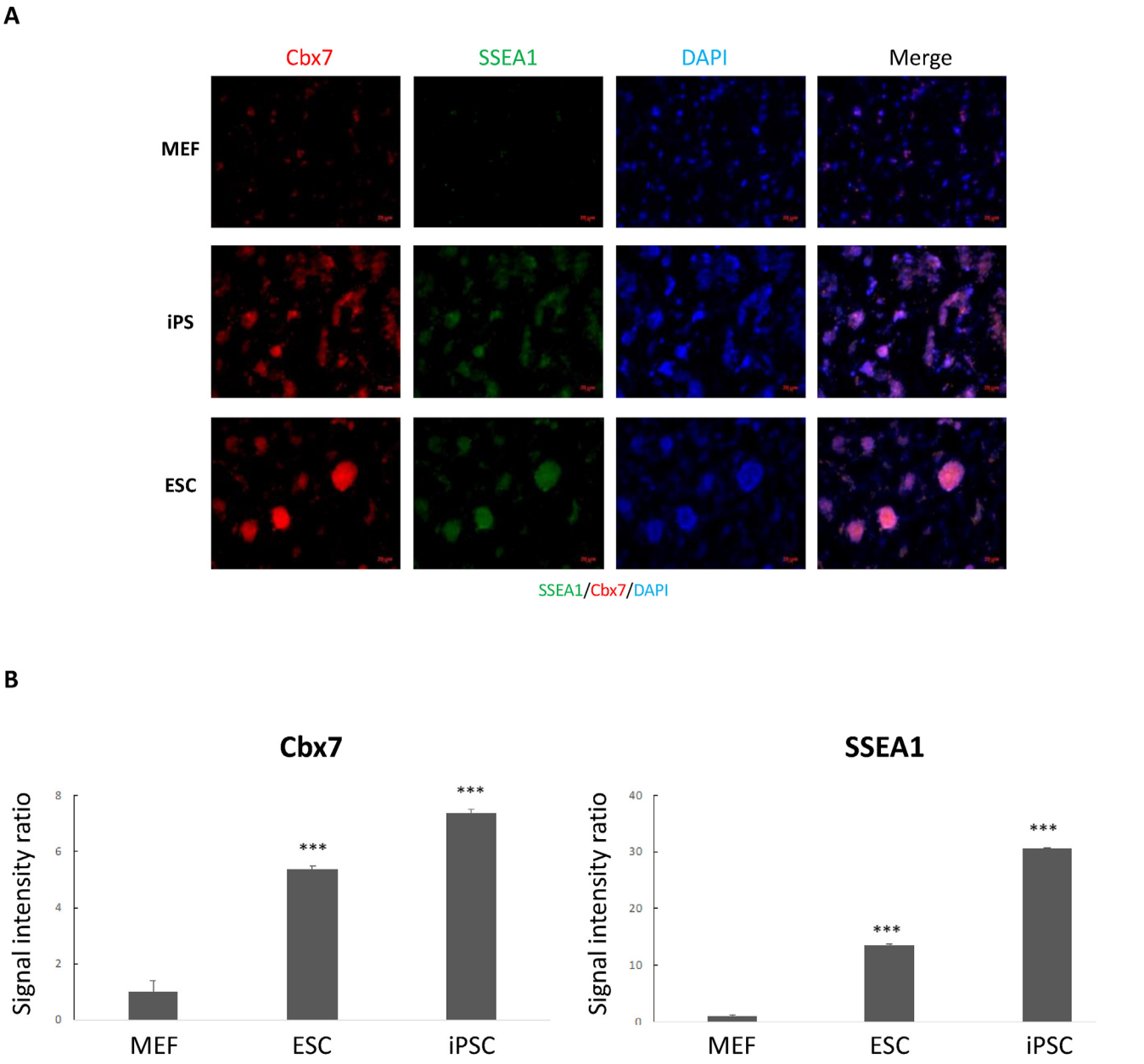

Fig. 1. The expression levels of Cbx7 and SSEA1 in MEF, iPSCs, and ESCs. (A) Immunofluorescent staining showed that Cbx7 and SSEA1 were highly expressed in iPSCs and ESCs, but not in MEFs. Digital images were taken at a magnification of 40×. (B) The quantitative results showed Cbx7 and SSEA1 levels significantly higher in iPSCs and ESCs than in MEFs. Bars represented mean and SD. Differences between MEF and ESC or iPSC groups evaluated by two-tailed Student's t-test. *$P < 0.05$ indicates statistical significance (***$P < 0.001$).

expression, we performed real-time PCR experiments in Cbx7-overexpressing MEFs to determine the levels of Oct4, Klf-4, C-Myc, and Sox2. We observed that MEFs expressing Cbx7 showed elevated expression levels of Oct4, Klf-4, C-Myc, and Sox2. As a result of the increased expression of Yamanaka factors, there was an average increase of two to three times (Fig. 3).

3.4. Verification of stem cell-specific gene expression levels in iPS-OKMS and iPS-OKMSC cells

We also determined the expression of several embryonic stem cell marker genes, including Nanog, Ecat1, Eras, Sox2, Oct4, Zfp, Dax1, Rex1, and Gdf3 in MEFs, iPS-OKMS cells, iPS-OKMSC cells, and ES cells by reverse transcription PCR (Fig. 4). Nat1 was used as a loading control. The expression of Nanog, Ecat1, Eras, Oct4, Zfp, Dax1, Rex1, and Gdf3 were detected in iPS-OKMS, iPS-OKMSC, and ES cells, but not in MEFs. Further, the expression levels of Ecat1, Sox2, Oct4, and Zfp were lower than those in ES cells. Collectively, these expression patents of embryonic stem cell markers demonstrated the pluripotency of iPS-OKMS and iPS-OKMSC cells.

3.5. Stem cell marker staining

These iPS-OKMS and iPS-OKMSC cells were cultured for more than 10 passages without spontaneous differentiation. As a result, these iPSC lines lost the morphology characteristics of fibroblasts and their morphology and growth dynamics resembled that of natural, *bona fide* mouse ES cells. First, we assessed the activity of alkaline phosphatase (ALP) in these iPS cells to confirm their stemness. The iPS-OKMSC cells formed typical ES-like colonies and

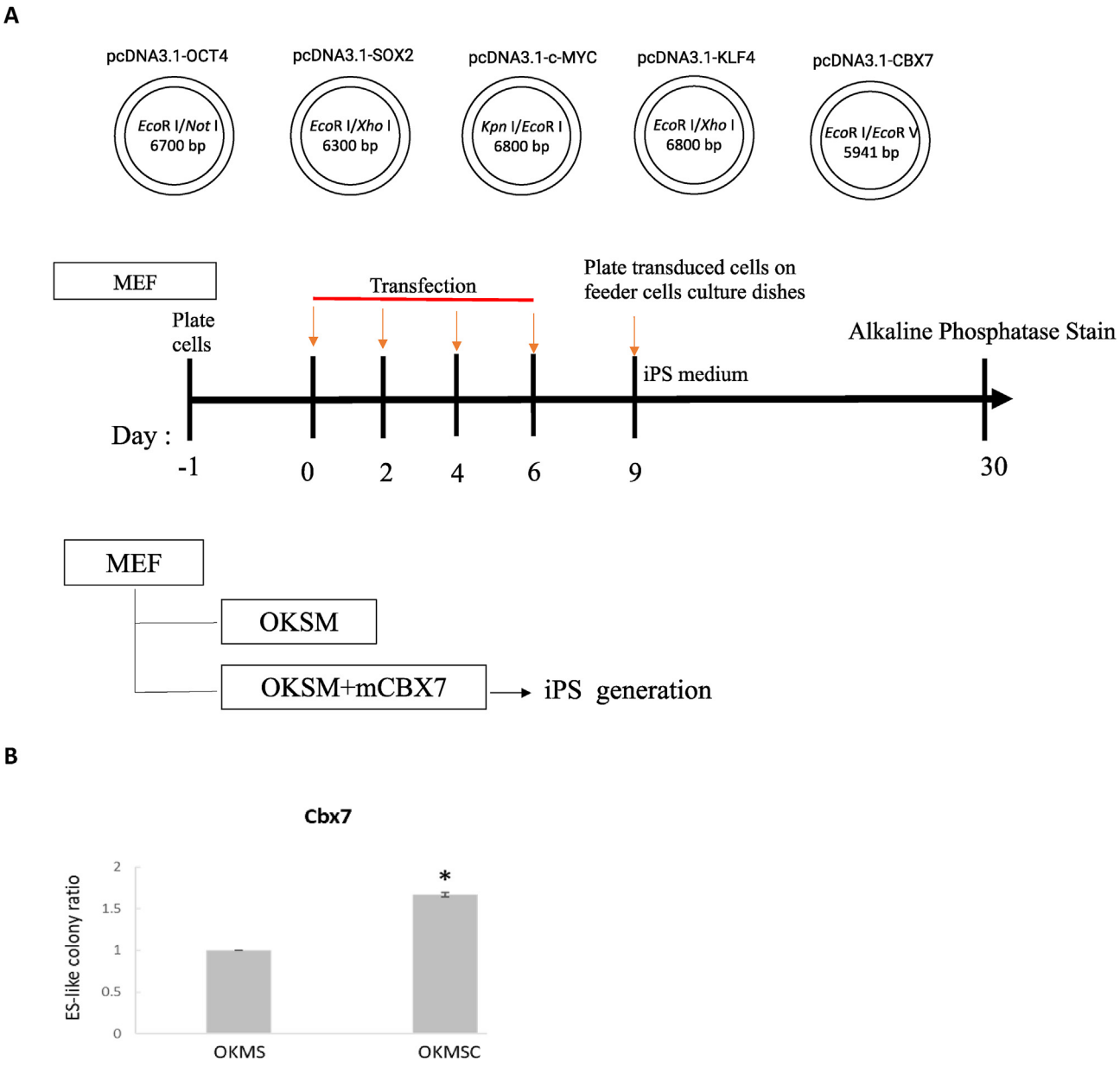

Fig. 2. Schematic illustration of the reprogramming protocol. (A) The reprogramming strategy of iPS-OKMS and iPS-OKMSC cells. (B) Comparison of the reprogramming efficiency of iPS-OKMS and iPS-OKMSC cells. The ES-like colonies were counted on day 30. Bars represented mean and SD. Differences between OKMS and OKMSC groups were evaluated by a two-tailed Student's t-test. *$P < 0.05$ indicates statistical significance.

exhibited positive staining for ALP as well as the iPS-OKMS cells (Fig. 5A). As a next step, immunofluorescent staining was used to determine the expression of stem cell marker genes in the iPS-OKMS and iPS-OKMSC cells. Both of the iPS cells were positively stained with antibodies against Oct4, Sox2, and SSEA1 (Fig. 5B). Images of immunofluorescence-stained Oct4, Sox2, and SSEA1 showed that these three markers were ubiquitously expressed in both iPS-OKMS and iPS-OKMSC cells. Based on these results, the iPS-OKMS and iPS-OKMSC cells could be identified as stem cells.

3.6. In vitro iPS cell pluripotency

In vitro embryoid body formation was used to determine the pluripotency of the iPS-OKMS and iPS-OKMSC cells. Experimental results show that iPS-OKMS and iPS-OKMSC cells form embryoid bodies in low-attached plates in vitro. After placing embryoid bodies on cell culture plates, the iPS-OKMS and iPS-OKMSC cells initiated differentiation. Using immunofluorescence staining, we then identified cells positive for Gata4 (a marker of the endoderm), alpha-smooth muscle actin (α-SMA, a marker of the mesoderm), and Tuj-1 (a marker of the ectoderm). The results in Fig. 6A indicated that iPS-OKMS and iPS-OKMSC cells differentiated into all three germ layer cells.

3.7. In vivo iPS cell pluripotency

To observe teratoma formation in vivo, we injected iPS-OKMS and iPS-OKMSC cells into the dorsal of nude mice. According to Fig. 6B, the cells differentiated into all three types of germ layer cells, including respiratory epithelial cells, gut epithelial cells,

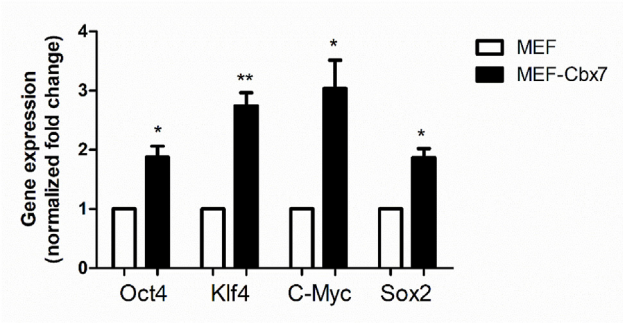

Fig. 3. Real-time PCR analysis of the expression levels of Yamanaka factors in Cbx7-overexpressing MEFs. The expression of Gapdh was detected as the reference gene. Bars represent mean and SD. The difference between groups was evaluated by a two-tailed Student's t-test. *$P < 0.05$ indicates statistical significance. (*$P = 0.01–0.05$; **$P = 0.001–0.01$).

muscle cells, cartilage cells, epidermal cells, and neural cells. Based on these data, it can be concluded that both iPS-OKMS and iPS-OKMSC cells are pluripotent in vitro and in vivo.

4. Discussion

A revolutionary technology was developed 17 years ago in 2006, called iPS [15]. IPS cells are still mostly generated using Yamanaka factors (Oct4, Sox2, c-Myc, and Klf4) today. An iPS clinical trial published in 2017 also used Yamanaka factors to generate iPS cells to treat macular degeneration [16]. Genes that may be useful in generating iPS have not yet been identified. Even though several studies have used genes other than Yamanaka factors to generate iPS cells [17,18], no polycomb-group gene has been published for generating iPS cells.

Cell fate decisions are regulated by polycomb group proteins during embryogenesis [12]. It has been found that five different Cbx proteins (Cbx2, Cbx4, Cbx6, Cbx7, and Cbx8) are associated with Polycomb repressive complex 1 (PRC1) in mammals. During the differentiation of ESC, Cbx7 is downregulated, and when it is overexpressed in hematopoietic stem cells, it enhances self-renewal through the binding of H3K27me3 to repress progenitor-specific genes [11]. Additionally, Morey et al. found that distinct Cbx-associated PRC1 complexes regulate mouse ESC pluripotency and differentiation [12]. A previous study indicated that Cbx7 is primarily responsible for maintaining pluripotency in ESCs [12]. Knocking down Cbx7 leads to the differentiation and the derepression of lineage-specific markers in ES cells [13]. As shown in Fig. 1, Cbx7 was highly expressed in both pluripotent stem cells (iPS and ES cells). Based on these data, Cbx7 may be an important factor in generating iPS cells. We generated the iPS cells by combining Cbx7 with Yamanaka factors (Oct4, Sox2, c-Myc, and Klf4). As compared to using Yamanaka factors alone, the generation efficiency was much higher. As a result of published studies, we hypothesized that Cbx7 plays a role in the methylation of differentiation-related genes and inhibits their expression.

In clinical applications, the goal is to establish a safe strategy for reprogramming somatic cells without the presence of oncogenic

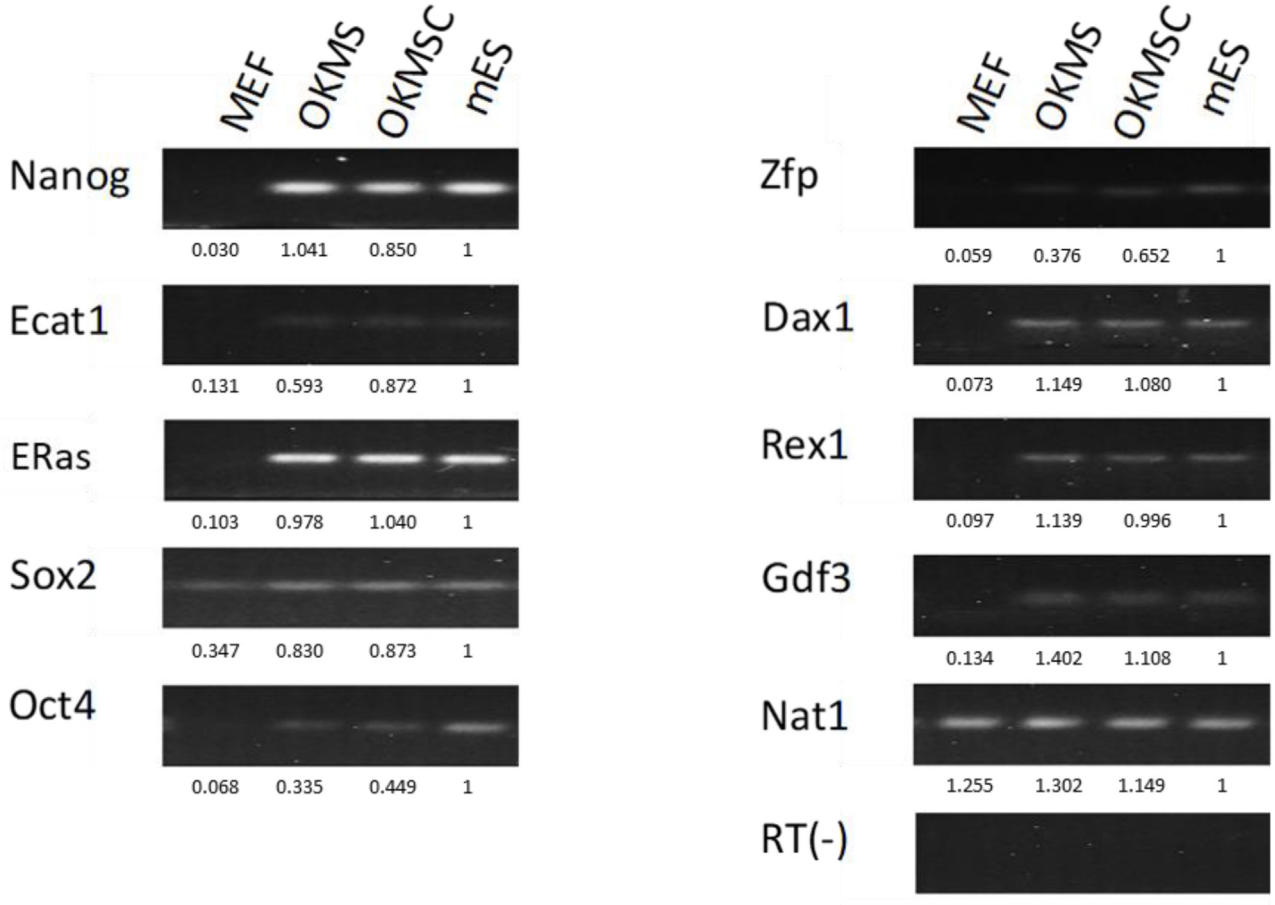

Fig. 4. RT-PCR analysis of stem cell-related genes in MEFs, iPS-OKMS cells, iPS OKMSC cells, and mouse ESCs. The expression levels were normalized to the mES group. RT(-): real-time PCR negative control. The signal was quantified with ImageJ software.

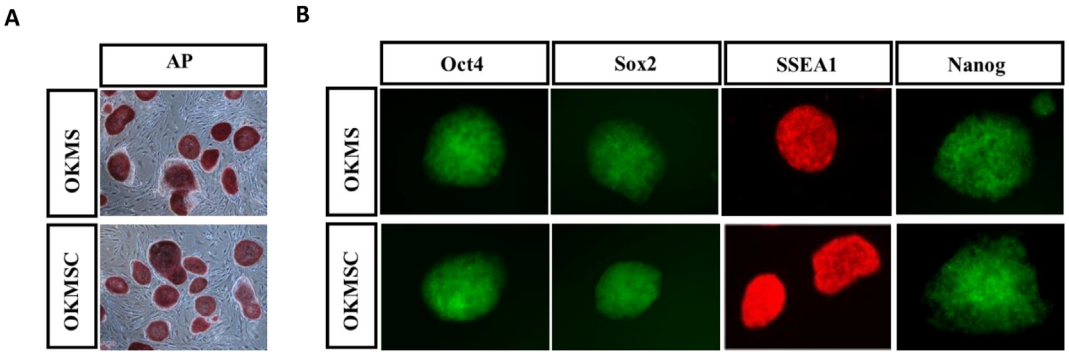

Fig. 5. The alkaline phosphatase staining and immunofluorescent staining of iPS-OKMS and iPS-OKMSC cells. (A) The activities of alkaline phosphatase were detected by immunocytochemistry staining in iPS-OKMS and iPS-OKMSC cells. Digital images were taken at a magnification of 100×. (B) The stem cell markers (Oct4, Sox2, and SSEA1) were explored with immunofluorescent staining. Digital images were taken at a magnification of 200×.

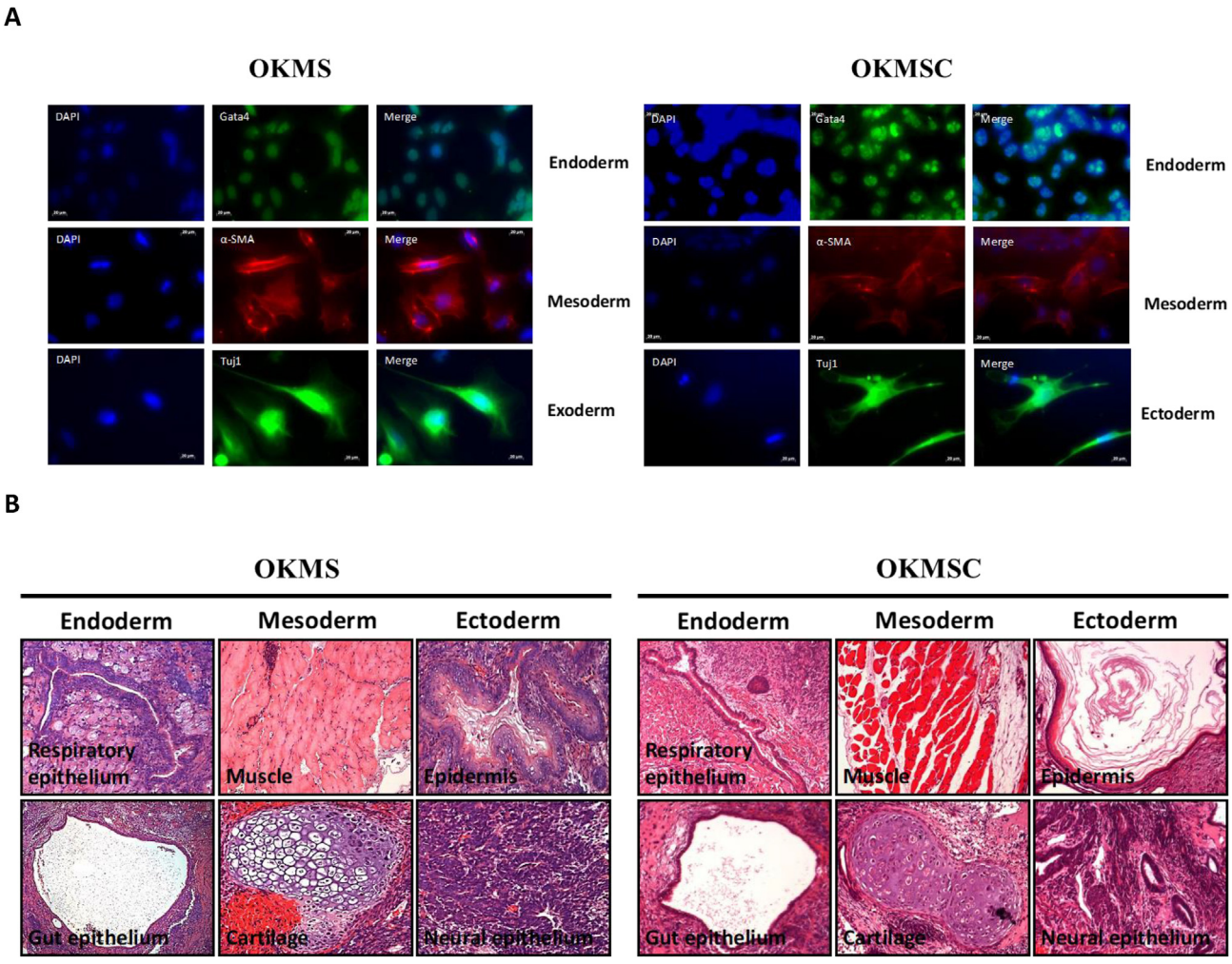

Fig. 6. Pluripotency assessment of iPS-OKMS and iPS-OKMSC cells. (A) iPS-OKMS and iPS-OKMSC cells were grown into embryoid bodies and seeded onto 0.1% gelatin-coated cell culture plates containing a differentiation medium. The expression of markers specific for the three germ-layer in EB-outgrowth cells was visualized by immunofluorescent staining: Gata4 (endoderm marker), alpha-smooth muscle actin (alpha-SMA) (mesoderm marker), and Tuj-1 (ectoderm marker). Nuclei were stained with DAPI (blue). Digital images were taken by a fluorescent microscope at a magnification of 400×. (B) The stem cell pluripotency was evaluated by in vivo teratoma formation assay. HE staining results indicated that iPS-OKMS and iPS-OKMSC cells possessed the ability to differentiate into all the 3-germ layer cells. Digital images were taken at a magnification of 200×.

factors. Several types of human cancers downregulate the polycomb group protein encoded by CBX7. Overall, our data indicated that co-transfection of Cbx7 with Yamanaka factors in mouse embryonic fibroblasts increased the reprogramming efficiency of iPS cells by up-regulating the expression of Oct4, Klf4, C-Myc, and Sox2.

Data availability

All of the data used to support the findings of this study are included in the article.

Author's contributions

Chie-Hong Wang wrote the manuscript. Yi-Fang Huang performed the experiments. Woei-Cherng Shyu performed the experiment and data analysis. Woei-Cherng Shyu, Long-Bin Jeng, and Shih-Ping Liu performed the conception and design. Shih-Ping Liu was also responsible for the manuscript as it moves through the entire publication process. The authors have read and approved the final manuscript.

Funding

This study is supported in part by the National Science and Technology Council, Taiwan (MOST 111-2320-B-039-051-), the National Science and Technology Coucil, Taiwan (NSTC 112-2320-B-039-051), and China Medical University Hospital, Taiwan (DMR-112-104).

Declaration of competing interest

The authors have no conflicts of interest to declare.

References

[1] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126(4):663–76. https://doi.org/10.1016/j.cell.2006.07.024. S0092-8674(06)00976-7 [pii].

[2] Giorgetti A, Montserrat N, Aasen T, et al. Generation of induced pluripotent stem cells from human cord blood using OCT4 and SOX2. Cell Stem Cell 2009;5(4):353–7. https://doi.org/10.1016/j.stem.2009.09.008.

[3] Montserrat N, Ramirez-Bajo MJ, Xia Y, et al. Generation of induced pluripotent stem cells from human renal proximal tubular cells with only two transcription factors, OCT4 and SOX2. J Biol Chem 2012;287(29):24131–8. https://doi.org/10.1074/jbc.M112.350413.

[4] Moon JH, Heo JS, Kim JS, et al. Reprogramming fibroblasts into induced pluripotent stem cells with Bmi1. Cell Res 2011;21(9):1305–15. https://doi.org/10.1038/cr.2011.107.

[5] Kim JB, Greber B, Arauzo-Bravo MJ, et al. Direct reprogramming of human neural stem cells by OCT4. Nature 2009;461(7264). https://doi.org/10.1038/nature08436. 649-643.

[6] Liu SP, Fu RH, Huang YC, et al. Induced pluripotent stem (iPS) cell research overview. Cell Transplant 2011;20(1):15–9. https://doi.org/10.3727/096368910X532828.

[7] Zhou Z, Yang X, He J, et al. Kdm2b regulates somatic reprogramming through variant PRC1 complex-dependent function. Cell Rep 2017;21(8):2160–70. https://doi.org/10.1016/j.celrep.2017.10.091.

[8] Grossniklaus U, Paro R. Transcriptional silencing by polycomb-group proteins. Cold Spring Harbor Perspect Biol 2014;6(11):a019331. https://doi.org/10.1101/cshperspect.a019331.

[9] Schoenfelder S, Sugar R, Dimond A, et al. Polycomb repressive complex PRC1 spatially constrains the mouse embryonic stem cell genome. Nat Genet 2015;47(10):1179–86. https://doi.org/10.1038/ng.3393.

[10] Delgado-Olguin P, Recillas-Targa F. Chromatin structure of pluripotent stem cells and induced pluripotent stem cells. Brief Funct Genomics 2011;10(1):37–49. https://doi.org/10.1093/bfgp/elq038.

[11] Klauke K, Radulovic V, Broekhuis M, et al. Polycomb Cbx family members mediate the balance between haematopoietic stem cell self-renewal and differentiation. Nat Cell Biol 2013;15(4):353–62. https://doi.org/10.1038/ncb2701.

[12] Morey L, Pascual G, Cozzuto L, et al. Nonoverlapping functions of the Polycomb group Cbx family of proteins in embryonic stem cells. Cell Stem Cell 2012;10(1):47–62. https://doi.org/10.1016/j.stem.2011.12.006.

[13] O'Loghlen A, Munoz-Cabello AM, Gaspar-Maia A, et al. MicroRNA regulation of Cbx7 mediates a switch of Polycomb orthologs during ESC differentiation. Cell Stem Cell 2012;10(1):33–46. https://doi.org/10.1016/j.stem.2011.12.004.

[14] Liu SP, Fu RH, Wu DC, et al. Mouse-induced pluripotent stem cells generated under hypoxic conditions in the absence of viral infection and oncogenic factors and used for ischemic stroke therapy. Stem Cell Dev 2014;23(4):421–33. https://doi.org/10.1089/scd.2013.0182.

[15] Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131(5):861–72. https://doi.org/10.1016/j.cell.2007.11.019. S0092-8674(07)01471-7 [pii].

[16] Mandai M, Watanabe A, Kurimoto Y, et al. Autologous induced stem-cell-derived retinal cells for macular degeneration. N Engl J Med 2017;376(11):1038–46. https://doi.org/10.1056/NEJMoa1608368.

[17] Heng JC, Feng B, Han J, et al. The nuclear receptor Nr5a2 can replace Oct4 in the reprogramming of murine somatic cells to pluripotent cells. Cell Stem Cell 2010;6(2):167–74. https://doi.org/10.1016/j.stem.2009.12.009. S1934-5909(09)00632-8 [pii].

[18] Rais Y, Zviran A, Geula S, et al. Deterministic direct reprogramming of somatic cells to pluripotency. Nature 2013;502(7469):65–70. https://doi.org/10.1038/nature12587.