Effects of hypoxia inducible factors on pluripotency in human iPS cells

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Abstract
A hypoxic condition is known to contribute to pluripotency. In the present article, the effects of transcription factors were first assessed regarding the proliferation and differentiation of human induced pluripotent stem (iPS) cells under hypoxic conditions using cell morphology and real-time polymerase chain reaction (RT-PCR). Morphology evaluations and RT-PCR revealed that the colony formation was promoted and the expression of pluripotent markers was increased under hypoxic conditions. In addition, the function of hypoxia inducible factors (HIFs) in human iPS cells under hypoxic conditions was evaluated in relation to the morphology and the expression of pluripotency markers by siRNA and RT-PCR. The HIF-2α silencing group showed a reduction in the colony size of human iPS cells and a statistically significant reduction in the expression of undifferentiation markers compared to the control group. Furthermore, the expression of HIF-2α was decreased when signal transducer and activator of transcription 3 (STAT3) was suppressed by its inhibitor, Stattic or S31 201. The inhibition using Stattic did not produce colony formation. The expression of pluripotent markers was also decreased using Stattic or S31 201. This study indicates that the HIF-2α expression in human iPS cells was activated under hypoxic conditions, similarly to that in murine iPS cells, and that HIF-2α among HIFs is the most effective compound for maintaining the pluripotency of human iPS cells. Furthermore, the STAT3 signal pathway regulates the expression of HIF-2α.

KEYWORDS
pluripotency marker, hypoxic condition, STAT3, transcription factors

1 | INTRODUCTION

Hypoxia inducible factors (HIFs) contribute to the transcriptional networks controlling stemness under hypoxic conditions (Bertout, Patel, & Simon, 2008). Hypoxia induces dental stem cells to differentiate into cementoblasts in an HIF-1α-dependent manner (Choi et al., 2014). HIF-2α regulates the pluripotency of human embryonic stem cells and enhances stemness through binding to the Nanog promoter (Forristal, Wright, Hanley, Oreffo, & Houghton, 2010; Petruzelli, Christensen, Parry, Sanchez-Elsner, & Houghton, 2014).

The activation of signal transducer and activator of transcription 3 (STAT3) can reprogram human ES cells in a Leukemia Inhibitory Factor-dependent manner (Chen et al., 2015). When the STAT3 function is suppressed, cell immaturity is not maintained in human ES cells (Wang et al., 2017). Hypoxia activates STAT3 in HeLa cells and endothelial cell lines (Ortega, Ondo-Mendez, & Garzon, 2017). HIF-1α also activates the JAK1/2-STAT3 signal pathway and promotes the self-renewal of glioma stem-like cells (Almiron Bonnin et al., 2017).

Complicated techniques are necessary to cultivate human induced pluripotent stem (iPS) cells for differentiation and freezing compared to murine iPS cells. Although LIF is not added to the culture medium, bFGF and feeder cells are generally necessary to maintain the pluripotent state of human iPS cells. Our previous study in murine iPS cells showed that the stemness was significantly maintained under hypoxic conditions compared to normoxia and HIF-2α among HIFs is the most influential factor for maintaining pluripotency (Sugimoto et al., 2013).
Nevertheless, little is known about how hypoxia can maintain the pluripotent state in human iPS cells through HIFs. It is hypothesized that human iPS cells can also maintain their stemness under hypoxia.

In the present study, the effects of transcription factors were measured with regard to the proliferation and pluripotency of human iPS cells under hypoxia by using real-time polymerase chain reaction (RT-PCR). Furthermore, the function of HIFs in human iPS cells under hypoxic conditions was directly evaluated in relation to the morphology and expression of pluripotency markers by siRNA and RT-PCR.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human iPS cells (201B7) induced four transcription factors were obtained from Riken CELL BANK (Tsukuba, Japan) for this research. The derivation of iPS cells was human skin fibroblasts, with a passage number of 30. These stem cells were seeded onto the feeder layer of Murine Embryonic Fibroblasts (MEFs) prepared from 12.5-day-old mouse embryos (ReproCELL, Yokohama, Japan). These stem cells were maintained in ReproStem (specific nutrient medium for human iPS cells; ReproCELL) with 5 ng/ml bFGF (ReproCELL).

2.2 | Cell proliferation and pluripotency

Human iPS cells were seeded onto MEFs in the maintenance medium. After one passage, five colonies of human iPS cells were seeded onto a 6-well plate. These cells were cultured in 20% or 5% oxygen for 14 days. The colony number was counted after alkaline phosphatase (ALP) staining (ALP Staining Kit; Stemgent, Cambridge, MA, USA) at 5 and 14 days. To evaluate the mRNA expression of Nanog, Sox2, and Oct4 as pluripotency marker genes, the iPS cells were harvested using the TriZol reagent (Life Technology, Carlsbad, CA, USA). RT-PCR was carried out using our previous methods (Sugimoto et al., 2013). The sequences of primers used were as follows: NANOG F, 5’-TGCAAGAACCTCTCCAACATCC-3’ and R, 5’-CCAGTTGTTTTCTGCCACCTC-3’; SOX2 F, 5’-CAAGATGCACAACTCGGAGA-3’ and R, 5’-GCTTAGCCTCGTCGATGAAC-3’; OCT4, 5’-AGTGCCCGAAACCCACACT-3’ and R, 5’-ACAGAACCACACTCGGACCA-3’. Glyceraldehyde phosphate dehydrogenase (GAPDH, F 5’-GAAGGTGAAGGTCGGAGTCA-3’ and R, 5’-AATGAAGGGGTCATGATGG-3’) was used as an internal control. RT-PCR was run at the Real-Time PCR System (Mx3000P; Agilent Technologies) using the following methods; 94°C for 5 min (denaturation), followed by 35 cycles at 94°C for 45 s, 60°C for 1 min and 72°C for 1 min. The reactions were performed in triplicate. The mRNA expression of each gene relative to GAPDH was calculated by the “Comparative Quantification” protocol using the Agilent kit.

2.3 | siRNA analyses

The siRNA transfection was performed using 201B7 iPS cells on 60-mm culture dishes at 10 colonies under 5% oxygen for one passage. These cells cultured under 5% oxygen were cultured for 7 days. Each transfection was done using siRNAs (Product Names: Mm_Hif1a_4FlexiTube siRNA, Mm_Epas1 (Hif2a)_5FlexiTube siRNA, Mm_Hif3a _5FlexiTube siRNA) (QIAGEN, Germany). siRNA analyses were carried out according to our previous study (Sugimoto et al., 2013). At 7 days after transfection, the cells were collected, and extracts were prepared for RT-PCR. AllStars Negative Control (QIAGEN) siRNA was used as the negative control for each transfection. Colony morphology was observed under a microscope. The pluripotency markers (Nanog, Sox2, and Oct4) were analyzed by RT-PCR. The silencing efficiency of siRNA was calculated in comparison with the mRNA expression of the control groups not subjected to the transfection method.

2.4 | STAT3 inhibition analyses

STAT3 in human iPS cells was inhibited by specific inhibitors, Stattic (Axon Medchem, Groningen, Netherlands) and S31 201 (Axon Medchem). Cell morphology was observed, and the expression of HIF-2α mRNA was analyzed using RT-PCR. Stattic and S31 201 were diluted with DMSO to a final concentration of 20 mM. Each diluted solution was added to ReproStem. Human iPS cells were cultured in 5% oxygen as the inhibitor un-added group (Control), the DMSO-added group and the DMSO-added group with each inhibitor.
(DMSO), the Stattic-added group (Stattic), and the 201 S31-added group (S31 201). After three days, the cells were harvested by Trizol reagent (Life Technologies). The mRNA expression of STAT3 under STA3 inhibition was analyzed by RT-PCR. Furthermore, the expression of HIF-2α mRNA under STAT3 inhibition (Stattic or S31 201) was also analyzed by RT-PCR. The sequences of primers used were as follows: Stat3 F, 5'-GGCATTCGGGAAGTATTGTCG-3' and R, 5'-GGTA-GGCGCCTCAGTCGTATC-3'; HIF-2α F, 5'-CGGAGGTGTTCTATGAGCTGG-3' and R, 5'-AGCTTGTGTTCGCAGGAA-3'; Glyceraldehyde phosphate dehydrogenase (GAPDH F, 5'-ATGGGGAAGGTGAGGTCG-3' and R, 5'-GGGGTCATTGATGGCAACAATA-3) was used as an internal control.

2.5 | Statistical analyses

Statistical analyses were performed by Student’s t-test. All data are presented as the results of at least triplicate experiments, except for the experiment regarding the knockdown efficiency of HIFs. All data are expressed as the mean ± the SD. A value of p < 0.05 was considered to be significant.

3 | RESULTS

3.1 | iPSC colony number

The stained colonies, which indicated an undifferentiated state, were significantly more numerous at 14 days’ culture under 5% oxygen in human iPSC cells than under 20% oxygen (Figure 1).

3.2 | Pluripotency marker expression

The expression levels of pluripotency marker mRNA (Nanog, Sox2, and Oct4) in the iPSC cells after 14 days’ culture in 5% oxygen were significantly increased than that in 20% oxygen (Figure 2).

3.3 | HIFs-knockdown efficiency

After siRNA transfection, RT-PCR confirmed an 85% silencing of HIF-1α compared to that following the transfection of control siRNA. The transfection demonstrated 73% silencing of HIF-2α and 78% silencing of HIF-3α at 7 days (Figure 3).
3.4 | Morphologic difference of the iPS cells in the HIFs silencing

Knockdown of HIF-1α or HIF-3α did not affect the morphology of these stem cells. The colony was a kind circular, and the cell density in the colony was high. Precisely, a normal colony formation of iPS cells was observed (Figure 4a,b,d). However, while colony formation did still occur after knockdown of HIF-2α, the cell density was decreased. Moreover, the reduction of colony size was observed (Figure 4c).

3.5 | Effects of HIFs on the expression of pluripotency markers

The expression levels of Nanog, Sox2, and Oct4 were significantly decreased when HIF-2α was silenced. The expression levels of Sox2 and Oct4 were significantly decreased when HIF-3α was silenced. Although the expression of Nanog showed a decreasing trend, there was no significant reduction compared to the control group (Figure 5a–c). Furthermore, there was no significant reduction in the expression levels of the pluripotency markers when HIF-1α was silenced compared to the control group (Figure 5a–c).

3.6 | STAT3 inhibition

The expression of STAT3 mRNA was decreased by 15%, 20% when Stattic or S31 201 was added to iPS cells (Figure 6a). Furthermore, there was a significant reduction in the HIF-2α expression when STAT3 was inhibited by Stattic or S31 201 (Figure 6b). We evaluated the colonies of human iPS cells under STAT3 inhibition by adding Stattic or S31 201. In the Stattic group, there was no colony formation. Furthermore, while colony formation was partly seen in the S31 201 group, the colony size was smaller than control group and DMSO group (Figure 7a). On RT-PCR, the mRNA expression of the undifferentiation marker genes, Nanog, Sox2, and Oct4 was significantly decreased in the Stattic and S31 201 group compared to the control group (Figure 7b–d).

4 | DISCUSSION

Given that early embryogenesis occurs under a hypoxic condition and multiple stem cell lineages seem to be in hypoxia niches (Ezashi, Das, & Roberts, 2005; Simon & Keith, 2008), the function of hypoxic condition in favoring the stemness and promoting stem cell growth has been evaluated in the setting of iPS cell generation (Yoshida, Takahashi, Okita, Ichisaka, & Yamanaka, 2009). The present study clearly demonstrated that hypoxia promotes colony formation in human iPS cells and promotes the expression levels of mRNA for pluripotency. In a previous study, the CCL2 protein, which was supplemented in the medium instead of bFGF, was able to keep the pluripotency in human iPS cells through STAT3 phosphorylation and the JAK/STAT signal pathway (Hasegawa et al., 2014). The CCL2 protein may activate genes for both pluripotency and HIFs, which means that CCL2 induces human iPS cells under hypoxic condition. The findings of this previous report are supported by the present findings, namely that hypoxia promotes immaturity in human iPS cells through the JAK/STAT signal pathway.

Human iPS cells under hypoxic conditions showed an increased expression of HIF-2α, and their immaturity was suggested to be maintained by the JAK/STAT signal pathway. Furthermore, the present siRNA findings together with the colony morphology in human iPS cells strongly suggest that HIF-2α is the strongest factor influencing the maintenance of pluripotency in human iPS cells, similar to our findings in murine iPS cells. Since Yamanaka’s factors (Sox2, Oct3/4, Klf4, and c-Myc) were discovered for cell reprogramming, many studies have been conducted to establish more effective methods for inserting the glis family zinc finger 1 (Maekawa et al., 2011). The STAT3 signal regulates the expression of HIF-2α, and this STAT3-HIF-2α pathway has been examined for its involvement in reprogramming and stemness processes in human iPS cells (Kuan et al., 2017). In this study, the relationship between STAT3 and the regulation of the pluripotency in human iPS cells was investigated using two specific inhibitors that suppressed the dimer formation and nuclear translocation of STAT3 (Schust, Sperl, Hollis, Mayer, & Berg, 2006; Siddiquee et al., 2007). The expression levels of both HIF-2α and pluripotency markers were clearly confirmed to be down-regulated under STAT3 inhibition.

The expression of HIF-2α or HIF-3α is hypothesized to be involved in ES cell biology (Mohyeldin, Garzon-Muvdi, & Quinones-Hinojosa, 2010). HIF-3α seems to be an upstream regulator of HIF-2α, since when the expression of HIF-3α was lost, the expression of HIF-2α was also significantly decreased in human ES cells. Thus, the
inhibition of the expression levels of Nanog, Oct4, and Sox2 mRNAs observed in HIF-3α silencing may be the result of the reduction of HIF-2α, not HIF-3α (Forristal et al., 2010). As the detailed relationship between HIF-2α and HIF-3α remains unclear, the present findings may provide basic foothold for further research.

In conclusion, the HIF-2α silencing group showed a reduction of colony size in human iPS cells and demonstrated a statistically significant reduction in the expression levels of the pluripotency markers compared to the control group. This study indicates that HIF-2α among HIFs is the most effective factor for maintaining the pluripotency of human iPS cells, and that STAT3 signal pathway regulates the HIF-2α expression.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest regarding the publication of this study.

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