Effects of hypoxia on pluripotency in murine iPS cells

杉本 浩司

NAOSITE: Nagasaki University’s Academic Output SITE
http://naosite.lb.nagasaki-u.ac.jp
Original Research Article

Effects of Hypoxia on Pluripotency in Murine iPS Cells

KOUJI SUGIMOTO, YUU YOSHIZAWA, SHIZUKA YAMADA, KAZUNARI IGAWA, YOSHIHIKO HAYASHI, AND HIDETAKA ISHIZAKI

Department of Cariology, Nagasaki University Graduate School of Biomedical Sciences, Sakamoto 1-7-1, Nagasaki 852-8588, Japan

Running title: PLURIPOTENCY IN iPS CELLS UNDER HYPOXIA

KEY WORDS: iPS CELLS; TRANSCRIPTION FACTORS; PLURIPOTENCY; HYPOXIA INDUCIBLE FACTORS; HYPOXIA

Corresponding to: Y. Hayashi; Department of Cariology, Nagasaki University Graduate School of Biomedical Sciences, Sakamoto 1-7-1, Nagasaki 852-8588, Japan. E-mail address: hayashi@nagasaki-u.ac.jp
**ABSTRACT**

Retroviral transduction of four transcription factors (Oct4, Sox2, Klf4 and c-Myc) or three factors, excluding c-Myc, has been shown to initiate a reprogramming process that results in the transformation of murine fibroblasts to induced pluripotent stem (iPS) cells, and there has been a rapid increase in the number of iPS cell-based preclinical trials. In this study, the effects of these transcription factors were evaluated regarding the growth and differentiation of murine iPS cells under hypoxia. Based on the results of RT-PCR and alizarin red S staining, there were no statistical differences in the growth and differentiation of iPS cells or the induction of iPS cells to osteoblasts under hypoxia between the transcription factor groups. Furthermore, the function of hypoxia inducible factors (HIFs) in murine iPS cells under hypoxia was investigated in relation to the morphology and expression of transcription factors using RT-PCR and Western blotting. The HIF-2α knockdown group exhibited a decrease in the colony size of the iPS cells. The HIF-2α or -3α knockdown group demonstrated a statistically significant decrease in the transcription factor expression compared to that observed in the control group. These results demonstrate that HIF-2α among HIFs is the most influential candidate for the maintenance of the pluripotency of murine iPS cells.
INTRODUCTION

The creation of pluripotent cells from somatic cells has great potential for clinical application and basic research in the near future (Hochedlinger and Jaenisch, 2003; Rideout et al., 2002). It has recently been shown that retroviral transduction of murine and human somatic cells with four transcription factors (Oct4, Sox2, Klf4 and c-Myc) initiates the gradual conversion of a small subpopulation of the infected cells into a pluripotent, embryonic stem cell (ESC)-like state (Maherali et al., 2007; Meissner et al., 2007; Okita et al., 2007; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007; Yu et al., 2007). More recently, iPS cells were isolated in the absence of transduced c-Myc (a carcinogenic factor), although with a significantly lower efficiency (Nakagawa et al., 2008; Wernig et al., 2008; Yu et al., 2007). Research using iPS cells has provided new and exciting opportunities for regenerative medicine.

Somatic stem cells reside in specific microenvironments, called niches (Schofield, 1978). The stem cell niche has come to be defined as an anatomical compartment that includes cellular and acellular components that integrate both systematic and local cues to regulate the biology of stem cells (Johnes and Wagers, 2008; Li and Xie, 2005; Scadden, 2006; Yin and Li, 2006). A specialized microenvironment is formed for stem cells (Scadden, 2006). Contact and communication in this microenvironment are critical for stem cell self-renewal and multipotency. Low oxygen tension also promotes the survival of neural crest and hematopoietic cells and prevents differentiation of human ESCs (Danet et al., 2003; Ezashi et al., 2005;
Morrison et al., 2000).

The transcriptional networks controlling stemness under hypoxia are controlled by HIFs (Bertout et al., 2008), the key regulators of cell reactions (e.g. erythropoiesis, apoptosis and proliferation) (Semenza, 2000) to the lack of cell oxygen. Environmental oxygen tension regulates the HIF expression (Mohyeldin et al., 2010). Stabilized HIF-1α subunits translate from the cytoplasm to the nucleus where they bind with HIF-1β to activate target genes (Wenger, 2002). HIF-1α, the first HIF-α subunit described, is thought to be the global regulator of the hypoxic response (Semenza and Wang, 1992). The contribution of HIF-2α and HIF-3α, which are believed to be regulated in a similar manner (Ivan et al., 2001; Masson et al., 2001), remains to be fully characterized. It is hypothesized that hypoxia is capable of regulating stemness and pluripotency via the HIF-2α or HIF-3α expression in ESC biology (Mohyeldin et al., 2010). However, little is known about how hypoxia is able to regulate pluripotency in iPS cells through HIFs. In the present study, the effects of four or three transcription factors were first evaluated regarding the growth and differentiation of murine iPS cells under hypoxia using RT-PCR and alizarin red S staining. Furthermore, the function of HIFs in murine iPS cells under hypoxia was directly investigated in relation to the morphology and expression of transcription factors using siRNA, RT-PCR and Western blotting.

MATERIALS AND METHODS
Cell Cultivation

Four transcription factors inducing murine iPS cells (iPS-MEF-Ng-20D-17) and three transcription factors inducing murine iPS cells (iPS-MEF-Ng-178B-5) were purchased from Riken CELL BANK (Tsukuba City, Japan) for this experiment. Both line of murine iPS cells were derived from murine embryonic fibroblasts (MEFs), with a passage number of 14. The iPS cells were seeded onto the feeder layer of MEFs prepared from 12.5-day-old murine embryos (ReproCELL, Japan). The iPS cells were maintained in DMEM supplemented with 0.1 mM NEAA, 1,000 U/ml mouse LIF, 15% FBS, 1% penicillin/streptomycin and 0.1 mM 2-mercaptoethanol.

Cell Proliferation and Differentiation

Murine iPS cells were fed on MEFs in the maintaining medium. After one passage, the murine iPS cells were seeded in 60-mm culture dishes at a density of $1.0 \times 10^5$ cell/dish. The iPS cells were cultured at 20% or 5% oxygen for seven days. In addition to performing morphologic observation, the cell number was counted at 3, 5 and 7 days using a hemocytometer under a phase contrast microscope. To assess the mRNA expression levels of Nanog, Sox2 and Oct4 as pluripotency markers, the cells were rinsed twice with PBS (-) and lysed by adding Trizol® reagent (Life Technologies, CA). Total RNA was prepared following the manufacturer’s instructions. The first strand cDNA was synthesized from the total RNA following the manufacturer’s instructions using the SuperScript™ First-Strand Synthesis System for RT-PCR (Life Technologies). For the PCR reaction, the Brilliant
SYBR Green QPCR Master Mix® (Life Technologies) containing the components necessary to carry out PCR amplification, including Taq DNA polymerase (Nippon gene, Japan) and SYBR Green I® (Agilent Technologies, USA) as the reporter fluorescent dye, ROX as the reference dye and cDNA as the PCR template, were added to 0.2-μl PCR tubes. The primers used for amplification were as follows: Nanog F, 5’-AGGGTCTCTGCTACTGAGATGCTCTG-3’, and R, 5’-CAACCACTGGTTTTTCTGCCACCG-3’; Oct4 F, 5’-CTGTAGGGAGGGCTTCGGGCACTT-3’, and R, 5’-CTGAGCATGAGGCGAGGCAGGCAGGACACGAG-3’; Sox2 F, 5’-GGCAGCTACAGCATGATGCAGGAGCC-3’, and R, 5’-CTGGTCATGGAGTTGTACTGCAGGAGCC-3’. Glyceraldehyde phosphate dehydrogenase (GAPDH, F, 5’-GCACAGTCAAGGCCGAGAAT-3’, and R, 5’-GCCTTCTCCATGGTGGTGAA-3’) was used as an internal control. The cDNA was amplified under the following conditions using a real-time PCR (RT-PCR) system (Mx3000P™, Agilent Technologies, CA): 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. A melting curve peak was observed for each sample, confirming the purity and specificity of both amplified products. The mRNA level of each gene relative to that of GAPDH was calculated using the “Comparative Quantification” method with the Agilent® kit. For osteogenic differentiation, iPS cells were seeded on MEFs in 6-well plates at a density of $1.0 \times 10^4$ cells per well and fed in both DMEM supplemented with 10% FBS, 10^{-7} M dexamethasone, 10 mM β-glycerophosphate and 50 μg/ml of ascorbate 2-phosphate and normal maintaining medium under 5% or 20%
oxygen. The medium was changed every three days. The cells were cultured for 14 days. The cells were washed with PBS (-), fixed with 100% methanol, and stained with 1% alizarin red S (Sigma Chemical, MO). The mRNA expression levels of Runx2 and osteocalcin were analyzed using quantitative RT-PCR after 5, 7 and 14 days in culture. The primers used for amplification were as follows: Runx2, F, 5'-GGACGAGGCAAGAGTTTCAC -3', and R, 5'-TGCCCTGCCC TGGGATCTGTAA-3'; Osteocalcin, F, 5'-CTTGGTGACACCTAGCAGA-3', and R: 5'-ACCTTATTGCCCTCCTGCTT-3'. The cDNA was amplified under the following conditions using RT-PCR (Mx3000P™, Agilent Technologies): 98°C for 10 s (denaturation), followed by 35 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 20 s.

**siRNA Analysis**

The siRNA experiments were carried out using three transcription factors inducing murine iPS cells plated on 60-mm culture dishes at 1.0 × 10^5 cell/dish under 5% oxygen for one passage. The iPS cells cultured under 5% oxygen were incubated for 48 hours. For each transfection, 50 nM of siRNAs {Product Names: Mm_Hif1α_4FlexiTube siRNA, Mm_Epas1 (Hif2α)_5FlexiTube siRNA, Mm_Hif3α_5FlexiTube siRNA} (QIAGEN, Germany) were added to each dish with 15 µl of HiPerfect transfection reagent (QIAGEN) and mixed into 100 µl of DMEM and added in a dropwise manner to 60-mm dishes. At 48 h after transfection, the cells were harvested and the extracts were prepared for mRNA and protein analyses. AllStars
Negative Controls (QIAGEN) siRNA that has no homology to any known mammalian genes was used as the negative control for each transfection. The cell morphology and effects of HIFs on the expressions of the pluripotency marker genes (Nanog, Oct4, Sox2) were observed and analyzed using RT-PCR. The knockdown efficiency of siRNA into iPS cells was calculated in comparison with the mRNA expression in control groups not treated with the transfection procedure.

**Western Blot Analysis**

Murine iPS cells were again seeded in 100mm culture dishes at a density of 1x10^6 cells, and the siRNA experiments were carried out under 5% oxygen. Following transfection of siRNA according to the above-mentioned protocols, murine iPS cells cultured under 5% oxygen were incubated. After 48 hours of cell culture, the cells were retrieved using a rubber scrapper (Thermo Fisher Scientific, MA). The cells in each group were lysed in lysis buffer (1M HEPES, 10% NP-40, 1mM NaF, 1 mM Na_3 VO_4, 0.5 M EDTA, 100% protease inhibitor cocktail, deionized water) and then sonicated with a cell disrupter for one min in ice-cold water. Following centrifugation of the lysates at 1,000 rpm for 10 min at 4°C, the supernatants were subjected to a Western blot analysis. The protein concentrations in each group were determined using the micro-Lowry method. A 30-μl sample was denatured in 2×SDS-PAGE sample buffer and separated using SDS-PAGE. The gels were transferred using the iBlot® Transfer Stack (Life Technologies, USA) on the iBlot® Gel Transfer Device (Life Technologies). The recommended P3
program (20 V for seven min) was used for all transfers. Anti-mouse-Nanog antibody (ReproCELL, Japan) diluted to 1:500, anti-mouse-Oct4 antibody (Cell Signaling Technology, CA) diluted to 1:500 and anti-mouse-Sox2 antibody (Cell Signaling Technology) diluted to 1:500 were used as the primary antibodies. The specific antibody bindings detected with horseradish peroxidase-conjugated secondary antibodies (iBlot® Western Detection Kits, Life Technologies) were visualized using the supplied reagents (iBlot® Chemiluminescent Kit, Life Technologies). The band density of each group was quantified using a densitometric analysis employing the Scion Image software program. The ratio of the densitometric value of the experiment group to that of the control group was then calculated.

**Statistical Analysis**

The statistical significance of differences was determined using Student’s $t$-test. All data were represented at least three independent experiments except the experiment of knockdown efficiency of HIFs. Data were expressed as the mean ± S.E. A value of $p < 0.05$ was considered to be significant.

**RESULTS**

**Cell Morphology and Number**

Differentiated cells with a large amount of cytoplasm were observed at the periphery of the iPS cells transduced with the four or three factors after
seven days of culture under 20% oxygen (Figs. 1A and 1B). In contrast, undifferentiated cells with an oval shape and smooth surface were observed in the iPS cells transduced with the four or three factors after seven days of culture under 5% oxygen (Figs. 1A and 1B). Both the four- (at 5 days) and three- (at 3 and 5 days) factor-transduced iPS cells cultured under 5% oxygen demonstrated significantly rapid cell growth compared to that observed under 20% oxygen ($P<0.01$; Figs. 2A and 2B). The growth of both the four- and three-factor-transduced iPS cells was similar under same oxygen level at same day of culture ($P>0.05$; Figs. 2A and 2B). Under 5% oxygen, necrotic cells appeared and the number of iPS cells significantly decreased after seven days of culture ($P<0.001$; Figs. 2A and 2B).

**Pluripotency Marker Expression**

The expression of Nanog mRNA in the iPS cells transduced with four or three factors after five and seven days of culture under 5% oxygen was significantly higher than that observed under 20% oxygen ($P<0.05$; Fig. 3A). The expression of Oct4 mRNA in the iPS cells transduced with four or three factors after seven days of culture under 5% oxygen was significantly higher than that observed under 20% oxygen ($P<0.01$; Fig. 3B). The expression of Sox2 mRNA in the iPS cells transduced with three factors after five and seven days of culture under 5% oxygen was significantly higher than that observed under 20% oxygen. The expression of Sox2 mRNA in the iPS cells transduced with four factors after seven days of culture under 5% oxygen was significantly higher than that observed under 20% oxygen ($P<0.05$; Fig.
3C).

**Osteogenic Differentiation**

With respect to osteogenic differentiation, alizarin red S staining was observed at day 14 only under 20% oxygen in the iPS cells transduced with four or three factors (Fig. 4A). No alizarin red S staining was observed at day 14 under 5% oxygen in the iPS cells transduced with four or three factors (Fig. 4A). The expressions of Runx2 (at 5 and 7 days) and osteocalcin (at 7 and 14 days) mRNAs were significantly suppressed under 5% oxygen compared to those observed under 20% oxygen (\(P<0.05\); Fig. 4B, C).

**Knockdown Efficiency of HIFs**

Following siRNA transfection, quantitative RT-PCR confirmed 84% and 82% knockdown of HIF-1\(\alpha\) at 24 and 48 hours, respectively, compared to that observed following the transfection of control siRNA (Fig. 5). There were no significant differences between the two different times (\(P>0.05\)). The transfection demonstrated 84% and 81% knockdown of HIF-2\(\alpha\), and 71% and 72% knockdown of HIF-3\(\alpha\) at 24 and 48 hours, respectively (Fig. 5). There were also no significant differences between these two different times (\(P>0.05\)).

**Effects of the HIF Expression on iPS Cell Morphology**

Silencing HIF-1\(\alpha\) or HIF-3\(\alpha\) did not affect the iPS cell morphology and normal colony formation was observed (Fig. 6). Although colony formation
occurred after silencing HIF-2α, the size of the colonies was decreased, and only small (approximately 50 µm in diameter) colonies were observed (Fig. 6).

**Effects of HIFs on the Pluripotency Marker Expression**

Following real-time RT-PCR, there were no significant reductions in the expressions of the pluripotency marker genes (Nanog, Oct4 and Sox2) compared to those observed in the control group when HIF-1α was silenced ($P>0.05$; Figs. 7A-7C). There was a significant reduction in the expressions of the pluripotency marker genes compared to those observed in the control group when HIF-2α or HIF-3α was silenced independently ($P<0.01$; Figs. 7A-7C). According to a Western blotting analysis, there were no significant reductions in the expressions of the pluripotency marker proteins (NANOG, OCT4 and SOX2) compared to those observed in the control group when HIF-1α was silenced ($P>0.05$; Figs. 8A-8C). There was a significant reduction in the expressions of the pluripotency marker proteins compared to those observed in the control group when HIF-2α or HIF-3α was silenced independently ($P<0.05$; Figs. 8A-8C).

**DISCUSSION**

c-Myc is a major human oncogene that is frequently altered in many forms of cancer (Liano and Dickson, 2000; Weng et al., 2011; Dang et al., 2006; Adhikary and Eilers, 2005). c-Myc modulates the cell cycle and cell
proliferation, increases cell metabolism and stimulates cell differentiation (Dang, 2012; Takahashi and Yamanaka, 2006; Osthus et al., 2000; Yuneva et al., 2007). Three factors are able to initiate slower reprogramming processes sufficient to fully reprogram fibroblasts after a longer time period (Meissner et al., 2007; Wernig et al., 2007). In one study, the reprogramming process was substantially delayed (~30 versus ~6 days after injection) and the overall efficiency was reduced by one to two orders of magnitude when c-Myc was not transduced into iPS cells (Wernig et al., 2008). Many iPS cell-derived animals develop tumors due to the reactivation of the c-Myc gene (Okita et al., 2007), which represents a major safety concern with respect to human applications. The present findings showed that there were no statistical differences in either the growth and differentiation of iPS cells or the induction of iPS cells to osteoblasts between the four and three transcription factor groups. This suggests that the approach used to achieve reprogramming without this particular oncogene (c-Myc) could be translated to clinical regenerative medicine.

Since early embryogenesis occurs in an oxygen-poor environment and multiple stem cell lineages appear to reside in hypoxic niches (Ezashi et al., 2005; Simon and Keith, 2008), the function of hypoxia in favoring the stem cell state and promoting stem cell proliferation has been investigated in the setting of iPS cell generation (Yoshida et al., 2009). These data showed that the formation of Nanog-positive iPS cell colonies (via the transfection of four transcription factors) was significantly higher under mild hypoxia conditions (5% O₂) compared to that observed under normoxia and strict
hypoxia (1% O₂). In addition, the iPS cells arose earlier under hypoxia. By comparing the efficiency of iPS cell induction under normoxic and hypoxic conditions, hypoxic conditions can be used to improve the efficiency of iPS cell generation from murine and human somatic cells, especially under 5% oxygen (Yoshida et al., 2009). In general, somatic cells, such as dental pulp cells (DPC), under hypoxia enhance the angiogenic potential of DPC (Aranha et al., 2010) and promote DPC mineralization (Li et al., 2011). Although bone marrow-derived mesenchymal stem cells (BM-MSCs) proliferate faster, exhibit a greater colony-forming ability and maintain their stemness better under hypoxia (<3% oxygen) compared to those observed under normoxic conditions (20% oxygen) (Grayson et al., 2007; Volkmer et al., 2009; Dos Santos et al., 2010), hypoxia inhibits the spontaneous calcification of BM-MSCs (Huang et al., 2012). The present study demonstrated that although the expressions of osteogenic differentiation markers were confirmed to be inhibited in the hypoxic culture of iPS cells, the increased number of detached cells made it difficult to maintain a long-term culture (maximum: approximately 20 days) of iPS cells.

In ESCs, the Oct4 expression is upregulated by the overexpression of HIF-2α. This upregulation results in the maintenance of the stem cell state as well as the formation of ESC-derived tumors (Covello et al., 2006). HIF-2α has been found to be a direct upstream regulator of Oct4 in murine ES cells, suggesting that HIF-2α is involved in the regulation of stem cell maintenance (Covello et al., 2006). Moreover, chromatin immunoprecipitation data, together with the significant presence of
functional hypoxia response element consensus sequences in the promoter region of Sox2, strongly validate the hypothesis that this factor behaves as a target gene of HIF-2α in ependymal progenitor stem cells obtained from rats (Moreno-Manzano et al., 2010). Furthermore, the culture of ESCs under normal oxygen conditions leads to a significant decrease in the expressions of embryonic stem cell markers (Nanog, Oct4 and Sox2), and upon silencing of HIF-2α or HIF-3α, but not HIF-1α, the expressions of NANOG, OCT4 and SOX2 decrease significantly (Forristal et al., 2010; Moreno-Manzano et al., 2010). HIF-3α appears to be an upstream regulator of HIF-2α since when the HIF-3α expression is lost, the HIF-2α expression significantly decreases in human ESCs. Therefore, the decrease in the expressions of Nanog, Oct4 and Sox2 mRNAs and proteins observed in HIF-3α knockdown may be the result of the loss of HIF-2α, not HIF-3α [Forristal et al., 2010]. The present siRNA experiments together with the iPS cell morphology clearly demonstrate that HIF-2α is the most influential candidate for the maintenance of pluripotency in iPS cells.

In conclusion, there were no statistical differences in either the growth and differentiation of iPS cells or the induction of iPS cells to osteoblasts between the four and three transcription factor groups cultured under hypoxia. Furthermore, the function of HIFs in murine iPS cells under hypoxia was investigated in relation to the morphology and expression of the transcription factors. The HIF-2α knockdown group exhibited a decreased colony size in the iPS cells. The HIF-2α or -3α knockdown group demonstrated a statistically significant decrease in the expressions of the
transcription factors compared to those observed in the control group. These results demonstrate that HIF-2α among HIFs is the most influential candidate for the maintenance of the pluripotency of murine iPS cells.

ACKNOWLEDGMENTS

This research was supported by Grants-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science funded by the Ministry of Education, Science, Sports and Culture of Japan (grant number: 22390360).

REFERENCES


FIGURE LEGENDS

Fig. 1. Representative phase contrast photographs of cultured murine iPS cells. A: iPS cells transduced using four transcription factors under 5% and 20% oxygen on days 3, 5 and 7. Scale bar = 100 µm. B: iPS cells transduced using three transcription factors under 5% and 20% oxygen on days 3, 5 and 7. Scale bar = 100 µm.

Fig. 2. The number of murine iPS cells under 5% and 20% oxygen on days 3, 5 and 7 (data from triplicate samples. **P<0.01, ***P<0.001). A: iPS cells transduced using four transcription factors. B: iPS cells transduced using three transcription factors.

Fig. 3. The pluripotency marker mRNAs expression under 5% and 20% oxygen on days 3, 5 and 7 (data from triplicate samples. *<0.05, **P<0.01, ***P<0.001). A: The mRNA expression of Nanog. B: The mRNA expression of Oct4. C: The mRNA expression of Sox2.

Fig. 4. Osteogenic differentiation of murine iPS cells under 5% and 20% oxygen (data from triplicate samples. *<0.05, **P<0.01, ***P<0.001). A: Representative photographs of mineralized nodules in the four and three transcription factor-induced iPS cells stained with alizarin red S staining after 14 days of culture. B: The mRNA expression of Runx2 in the three transcription factor-induced iPS cells on days 5, 7 and 14. C: The mRNA
expression of osteocalcin in the three transcription factor-induced iPS cells on days 5, 7 and 14.

Fig. 5. The knockdown efficiency of HIFs in the three transcription factor-induced murine iPS cells 24 and 48 hours after knockdown (data from duplicate samples).

Fig. 6. Representative phase contrast photographs of colony morphology 48 h after HIF knockdown in the three transcription factor-induced murine iPS cells under 5% oxygen. A: Control group. B: HIF-1α knockdown group. C: HIF-2α knockdown group. D: HIF-3α knockdown group. Scale bar = 100 µm.

Fig. 7. The pluripotency marker mRNA expression 48 h after HIF knockdown in the three transcription factor-induced murine iPS cells under 5% oxygen (data from triplicate samples. **P<0.01, ***P<0.001). A: The mRNA expression of Nanog. B: The mRNA expression of Oct4. C: The mRNA expression of Sox2.

Fig. 8. The pluripotency marker protein expression 48 h after HIF knockdown in the three transcription factor-induced murine iPS cells under 5% oxygen (data from triplicate samples. *P<0.05, ***P<0.001). A: The protein expression of Nanog. B: The protein expression of Oct4. C: The protein expression of Sox2.
Figure 1.
Figure 2.
Figure 3.
Figure 5.
Figure 6.

[Images of cell cultures labeled A, B, C, D]
Figure 8.