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Developmental Biology:
**High Oxygen Condition Facilitates the
Differentiation of Mouse and Human
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High Oxygen Condition Facilitates the Differentiation of Mouse and Human Pluripotent Stem Cells into Pancreatic Progenitors and Insulin-producing Cells^{*[5]}

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Background: Oxygen plays a key role in organ development, including pancreatic β -cells.

Results: High oxygen conditions increase Ngn3-positive and insulin-positive cells from both mouse and human pluripotent stem cells.

Conclusion: Culturing under high oxygen conditions has a facilitative effect on pancreatic differentiation.

Significance: This new technique provides an efficient method to utilize patient-specific iPS cells for the treatment of diabetes.

Pluripotent stem cells have potential applications in regenerative medicine for diabetes. Differentiation of stem cells into insulin-producing cells has been achieved using various protocols. However, both the efficiency of the method and potency of differentiated cells are insufficient. Oxygen tension, the partial pressure of oxygen, has been shown to regulate the embryonic development of several organs, including pancreatic β -cells. In this study, we tried to establish an effective method for the differentiation of induced pluripotent stem cells (iPSCs) into insulin-producing cells by culturing under high oxygen (O_2) conditions. Treatment with a high O_2 condition in the early stage of differentiation increased insulin-positive cells at the terminus of differentiation. We found that a high O_2 condition repressed Notch-dependent gene *Hes1* expression and increased *Ngn3* expression at the stage of pancreatic progenitors. This effect was caused by inhibition of hypoxia-inducible factor-1 α protein level. Moreover, a high O_2 condition activated Wnt signaling. Optimal stage-specific treatment with a high O_2 condition resulted in a significant increase in insulin production in both mouse embryonic stem cells and human iPSCs and yielded populations containing up to 10% C-peptide-positive cells in human iPSCs. These results suggest that culturing in a high O_2 condition at a specific stage is useful for the efficient generation of insulin-producing cells.

Cell replacement therapy has become possible by utilizing artificially generated cells or organs from embryonic stem cells (ESCs),³ induced pluripotent stem cells (iPSCs), and adult stem cells. These stem cells have marked potential to develop into many different cell types in the body during early life and growth. Over the last decade, with the advent of new techniques and technologies in modern molecular biology, understanding of the underlying mechanism responsible for organ differentiation has developed rapidly. This knowledge has given rise to various new methods of manipulating stem cells in order to generate deficient organs in various diseases. To date, various differentiation methods have been developed for each cell type, including neurons, cardiomyocytes, and pancreatic endocrine cells. Many of these methods are based on mimicking the *in vivo* development. The development of efficient and safe methods is desired for clinical applications and studying the cause of disease.

Pluripotent stem cells are capable of spontaneous differentiation into insulin-producing cells. This is mainly carried out by preferential differentiation of stem cells into insulin-producing cells by changing the composition of the culture medium and causing the expression of dominant transcription factor genes, which are mainly involved in pancreatic development. Several groups have reported methods of generating pancreatic cell lineages from ESCs and iPSCs (1–8). These methods induce definitive endoderm differentiation in the first stage and then pancreatic specialization and maturation in the following stages, using combinations of growth factors, small molecules, and extracellular matrix. Lumelsky *et al.* (6) first demonstrated the successful differentiation of mouse ESCs (mESCs) to insulin-

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³ The abbreviations used are: ESC, embryonic stem cell; mESC, mouse embryonic stem cell; iPSC, induced pluripotent stem cell; hiPSC, human induced pluripotent stem cell; HIF, hypoxia-inducible factor; MEF, mouse embryo fibroblast; NEAA, nonessential amino acid(s); β -ME, β -mercaptoethanol; bFGF, basic fibroblast growth factor; qPCR, quantitative PCR.

High Oxygen Facilitates Pancreatic Differentiation

secreting structures, which was concluded to be similar to that of pancreatic islets. However, the limiting factor of this method is that the abundance of differentiated cells is relatively low. Moreover, several reports had the same issue that the differentiated cells are immature and/or not fully functional in culture. Some reports succeeded in generating functional insulin-secreting cells utilizing differentiation under implantation or coculture with organ-matched mesenchyme (7, 8). However, such methods have a risk of teratoma or teratomatous tissue element formation in their grafts. Fifteen percent of grafts showed teratoma or a teratomatous tissue element (7). To improve this issue, establishment of safer and more efficient methods is desired.

Oxygen (O_2) plays a crucial role in cellular homeostasis (9, 10). In normal tissues, the lack of oxygen contributes to cell death, whereas in stem cells, lack of O_2 controls stem cell self-renewal and pluripotency by activating specific signaling pathways, such as Notch, and the expression of transcriptional factors, such as Oct4 (11, 12). Hypoxia is accompanied by the stabilization of hypoxia-inducible factors (HIFs), O_2 -regulated transcriptional factors that regulate an ever increasing number of genes involved in glycolytic metabolism, angiogenesis, erythropoiesis, and metastasis and mediate the adaptation of cells to decreased O_2 availability (13, 14). O_2 tension, the partial pressure of O_2 , has been shown to regulate the embryonic development of several organs, including the trachea, heart, lung, limb bud, and bone (15–19). It is also reported that O_2 tension plays a key role in pancreatic development (20–23). The embryonic pancreas early in development is poorly vascularized and has a paucity of blood flow, and, at later stages, blood flow increases, and endocrine differentiation occurs at the same time (21). It has also been shown that HIF-1 α protein is highly expressed in the embryonic pancreas early in development and that increasing concentrations of O_2 *in vitro* represses HIF-1 α expression and fosters the development of endocrine progenitors (22, 23). Suitable O_2 concentrations should be tested for the differentiation efficiency of ESC and iPSC into pancreatic lineages. However, until now, there has been no report of such an effect on ESC and iPSC differentiation *in vitro*.

Here we studied the effect of increasing concentrations of O_2 on the differentiation efficiency of mESC and human iPSC (hiPSC) into pancreatic lineages. A high O_2 condition (60% O_2) in early stages of differentiation increased the percentage of Ngn3-expressing endocrine progenitors and insulin-positive cells in both mESC and hiPSC. This effect was mediated via the inhibition of HIF-1 α expression and increase of *Ngn3* gene expression. Moreover, a high O_2 condition was found to induce the activation of Wnt signaling. In this study, we demonstrated that culturing ESC and iPSC in a high O_2 condition improved differentiation efficiency into endocrine progenitors and insulin-producing cells compared with normoxic conditions.

EXPERIMENTAL PROCEDURES

mESC and hiPSC Lines—The mESC line ING112, containing an *Ins1* promoter-driven GFP reporter transgene, was established by culturing blastocysts obtained from transgenic mice homozygous for the *Ins1*-GFP gene (3, 24). ING112 cells were maintained on mouse embryonic fibroblast (MEF) feeders in

high glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 1000 units/ml leukemia inhibitory factor (Wako, Osaka, Japan), 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT), 100 μ M nonessential amino acids (NEAA), 2 mM L-glutamine (L-Gln), 1 mM sodium pyruvate, 50 units/ml penicillin and 50 μ g/ml streptomycin, and 100 μ M β -mercaptoethanol (β -ME). The hiPSC clone 23 (C23) was previously produced by Sendai virus vector expressing OCT3/4, SOX2, KLF4, and c-MYC (25). C23 cells were maintained on mitomycin C (Nacalai Tesque, Kyoto, Japan)-treated MEF feeders in DMEM/F-12 HAM (Sigma) supplemented with 100 μ M NEAA, 2 mM L-Gln, 20% knock-out serum replacement (Invitrogen), 25 units/ml penicillin and 25 μ g/ml streptomycin, 100 μ M β -ME, and 5 ng/ml bFGF (Peprotech, Rocky Hill, NJ).

Differentiation of mESC and hiPSC into Insulin-producing Cells—Prior to differentiation, mESCs were passaged using 0.25% trypsin and seeded at 35,000 cells/well on 4- or 24-well plates coated with 50 μ g/ml poly-L-lysine (Sigma) and 2.5 μ g/well laminin (Roche Applied Science). After overnight culture, the cells were cultured in high glucose (4500 mg/liter) DMEM supplemented with 10 mg/liter insulin, 5.5 mg/liter transferrin, 6.7 μ g/ml sodium selenite (insulin-transferrin-selenium-G supplement, Invitrogen), 2.5 mg/ml ALBUMAX II (Invitrogen), NEAA, L-Gln, 50 units/ml penicillin and 50 μ g/ml streptomycin, β -ME, 10 ng/ml activin A (R&D Systems, Minneapolis, MN), and 5 ng/ml bFGF (Peprotech) from day 1 to day 6. From day 7 to 10, the culture medium was changed to RPMI supplemented with NEAA, L-Gln, 50 units/ml penicillin, and 50 μ g/ml streptomycin, β -ME, B27 supplement (Invitrogen), 50 ng/ml FGF10 (Peprotech), 250 nM KAAD-cyclopamine (Calbiochem), and 1 μ M retinoic acid (Sigma). At day 11, the medium was switched to low glucose (1000 mg/liter) DMEM supplemented with insulin-transferrin-selenium-G supplement, ALBUMAX II, NEAA, L-Gln, penicillin, streptomycin, β -ME, 10 mM nicotinamide (Sigma), and 10 nM glucagon-like peptide (Sigma), and the cells were cultured until day 17. hiPSCs were passaged as small clusters using 1 unit/ml dispase (Roche Applied Science) at a 1:5 split ratio weekly and plated on mitomycin C-treated MEF feeders of 4- or 24-well plates. After a 1-week culture, the cells were cultured in differentiation medium in the same way as mouse ESCs.

Cells were cultured under high O_2 concentration conditions (60% O_2) for the indicated periods in a multigas incubator (APM-30DR, ASTEC, Fukuoka, Japan).

Immunostaining—Cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 30 min at room temperature (22–24 °C). Immunostaining was carried out with the standard protocol. The following primary antibodies were used: mouse anti-GFP (1:1000; Medical and Biological Laboratories, Nagoya, Japan), goat anti-Sox17 (1:200; R&D Systems), rabbit anti-Pdx1 (1:1000; Upstate Biotechnology, Inc., Lake Placid, NY), goat anti-Ngn3 (1:100; Santa Cruz Biotechnology, Inc.), goat anti-Foxa2 (1:300; Santa Cruz Biotechnology, Inc.), mouse anti-insulin (1:1000; Sigma), and rabbit anti-C-peptide (Cell Signaling Technology, Beverly, MA). Alexa488- or Alexa568-conjugated secondary antibodies were used at 1:500 dilution (Molecular Probes, Inc., Eugene, OR). Cells were counterstained with DAPI (Roche Applied Science).

TABLE 1**Primers used in quantitative real-time PCR analysis**

Genes	Sequences (forward and reverse)	Product bp
Mouse		
<i>Act</i>	CCTCATGAAGATCCTGACCGA TTGCCAATAGTGATGACCTGG	192
<i>Oct4</i>	GAGGAAGCCGACAACAATGAGAACCTTCAG TTCTGGCGCCGGTTACAGAACCATACTCGA	227
<i>Sox17</i>	GAACAGTTGAGGGGCTACAC GTTTAGGGTTTCTTAGATGC	322
<i>Foxa2</i>	TGGTCACTGGGGACAAGGGAA GCAACAACAGCAATAGAGAAC	289
<i>Pdx1</i>	TCACTGGAGCAGGGAAGTCCT TTCCGCTGTGTAAGCACCTCC	264
<i>Ngn3</i>	ACTGCAGCAGTGGTCGAGTAC AAGGGATGAGGCCCATCTCA	225
<i>Ins1</i>	CAGCCCTTAGTAGCAGCTA ATGCTGGTGCAGCACTGATC	348
<i>Vegfa</i>	GCTACTGCGCTCCGATTGAGA AGGTTTGATCCGCATGATCTGC	185
<i>Hes1</i>	TCAACACGACACCGGACAAACC GGTATTTCCCAACACGCTCG	270
Human		
<i>ACT</i>	CCTCATGAAGATCCTCACCGA TTGCCAATGGTGATGACCTGG	192
<i>SOX17</i>	GCATGACTCCGGTGTGAATCT TCACACGTACAGGATAGTTGCAGT	103
<i>FOXA2</i>	ATTGCTGGTTCGTTGTTGTG TACGTGTTTCATGCCGTTTCA	187
<i>PDX1</i>	CCTTCCCATGGATGAAGTC GGAATCCTTCTCCAGCTCTA	145
<i>NGN3</i>	TTGCGCCGGTAGAAGGATGAC TCAGTGCCAACTCGCTCTTAGG	249
<i>NEUROD1</i>	CCCATGGTGGGTTGTCATATATTCA CCAGCATCACATCTCAACAGCAC	200
<i>MAFA</i>	TGCAGCAGCGGCACATTC CGCCAGCTTCTCGTATTCTCCTTGT	128
<i>INS</i>	GAGGCCATCAAGCAGATCAC GGCTGCGTCTAGTTGCAGTA	373
<i>VEGFA</i>	CCCTGATGAGATCGAGTACAT CGGCTTGTCACATCTGCAAGT	496
<i>HES1</i>	TCAACACGACACCGGATAAACC GGTACTTCCCAAGCACACTTG	270

Images were taken with an Olympus IX81 fluorescence microscope (Olympus Optical Co. Ltd., Tokyo, Japan). Quantification was carried out using MetaMorph software (Molecular Devices). After images of marker fluorescence and DAPI fluorescence were taken in defined areas of wells in the cell culture plate, each image was thresholded to exclude background noise. The area was quantified, and the percentage of marker-positive cells was calculated by dividing the DAPI-positive area (total cell number) into the marker-positive area (supplemental Fig. S1).

Quantitative Real-time PCR—Total RNA was isolated from cells using a TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using a Superscript III first strand synthesis system (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) analysis was performed on an ABI Prism 7300 (Applied Biosystems, Foster City, CA) using a SYBR Premix ExTaq GC (Takara, Shiga, Japan). The primer sequences for each primer set are shown in Table 1. mRNA expression data were normalized against actin expression in a corresponding sample. The data were analyzed using the relative quantification study in Sequence Detection software version 1.2 (Applied Biosystems).

Immunoblotting—HIF-1 α levels were determined using immunoblotting. Cells were washed with PBS, scraped, and lysed in radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.5, 4 mM EGTA, 150 mM NaCl, 50 mM NaF, 1 mM Na₃VO₄, 30 mM Na₄P₂O₇, 10 mM EDTA, 1% Triton X-100). After soni-

High Oxygen Facilitates Pancreatic Differentiation

cation, the insoluble materials were removed by centrifugation at 15,000 rpm for 15 min. The supernatants were then mixed with a 5-fold amount of Laemmli sample buffer (0.38 M Tris-HCl, pH 6.8, 12% SDS, 30% β -mercaptoethanol, 10% glycerol, 0.05% bromophenol blue) and boiled for 4 min. Samples were loaded and subjected to SDS-PAGE and then transferred to a nitrocellulose membrane (Bio-Rad) and then blocked for nonspecific binding in a 5% skim milk solution. Membranes were incubated with mouse anti-HIF-1 α antibody (1:1000; R&D Systems) or mouse anti- β -actin (1:1000; Chemicon, Temecula, CA) overnight at 4 °C. Then membranes were washed and incubated for 1 h with HRP-conjugated goat anti-mouse IgG antibody (1:2000; Dako, Carpinteria, CA). After washing, membranes were incubated with enhanced chemiluminescence reagent (GE Healthcare), and the immunoreactive proteins were visualized by an ImageQuant400 (GE Healthcare).

Microarray—Microarray analysis was performed on total RNA samples using a TORAY 3D-gene oligo chip (TORAY, Tokyo, Japan), according to the manufacturer's instructions. The genes induced by a high O₂ environment were determined by global normalization after excluding genes of <100 intensity in the high O₂ condition-treated group. Genes increased over 8-fold in the ratio of high O₂ condition to normoxia are listed in Fig. 6A. Pathway analysis was performed on up-regulated genes in the high O₂ condition-treated or echinomycin-treated group using GenMapp/MAPP Finder software.

Statistics—Data are shown as the mean $\pm$ S.E. Student's *t* test was used to identify significant differences between two conditions, and one-way analysis of variance or two-way analysis of variance followed by Tukey-Kramer's post hoc analysis was used to compare multiple conditions. *p* < 0.05 was considered to be significant.

RESULTS

High Oxygen Condition Facilitates the Differentiation of mESC into Insulin-producing Cells—We used a modified protocol from a previous report of three-stage stepwise differentiation into insulin-producing cells (25, 26) (Fig. 1A). First, mESC ING112 cells were treated with activin A and bFGF to direct the differentiation into definitive endoderm from day 1 to day 7. By day 7, there was a steep reduction in the expression of *Oct4* relating to the fact that the cells have transitioned from pluripotency to an endodermal progenitor (Fig. 1B). This is evident from the increased expression of *Sox17* and *Foxa2* on day 7, both of which are markers of a definitive endoderm. With the change in the composition of the medium containing B27, FGF10, KAAD-cyclopamine, and retinoic acid from day 7 onward, there was a gradual decrease in the expression of *Sox17* and *Foxa2*. Subsequently, there was a marked increase in the expression of *Pdx1* and *Ngn3* on day 11, indicating the prevalence of pancreatic progenitors and endocrine progenitors in the population of the culture. With the change in the medium composition containing nicotinamide and GLP-1 on day 11, *Pdx1* and *Ngn3* expressions decreased, whereas the maximum level of *Ins1* expression was reached.

Next we examined the effects of a high O₂ concentration condition on the differentiation efficiency of mESC ING112 cells. We cultured cells in a high O₂ condition (60% O₂) under

High Oxygen Facilitates Pancreatic Differentiation

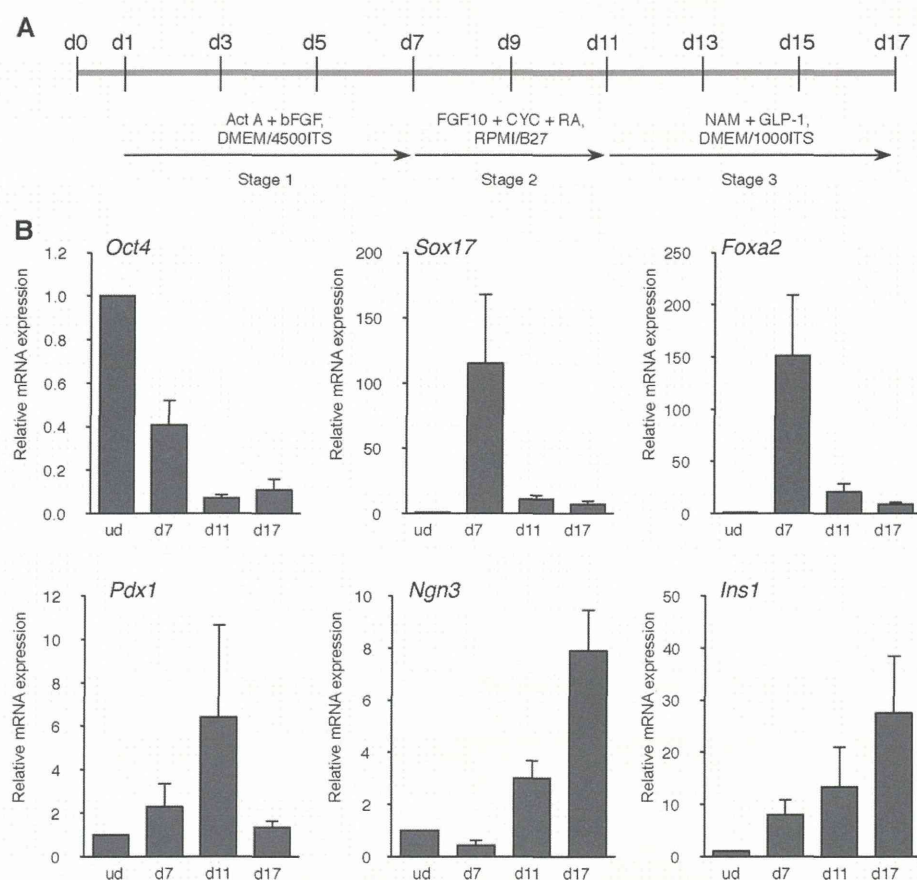


FIGURE 1. Stepwise differentiation of mESCs into insulin-producing cells. A, scheme of the stepwise differentiation protocol used to generate insulin-producing cells from mouse embryonic stem ING112 cells. Act A, activin A; bFGF, basic FGF; CYC, KAAD-cyclopamine; RA, retinoic acid; NAM, nicotinamide; GLP-1, glucagon-like peptide; ITS, insulin-transferrin-selenium. B, the dynamics of *Oct4*, *Sox17*, *Foxa2*, *Pdx1*, *Ngn3*, and *Ins1* gene expression, several key markers in pancreatic differentiation, were analyzed at different stages in normoxic conditions by qPCR. $n = 8$ each. ud, undifferentiated cells. Error bars, S.E.

stepwise differentiation during stage 1 (days 3–7), stage 2 (days 7–11), or stage 3 (days 11–17) (Fig. 2A). It was observed that a high O_2 condition during stage 1, the early phase of differentiation, had the greatest effect on differentiation efficiency with an almost 8-fold increase ($p < 0.05$) in the percentage of *Ins1*-GFP-positive cells (Fig. 2, B and C). However, a high O_2 condition during stage 2 or stage 3 had no effect on differentiation efficiency. At the beginning of these experiments, we cultured under high O_2 conditions from day 1; however, earlier treatment had a deleterious effect on the percentage of surviving cells (Fig. 2F). Therefore, we used this protocol as the high O_2 condition from day 3. To determine whether 60% high O_2 is the best condition, we tested different levels of O_2 condition. As a result, 40% O_2 during stage 1 also increased the percentage of *Ins1*-GFP-positive cells by 4-fold ($p < 0.05$; Fig. 2G), but this effect was less than that of 60% O_2 . Instead of high O_2 , we used a hypoxic condition during differentiation, but there was no change compared with the normoxic condition (Fig. 2D). The high O_2 condition also increased the percentage of C-peptide-positive cells in a different mESC line, SK7 (27, 28) (Fig. 2E).

High Oxygen Condition Facilitates Differentiation into Endocrine Progenitors—Based on the results in Fig. 2, B and C, we compared gene expression levels between the normoxia and high O_2 condition groups on day 6 of stage 1 (Fig. 3A). We

observed a significant decrease ($p < 0.0005$) in the expression of *Oct4*, which is indicative of the fact that the cells lost their pluripotency (Fig. 3B). Whereas there was no difference in the expression of *Sox17*, a marker gene of definitive endoderm, there was an almost 6-fold ($p < 0.005$) and 7-fold increase ($p < 0.0005$) in the expressions of *Pdx1* and *Ngn3*, respectively (Fig. 3B). To determine the proportion of cells expressing each marker, immunofluorescence analysis was performed. There was no marked difference in the number of *Sox17*-positive cells on day 6 (Fig. 3C), and *Pdx1*-positive cells on day 11 (Fig. 3D), although induction of its gene expression was observed (Fig. 3B). It was confirmed that the number of *Ngn3*-positive cells was significantly increased on day 11 (Fig. 3E). Quantification of the percentage of *Ngn3*-positive cells showed an almost 3-fold ($p < 0.0005$) increase (Fig. 3F). These results show that the high O_2 condition reduced the pluripotency of the cells and directed them markedly toward endocrine progenitors.

In our differentiation protocol, stage 1 contained supplements, such as activin A and bFGF, in the medium to direct toward a definitive endoderm. Next, to clarify which high O_2 condition affected undifferentiated mESC or differentiating cells, we examined the effect of a high O_2 condition on *Ngn3* expression in undifferentiated cells. Treatment with a high O_2 condition for 3 days did not affect *Ngn3* expression in the undif-

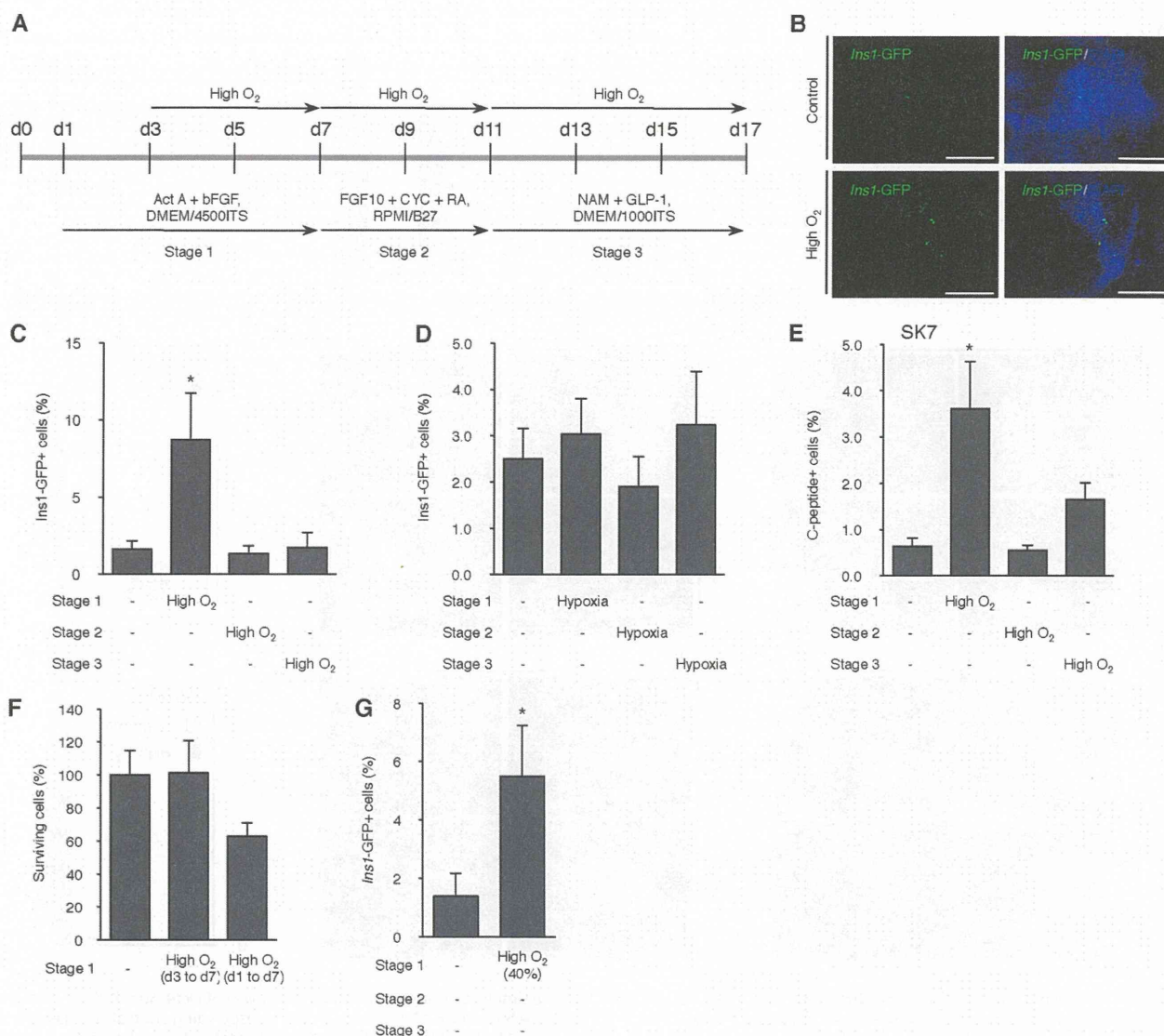


FIGURE 2. Effect of high O₂ condition on differentiation efficiency of mESCs. *A*, scheme of the timeline of high O₂ condition. *B*, immunofluorescence for *Ins1*-GFP on day 17 of differentiation in ING112 cells treated with a high O₂ condition during stage 1. Scale bars, 200 μ m. *C*, values are the percentage of *Ins1*-GFP-positive cells per well of the cells treated with high O₂ condition (60% O₂) during three different stages. *, $p < 0.05$ versus corresponding control. $n = 8$ each. *D*, values are the percentage of *Ins1*-GFP-positive cells per well of the cells treated with hypoxic condition (5% O₂) during three different stages. $n = 20$ each. *E*, values are the percentage of C-peptide-positive cells per well of the cells treated with the high O₂ condition (60% O₂) during three different stages in mESC line SK7. *, $p < 0.05$ versus corresponding control. $n = 6$ each. *F*, cell viability assay on the number of viable cells after treatment with the high O₂ condition (60% O₂) during day 1 to day 7 or day 3 to day 7. $n = 6$ each. *G*, values are the percentage of *Ins1*-GFP-positive cells per well of the cells treated with the high O₂ condition (40% O₂) during stage 1. *, $p < 0.05$ versus corresponding control. $n = 6$ each. Error bars, S.E.

differentiated state maintained in mESC medium compared with treatment in differentiation medium, showing that the high O₂ condition affected differentiating cells (Fig. 3G).

High Oxygen Condition Represses HIF-1 α Protein Level and *Hes1* Gene Expression—Even under normoxia, HIF-1 α is reported to be expressed at a detectable level and participate in the expression of hypoxia-inducible genes in mESCs (29). Therefore, we compared HIF-1 α protein level and its target gene expression between normoxic and high O₂ condition groups. High O₂ condition during days 3–6 of differentiation repressed HIF-1 α protein level (Fig. 4, *A* and *B*). Under normoxic conditions, expression of *Vegfa*, a HIF-1 α targeting gene, increased from day 4 with a peak level on day 5 of differentiation, showing that activation of HIF-1 α occurs

during differentiation, whereas the high O₂ condition significantly decreased *Vegfa* expression on days 5 and 6 ($p < 0.05$, respectively; Fig. 4C). It is reported that HIF-1 α activates Notch signaling in stem cells and embryonic pancreatic cells (11, 22). Hypoxia and subsequent HIF-1 α expression induced expression of *Hes1* (hair and enhancer of split 1), a Notch downstream gene, and repressed *Ng3* expression, leading to the inhibition of β -cell development (22). Therefore, we compared the kinetics of *Hes1* and *Ng3* expression in normoxia with those in the high O₂ condition. Under differentiation, *Hes1* expression was slightly increased at day 4 and gradually decreased from day 5, whereas high O₂ condition significantly repressed its expression on days 5 and 6 ($p < 0.01$ and $p < 0.05$, respectively; Fig. 4D). In contrast,

High Oxygen Facilitates Pancreatic Differentiation

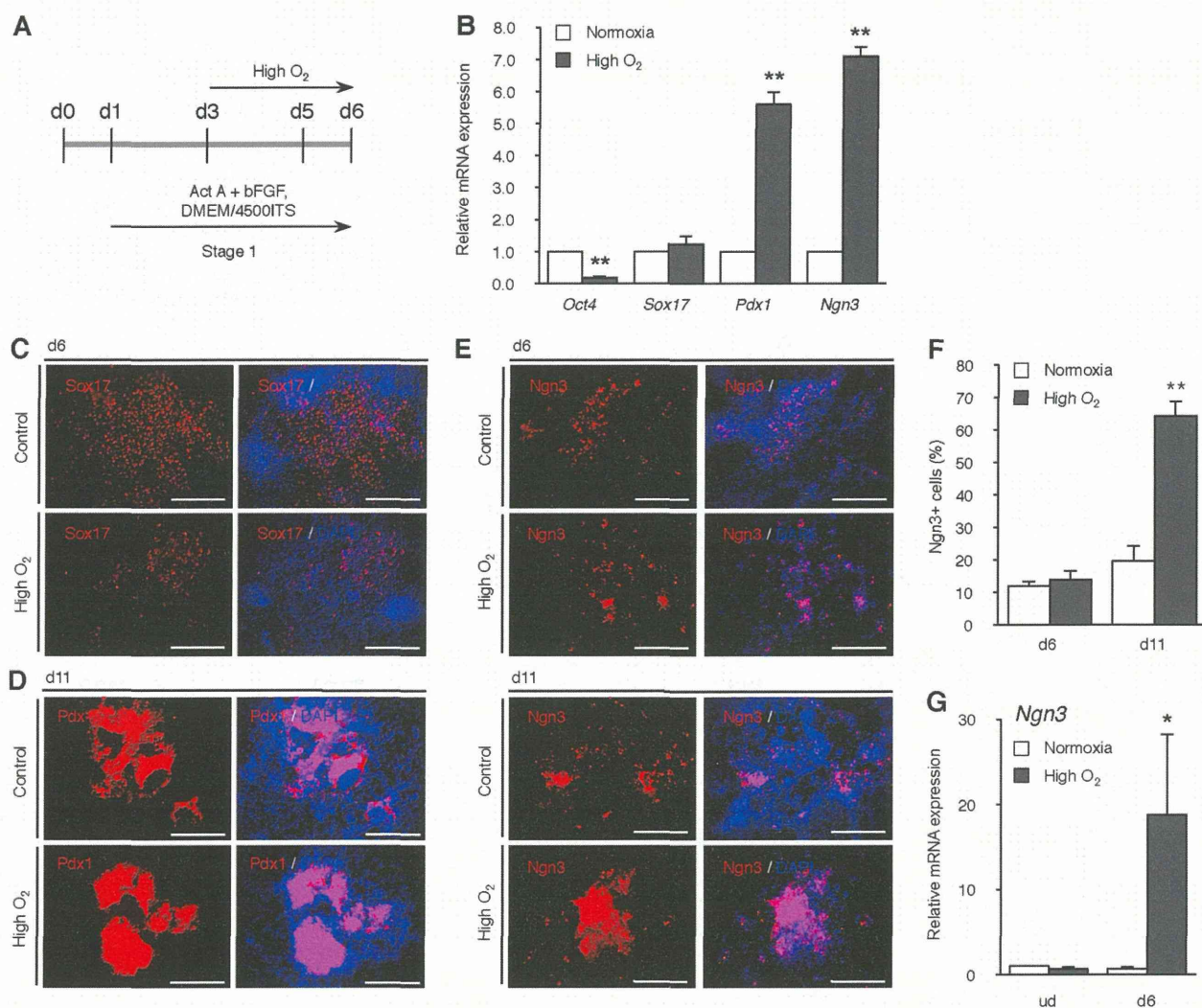


FIGURE 3. Effect of high O₂ condition on differentiation markers. A, scheme of the timeline of high O₂ condition. B, levels of *Oct4*, *Sox17*, *Pdx1*, and *Ngn3* genes were analyzed at day 6 of differentiation in normoxia or high O₂-treated ING112 cells by qPCR. **, $p < 0.01$ versus corresponding control. $n = 4$ each. C–E, immunofluorescence for *Sox17* on day 6 (C), *Pdx1* on day 11 (D), and *Ngn3* on day 6 or day 11 (E) of differentiation in ING112 cells treated with high O₂ condition during stage 1. Scale bars, 200 μ m. F, values are the percentage of *Ngn3*-positive cells per well at day 6 or day 11 in normoxia or high O₂-treated ING112 cells. **, $p < 0.01$ versus corresponding control. $n = 4$ each. G, levels of the *Ngn3* gene were analyzed on undifferentiated ING112 cells treated with high O₂ condition for 3 days by qPCR. The effect of high O₂ condition on differentiating cells is shown as a positive control. *, $p < 0.05$ versus corresponding control. $n = 4$ each. Error bars, S.E.

Ngn3 expression significantly increased on both day 5 and 6 in the high O₂ condition ($p < 0.05$, respectively; Fig. 4E).

Next we examined the effect of HIF-1 α inhibition on *Ngn3* expression under differentiation. Cells were treated with 1 nM echinomycin, an inhibitor of HIF-1 α , from day 3 to day 6. On day 6, *Ngn3* expression was significantly increased by echinomycin treatment, whereas *Vegfa* and *Hes1* expressions were decreased ($p < 0.0005$, $p < 0.05$, and $p < 0.05$, respectively; Fig. 4F). These expression profiles were similar to those in the high O₂ condition. Hence, it was shown that HIF-1 α inhibition and subsequent repression of Notch signaling play a role in facilitated differentiation in the high O₂ condition.

High Oxygen Condition Activates Wnt Signaling Pathway—To further clarify the effect of the high O₂ condition on differentiating cells, we performed microarray analysis on normoxia or high O₂-treated cells. As a result, many genes were up-regu-

lated by the high O₂ condition, and genes showing over 8-fold expression in the high O₂ group compared with the normoxia group are listed in Fig. 5A. Pathway analysis using up-regulated genes in the high O₂ group indicated that several genes were involved in the Wnt signaling pathway, and this pathway was ranked first ($p < 0.01$; Fig. 5B). *Wnt3*, *Wnt6* (over 8-fold), *Wnt5a*, *Wnt10a* (over 4-fold; data not shown), *Wnt4*, *Wnt7b*, *Wnt10b*, *Fzd1*, *Myc*, and *Ccnd2* (over 2-fold; data not shown) were increased in the high O₂ group and detected as the Wnt signaling pathway. We also performed microarray analysis on the echinomycin-treated group. Similarly, the Wnt signaling pathway was ranked first when analyzed using up-regulated genes ($p < 0.001$; Fig. 5C), suggesting that HIF-1 α inhibition led to the activation of Wnt signaling.

The Wnt/ β -catenin pathway plays an important role in the regulation of pluripotency and pancreatic development and dif-

High Oxygen Facilitates Pancreatic Differentiation

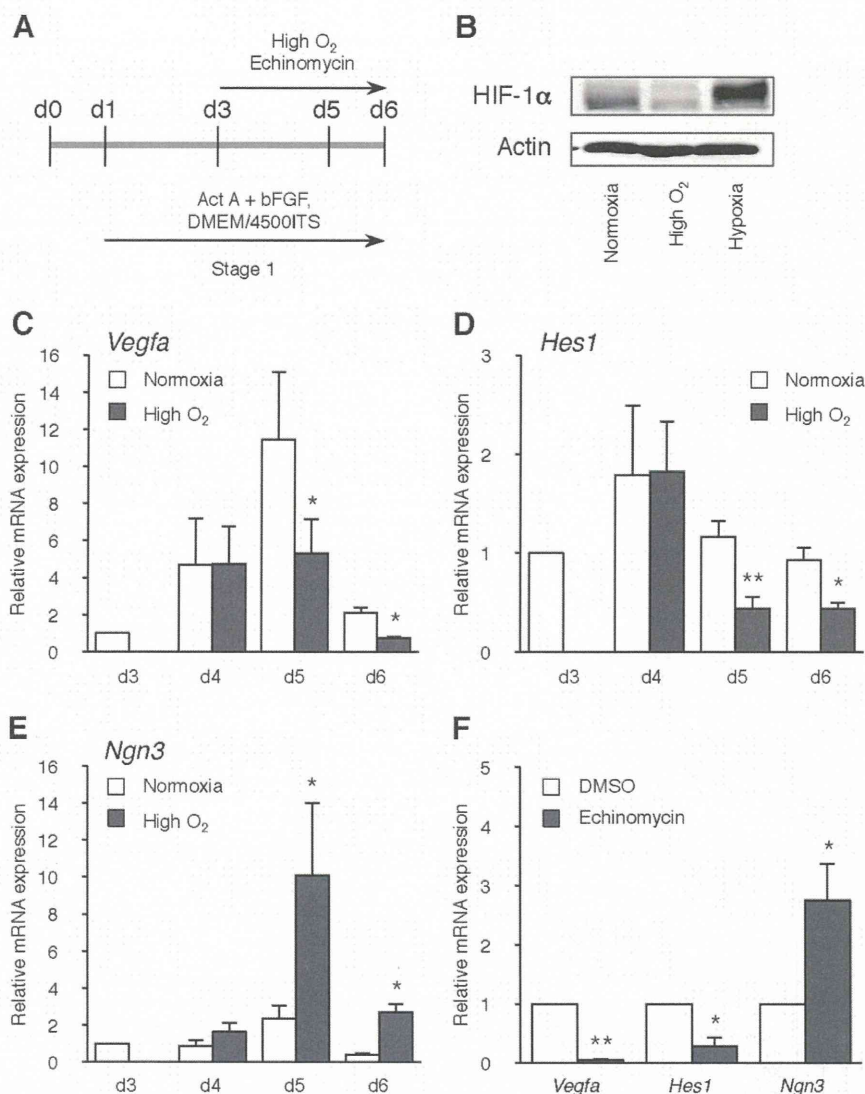


FIGURE 4. Effect of high O₂ condition on Notch signaling. *A*, scheme of the timeline of high O₂ condition. *B*, levels of HIF-1α and β-actin (loading control) were analyzed on day 6 of differentiation in normoxia, high O₂, or hypoxia (1% O₂; positive control)-treated ING112 cells by immunoblotting. *C–E*, levels of *Vegfa* (*C*), *Hes1* (*D*), and *Ngn3* (*E*) gene were analyzed on days 3, 4, 5, and 6 of differentiation in normoxia or high O₂-treated cells by qPCR. *, $p < 0.05$; **, $p < 0.01$ versus corresponding control. $n = 8$ each. *F*, levels of *Vegfa*, *Hes1*, and *Ngn3* genes were analyzed on day 6 of differentiation in DMSO or 1 nM echinomycin-treated ING112 cells by qPCR. *, $p < 0.05$; **, $p < 0.01$ versus corresponding control. $n = 3$ each. Error bars, S.E.

ferentiation (1, 30–32). Hence, we examined the effect of Wnt inhibitor Dkk-1 on high O₂-induced *Ngn3* expression. The application of Dkk-1 led to significant repression of high O₂-induced *Ngn3* expression ($p < 0.05$; Fig. 5D), showing that activated Wnt signaling is involved in facilitated differentiation in a high O₂ condition.

High Oxygen Condition Facilitates Differentiation of hiPSC into Insulin-producing Cells—We performed pancreatic differentiation from hiPSC clone 23 (25) by our stepwise protocol (Fig. 6A) and analyzed marker expression by immunofluorescence analysis. It was confirmed that the expression of SOX17, a definitive endoderm marker, was not detected in undifferentiated cells (*ud*) but was markedly expressed on day 7 (stage 1) during differentiation. That signal continued to appear on day 11 (stage 2) to 17 (stage 3) (Fig. 6B). Another definitive endoderm marker, FOXA2, also began to be expressed at stage 1 and

also appeared in later periods (Fig. 6C). Expression of PDX1, a marker of pancreatic progenitors, was not detected in undifferentiated and stage 1 cells (data not shown), whereas some signals were detected at stage 2 with a peak signal at stage 3 (Fig. 6D). NGN3, a marker of endocrine progenitors, was also not detected in undifferentiated and stage 1 cells (data not shown), whereas robust signals were detected at stage 2 and continued to be expressed at stage 3 (Fig. 6E). At stage 3, the termination of this differentiation protocol, several insulin- and C-peptide-positive cells were detected (Fig. 6E). Next, we examined the expression dynamics of marker genes by qPCR analysis and concurrently compared our expression dynamics with that of a previously reported protocol (32) (Fig. 7A). It was revealed that the expression dynamics of analyzed genes during differentiation by our protocol (three-step protocol) was similar to that of pancreatic β-cell development (33, 34).

High Oxygen Facilitates Pancreatic Differentiation

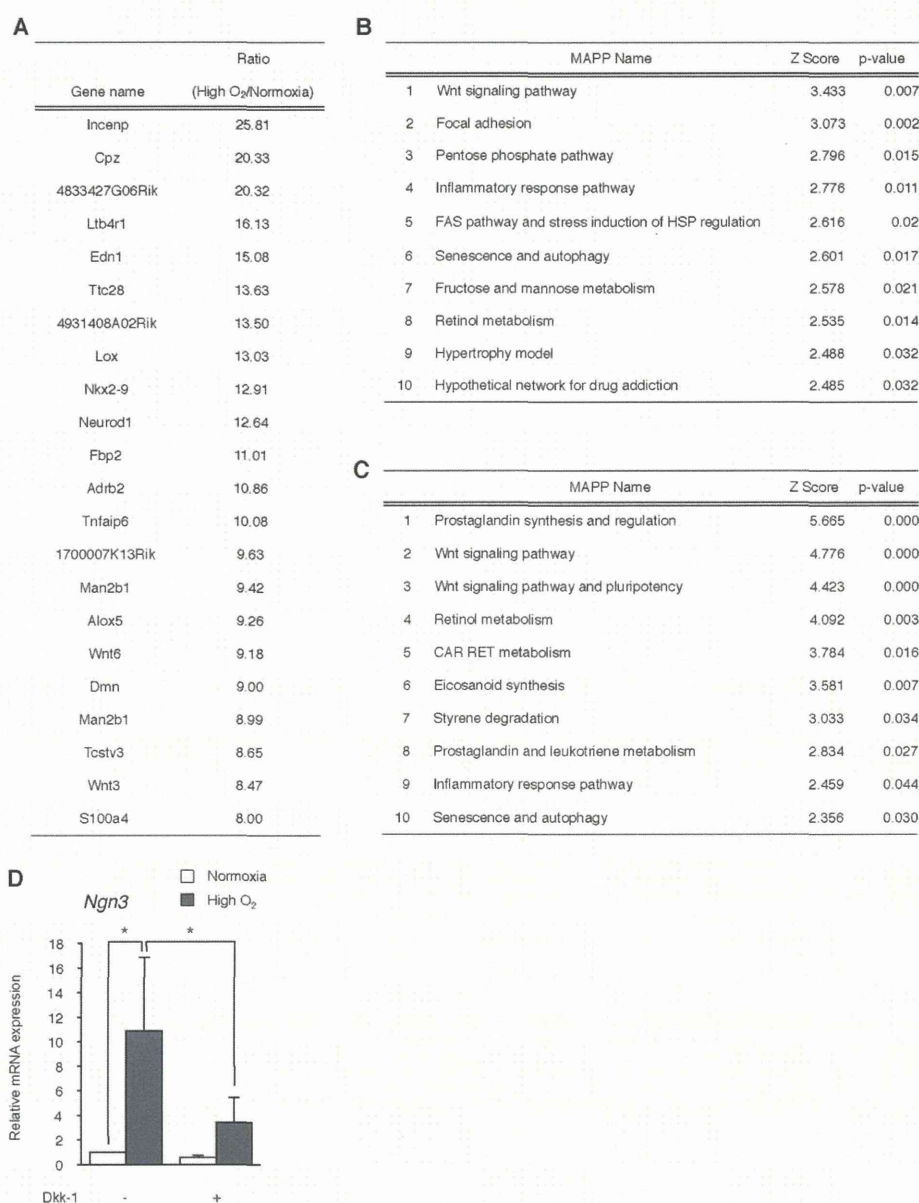


FIGURE 5. Microarray analysis on genes induced by high O₂ condition. A, microarray analysis was performed on ING112 cells treated with high O₂ condition during days 3–5 of differentiation using a TORAY 3D-gene oligo chip. The genes induced by high O₂ condition are shown determined by global normalization after excluding genes of <100 intensity in the high O₂ condition group. Genes increased over 8-fold in the ratio of high O₂ to normoxia are listed. B and C, pathway analysis was performed on up-regulated genes in the high O₂-treated (B) or echinomycin-treated (C) group using GenMapp/MAPP Finder software. D, levels of the *Ng3* gene were analyzed on day 6 of differentiation in normoxia or high O₂-treated ING112 cells with or without Wnt signaling inhibitor Dkk-1 by qPCR. *, $p < 0.05$ versus corresponding control. $n = 11$ each. Error bars, S.E.

The D'Amour protocol consists of stage 1 to stage 5. Stage 1 guides pluripotent cells to definitive endoderm, stage 2 and 3 to pancreatic progenitors, stage 4 to endocrine progenitors, and stage 5 to hormone-expressing endocrine cells. By the D'Amour protocol, *SOX17* and *FOXA2* were expressed higher than in our protocol at stage 1 (Fig. 7A). In contrast, *PDX1* gene expression was very high at stage 2 of our protocol and was higher than in the D'Amour protocol. Moreover, robust increases of *NGN3*, *NEUROD1*, *MAFA*, and *INS* expression were detected at the termination of our protocol (Fig. 7A). The percentages of *PDX1*-, *NGN3*-, and C-peptide-positive cells in the population of differentiated cells by

our protocol were higher than those by the D'Amour protocol (Fig. 7B). Different protocols are usually used for mESC and hESC/iPSC, especially different lengths of time. Therefore, we examined the effect of altered culture times on pancreatic differentiation of hiPSC. A shorter time frame decreased the percentage of C-peptide-positive cells ($p < 0.05$), whereas a longer time had no effect (Fig. 7C).

Next we investigated whether the high O₂ condition has an effect on the pancreatic differentiation of hiPSC (Fig. 8A). The effect of the high O₂ condition during differentiation showed a similar result to that of mESC. Immunofluorescence analysis revealed a significant increase of the percent-