High oxygen condition facilitates the differentiation of mouse and human pluripotent stem cells into pancreatic progenitors and insulin-producing cells*

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Key words: Differentiation; Embryonic stem cell; Hypoxia-inducible factor; Induced pluripotent stem cell; Regenerative medicine.

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

Results: High oxygen condition increases 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 method provides an efficient method to utilize patient-specific iPS cells for the treatment of diabetes.

ABSTRACT

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 (O₂) conditions. Treatment with a high O₂ condition in the early stage of differentiation increased insulin-positive cells at the terminus of differentiation. We found that a high O₂ 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-1α (HIF-1α) protein level. Moreover, a high O₂ condition activated Wnt signaling. Optimal stage-specific treatment with a high O₂ condition resulted in a significant increase in insulin production in both mouse embryonic stem cells (mESCs) and human iPSCs (hiPSCs), and yielded populations containing up to 10% C-peptide-positive cells in hiPSCs. These results suggest that culturing in a high O₂ 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), 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, then pancreatic specialization and maturation in following stages, using combinations of growth factors, small molecules and extracellular matrix. Lumelsky et al. first demonstrated the successful differentiation of mouse ESCs (mESCs) to insulin-secreting structures, which was concluded to be similar to pancreatic islets (6). 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 more safe and efficient methods is desired.

Oxygen (O2) plays a crucial role in cellular homeostasis (9,10). In normal tissues, the lack of oxygen contributes to cell death, while in stem cells, lack of O2 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), O2-regulated transcriptional factors regulate an ever-increasing number of genes involved in glycolytic metabolism, angiogenesis, erythropoiesis, and metastasis, and mediate the adaptation of cells to decreased O2 availability (13,14). O2 tension, the partial pressure of O2, 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 O2 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 is also shown that HIF-1α protein is highly expressed in the embryonic pancreas early in development, and that increasing concentrations of O2 in vitro repressed HIF-1α expression and fostered the development of endocrine progenitors (22,23). Suitable O2 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 O2 on the differentiation efficiency of mESC and human iPSC (hiPSC) into pancreatic lineages. A high O2 condition (60% O2) 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-1α expression and increase of Ngn3 gene expression. Moreover, a high O2 condition was found to induce the activation of Wnt signaling. In this study, we demonstrated that culturing ESC and iPSC in a high O2 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, Carlsbad, CA, USA) supplemented with 1,000 units/ml leukemia inhibitory factor (LIF; Wako, Osaka, Japan), 10% fetal bovine serum (FBS; HyClone Laboratories, Losan, UT, USA), 100 µM nonessential amino acids (NEAA), 2 mM L-glutamine (L-Gln), 1 mM sodium pyruvate, 50 units/ml penicillin and 50 µg/ml streptomycin (P/S), and 100 µM 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 (mmc; Nacalai Tesque, Kyoto, Japan)-treated MEF feeders in DMEM/F12 HAM (Sigma, St. Louis, MO, USA) supplemented with 100 µM NEAA, 2 mM L-Gln, 20% Knockout Serum Replacement (KSR; Invitrogen), 25 units/ml penicillin and 25 µg/ml streptomycin, 100 µM β-ME, and 5 ng/ml bFGF (Peprotech, Rocky Hill, NJ, USA).

**Differentiation of mESC and hiPSC into insulin-producing cells** - Prior to differentiation, mESCs were passaged using 0.25% trypsin and seeded at 35000 cells per well on 4-well or 24-well plates coated with 50 µg/ml poly-L-lysine (Sigma) and 2.5 µg/ml laminin (Roche Applied Science, Indianapolis, IN, USA). After overnight culture, the cells were cultured in high-glucose (4500 mg/l) DMEM supplemented with 10 mg/l insulin, 5.5 mg/l transferrin, 6.7 µg/ml sodium selenite (Insulin-Transferrin-Selenium-G Supplement, ITS; Invitrogen), 2.5 mg/ml ALBUMAX II (Invitrogen), NEAA, L-Gln, P/S, β-ME, 10 ng/ml activin A (Act A; R&D Systems, Minneapolis, MN, USA), 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, P/S, β-ME, B27 supplement (Invitrogen), 50 ng/ml FGF10 (Peprotech), 250 nM KAAD-cyclomycin (CYC; Calbiochem, San Diego, CA, USA), and 1 µM retinoic acid (RA; Sigma). At day 11, the medium was switched to low-glucose (1000 mg/l) DMEM supplemented with ITS, ALBUMAX II, NEAA, L-Gln, PS, β-ME, 10 mM nicotinamide (NAM; Sigma), and 10 nM glucagon-like peptide (GLP1; Sigma), and the cells were cultured until day 17. hiPSCs were passaged as small clusters using 1 U/ml dispase (Roche Applied Science) at a 1:5 split ratio weekly and plated on mmc-treated MEF feeders of 4-well or 24-well plates. After one week culture, the cells were cultured in differentiation medium in the same way as mouse ESCs.

Cells were cultured under high O₂ concentration conditions (60% O₂) for the indicated periods in a multi-gas 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), anti-Pdx1 (1:1000; Upstate Biotechnology, Lake Placid, NY, USA), anti-Ngn3 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-Foxa2 (1:300; Santa Cruz Biotechnology, mouse anti-Insulin (1:1000; Sigma), and rabbit anti-C-Peptide (Cell Signaling Technology, Beverly, MA, USA). Alexa488 or Alexa568-conjugated secondary antibodies were used at 1:500 dilution (Molecular Probe, Eugene, OR, USA). Cells were counterstained with DAPI (Roche Applied Science). 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 (supplementary 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, USA) using a SYBR Premix Ex Taq GC (Takara, Shiga, Japan). The primer sequences for each primer set shown in Table 1. mRNA expression data were normalized against Actin expression in a corresponding sample. The data were analyzed using the relative Quantification (rQ) study in Sequence Detection Software V. 1.2 (Applied Biosystems).

Immunoblotting - HIF-1α levels were determined using immunoblotting. Cells were washed with PBS, scraped and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 4 mM EGTA, 150 mM NaCl, 50 mM NaF, 1 mM Na3VO4, 30 mM Na2P2O7, 10 mM EDTA, 1% Triton X-100). After sonication, 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’s 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 Laboratories, Hercules, CA, USA), and then blocked for nonspecific binding in a 5% skim milk solution. Membranes were incubated with mouse anti-HIF-1α antibody (1:1000; R&D Systems) or mouse anti-β-actin (1:1000; Chemicon, Temecula, CA, USA) 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, USA). After washing, membranes were incubated with enhanced chemiluminescence reagent (GE Healthcare, Piscataway, NJ, USA) and the immunoreactive proteins were visualized by a ImageQuart400 (GE).

MicroArray - Microarray analysis was performed on total RNA samples using 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 upregulated genes in the high O2 condition-treated or echinomycin-treated group using GenMapp/MAPP Finder software.

Statistics - Data are shown as the mean ± S.E.M. Student’s t-test was used to identify significant differences between two conditions and one-way ANOVA or two-way ANOVA 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 ActA 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, CYC, and RA from day 7 onwards, 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 NAM and GLP-1 on day 11, Pdx1 and Ngn3 expressions decreased while 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 stepwise differentiation during stage 1 (day 3-7), stage 2 (day 7-11), or stage 3 (day 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 Figure 2B 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 towards endocrine progenitors.

In our differentiation protocol, stage 1 contained supplements such as Activin A (ActA) 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 undifferentiated state maintained in mESC medium compared to treatment in differentiation medium, showing that the high O_2 condition affected differentiating cells (Fig. 3G).

**High oxygen condition represses HIF-1α protein level and Hes1 gene expression** - Even under normoxia, HIF-1α 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-1α protein level and its target gene expression between normoxic and high O_2 condition groups. High O_2 condition during day 3 to day 6 of differentiation repressed HIF-1α protein level (Fig. 4, A and B). Under normoxic conditions, Vegfa expression, a HIF-1α targeting gene, increased from day 4 with a peak level on day 5 of differentiation, showing that activation of HIF-1α occurs during differentiation, while the high O_2 condition significantly decreased Vegfa expression on day 5 and 6 (P <0.05, respectively; Fig. 4C). It is reported that HIF-1α activates Notch signaling in stem cells and embryonic pancreatic cells (11,22). Hypoxia and subsequent HIF-1α expression induced hairy and enhancer of split (Hes) 1 expression, Notch downstream gene, and repressed Ngn3 expression, leading to the inhibition of β-cell development (22). Therefore, we compared the kinetics of Hes1 and Ngn3 expression in normoxia with those in the high O_2 condition. Under differentiation, Hes1 expression was slightly increased at d4, and gradually decreased from day 5, while high O_2 condition significantly repressed its expression on day 5 and 6 (P <0.01 and P <0.05, respectively; Fig. 4D). In contrast, Ngn3 expression significantly increased on both day 5 and 6 in the high O_2 condition (P <0.05, respectively; Fig. 4E).
Next we examined the effect of HIF-1α inhibition on Ngn3 expression under differentiation. Cells were treated with 1 nM echinomycin, an inhibitor of HIF-1α, from day 3 to 6. On day 6, Ngn3 expression was significantly increased by echinomycin treatment, while 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-1α 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 upregulated by the high O2 condition, and genes showing over 8-fold expression in the high O2 group compared to the normoxia group are listed in Figure 5A. Pathway analysis using upregulated genes in the high O2 group indicated that several genes were involved in the Wnt signaling pathway, and this pathway was ranked first (P <0.1; 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 upregulated genes (P <0.001; Fig. 5C), suggesting that HIF-1α inhibition led to the activation of Wnt signaling.

The Wnt/beta-catenin pathway plays an important role in the regulation of pluripotency and pancreatic development and differentiation (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). PDX1 expression, a marker of pancreatic progenitors, was not detected in ud and stage 1 (data not shown), while 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 ud and stage 1 (data not shown), while 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 (3-step protocol) was similar to that of pancreatic β-cell development (33,34). 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 timeframe decreased the
percentage of C-PEPTIDE-positive cells (P <0.05), while 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 percentage of C-peptide-positive cells by the high O2 condition during stage 1 (P <0.05; Fig. 8B). It was confirmed that there was significant increase in INS gene expression (P <0.05; Fig. 8C). These effects were also observed in an other hiPSC line, 201B7 (34) (P <0.05; Fig. 8D). We performed immunostaining for glucagon to determine whether differentiated cells are monohormonal or polyhormonal by our protocol. A few glucagon-positive cells were observed in the differentiated cells treated with the high O2 condition (Fig. 8E). Moreover, co-expression with C-PEPTIDE appeared in a few cells, suggesting that some cells were polyhormonal for insulin and glucagon.

High oxygen condition facilitates differentiation of hiPSC into endocrine progenitors - Corresponding with our results on mESC, we observed that NGN3 expression was significantly increased on day 7 by the high O2 condition during stage 1, while VEGFA and HES1 expression were significantly decreased (P <0.0000005 and P <0.000005, respectively; Fig. 9, A and B). Immunofluorescence analysis confirmed that the percentage of NGN3-positive cells was increased by this treatment (Fig. 9C). To further determine whether HIF-1α inhibition and Wnt signaling activation are involved in the case of hiPSC, we performed Western blot analysis of HIF-1α and microarray analysis of high O2-treated hiPSCs. The high O2 condition repressed the HIF-1α protein level (Fig. 9D). By microarray analysis, many genes were found to be upregulated by the high O2 condition, as listed in supplementary Figure S2A. Some of these genes were determined as the Wnt receptor signaling pathway in the GO biological process (Supplementary Fig. S2B), suggesting that Wnt signaling was also activated in high O2-treated hiPSCs. In addition, Dkk-1 treatment weakened the high O2-induced NGN3 expression (Fig. 9E). These results in hiPSC are similar to those observed in mouse ESC, thereby indicating that both human and mouse pluripotent cells follow a similar pathway in a high O2 condition.

We tested whether the high O2 condition had an effect even in the D’Amour protocol and Nostro protocol (1) (Fig. 10A). Using this protocol, we also saw a significant increase in the percentage of C-PEPTIDE-positive cells by the high O2 condition from day 1 to 4 (Stage 1; P <0.05) and also in the INS expression by high O2 condition from day 1 to 4 (Stage 1; P <0.05) and day 4 to 7 (Stage 2; P <0.05) (Fig. 10, B and C). However, in the Nostro protocol, we did not observe any facilitative effect of the high O2 condition on the percentage of C-PEPTIDE-positive cells (Fig. 10, D and E).

DISCUSSION

Insulin-secreting pancreatic β-cells are essential regulators of the mammalian metabolism. The absence of functional β-cells leads to hyperglycemia and diabetes, making patients dependent on exogenously supplied insulin. Recent insights into β-cell development, combined with the discovery of pluripotent stem cells, have led to an unprecedented opportunity to generate new β-cells for transplantation therapy and drug screening (36,37). It is important to mimic the in vivo developmental stages of pancreatic organogenesis in which cells are transitioned through mesendoderm, definitive endoderm, foregut endoderm, pancreatic progenitor, and the endocrine progenitor stage, until mature β-cells are obtained from pluripotent stem cells (38). Oxygen tension, the partial pressure of oxygen, has been shown to regulate the stem cell function and embryonic development of several organs, including pancreas (9,10,15-23). In the present study, we demonstrated that a high O2 condition during the in vitro differentiation of ESC and iPSC has a facilitative effect on generating pancreatic progenitors and insulin-producing cells.

In our stepwise differentiation protocol, the cells transitioned through definitive endoderm, pancreatic progenitor, endocrine progenitor, and insulin-producing cells as revealed by qPCR analysis. Induction of Pdx1 and Ngn3 gene expressions appeared on day 7 (stage 1), showing that slight transition to pancreatic progenitors and
endocrine progenitors had already started during stage 1 (Fig. 1B). With this protocol, treatment with a high O2 condition during stage 1 (toward to definitive endoderm) increased differentiation efficiency into endocrine progenitors, and subsequent insulin-producing cells. This was demonstrated by a significant increase of Ngn3-positive cells and Ngn3 gene expression (Fig. 3, B, E and F). Ngn3 is a basic helix-loop-helix transcription factor expressed in cell progenitors that is necessary to initiate the endocrine differentiation program in pancreatic development (39,40), and its gene expression is inversely regulated by HIF-1α (22). Down regulation of Notch signaling will yield cells that express Ngn3 (41). Ngn3 gene expression and pancreatic endocrine development are tightly regulated by Hes1, which is an inhibitory bHLH factor activated by Notch signaling, and binds to the proximal promoter of Ngn3 and specifically blocks promoter activity (39,42). It has been shown that HIF-1α activates Notch-responsive promoters and increases the expression of Notch direct downstream genes, including Hes1 (11). During differentiation, HIF-1α signaling is moderately activated even in normoxic conditions, revealed by HIF-1α protein expression and increase of its target gene Vegfa, while a high O2 condition marked repressed both expressions (Fig. 4, B and C). Supporting our results, it is reported that HIF-1α signaling of cultured stem cells is activated during spontaneous differentiation even in normoxic conditions, showing a time-dependent increase of Vegfa (43). A high O2 condition might increase cellular O2 concentration and lead to inhibition of HIF-1α signaling. The high O2 condition significantly repressed Hes1 gene expression on day 5 and 6 (Fig 4D). Consistent with previous reports, inhibition of HIF-1α signaling might lead to repression of Hes1 expression and subsequent induction of Ngn3 expression in a high O2 condition. Furthermore, HIF-1α inhibitor echinomycin had a similar effect to the high O2 condition (Fig 4F). These data indicate that inhibition of HIF-1α signaling is involved in the facilitative effect of the high O2 condition on pancreatic differentiation. The high O2 condition had no effect on Sox17 gene expression and the number of immunoreactive cells (Fig. 3, B and C), suggesting that its treatment might affect transition from definitive endoderm to pancreatic progenitor or endocrine progenitor. This is supported by the result that its treatment did not increase Ngn3 expression in the undifferentiated state (Fig 3G). The high O2 condition significantly decreased Oct4 gene expression (Fig. 3B). Oct4 gene expression is directly regulated by HIF-2α, also a hypoxia-dependent factor (12); therefore, it is considered that the high O2 condition might repress Oct4 gene expression via HIF-2α inhibition.

By microarray analysis, we found that the Wnt signaling pathway is activated in high O2 condition-treated cells (Fig. 5B). Wnt inhibitor Dkk-1 partially repressed high O2 condition-induced Ngn3 expression (Fig 5D). It is reported that hypoxia inhibits Wnt signaling via HIF-1α, competing with T-cell factor-4 (TCF-4) for direct binding to β-catenin (44). The high O2 condition might inhibit HIF-1α signaling and compensatory Wnt signaling was activated. This is supported by the result that genes induced by the HIF-1α inhibitor echinomycin are also involved in the Wnt signaling pathway (Fig. 5C). The canonical Wnt cascade has emerged as a critical regulator of self-renewal and pluripotency in stem cells (30,45-49). In contrast, it is also reported that Wnt/β-catenin signaling promotes the differentiation, not self-renewal, of embryonic stem cells (1,32,50,51). Nostro et al. showed that Wnt signaling induce a posterior endoderm fate, the primed stage from definitive endoderm, and at optimal concentrations enhanced the development of pancreatic lineage cells (1). In this report, Wnt signaling did not affect the levels of PDX1, but did increase INS expression in hiPSC with Wnt3a treatment at the stage of definitive endoderm to pancreatic endoderm. This report is consistent with our results that a high O2 condition activates Wnt signaling and facilitates differentiation from definitive endoderm into pancreatic fate. In the developing embryo, a key step in the generation of endoderm-derived cell types is patterning the appropriate region of the gut tube along the anterior-posterior axis. Studies using different model systems have shown that in gastrulation, Wnt signaling is restricted to the posterior region of the embryo and, together with FGF signaling, is responsible for the induction of a posterior phenotype (52,53). Activated Wnt signaling by a
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The high O₂ condition might function to promote the development of a posterior phenotype in mESC and hiPSC cultures.

The high O₂ condition also facilitates pancreatic differentiation of hiPSC. Our stepwise differentiation protocol generated insulin-producing cells larger than the D’Amour protocol (32). However, in the case of SOX17 and FOXA2, a robust increase was observed in the D’Amour protocol (Fig. 7A), indicating that the induction of definitive endoderm was more efficient than our three-step protocol. This might have been due to the difference of the Activin A (ActA) concentration (D’Amour protocol, 100 ng/ml; our protocol, 10 ng/ml), because induction of definitive endoderm by ActA is reported to increase in a dose-dependent fashion (54-56). However, at stage 2 of our protocol, there was a marked increase in the expression of PDX1, greater than at stage 4 of the D’Amour protocol. Furthermore, NGN3, NEUROD1, MAFA and INS gene expressions were higher in our protocol, indicating that the induction of pancreatic fate from definitive endoderm in our protocol was more efficient than with the D’Amour protocol. Using this protocol, hiPSCs more efficiently differentiated into endocrine progenitors and insulin-producing cells in the high O₂ condition during stage 1, similar to mESCs. The effect of the high O₂ condition was also observed in the D’Amour protocol, but not in the Nosto protocol (1) (Fig. 10, A-E). Nosto et al. included VEGF during stage 1, probably to support endothelial development for pancreatic differentiation of hESC (1, 57). The high O₂ condition repressed VEGF expression in our study (Fig. 9B), and a similar effect is expected to occur in the Nosto protocol. This effect may compete with the addition of VEGF. Therefore, the high O₂ condition seems to have no facilitative effect in the Nosto protocol.

Insulin-producing cells obtained in our study did not secrete insulin by high-glucose stimulation (data not shown), and some cells were polyhormonal because co-expression of insulin and glucagon occurred. During normal human embryogenesis, β-cells are not generated until ~10 weeks after endoderm specification (58), while in hiPSC differentiation cultures, this typically occurs in 2 to 3 weeks (32). It is possible that pancreatic differentiation in human ESC/iPSC culture may be accelerated by rapid changing of the transcriptional network and/or epigenetic modifications by changing supplements (growth factor and inhibitors, etc). For proper β-cells it may be necessary to change the extracellular environment and signal more precisely to mimic normal human embryogenesis.

In the previous study, Shah et al. stated that the early mammalian embryo is located within the uterus, with a non-existent or immature cardiovascular system and blood supply but, despite this hypoxic environment, the embryo is still able to undergo rapid growth and organogenesis (21). Furthermore, they showed that the number of Ngn3-positive cells was not altered by hypoxia treatment in pancreatic explants, while the number of insulin-positive cells was decreased by hypoxia, implying that high oxygen may only be required at later stages during pancreatic differentiation, namely endocrine progenitor to β-cell. However, in our study, the number of Ngn3-positive cells differentiated from mESCs and hiPSCs was increased by the high O₂ condition (Fig. 3, E, F and Fig. 9C). There are some differences between the culture environments of dissociated mESCs and pancreatic explants. (1) HIF-1α levels seem to be different between mESC culture and pancreatic explants because of spatial and temporal patterns of cell-cell interactions. (2) Moreover, the external signals were different, as our study used chemically defined medium, whereas pancreatic explants were maintained in serum-containing medium. These differences seemed to have caused the discrepancy.

A previous study modulated the O₂ environment for pancreatic differentiation (59). Cheng et al. used a 5% O₂ environment for maintaining and differentiating human endodermal progenitor (EP) cells into β-cells. However, they did not mention the reason for using a hypoxic environment and did not compare the effect on differentiation cultured under hypoxia with normoxia. In our study, a hypoxic condition (5% O₂) during differentiation had no facilitative effect on the number of Ins1-GFP-positive cells of mESCs. This discrepancy seems to have been particularly caused by the cell density during differentiation. In their studies, EP cells were plated in 12-well dishes at 3-4 x 10⁵
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cells per well as dissociated at the start of
differentiation, whereas in our study, hiPSCs were
grown for 7 days as a colony before the start of
differentiation. In the colony state, cells appeared
to promote a hypoxic phenotype because HIF-1α
is expressed at a detectable level even in normoxia
(Fig. 9D). Therefore, in our case, the high O₂
condition rather than the hypoxic condition
facilitated pancreatic differentiation.

In conclusion, the present study showed that a
high O₂ condition during differentiation has
facilitative effects on generating insulin-producing
cells from mESCs and hiPSCs. This effect was
due to the inhibition of Notch signaling and
activation of Wnt signaling during definitive
endoderm to pancreatic fate. We also found that
HIF-1α inhibition during differentiation
accelerated the generation of pancreatic lineages.
These observations would provide an efficient
method of utilizing patient-specific iPS cells for
the treatment of diabetes.
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Abbreviations: mESC, mouse embryonic stem cell; hiPSC, human induced-pluripotent stem cells; HIF-1α, hypoxia-inducible factors-1α

FIGURE LEGENDS

FIGURE 1. Stepwise differentiation of mESCs into insulin-producing cells. A, Scheme of the stepwise differentiation protocol used to generate insulin-producing cells from mES ING112 cells. Act A, ActivinA; 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.

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 µm. 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.

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 gene 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 µm. 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 Ngn3 gene were analyzed on undifferentiated ING112 cells treated with high O₂ condition for 3 days by qPCR. Effect of high O₂ condition on differentiating cells is shown as a positive control. *P <0.05 versus corresponding control. n = 4 each.

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 day 3, 4, 5 and 6 of differentiation in normoxia or high O₂-treated ING112 cells by qPCR. *P <0.05 or **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 or **P <0.01 versus corresponding control. n = 3 each.

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 day 3 to 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, C, Pathway analysis was performed on upregulated genes in the high O₂-treated (B) or echinomycin-treated (C) group using GenMapp/MAPP Finder software. D, Levels of Ngn3 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.

FIGURE 6. Stepwise differentiation of hiPSCs into insulin-producing cells. A, Scheme of the stepwise differentiation protocol used to generate insulin-producing cells from hiPS C23 cells. B-E, Immunofluorescence for SOX17 (B), FOXA2 (C), PDX1 (D), NGN3 (E), INSULIN (E) and C-PEPTIDE (E) at day 7 (stage 1), 11 (stage 2) or 17 (stage 3) of differentiation in C23 cells in normoxic condition. Scale bars = 200 µm.

FIGURE 7. Dynamics of pancreatic differentiation marker genes. A, The dynamics of SOX17, FOXA2, PDX1, NGN3, NEUROD1, MAFa and INS gene expression were analyzed at different stages in normoxic conditions under the D’Amour protocol or our 3-step protocol by qPCR. n = 3 each. ud, undifferentiated; st, stage. B, Values are the percentage of PDX1-, NGN3-, or C-PEPTIDE-positive cells per well of the cells differentiated under the D’Amour protocol or our 3-step protocol. n = 3 each. C, Values are the percentage of C-PEPTIDE-positive cells per well of the cells differentiated under different timeframe of our 3-step protocol. *P <0.05 versus corresponding control. n = 4 each. d, day.

FIGURE 8. Effect of high O₂ condition on differentiation efficiency of hiPSCs. A, Scheme of the timeline of high O₂ condition. B, Immunofluorescence for C-PEPTIDE on day 17 of differentiation in C23 cells treated with high O₂ condition during stage 1. Scale bars = 200 µm. Right graph, values are the percentage of C-PEPTIDE-positive cells per well of the cells treated with high O₂ condition during three different stages. *P <0.05 versus corresponding control. n = 11 each. C, Level of INS gene was analyzed at day 17 of differentiation in normoxia or high O₂-treated C23 cells by qPCR. *P <0.05 versus corresponding control. n = 4 each. D, Values are the percentage of C-PEPTIDE-positive cells per well of
the cells treated with high O$_2$ condition during three different stages in hiPSC line 201B7. *P <0.05 versus corresponding control. n = 8 each. E, Immunofluorescence for C-PEPTIDE and glucagon on day 17 of differentiation in C23 cells treated with high O$_2$ condition during stage 1. Scale bars = 200 µm.

FIGURE 9. Effect of high O$_2$ condition on NGN3 gene expression of hiPS cells. A, Scheme of the timeline of high O$_2$ condition. B, Levels of VEGFA, HES1 and NGN3 gene were analyzed at day 7 of differentiation in normoxia or high O$_2$-treated C23 cells by qPCR. *P <0.05 or **P <0.01 versus corresponding control. n = 4 each. C, Immunofluorescence for NGN3 on day 11 of differentiation in C23 cells treated with high O$_2$ condition during stage 1. Scale bars = 200 µm. Right graph, values are the percentage of NGN3-positive cells per well of the cells treated with high O$_2$ condition during stage 1. *P <0.05 versus corresponding control. n = 3 each. D, Levels of HIF-1α and β-actin (loading control) were analyzed on day 6 of differentiation in normoxia, high O$_2$, or hypoxia (1% O$_2$; positive control)-treated C23 cells by immunoblotting. E, Levels of NGN3 gene were analyzed on day 6 of differentiation in normoxia or high O$_2$-treated C23 cells with or without Wnt signaling inhibitor Dkk-1 by qPCR. n = 4 each.

FIGURE 10. Effect of high O$_2$ condition on differentiation efficiency of hiPSCs in D’Amour and Nostro protocol. A, Scheme of the D’Amour differentiation protocol and the timeline of high O$_2$ condition. FBS, fetal bovine serum; Ex-4, exendin-4; HGF, hepatocyte growth factor; IGF1, insulin-like growth factor 1. B, Values are the percentage of C-PEPTIDE-positive cells per well of the cells treated with high O$_2$ condition during three different stages. *P <0.05 versus corresponding control. n = 6 each. C, Levels of INS gene were analyzed on day 17 of differentiation in cells treated with high O$_2$ condition during five different stages by qPCR. *P <0.05 versus corresponding control. n = 7 each. D, Scheme of the Nostro differentiation protocol and the timeline of high O$_2$ condition. E, Values are the percentage of C-PEPTIDE-positive cells per well of the cells treated with high O$_2$ condition during three different stages. n = 3 each.
Table 1. Primers used in quantitative real-time PCR analysis.

| Mouse         | Genes | Sequences (forward and reverse) | Product (bp) |
|---------------|-------|---------------------------------|--------------|
|               | Act   | CCTCATGAGATGCTCTCCGGA TGGCAATGTGATGACTCTGG | 192          |
|               | Oct4  | GAGGAAGGCCGAACCAATGAGAACCCTTCAG TTCTGGCAGCGGTTACAGAACACTCTGA | 227          |
|               | Sox17 | GAACAGTGAGGCGCTACAC GTTAGGTTTCTCTAGATGC | 322          |
|               | Foxa2 | TGTCATCGGTCAGAAGGAA GCAACACAGCAATAGAGA | 289          |
|               | Pdx1  | TCACAGGAGACAGAATGCTCT TTCCGCTGTTAGCAGCCTC | 264          |
|               | Ngn3  | ACTGACGCGTGGTGCGAGTAC AAGGGATGAGGCCGACATCT | 225          |
|               | Ins1  | CAGGGCGGGGAGACGGCTA ATGGTGCCAGGAGACATCT | 348          |
|               | Vegfa | GTCTCGGTCAGGATGAGA AGGTGGATGCAATGGCTC | 185          |
|               | Hes1  | TCAACAGCAACCCGACAAACC GGTATTTCCCCAACACGCT | 270          |

| Human         | Genes | Sequences (forward and reverse) | Product (bp) |
|---------------|-------|---------------------------------|--------------|
|               | ACT   | CCTCATGAGATGCTCTCCGGA TGGCAATGTGATGACTCTGG | 192          |
|               | SOX17 | GCATGACTCGGGTTGAGACTCT | 103          |
|               | FOXA2 | ATTGCTTGCTGTGGTTTGTTG TACGTGGTTATGCGCTTCAT | 187          |
|               | PDX1  | CTTTTTCCTGAGATGAGTC TACGTGGTTATGCGCTTCAT | 145          |
|               | NGN3  | TTGGCAGCGAGAAAGATGAC TCAGTGGCAACTCTGCTTCTTC | 249          |
|               | NEUROD1 | CCATGGTGGGTTGTCATAATCT | 200          |
|               | MAFA  | TGCAAGGCGGCCATATC TCCAGCTTCTCTGTATTTCCTTGT | 128          |
|               | INS   | GAGGCCATCAAGCAGATCAC GGCCTGCTTCTGTTTGG | 373          |
|               | VEGF4 | CCCTGATGAGATGAGTACAT CGCCTACTGACATGGCTC | 496          |
|               | HES1  | TCAACAGGAACCCGGAATCC GGTATTTCCCCAACACGCT | 270          |
Figure 1

High oxygen facilitates pancreatic differentiation

A

| d0 | d1 | d3 | d5 | d7 | d9 | d11 | d13 | d15 | d17 |
|----|----|----|----|----|----|------|-----|-----|------|

Act A + bFGF, DMEM/4500ITS  FGF10 + CYC + RA, RPMI/B27  NAM + GLP-1, DMEM/1000ITS

Stage 1  Stage 2  Stage 3

B

Relative mRNA expression

Oct4  Sox17  Foxa2

Pdx1  Ngn3  Ins1

ud  d7  d11  d17

ud  d7  d11  d17

ud  d7  d11  d17

ud  d7  d11  d17
High oxygen facilitates pancreatic differentiation

Figure 2

A

Stage 1

- High O₂

Stage 2

- High O₂

Stage 3

- High O₂

ActA + bFGF, DMEM/500ITS

FGF10 + CYC + RA, RPM/B27

NAM + GLP-1, DMEM/1000ITS

B

Control

High O₂

Ins1-GFP

Ins1-GFP/DAPI

C

Ins1-GFP+ cells (%)

Stage 1 - High O₂

Stage 2 - High O₂

Stage 3 - High O₂

D

Ins1-GFP+ cells (%)

Stage 1 - Hypoxia

Stage 2 - Hypoxia

Stage 3 - Hypoxia

E

SK7

C-peptide+ cells (%)

Stage 1 - High O₂

Stage 2 - High O₂

Stage 3 - High O₂

F

Surviving cells (%)

Stage 1 - High O₂ (d3 to d7)

Stage 2 -

Stage 3 -

G

Ins1-GFP+ cells (%)

Stage 1 - High O₂ (40%)

Stage 2 -

Stage 3 -

*
Figure 3

High oxygen facilitates pancreatic differentiation
High oxygen facilitates pancreatic differentiation

Figure 4

A

B

C

D

E

F

Echinomycin

Actin

Stage 1

Vegfa

Hes1

Ngn3

Relative mRNA expression

Stage 1

High O2

DMEM/4500ITS

Act A + bFGF

Echinomycin

High O2

Echinomycin

DMSO
Figure 5

High oxygen facilitates pancreatic differentiation

| MAPP Name | Z Score | p-value |
|-----------|---------|---------|
| 1 Wnt signaling pathway | 3.433 | 0.007 |
| 2 Focal adhesion | 3.073 | 0.002 |
| 3 Pentose phosphate pathway | 2.796 | 0.015 |
| 4 Inflammatory response pathway | 2.776 | 0.011 |
| 5 FAS pathway and stress induction of HSP regulation | 2.616 | 0.02 |
| 6 Senescence and autophagy | 2.601 | 0.017 |
| 7 Fructose and mannose metabolism | 2.578 | 0.021 |
| 8 Retinol metabolism | 2.535 | 0.014 |
| 9 Hypertrophy model | 2.488 | 0.032 |
| 10 Hypothetical network for drug addiction | 2.485 | 0.032 |

A

| Gene name | Ratio (High O2/Normoxia) |
|-----------|--------------------------|
| Incenp | 25.81 |
| Cpz | 20.33 |
| 4833427G06Rik | 20.32 |
| Lb4r1 | 16.13 |
| Edn1 | 15.08 |
| Ttc28 | 13.63 |
| 4931408A02Rik | 13.50 |
| Lox | 13.03 |
| Nkx2-9 | 12.91 |
| Neurod1 | 12.64 |
| Flp2 | 11.01 |
| Adrb2 | 10.86 |
| Tnflap6 | 10.08 |
| 1700007K13Rik | 9.63 |
| Man2b1 | 9.42 |
| Alox5 | 9.26 |
| Wnt6 | 9.18 |
| Dmn | 9.00 |
| Man2b1 | 8.99 |
| Tcstv3 | 8.65 |
| Wnt3 | 8.47 |
| S100a4 | 8.00 |

B

| MAPP Name | Z Score | p-value |
|-----------|---------|---------|
| 1 Prostaglandin synthesis and regulation | 5.665 | 0.000 |
| 2 Wnt signaling pathway | 4.776 | 0.000 |
| 3 Wnt signaling pathway and pluripotency | 4.423 | 0.000 |
| 4 Retinol metabolism | 4.092 | 0.003 |
| 5 CAR RET metabolism | 3.784 | 0.016 |
| 6 Eicosanoid synthesis | 3.581 | 0.007 |
| 7 Styrene degradation | 3.033 | 0.034 |
| 8 Prostaglandin and leukotriene metabolism | 2.834 | 0.027 |
| 9 Inflammatory response pathway | 2.459 | 0.044 |
| 10 Senescence and autophagy | 2.356 | 0.030 |

C

| MAPP Name | Z Score | p-value |
|-----------|---------|---------|
| 1 Prostaglandin synthesis and regulation | 5.665 | 0.000 |
| 2 Wnt signaling pathway | 4.776 | 0.000 |
| 3 Wnt signaling pathway and pluripotency | 4.423 | 0.000 |
| 4 Retinol metabolism | 4.092 | 0.003 |
| 5 CAR RET metabolism | 3.784 | 0.016 |
| 6 Eicosanoid synthesis | 3.581 | 0.007 |
| 7 Styrene degradation | 3.033 | 0.034 |
| 8 Prostaglandin and leukotriene metabolism | 2.834 | 0.027 |
| 9 Inflammatory response pathway | 2.459 | 0.044 |
| 10 Senescence and autophagy | 2.356 | 0.030 |
Figure 6

High oxygen facilitates pancreatic differentiation

A

|   | d0 | d1 | d3 | d5 | d7 | d9 | d11 | d13 | d15 | d17 |
|---|----|----|----|----|----|----|-----|-----|-----|-----|
|   | Act A + bFGF | FGF10 + CYC + RA | NAM + GLP-1 |
|   | DMEM/4500ITS (Stage 1) | RPMI/B27 (Stage 2) | DMEM/1000ITS (Stage 3) |

B

SOX17 | SOX17 / DAPI

C

FOXA2 | FOXA2 / DAPI

D

PDX1 | PDX1 / DAPI

E

NGN3 | INS | NGN3 / INS / DAPI

C-PEP | C-PEP / DAPI
Figure 7

A

High oxygen facilitates pancreatic differentiation

B


C

Stage 1
Stage 2
Stage 3

D'Amour protocol
3-step protocol

n.d.

St1
St2
St3
St4
St5
High oxygen facilitates pancreatic differentiation

**A**

![Diagram showing time points and treatments](image)

- d0 to d17
- d0: Act A + bFGF, DMEM/4500ITS
- d7: FGF10 + CYC + RA, RPMI/B27
- d13: NAM + GLP-1, DMEM/1000ITS
- Stage 1: FGF10 + CYC + RA
- Stage 2: NAM + GLP-1
- Stage 3: DMEM/1000ITS

**B**

![Images and bar graph showing C-PEP and C-PEPTIDE+ cells](image)

**C**

![Bar graph showing INS expression](image)

**D**

![Bar graph showing C-PEPTIDE+ cells](image)

**E**

![Images showing C-PEP and GCG](image)
High oxygen facilitates pancreatic differentiation

Figure 9

A

Stage 1

Act A + bFGF, DMEM/4500ITS

B

Relative mRNA expression

VEGFA  HES1  NGN3

Normoxia  High O2

C

NGN3

Control  HIF-1α

D

HIF-1α

Actin

E

NGN3

Normoxia  High O2

Dkk-1

Relative mRNA expression
Figure 10

A

Stage 1 → High O2 → Stage 2 → High O2 → Stage 3 → High O2 → Stage 4 → High O2 → Stage 5

Stage 1
Stage 2
Stage 3
Stage 4
Stage 5

ActA + Wnt3a, RPMI
FGF10 + CYC, RPMI/FBS
FGF10 + CYC + RA, DMEM4500/B27
Ex-4 + DAPT, DMEM4500/B27
Ex-4 + HGF + IGF1, CMRL1066

B

C

D

E

Stage 1
Stage 2
Stage 3
Stage 4
Stage 5

Stage 1
Stage 2
Stage 3
Stage 4

SFD+ActA+Wnt3a +BMP4+bFGF+VEGF
SFD+Wnt3a+FGF10 +Dorso
DMEM+NCR +FGF10+bB27
DMEM+B27

Stage 1
Stage 2
Stage 3
Stage 4

C-PEPTIDE+ cells (%)
C-PEPTIDE+ cells (%)
Relative mRNA expression
Relative mRNA expression

Stage 1: High O2
Stage 2 & 3: High O2
Stage 4: High O2
Stage 5: High O2