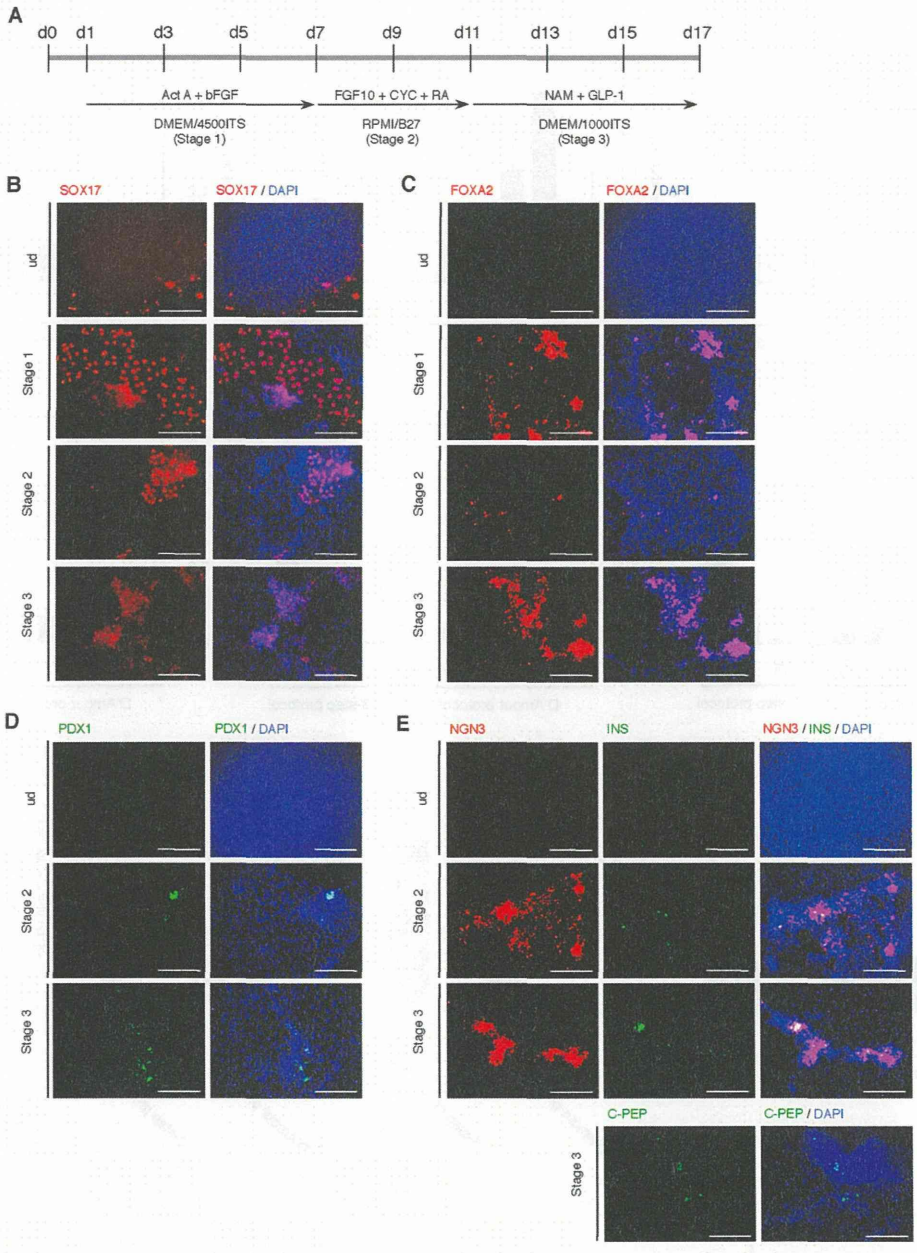

FIGURE 6. **Stepwise differentiation of hiPSCs into insulin-producing cells.** *A*, scheme of the stepwise differentiation protocol used to generate insulin-producing cells from human induced pluripotent stem C23 cells. *B–E*, immunofluorescence for SOX17 (*B*), FOXA2 (*C*), PDX1 (*D*), NGN3 (*E*), insulin (*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.

age of C-peptide-positive cells by the high O_2 condition during stage 1 ($p < 0.05$; Fig. 8*B*). It was confirmed that there was a significant increase in *INS* gene expression ($p < 0.05$; Fig. 8*C*). These effects were also observed in another hiPSC line, 201B7 (35) ($p < 0.05$; Fig. 8*D*). 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 O_2 condition (Fig. 8*E*). 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 O_2 condition during stage 1, whereas *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. 9*C*). 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 O_2-treated hiPSCs. The high O_2 condition

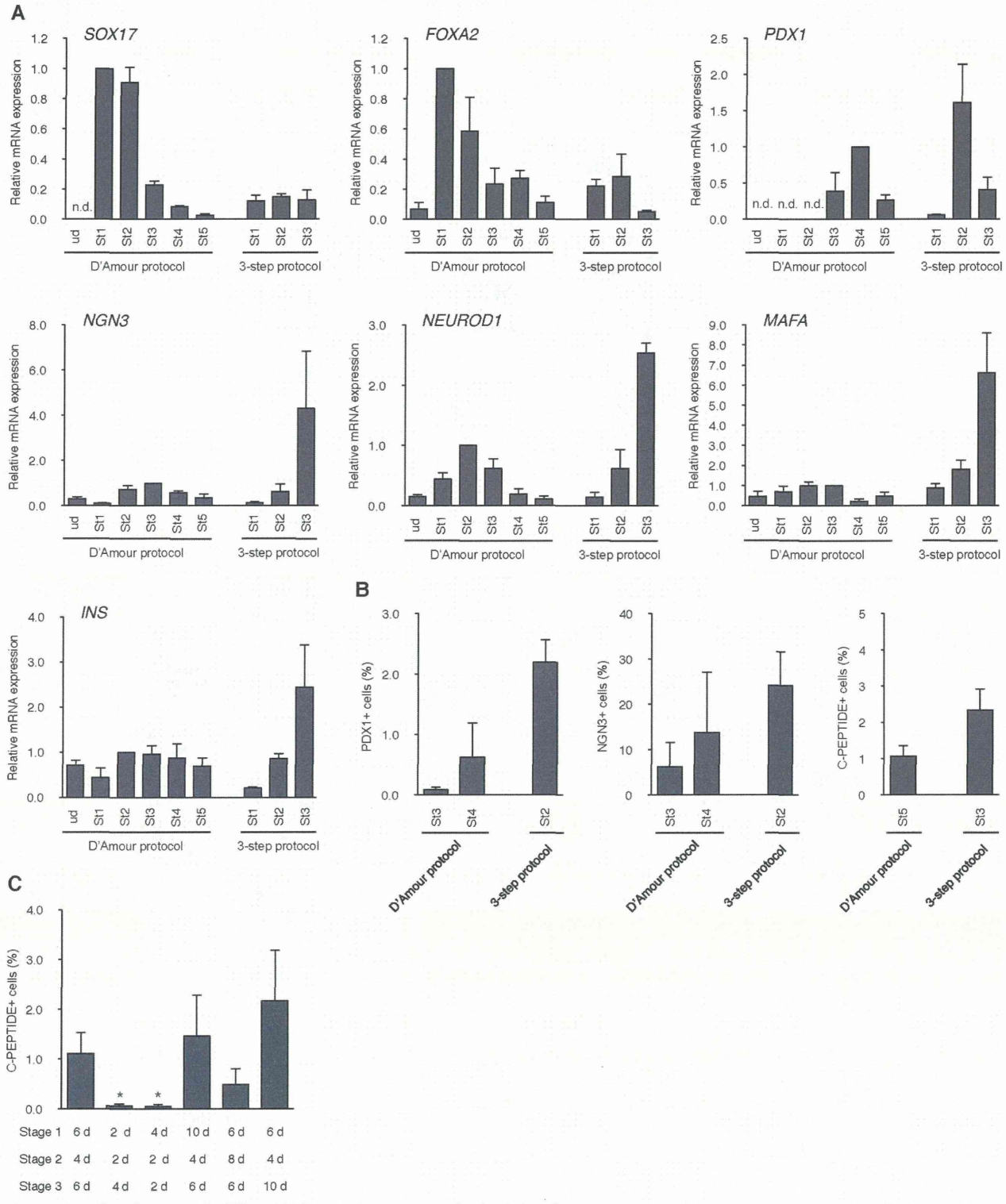

FIGURE 7. **Dynamics of pancreatic differentiation marker genes.** *A*, the dynamics of *SOX17*, *FOXA2*, *PDX1*, *NGN3*, *NEUROD1*, *MAFA*, and *INS* gene expression were analyzed at different stages in normoxic conditions under the D'Amour protocol or our three-step protocol by qPCR. $n = 3$ each. *ud*, undifferentiated; *st*, stage. *B*, values are the percentage of PDX1-, NGN3-, or C-peptide-positive cells/well of the cells differentiated under the D'Amour protocol or our three-step protocol. $n = 3$ each. *C*, values are the percentage of C-peptide-positive cells/well of the cells differentiated under a different time frame of our three-step protocol. *, $p < 0.05$ *versus* corresponding control. $n = 4$ each. *d*, day. *Error bars*, S.E.

repressed the HIF-1α protein level (Fig. 9*D*). By microarray analysis, many genes were found to be up-regulated by the high O_2 condition, as listed in supplemental Fig. S2*A*. Some of these genes were determined as the Wnt receptor signaling pathway in the GO biological process (supplemental Fig. S2*B*), suggesting that Wnt signaling was also activated in high O_2-treated

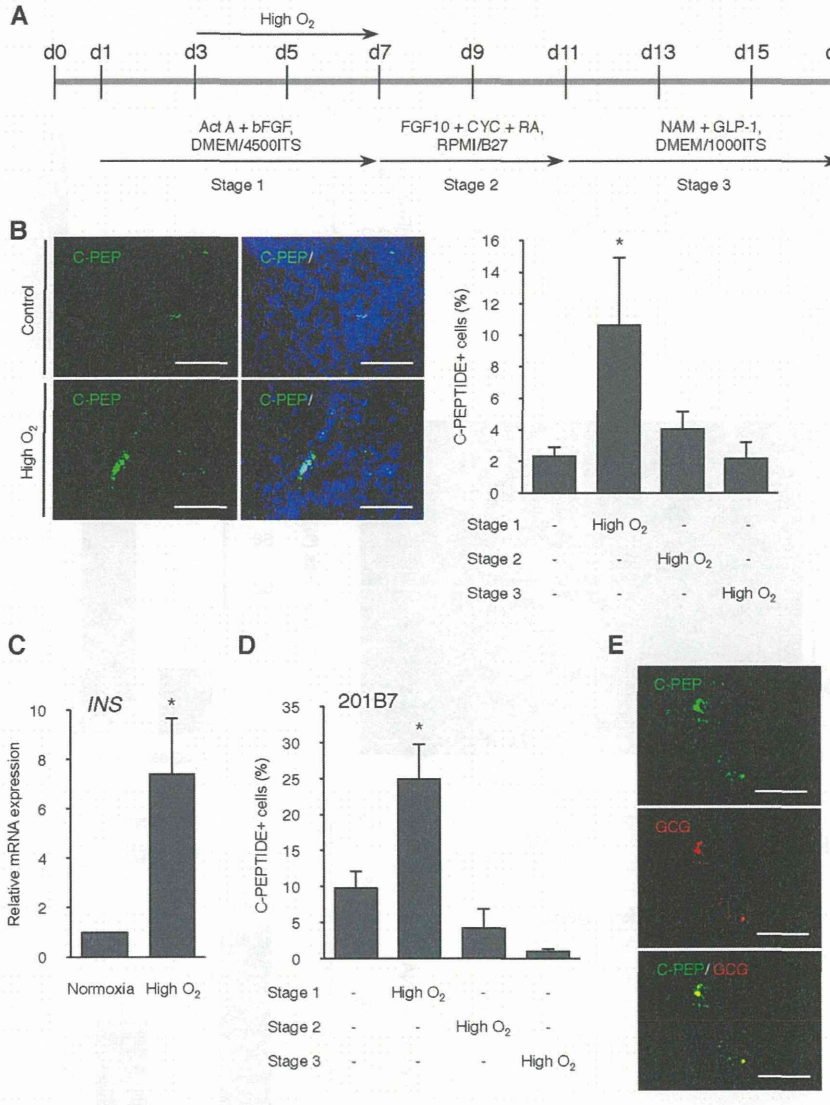

FIGURE 8. **Effect of high O_2 condition on differentiation efficiency of hiPSCs.** *A*, scheme of the timeline of high O_2 condition. *B*, immunofluorescence for C-peptide on day 17 of differentiation in C23 cells treated with high O_2 condition during stage 1. *Scale bars*, 200 μm. *Right graph*, values are the percentage of C-peptide-positive cells/well of the cells treated with high O_2 condition during three different stages. *, $p < 0.05$ *versus* corresponding control. $n = 11$ each. *C*, level of the *INS* gene was analyzed at day 17 of differentiation in normoxia or high O_2-treated C23 cells by qPCR. *, $p < 0.05$ *versus* corresponding control. $n = 4$ each. *D*, values are the percentage of C-peptide-positive cells/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. *Error bars*, S.E.

hiPSCs. In addition, Dkk-1 treatment weakened high O_2-induced *NGN3* expression (Fig. 9*E*). 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 O_2 condition.

We tested whether the high O_2 condition had an effect even in the D'Amour protocol and Nostro protocol (1) (Fig. 10*A*). Using this protocol, we also saw a significant increase in the percentage of C-peptide-positive cells by the high O_2 condition from day 1 to day 4 (Stage 1; $p < 0.05$) and also in the *INS* expression by high O_2 condition from day 1 to day 4 (Stage 1; $p < 0.05$) and from day 4 to day 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 O_2 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

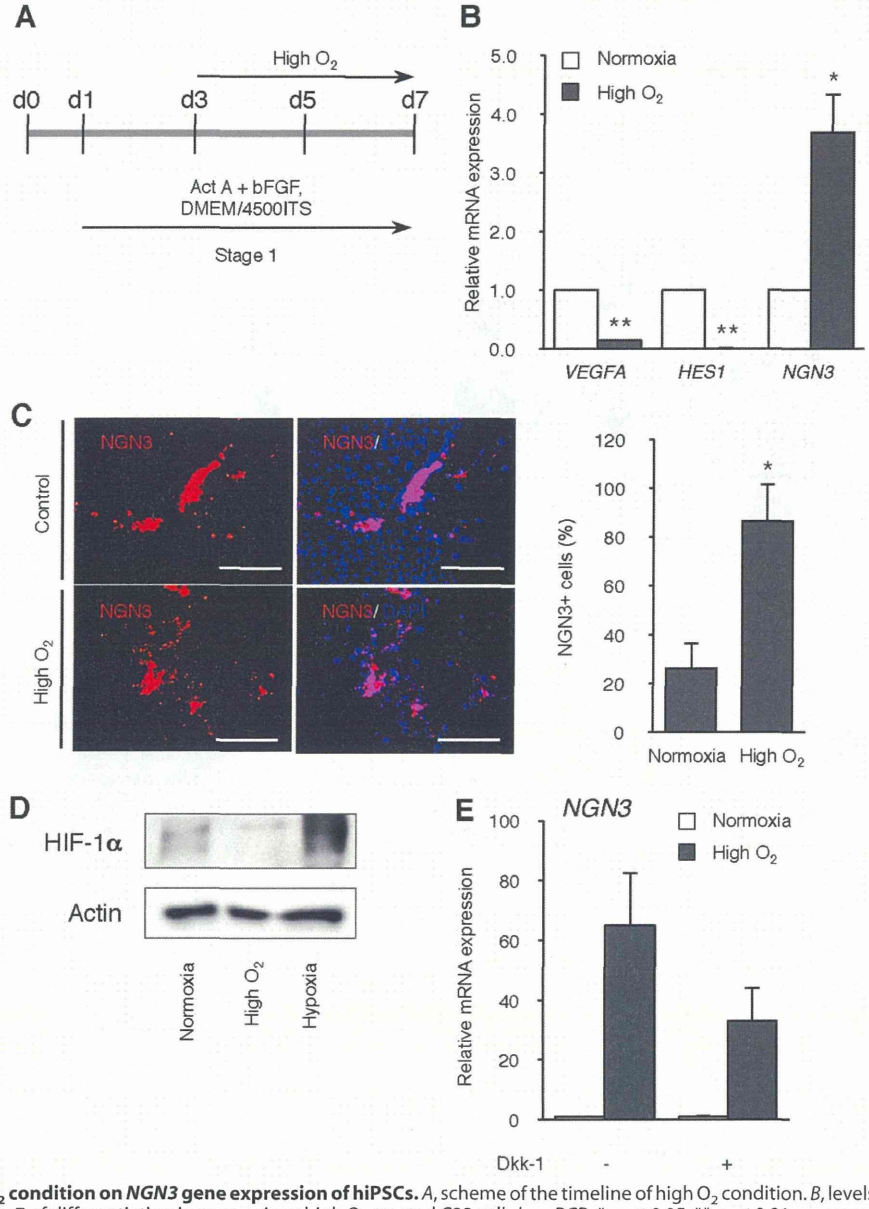

FIGURE 9. **Effect of high O_2 condition on *NGN3* gene expression of hiPSCs.** *A*, scheme of the timeline of high O_2 condition. *B*, levels of *VEGFA*, *HES1*, and *NGN3* genes were analyzed at day 7 of differentiation in normoxia or high O_2-treated C23 cells by qPCR. *, $p < 0.05$; **, $p < 0.01$ *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/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 the *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. *Error bars*, S.E.

embryonic development of several organs, including the pancreas (9, 10, 15–23). In the present study, we demonstrated that a high O_2 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 a slight transition to pancreatic progenitors and endocrine progenitors had already started during stage 1 (Fig. 1*B*). With this protocol, treatment with a high O_2 condition during stage 1 (toward 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 fac-

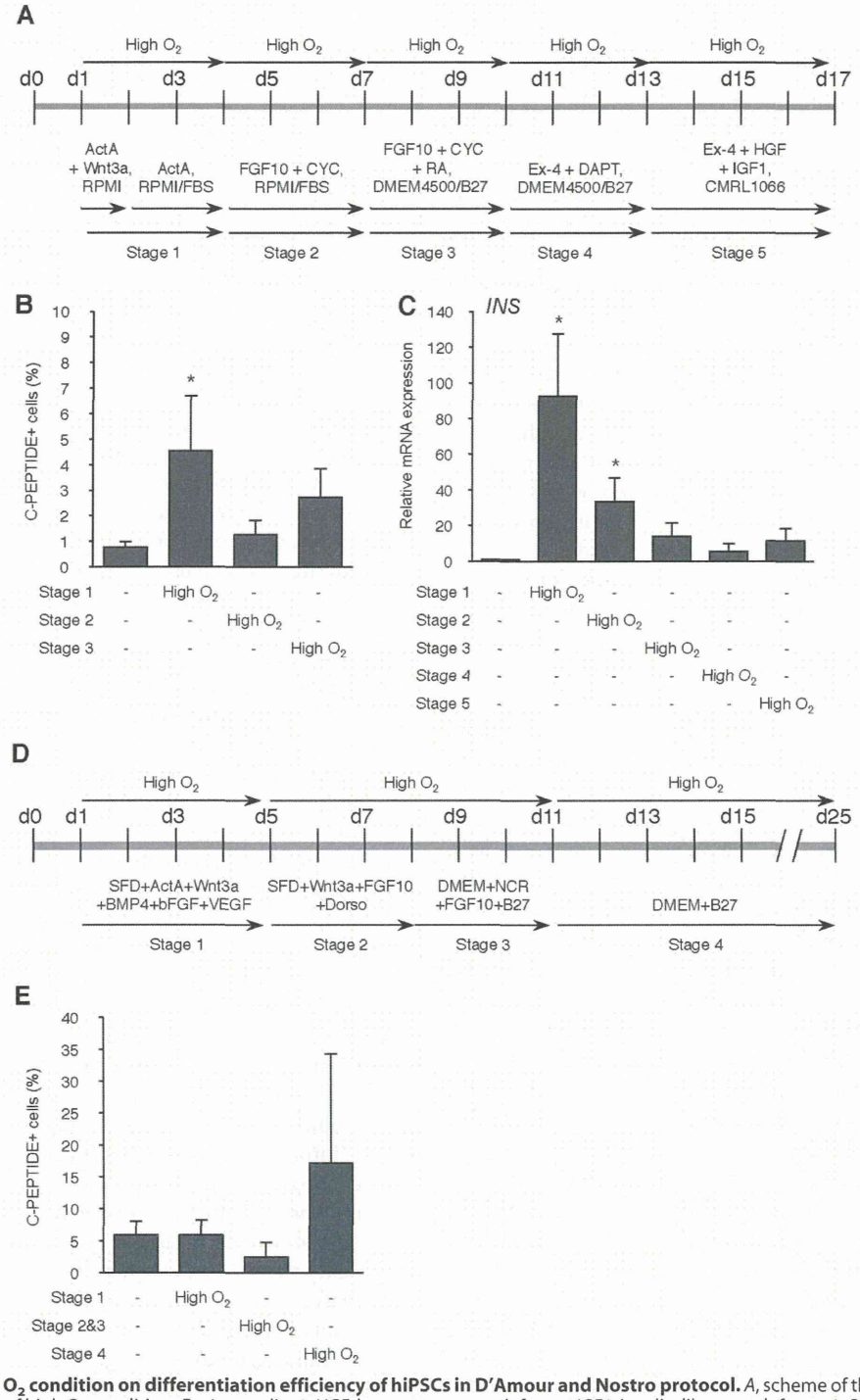

FIGURE 10. **Effect of high O_2 condition on differentiation efficiency of hiPSCs in D'Amour and Nostro protocol.** *A*, scheme of the D'Amour differentiation protocol and the timeline of high O_2 condition. *Ex-4*, exendin-4; *HGF*, hepatocyte growth factor; *IGF1*, insulin-like growth factor 1. *B*, values are the percentage of C-peptide-positive cells/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/well of the cells treated with high O_2 condition during three different stages. $n = 3$ each. *Error bars*, S.E.

tor 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 an increase of its target gene *Vegfa*, whereas a high O_2 condition markedly 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 O_2 condition might increase cellular O_2 concentration and lead to inhibition of HIF-1α signaling. The high O_2 condition significantly repressed *Hes1* gene expression on days 5 and 6 (Fig. 4*D*). 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 O_2 condition. Furthermore, the HIF-1α inhibitor echinomycin had an effect similar to that of the high O_2 condition (Fig. 4*F*). These data indicate that inhibition of HIF-1α signaling is involved in the facilitative effect of the high O_2 condition on pancreatic differentiation. The high O_2 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 the transition from definitive endoderm to pancreatic progenitor or endocrine progenitor. This is supported by the finding that its treatment did not increase *Ngn3* expression in the undifferentiated state (Fig. 3*G*). The high O_2 condition significantly decreased *Oct4* gene expression (Fig. 3*B*). *Oct4* gene expression is directly regulated by HIF-2α, also a hypoxia-dependent factor (12); therefore, it is considered that the high O_2 condition might repress *Oct4* gene expression via HIF-2α inhibition.

By microarray analysis, we found that the Wnt signaling pathway is activated in high O_2 condition-treated cells (Fig. 5*B*). Wnt inhibitor Dkk-1 partially repressed high O_2 condition-induced *Ngn3* expression (Fig. 5*D*). 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 O_2 condition might inhibit HIF-1α signaling, and compensatory Wnt signaling was activated. This is supported by the finding that genes induced by the HIF-1α inhibitor echinomycin are also involved in the Wnt signaling pathway (Fig. 5*C*). 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.* (1) showed that Wnt signaling induces a posterior endoderm fate, the primed stage from definitive endoderm, and at optimal concentrations enhances the development of pancreatic lineage cells. 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 findings that a high O_2 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). Wnt signaling activated by a high O_2 condition might function to promote the development of a posterior phenotype in mESC and hiPSC cultures.

The high O_2 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. 7*A*), 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 concentration (D'Amour protocol, 100 ng/ml; our protocol, 10 ng/ml), because induction of definitive endoderm by activin A 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 O_2 condition during stage 1, similar to mESCs. The effect of the high O_2 condition was also observed in the D'Amour protocol but not in the Nostro protocol (1) (Fig. 10, *A–E*). Nostro *et al.* included VEGF during stage 1, probably to support endothelial development for pancreatic differentiation of hESC (1, 57). The high O_2 condition repressed *VEGF* expression in our study (Fig. 9*B*), and a similar effect is expected to occur in the Nostro protocol. This effect may compete with the addition of VEGF. Therefore, the high O_2 condition seems to have no facilitative effect in the Nostro 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), whereas in hiPSC differentiation cultures, this typically occurs in 2–3 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 a previous study, Shah *et al.* (21) 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. Furthermore, they showed that the number of Ngn3-positive cells was not altered by hypoxia treatment in pancreatic explants, whereas 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 O_2 condition (Figs. 3 (*E* and *F*) and 9*C*). There are some differences between the culture environments of dissociated mESCs and pancreatic explants. HIF-1α levels seem to be different between mESC culture and pancreatic explants because

of spatial and temporal patterns of cell-cell interactions. Moreover, the external signals were different because 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 O_2 environment for pancreatic differentiation (59). Cheng *et al.* used a 5% O_2 environment for maintaining and differentiating human endodermal progenitor 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% O_2) 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, endodermal progenitor cells were plated in 12-well dishes at $3-4 \times 10^5$ cells/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 was expressed at a detectable level even in normoxia (Fig. 9*D*). Therefore, in our case, the high O_2 condition rather than the hypoxic condition facilitated pancreatic differentiation.

In conclusion, the present study showed that a high O_2 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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Cyclin G2 Promotes Hypoxia-Driven Local Invasion of Glioblastoma by Orchestrating Cytoskeletal Dynamics[1,2]

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Abstract

Microenvironmental conditions such as hypoxia potentiate the local invasion of malignant tumors including glioblastomas by modulating signal transduction and protein modification, yet the mechanism by which hypoxia controls cytoskeletal dynamics to promote the local invasion is not well defined. Here, we show that cyclin G2 plays pivotal roles in the cytoskeletal dynamics in hypoxia-driven invasion by glioblastoma cells. Cyclin G2 is a hypoxia-induced and cytoskeleton-associated protein and is required for glioblastoma expansion. Mechanistically, cyclin G2 recruits cortactin to the juxtamembrane through its SH3 domain–binding motif and consequently promotes the restricted tyrosine phosphorylation of cortactin in concert with src. Moreover, cyclin G2 interacts with filamentous actin to facilitate the formation of membrane ruffles. In primary glioblastoma, cyclin G2 is abundantly expressed in severely hypoxic regions such as pseudopalisades, which consist of actively migrating glioma cells. Furthermore, we show the effectiveness of dasatinib against hypoxia-driven, cyclin G2–involved invasion *in vitro* and *in vivo*. Our findings elucidate the mechanism of cytoskeletal regulation by which severe hypoxia promotes the local invasion and may provide a therapeutic target in glioblastoma.

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Introduction

Glioblastoma multiforme (GBM), the most common and malignant primary tumor arising in the central nervous system, is characterized by a variety of pathologic findings including vascular proliferation, aggressive invasion, and pseudopalisades [1], which are promoted by hypoxic conditions [2,3]. Pseudopalisades consist of necrotic foci and surrounding glioma cells and are a specific finding that distinguishes GBM from lower grade astrocytomas [4]. They were also shown to consist of hypoxic and actively migrating glioma cells [5].

In the expansion of GBM, hypoxia contributes to the maintenance of glioma stem cells, angiogenesis, and cellular migration through the accumulation of hypoxia-inducible factor 1α (HIF-1α) and consequent activation of several genes [6–8]. Studies using microarray-based gene expression profiling in glioma or neuroblastoma cells have

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indicated the knockdown of HIF-1α to result in reduced expression of *ccng2* mRNA (which encodes cyclin G2), suggesting that cyclin G2 expression is hypoxia-responsive [9–11]. Cyclin G2 was first identified as a negative regulator of cell cycle progression [12] and shown to bind to an active complex of protein phosphatase 2A (PP2A) and induce a p53-dependent cell cycle arrest [13]. Moreover, cyclin G2 interacts with and stabilizes microtubules, suggesting that cyclin G2 contributes to the regulation of cytoskeletal dynamics [13]. Despite evidence that the expression and subcellular distribution of cyclin G2 were related to epithelial cancer progression and metastasis [14–16], the precise mechanism by which cyclin G2 controls tumor expansion is unclear, especially in GBM.

Although several studies have shown that hypoxia induced by tumor enlargement or antiangiogenic therapy stimulated the local invasion of glioblastoma cells by modulating signal transduction [8], we do not have sufficient evidence that hypoxia regulates cytoskeletal dynamics, which is required for cellular motility, to promote the local invasion of glioblastoma. Accordingly, the aim of this study is to elucidate the precise mechanism of cytoskeletal regulation in the hypoxia-driven invasion and thereby provide evidence of a therapeutic strategy in glioblastoma. Especially, we here focus on the roles of cyclin G2 in cellular migration in response to hypoxia from the viewpoint of cytoskeletal regulation.

Materials and Methods

Cell Culture and Reagents

Human glioma cell lines U87MG, U251MG, and LNZ308 and human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and penicillin/streptomycin (Life Technologies, Carlsbad, CA). Murine glioma-initiating 005 cells were cultured as previously reported [17]. For hypoxic stimulation, cells were cultured in medium containing 100 μM deferoxamine mesylate (Sigma-Aldrich, St Louis, MO) or hypoxic chamber maintained at 5% or 1% O_2. For the analysis of tyrosine phosphorylation, 10 μM erlotinib (LKT Laboratories, Inc, St Paul, MN) or 1 μM dasatinib (Selleck Chemicals, Houston, TX) was added before the hypoxic stimulation (Figure W7).

For the immunoblot analysis, the following antibodies were used as the primary antibody: anti–cyclin G2, anti-Src, anti-p21, anti-p53, anti-green fluorescent protein (GFP; Santa Cruz Biotechnology, Inc, Dallas, TX), anti–HIF-1α (Novus Biologicals, LLC, Littleton, CO), anti–α-tubulin, anti–β-actin (Sigma-Aldrich), anti-cortactin, anti-phosphotyrosine (Millipore, Billerica, MA), and anti-Src (Cell Signaling Technology, Inc, Danvers, MA). For the immunohistochemistry and immunofluorescence, the following antibodies were used: anti–cyclin G2, anti-Src, anti-CD68 (Santa Cruz Biotechnology, Inc), anti–HIF-1α (Novus Biologicals, LLC), anti-human glial fibrillary acidic protein (GFAP), anti-cortactin (Abcam, Cambridge, United Kingdom), anti–α-tubulin (Sigma-Aldrich), anti-cortactin, anti-phosphotyrosine (Millipore), and anti–phospho-Src (Y417) (Life Technologies). Alexa Fluor 555–conjugated phalloidin and Hoechst (Life Technologies) were used for the detection of F-actin and nuclei, respectively. Validation of the use of the cyclin G2 antibody in immunoblot, immunofluorescent, and immunostaining analyses was reported in the past studies [14,18,19] and was further confirmed as shown in Figure W7.

For the analysis of cell proliferation and viability, the water soluble tetrazolium-1 (WST-1) assay was performed according to the manufacturer's protocol (Roche, Tokyo, Japan).

Luciferase Reporter Assay

The human cyclin G2 promoter region (−1600~0) was amplified from genomic DNA of U87MG cells and cloned into a pGL4.14 luciferase reporter vector (Promega, Madison, WI). The vectors were transfected into U87MG cells before hypoxic stimulation and cultured in a hypoxic chamber for 24 hours. The cells were lysed and incubated with the Luciferase Assay System (Promega) before measurements were made. Oligonucleotides for amplification of human cyclin G2 promoter region were given as follows: forward, 5′-CAAAACCCTCACCAAGCTCACACCTCTCTG-3′; reverse, 5′-CCCCTTGTTTTTGTTAAGAGTTTCGACGCC-3′.

Wound-Healing Assay

Cells were plated on Matrigel-coated glass slips at 90% confluence and cultured for 24 hours in DMEM with 10% FBS. The next day, the slips were scratched with white tips (200 μl), and the medium was switched to DMEM with 0.1% FBS. Then, the cells were incubated in normoxic or hypoxic conditions for 24 hours and observed. To examine the effects of ectopic expression of cyclin G2 on cellular motility, plasmids encoding cyclin G2–enhanced green fluorescent protein (EGFP), EGFP, $G2^{K219Q}$-EGFP, or $G2^{P291A}$-EGFP were transfected before the scratching.

Plasmids, Small Interfering RNA, and Retroviruses

The human cyclin G2 cDNA was purchased from OriGene (Rockville, MD; Cat. No. SC117452), and the open reading frame was cloned into a pEGFP-N3 (Clontech, Mountain View, CA) or a pcDNA3.1/V5-His TOPO vector (Life Technologies). The K219Q and P291A mutants were generated using the site-directed mutagenesis kit (Stratagene, La Jolla, CA). Small interfering RNA (siRNA) and scrambled RNA were obtained from Thermo Fisher Scientific Inc (Waltham, MA; Dharmacon, ON-TARGET*plus* siRNA Reagents). The retrovirus encoding scrambled shRNA or shRNA against human *ccng2* was prepared using the HuSH-29 plasmids (OriGene) and PT67 cells (Clontech) according to the manufacturer's recommendation.

RNA Purification, cDNA Synthesis, and Real-Time Reverse Transcription–Polymerase Chain Reaction

For reverse transcription–polymerase chain reaction (PCR) analysis, total RNA was isolated from GBM and cancer cells using an RNeasy kit (Qiagen, Santa Clarita, CA) and subjected to on-column digestion of genomic DNA with RNase-free DNase (TaKaRa, Otsu, Japan). With 1 μg of purified total RNA, cDNA was synthesized using a Maxima cDNA synthesis kit (Thermo Fisher Scientific Inc) Quantitative analyses of *CCNG2*, *FYN*, *CSK*, *SRC*, *LYN*, and *LCK* mRNA were performed with RT^2 SYBR Green master mixes (Qiagen) and the ABI PRISM 7000 Sequence Detection System (Life Technologies). As an internal control, the copy number of *ACTB* (β-actin) mRNA was determined. We obtained predesigned and certified primers from TaKaRa. All data were analyzed using software (7000v1.1; Life Technologies).

Western Blot Analysis and Preparation of Cytoskeletal Pellets

Immunoblot analysis was performed as described previously [20]. Cells were lysed in cell lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 0.5% Triton X-100) containing Complete Proteinase Inhibitor Cocktail (Roche). For the detection of phosphoprotein, PhosSTOP Phosphatase Inhibitor Cocktail (Roche)