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Identification of Oxygen-Sensitive Transcriptional Programs in Human Embryonic Stem Cells

Suzanne D. Westfall,^{1,5} Shrikesh Sachdev,² Padmalaya Das,^{1,4} Leonard B. Hearne,³ Mark Hannink,²
R. Michael Roberts,^{1,2} and Toshihiko Ezashi¹

To realize the full potential of human embryonic stem cells (hESCs), it is important to develop culture conditions that maintain hESCs in a pluripotent, undifferentiated state. A low O₂ atmosphere (~4% O₂), for example, prevents spontaneous differentiation and supports self-renewal of hESCs. To identify genes whose expression is sensitive to O₂ conditions, microarray analysis was performed on RNA from hESCs that had been maintained under either 4% or 20% O₂. Of 149 genes differentially expressed, 42 were up-regulated and 107 down-regulated under 20% O₂. Several of the down-regulated genes are most likely under the control of hypoxia-inducing factors and include genes encoding enzymes involved in carbohydrate catabolism and cellular redox state. Although genes associated with pluripotency, including *OCT4*, *SOX2*, and *NANOG* were generally unaffected, some genes controlled by these transcription factors, including *LEFTY2*, showed lowered expression under 20% O₂, while a few genes implicated in lineage specification were up-regulated. Although the differences between O₂ conditions were generally subtle, they were observed in two different hESC lines and at different passage numbers. The data are consistent with the hypothesis that 4% O₂ favors the molecular mechanisms required for the maintenance of pluripotency.

Introduction

HUMAN EMBRYONIC STEM CELLS (hESCs) are generally derived from the inner cell mass (ICM) of preimplantation embryos and retain the potential of their embryonic founder cells to differentiate into cell types representing all three germ layers, ectoderm, mesoderm, and endoderm [1,2]. The pluripotent nature of hESCs and the study of their directed differentiation are beginning to provide insights into the process of lineage specification and the accompanying events that lead to functional differentiation [1,3]. In this regard, hESCs provide a unique model for the study of basic human developmental biology. In addition, hESCs have tremendous potential as a source of cells for tissue replacement and repair [1,4]. However, a serious hindrance to the use of hESCs for either of these purposes is the tendency of these cells to undergo spontaneous differentiation in culture [4,5]. As a result, hESC research has focused as much on the ability to propagate hESCs in an undifferentiated state

as to the development of techniques that direct differentiation along specific cell lineages. It is clear that the full potential of hESCs will not be realized until the delicate balance between the processes of self-renewal and early differentiation are elucidated.

Since the derivation of hESCs was first reported in 1998 [6], it has been routine practice to culture these cells under atmospheric oxygen (20% O₂). The efficacy of this practice is questionable as a preimplantation human conceptus would most likely be exposed to O₂ tensions well below this concentration in utero [7,8]. One consequence of this culture practice is that overt differentiation within hESC colonies generally becomes visible after about 1 week if the cultures are maintained under 20% O₂. Either undifferentiated cells must be separated from those already differentiated at the time the cells are passaged or else passage must be performed before differentiation is observable. In either case, pluripotent stem cells are likely to be contaminated with cells that are already

¹Department of Animal Sciences, ²Department of Biochemistry, and ³Department of Statistics, University of Missouri, Columbia, Missouri.

⁴Ontogeny and Reproduction, Centre de Recherche du Centre hospitalier de l'Université Laval (CHUL), Québec, Canada.

⁵Present address: Department of Pathology & Anatomical Sciences, University of Missouri School of Medicine, Columbia, Missouri.

cryptically differentiated and possibly committed to one lineage or another. We recently demonstrated that maintaining hESC cultures under low (physiological) O₂ (2–5% O₂) inhibits spontaneous differentiation and supports pluripotency [9]. Moreover, hESCs continuously maintained under 4% O₂ for more than a single passage prior to being placed under 20% O₂ conditions exhibit an even more prolonged delay before onset of differentiation [9]. These studies provided evidence that molecular processes that mediate pluripotency and suppress differentiation are supported under 4% O₂ conditions and that the cells retain a “memory” of their prior environment.

Hypoxia-inducible factors-1 α and -2 α (HIF1A and -2A) exhibit reduced stability as O₂ concentrations rise. They regulate transcriptional responses as heterodimers with a constitutively expressed protein, HIF1 β (ARNT) [10] through binding to cis-acting hypoxia-response elements (HREs) on their target genes when O₂ tensions are low [10–12] but may also have important roles in regulating pluripotent stem cell self-renewal and differentiation [13–15]. In most mammalian species examined, physiological O₂ improves *in vitro* embryo development [16–18] and increases cell number, particularly of the ICM [13,19,20], suggesting that O₂-regulated gene expression supports the maintenance of pluripotent cells. Interestingly, bovine blastocysts express HIF2A rather than HIF1A in their ICM [13]. Presumably, the former is responsible for the transcriptional responses in these pluripotent cells to physiological oxygen concentrations. Recently, HIF1A and HIF2A have been implicated in the maintenance of hESC pluripotency [21,22]. HIF2A, for example, regulates *OCT4* expression [21], one of the core transcription factors long known to be essential for maintaining ESCs in an undifferentiated state [23]. A not unreasonable assumption, therefore, is that the HIF transcription factors play a role in suppressing the differentiation of hESCs under physiological oxygen concentrations.

The main objective of the experiments described in this paper was to examine the transcriptional profiles of two hESC lines, H1 and H9, which had been maintained under either 4% or 20% O₂ conditions. As part of our experimental design, hESC RNA was collected prior to the time when obvious morphological differentiation first became apparent in the hESC colonies cultured under 20% O₂. We addressed four main questions: (1) Are there consistent differences in gene expression that accompany culture in 20% O₂? (2) Are the transcriptional profiles more consistent in cells cultured under 4% O₂ as compared to cells cultured under 20% O₂? (3) Does transcriptome profiling reveal any indications of differentiation occurring under 20% O₂? (4) Is there evidence for the involvement of the HIF transcription factors in maintaining the pluripotency of hESCs cultured under 4% O₂?

Materials and Methods

Culture of human ESCs

Human ESCs H1 (WA01) at passage 23 and H9 (WA09) at passage 19 were purchased from WiCell Research Institute (Madison, WI, USA) and cultured in six-well tissue culture plates (Nunc, Sigma-Aldrich, St. Louis, MO, USA) on a monolayer of γ -irradiated (8,000 cGy) mouse embryonic fibroblast

(MEF) feeder cells until passage number 33 and 30, respectively, as previously described [9]. Thereafter, cells were cultured on a substratum of 1:30 dilutions of Matrigel™ (BD Biosciences, San Diego, CA, USA) in medium conditioned by MEF [9,24].

To identify differences in global gene expression between hESCs cultured under 20% and 4% O₂, hESCs were passaged into six-well culture dishes and placed in either a standard humidified tissue culture CO₂ incubator (NuAire, IR Autoflow) set at 5% CO₂ in air, or a tri-gas incubator (Heraeus HERAcCell 150; Kendro Laboratory Products GmbH, Langenselbold, Germany) at a 4% O₂, 5% CO₂ setting. Oxygen levels, measured by using a Bacharach CO₂/O₂ Analyzer (Bacharach, Model 2830, New Kensington, PA, USA), were ~19% in the standard incubator and 3.5–4.5% in the low O₂ incubator. To minimize differences unrelated to O₂ tension, experimental groups within replicates were cultured in the same medium and passaged at approximately the same time and as swiftly as possible. Cells maintained under 4% O₂ were never exposed to air for more than 30 min, which is more than enough time to passage a six-well plate. We have measured how rapidly the level of dissolved oxygen in the culture media changes following changes in the oxygen tension in the air above the cells using an oxygen polarographic electrode (Pinpoint II Oxygen Meter, Aquatic Eco-Systems Inc, Apopka, FL, USA). When culture medium is shifted from a 20% environment to a 4% environment, it takes about ~3 h for the DOC to decrease back to 3.5 ppm, with a half-maximal change taking about 40 min. The reverse change, that is, medium transferred from a 4% environment to a 20% environment, takes about ~100 min for equilibrium, with a half time of 28 min. The H1 and H9 hESC lines had been maintained in a physiological O₂ (3–5% O₂) atmosphere since passage 26 and 23, respectively (Fig. 1A). RNA was isolated from H1 cells after 7 days of culture at two independent passage numbers (p37 and p50) and had been maintained on Matrigel™ for 3 and 16 passages, respectively. RNA from H9 cells was isolated at passage 32 and had been grown on Matrigel™ for 3 passages (Fig. 1A).

RNA extraction and preparation for microarray analysis

RNA was isolated from hESCs by using RNA STAT-60 reagent (Tel-Test Inc, Friendswood, TX, USA). RNA (5 μ g) was used to prepare the biotin-labeled antisense RNA (aRNA) target by using the GeneChip® one-cycle target labeling and control reagents (#900493, Affymetrix, Santa Clara, CA, USA). The biotin-labeled aRNA (10 μ g) was then hybridized to the Affymetrix Human U133 Plus 2.0 gene chip (Affymetrix) [25]. Each sample was hybridized to an individual chip, for a total of six chips. After hybridization, the chips were washed and stained with R-phycoerythrin-streptavidin on an Affymetrix fluidics station 450 by the Fluidics Protocol EukGE-WS2v5. The image data were acquired on an Affymetrix Genechip Scanner 3000.

Microarray data analysis

The study comprised three independent experiments (two samples of H1 and one H9 sample) cultured under two

FIG. 1. (A) Schematic diagram depicting conditions under which human embryonic stem cells (hESCs) were maintained and passaged prior to RNA extraction. H1 and H9 ESCs were maintained under 4% O₂ condition from p26 and p23, respectively. The H1 and H9 cells were propagated under 4% O₂ (represented by upper open box) for seven passages on MEF feeder layer (represented by solid arrow lines); thereafter the both cells were switched to Matrigel coated culture wells for at least three passages (represented by broken arrow lines) prior to RNA collection. RNA extraction was performed within 3 min following removal of the culture plates from respective O₂ conditions and well before dissolved O₂ in the culture medium showed any detectable changes. Total six RNA samples were collected from H1 cells at two different passage numbers (p37 and p50) and H9 cells at passage 32 (p32) under 4% and 20% O₂ (represented by lower gray box) conditions, respectively. The cells maintained under 4% O₂ were split into both 4% and 20% O₂ conditions and cultured for 7 days before of RNA collection. (B) Phase contrast images of H1p50 hESC colonies under 4% (left) and 20% (right) O₂ as they appeared prior to RNA extraction. RNA was isolated at 7 days postpassage, prior to the time at which morphological differentiation is apparent in H1 hESCs maintained under 20% O₂. Bar 1 mm.

different O₂ conditions, 4% and 20% O₂. An absolute analysis of the resulting output was performed with Affymetrix Data Mining Tool, Version 3.0 software. The relative abundance of the transcripts based on signal and detection (present, absent or marginal) was assessed and a preliminary assessment of changes in relative transcript concentrations was performed. In addition, Affymetrix data were imported into Genespring 7.2 software (Silicon Genetics; Redwood City, CA, USA) for default normalization, that is, setting signal values to below 0.01 to 1.0, total chip normalization to the 50th percentile, and normalization of each gene to the median based on its measured expressed values. Those probe sets that did not meet the following criteria: (1) a signal value of at least 100 in either 20% or 4% O₂, and (2) a consistent response to O₂ conditions, that is, an increase or decrease in expression under

20% O₂ in all three paired comparisons were removed from further analysis.

A separate statistical assessment was performed by first computing the logarithm of the intensity as a variance stabilizing transformation. The transformed intensity was then analyzed in a two stage mixed linear model. In the first stage, the across-array fixed effect treatment as well as random model effect array were modeled. The residuals were then modeled by probe by using a fixed effect model for treatment. A *t*-test for between-treatment comparisons within each probe was calculated. The Wilcoxon rank sum test was also calculated for each probe to add confidence for significant results.

Quantitative real-time PCR

Two-step real-time PCR was carried out to analyze and confirm the expression of select genes. RNA (1.2 µg) from different hESC treatment groups was reverse transcribed into cDNA in a reaction primed by oligo deoxynucleotide T (dT) 12–15 primer by using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative TaqMan real-time PCR analyses were conducted on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with the following PCR conditions: (50°C for 2 min; 95°C for 10 min; 45 cycles at 95°C for 15 s; 60°C for 1 min). Each 50 µl real-time PCR reaction contained 20 ng of reverse-transcribed cDNA and 25 µl of 2× TaqMan Universal Master Mix (4304437, Applied Biosystems). The final concentration of gene-specific forward and reverse primers (Sigma-Genosys, Sigma-Aldrich) and gene-specific Taqman FAM-MGB probe (Applied Biosystems) was 20 nM and 100 nM, respectively. Each target transcript was analyzed in triplicate and independently normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM_002046) and ribosomal protein L19 (NM_000981). Supplemental Table 1 (supplementary table 1 is available online at <http://www.liebertpub.com/scd>) lists the sequences of the gene-specific primers and probes that were used. Fold changes were calculated according to the formula, Fold Change = 2^{ΔΔCt} where ΔΔCt = ΔCt L – ΔCt A and ΔCt = Ct target gene – Ct reference (GAPDH or RPL19).

Immunocytochemistry

H1 ESCs (p33) were cultured for 10 days under 20% and 4% O₂ conditions, at which time spontaneous differentiation in colonies under 20% O₂ conditions can be easily observed [9]. Cell colonies were fixed by immersing the coverslips in 2% paraformaldehyde/PBS and permeabilized in 1.0% Triton X-100/PBS. Nonspecific immunoglobulin binding sites were blocked by incubation in 5% goat serum and 5% BSA prior to the addition of primary antibodies. The serological reagents used were a rabbit anti-HIF1A polyclonal antibody (NB100-134, Novus Biologicals, Littleton, CO, USA), a mouse anti-HIF2A monoclonal antibody (NB100-132, Novus Biologicals), mouse antistage-specific embryonic antigen (anti-SSEA)-4 (MC813-70, Developmental Studies Hybridoma Bank, IA, USA) and anti-Oct4 [9]. Secondary

antibody staining was performed with Alexa Fluor 568 and 488 goat antirabbit, goat antimouse and/or goat antirat antibodies (Molecular Probes, Invitrogen). Nuclei were labeled with TO-PRO-3 (Molecular Probes, Invitrogen) or Hoechst 33258 (Sigma-Aldrich). Images were captured on a Zeiss META NLO two photon confocal system (Carl Zeiss, Oberkochen, Germany) coupled with a Zeiss Axiovert 200M microscope.

Results

General features of gene expression in H1 and H9 ESCs under 20% and 4% O₂ conditions

We compared the transcriptional profiles of hESCs after 1 week of culture under 20% and 4% O₂ conditions after initial maintenance of the cell lines for several passages on a Matrigel substratum under 4% O₂ conditions (Fig. 1A). Despite the differences in O₂ atmosphere, the hESC colonies were indistinguishable morphologically and size ($658,000 \pm 108,764 \mu\text{m}^2$ and $636,614 \pm 220,157 \mu\text{m}^2$ in 20% and 4% O₂, respectively), with no signs of differentiation evident under 20% O₂ (Fig. 1B). A total of 21,891 and 22,534 (out of 54,675) probe-sets were assessed as “present” in all three samples by Genespring software, under 20% and 4% O₂, respectively.

The overall gene expression profiles of the H1 and H9 ESCs used for these experiments are similar to those previously reported for other hESCs, including the H1, BG01, BG02 and BG03 lines [26–31]. Several genes highly expressed in these cell lines [26,28,31,32], for example, most ribosomal proteins, *MTCO1* (cytochrome c oxidase), *ACTB* (beta-actin), and *EEF1A1L14* (eukaryotic translation elongation factor alpha 1), were also found to be amongst the top 50 genes expressed in the H1 and H9 samples analyzed here (Supplemental Table 2; supplementary table 2 is available online at <http://www.liebertpub.com/scd>) and showed no significant changes in expression in the shift from 4% to 20% O₂ conditions. Other genes reported to be highly expressed in hESCs [28,29,32] that were not amongst the top 50 genes in our analysis, for example, *HMG1*, *FLJ10713*, *USP9X*, *CDC2*, and *HSPCA* (Supplemental Table 2) were also not regulated by the increase in O₂. These data tend to confirm the general similarity of the H1 and H9 cells to other undifferentiated hESC lines, irrespective of they are cultured under 20% or 4% O₂ conditions.

The transcriptional profiles of the three samples of RNA collected from cultures maintained continuously under 4% O₂ conditions as assessed as a Pearson correlation coefficient (PCC) over 54,675 probe-sets were quite similar to each other (PCC = 0.87) (Supplemental Fig. 1; supplementary figure 1 is available online at <http://www.liebertpub.com/scd>). By contrast, the hESC samples cultured under 20% O₂ exhibited a greater variance in their gene expression profiles (PCC = 0.67) (Supplemental Fig. 1). In other words, H1 and H9 ESC samples cultured under 4% O₂ clustered more tightly with each other than with their respective partner samples under 20% O₂.

A total of 149 transcripts (42 with increased expression, 107 with decreased expression under 20% O₂ conditions) demonstrated consistent differences in all three samples sets

(Supplemental Table 3; supplementary table 3 is available online at <http://www.liebertpub.com/scd>). Tables 1 and 2 list the top 25 genes within each class that displayed the greatest changes in expression. Over 400 regulated genes were identified (Supplementary Table 4; supplementary table 4 is available online at <http://www.liebertpub.com/scd>) if a less stringent selection criterion was employed, namely a 1.5-fold or greater average change in expression but not necessarily in all three sample comparisons. Of these genes, 123 increased expression while 301 transcripts were down-regulated under 20% O₂ culture conditions.

Changes in expression of genes associated with pluripotency

Several genes associated with pluripotency [27,33–35], namely *OCT4* [36], *NANOG* [37], *LIN28* [38], *SOX2* [39], *ZFP42/REX1* [40], *TERT* [41], and *PODXL* [27] exhibited expression levels that did not vary greatly according to the O₂ conditions under which the cells were maintained (Table 2). In particular, *OCT4*, *NANOG*, and *SOX2* are believed to function as core transcription factors in maintaining pluripotency of ESCs [33,34,42]. Although the expression of *SOX2* varied somewhat between paired comparisons, that of *OCT4* and *NANOG* remained relatively constant (Table 2).

On the other hand, several genes considered to be under the transcriptional control of the *OCT4/NANOG/SOX2* triad [33,34,43] displayed markedly lower expression in cells cultured under 20% O₂ as compared to parallel cultures maintained under 4% O₂ conditions. These down-regulated genes included *LEFTY2* [33] and endothelin receptor type B (*ENDRB*) (Table 2). In addition, genes known to be regulated by *OCT4*, such as *SALL1* [34,43], *TRIM2* [43], *ZIC2* [43], and *FGFR2* [33,34,43], also exhibited decreased expression in hESCs cultured under 20% O₂ (Table 3). These data suggest that although transcriptional profiles of the core pluripotency genes may not be greatly affected by O₂ conditions, the expression of their downstream targets might well be.

Transforming growth factor beta (TGFβ) signaling pathway regulated genes

The TGFβ/ACTIVIN/NODAL signaling pathways play a critical role in maintaining hESC pluripotency [44–46]. Expression of a member of the TGFβ family of genes, *LEFTY2* was reduced under 20% O₂ (Table 2). The TGFβ-inducible protein, *SERPINE1* (PAI-1) [47] and transcription factor *KLF11* [48] also exhibited reduced expression (1.76- and 1.68-fold, respectively) in cells cultured under 20% O₂.

Changes in expression of genes associated with differentiation

Several genes previously associated with hESC differentiation [26,27,49,50] were up-regulated in at least one of the three replicates under 20% O₂ (Supplemental Table 4). For example, *HAND1* exhibited a 2-fold and 2.5-fold increase in the H1p37 and H9 samples, respectively. *EOMES* and *H19* (imprinted maternally expressed untranslated mRNA), *GATA6* and *MSX2* were increased in the H9 ESC comparison.

TABLE 1. LIST OF TOP 25 GENES UP AND DOWN-REGULATED IN 20% O₂

<i>Probe Set ID</i>	<i>Accession</i>	<i>Unigene</i>	<i>Symbol</i>	<i>Descriptions</i>	<i>Fold</i>
Up-regulated in 20% O ₂					
209160_at	NM_003739	Hs.78183	AKR1C3	Aldo-keto reductase family 1, member C3	2.2
225809_at	AI659927	Hs.105460	—	DKFZP564O0823 protein	2.1
207057_at	NM_004731	Hs.439643	SLC16A7	Solute carrier family 16 (monocarboxylic acid transporters), member 7	2.0
226278_at	AI150224	Hs.349096	—	Hypothetical protein DKFZp313A2432	2.0
226751_at	AW193693	Hs.212885	C2orf32	Chromosome 2 open reading frame 32	1.8
230960_at	AI740721	Hs.128292	—	Transcribed locus	1.7
211075_s_at	Z25521	Hs.446414	CD47	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)*	1.7
235371_at	AI452595	Hs.431092	PPP4R2	Protein phosphatase 4, regulatory subunit 2	1.7
230130_at	AI692523	Hs.29802	—	Transcribed locus	1.7
209459_s_at	AF237813	Hs.336768	ABAT	4-aminobutyrate aminotransferase	1.6
205328_at	NM_006984	Hs.26126	CLDN10	Claudin 10	1.6
201467_s_at	NM_000903	Hs.406515	NQO1	NAD(P)H dehydrogenase, quinone 1*	1.6
204493_at	NM_001196	Hs.172894	BID	BH3 interacting domain death agonist	1.6
48808_at	NM_000791	Hs.83765	DHFR	Dihydrofolate reductase*	1.6
223122_s_at	AF311912	Hs.481022	SFRP2	Secreted frizzled-related protein 2	1.5
211737_x_at	BC005916	Hs.371249	PTN	Pleiotrophin (heparin binding growth factor 8)*	1.5
225688_s_at	AK025444	Hs.477114	PHLDB2	Pleckstrin homology-like domain, family B, member 2	1.5
209773_s_at	BC001886	Hs.75319	RRM2	Ribonucleotide reductase M2 polypeptide	1.4
232037_at	AW204060	Hs.567396	PUNC	Putative neuronal cell adhesion molecule	1.4
225418_at	AI520949	Hs.8372	UQCR	Ubiquinol-cytochrome c reductase (6.4 kD) subunit	1.4
1568678_s_at	BC037785	Hs.487175	FGFR1OP	FGFR1 oncogene partner	1.4
201117_s_at	NM_001873	Hs.75360	CPE	Carboxypeptidase E	1.3
215646_s_at	R94644	Hs.443681	CSPG2	Chondroitin sulfate proteoglycan 2 (versican)*	1.3
201202_at	NM_002592	Hs.147433	PCNA	Proliferating cell nuclear antigen	1.3
202345_s_at	NM_001444	Hs.408061	FABP5	Fatty acid binding protein 5 (psoriasis-associated)	1.3
Down-regulated in 20% O ₂					
1569287_at	BC017942	Hs.621322	—	Similar to otoconin 90*	−6.7
201848_s_at	NM_004052	Hs.144873	BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3	−4.6
201010_s_at	NM_006472	Hs.533977	TXNIP	Thioredoxin interacting protein*	−4.1
236180_at	W57613	Hs.419240	SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	−3.2
202718_at	NM_000597	Hs.162	IGFBP2	Insulin-like growth factor binding protein 2	−3.1
202887_s_at	NM_019058	Hs.523012	DDIT4	DNA-damage-inducible transcript 4	−3.0
1569453_a_at	BG772667	Hs.560022	—	Hypothetical locus LOC692247	−2.9
206701_x_at	NM_003991	Hs.82002	EDNRB	Endothelin receptor type B*	−2.8
225283_at	AV70117	Hs.6093	ARRDC4	Arrestin domain containing 4	−2.6
1557636_a_at	BC031107	Hs.258357	—	Hypothetical protein LOC136288	−2.5
1562529_s_at	BC040965	Hs.569497	RORA	RAR-related orphan receptor A	−2.4
206012_at	NM_003240	Hs.25195	LEFTY2	Left-right determination factor 2	−2.3
223734_at	AF329088	Hs.84549	OSAP	Corneal endothelium specific protein 1	−2.2
207543_s_at	NM_000917	Hs.500047	P4HA1	Procollagen-proline, 2-oxoglutarate 4-dioxygenase, alpha polypeptide I	−2.2
218717_s_at	NM_018192	Hs.374191	LEPREL1	Leprecan-like 1	−2.2
214624_at	NM_007000	Hs.150309	UPK1A	Uroplakin 1A	−2.2
223710_at	NM_006072	Hs.131342	CCL26	Chemokine (C-C motif) ligand 26	−2.1

(Continued)

TABLE 1. LIST OF TOP 25 GENES UP AND DOWN-REGULATED IN 20% O₂ (CONTINUED)

Probe Set ID	Accession	Unigene	Symbol	Descriptions	Fold
Down-regulated in 20% O ₂					
201968_s_at	NM_002633	Hs.1869	PGM1	Phosphoglucomutase 1*	-2.1
201105_at	NM_002305	Hs.227751	LGALS1	Lectin, galactoside-binding, soluble, 1 (galectin 1)	-2.1
200737_at	NM_000291	Hs.7877	PGK1	Phosphoglycerate kinase 1*	-2.0
227271_at	AU151265	Hs.380704	FGF11	Fibroblast growth factor 11	-2.0
206424_at	NM_000783	Hs.150595	CYP26A1	Cytochrome P450, subfamily XXVIA, polypeptide 1	-2.0
202022_at	NM_005165	Hs.155247	ALDOC	Aldolase C, fructose-bisphosphate	-2.0
227404_s_at	AI459194	Hs.326035	EGR1	Early growth response 1	-2.0
214823_at	AF033199	Hs.8198	ZNF204	Zinc finger protein 204	-1.9

Genes exhibiting an increase (Up-regulated) or decrease (Down-regulated) in all three samples (H1p37, H1p50, and H9p32) are represented. Fold changes shown are the average of the three paired comparisons. Positive or negative values indicate fold-increases or fold-decreases in gene expression, respectively, in human embryonic stem cells cultured under 20% O₂ compared to cells under 4% O₂. The complete list of genes which are consistently up- or down-regulated in 20% O₂ is provided in Supplemental Table 3.

*Genes represented by more than one probe-set, (redundant unigene entries were removed from list).

On the other hand, expression of the *TDGF1* (*CRIPTO*) gene, which has been shown to be down-regulated in association with the initial steps of hESC differentiation [27,49], was not altered under 20% O₂. Nor did we note any changes in expression of the gene for leukemia inhibitory factor (LIF) and its receptor, both of which have been reported to be increased during initial hESC differentiation [51]. In fact, the hybridization signal for LIF was sufficiently low that it was considered to be absent under both 4% and 20% O₂ conditions. The expression of several genes associated with more advanced stages of differentiation and early embryoid body formation, including *GATA4*, *SOX1*, *FGF5*, and *T* (Brachyury) [27,50] was either very low or assessed as "absent" under both 4% and 20% O₂ conditions.

Genes associated with an oxidative stress response

A number of genes associated with oxidative stress responses [52] were increased under 20% O₂ conditions (Supplemental Table 5; supplementary table 5 is available online at <http://www.liebertpub.com/scd>). *NRF2* (*NFE2L2*), a transcription factor responsible for the regulation of cytoprotective and antioxidant proteins [53,54] and at least two of its downstream targets, *NQO1* (NAD(P)H quinine oxidoreductase) [53] and *AKR1C3* (aldo-keto reductase family 1, member C3) [53], were among those exhibiting increased expression under 20% O₂ (Supplemental Table 5). In addition, expression levels of the mRNAs for *VCAN* (versican, *CSPG2*), a proteoglycan isoform that protects cells from oxidative stress-induced apoptosis [55], and *CTNS* (cystinosis), a gene encoding a lysosomal membrane transporter for cystine [56] and whose deficiency can lead to increased ratio of oxidized to reduced glutathione [57], were increased under 20% O₂ (Supplemental Table 5).

Functional classes of regulated genes

The major physiological pathway down-regulated in 20% O₂ was glycolysis (Table 4). Regulated genes included the

rate limiting enzymes *HK2* and *HK1* (hexokinase 2, -1), and *PKM2* (pyruvate kinase). A few of these genes, for example, those encoding *PGM1* (phosphoglucomutase 1), *ALDOA* (aldolase A), and *GPI* (glucose phosphate isomerase), are also linked to the pentose phosphate pathway (Table 4). These regulatory switches relating to O₂ conditions were not unexpected as most genes encoding enzymes of the glycolytic pathway are considered to be under the control of HIF1A [12].

Several genes believed to be under the control of HIF1A [11,22,58] and that contribute to apoptosis, cellular redox regulation, and proliferation were down-regulated under 20% O₂, for example, *BNIP3* (-4.6-fold) [59, 60], *TXNIP* (thioredoxin interacting protein; -4.5-fold), *DDIT4* (DNA-damage-inducible transcript 4, -3.0-fold) [61], *IGFBP2* (-3.1-fold), *LGALS1* (-2.1-fold) and *VEGF* (-1.6-fold) (Table 5).

Hypoxia-inducible factors and related genes

HIF1A mRNA (NM_001530) was present in relatively high amounts in H1 and H9 cells, and the transcript concentrations did not change in response to placing the cells under 20% O₂ conditions (12,998 ± 4,589 and 11,924 ± 3,863 in 20% and 4% O₂, respectively). This lack of change in *HIF1A* message in response to higher O₂ was further supported by quantitative real-time PCR analysis (Supplemental Table 6; supplementary table 6 is available online at <http://www.liebertpub.com/scd>) and is not unexpected as HIF1A is primarily regulated at the level of protein turnover [14]. The expression of *HIF2A* was much lower than that of *HIF1A* (586 ± 409 and 276 ± 68 in 20% and 4% O₂, respectively), but its transcript concentration was increased at 20 % O₂. Transcripts for *ARNT*, like those for *HIF2A*, were also present in low concentrations (463 ± 51 and 550 ± 21 in 20% and 4% O₂, respectively) and were unaffected by O₂. Finally, expression of the von Hippel-Lindau (*VHL*) tumor suppressor gene, the recognition component of an E3 ubiquitin-protein ligase complex that targets both HIF1A and -2A for

TABLE 2. EXPRESSION OF PLURIPOTENT MARKERS IN HUMAN EMBRYONIC STEM CELLS UNDER 20% AND 4% O₂ CONDITIONS

Probe Set ID	Accession	Unigene	Symbol	Description	20% O ₂		4% O ₂		Change call	Average fold change
					Signal	STDEV	Signal	STDEV		
220668_s_at	NM_006892	Hs.251673	DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta	28,878	2,796	26,378	2,086	NC (I)	
206012_at	NM_003240	Hs.25195	LEFTY2	Left-right determination factor 2	1,606	150	3,866	1,450	D	-2.4 ± 1.0
204271_s_at	NM_000115	Hs.82002	EDNRB	Endothelin receptor type B	6,282	3,308	13,223	841	D	-2.1 ± 1.4
206783_at	NM_002007	Hs.1755	FGF4	Fibroblast growth factor 4	476	77	602	271	NC	
220053_at	NM_020634	Hs.86232	GDF3	Growth differentiation factor 3	519	76	542	187	NC	
214022_s_at	NM_003641	Hs.458414	IFITM1	Interferon induced transmembrane protein 1	14,449	1,316	16,809	631	D	-1.2 ± 0.1
219823_at	NM_024674	Hs.86154	LIN28	Lin-28 homolog (C. elegans)	24,203	2,986	23,495	3,453	NC	
220184_at	NM_024865	Hs.504647	NANOG	Nanog Homeobox	11,469	1,626	11,871	2,177	NC	
230916_at	NM_018055	Hs.65853	NODAL	Nodal Homolog (mouse)	686	225	575	408	NC (MI)	
201578_at	NM_005397	Hs.16426	PODXL	Podocalyxin-like	26,319	3,328	27,443	3,908	NC	
208286_x_at	NM_002701	Hs.2860	POU5F1	POU domain, class 5, transcription factor 1, Oct4	11,180	3,548	11,283	3,417	NC	
213721_at	L07335	Hs.518438	SOX2	SRY (sex determining region 1) -box 2	243	89	332	133	NC (D)	
207199_at	NM_003219	Hs.492203	TERT	Telomerase reverse transcriptase	243	89	332	133	NC	
208275_x_at	NM_003577	Hs.158307	UTF1	Undifferentiated embryonic cell transcription factor 1	859	720	944	642	NC (D)	
1554777_at	AF450454	Hs.335787	ZFP42 (Rex1)	Zinc Finger Protein 42	1,935	1,362	2,184	1,277	NC (D)	

Change calls (NC = no change, D = decrease, MI = mild increase) and a brief description of the observed change are listed.

proteasomal degradation [10], was present in both H1 and H9 cells in similar concentrations and showed no differential expression between the two conditions ($6,833 \pm 461$ and $6,266 \pm 1134$, in 20% and 4% O₂, respectively).

Validation by real-time, quantitative PCR (RT-QPCR)

To validate the results from the microarray analyses, RT-QPCR was performed on the same RNA samples used in the array hybridization experiment. Selected genes included, *AKR1C3*, *FGF2*, *OCT4*, and *LEFTY2* (Supplemental Table 6). Data in RT-QPCRs were independently normalized to transcripts for either ribosomal protein L19 (RPL19) or GAPDH. The latter, despite being a component of the glycolytic pathway and regulated by oxygen in endothelial cells [62], showed no evidence for regulation by O₂ in the microarray analyses and was therefore a suitable standard to employ. Although minor discrepancies were observed, the overall changes in expression observed with real-time PCR were consistent with the microarray data (Supplemental Table 6).

Expression patterns of HIF1A and -2A in undifferentiated and differentiated hESCs

Since HIF gene products are largely regulated at the protein level, we examined the expression pattern of HIF1A and HIF2A by immunocytochemistry in H1 cell colonies cultured under 20% and 4% O₂ conditions for 10 days, a time when signs of overt differentiation are visible in the 20% O₂ but not the 4% O₂ cultures (Fig. 2). In contrast to SSEA-4, a marker of pluripotent, undifferentiated hESCs, HIF1A was expressed in both the undifferentiated and differentiated areas of H1 cells cultured under 20% O₂ (Fig. 2). Under both 20% and 4% O₂ conditions, the protein was primarily localized to the cytoplasm. In contrast, the HIF2A protein, like OCT4, was expressed only in undifferentiated cells, but, like HIF1A, was concentrated in the cytoplasm (Fig. 2).

Discussion

Despite a decade of research, optimal conditions required for maintaining pluripotency and avoiding spontaneous differentiation of hESCs have not been established, although it

TABLE 3. OCT4-REGULATED GENES EXHIBIT DECREASED EXPRESSION IN 20% O₂

<i>Probe Set ID</i>	<i>Accession</i>	<i>Unigene</i>	<i>Symbol</i>	<i>Descriptions</i>	<i>Fold</i>
202887_s_at	NM_019058	Hs.523012	DDIT4	DNA-damage-inducible transcript 4	-3.0
206012_at	NM_003240	Hs.25195	LEFTY2	Left-right determination factor	-2.3
209220_at	NM_004484	Hs.567276	GPC3	Glypican 3	-1.7
206893_at	NM_002968	Hs.135787	SALL1	Sal-like 1 (Drosophila)	-1.4
221605_s_at	AF136970	Hs.462585	PIPOX	Pipecolic acid oxidase	-1.4
223642_at	NM_007129	Hs.591205	ZIC2	Zic family member 2 (odd-paired homolog, Drosophila)	-1.4
208789_at	BC004295	Hs.437191	PTRF	Polymerase I and transcript release factor	-1.3
202342_s_at	NM_015271	Hs.435711	TRIM2	Tripartite motif protein	-1.3
203638_s_at	NM_022969	Hs.533683	FGFR2	Fibroblast growth factor receptor 2	-1.3
225992_at	AL562031	Hs.30385	MLLT10	Myeloid/lymphoid or mixed-lineage leukemia translocated to, 10	-1.3
228356_at	AV711366	Hs.335003	ANKRD11	Ankyrin repeat domain 11	-1.2

Fold decreases shown are the average of three paired comparisons.

is clear that reduced O₂ conditions are helpful in this regard. For example, hESCs under low O₂ exhibit a lowered tendency to differentiate spontaneously [9] and enhanced clonal recovery and genomic stability [63]. Physiological O₂ also prevents differentiation of a range of adult stem cells [64,65] and enhances the derivation of ESC lines from mouse blastocysts [66]. Clearly there are benefits to culturing stem cells under physiological O₂, but the molecular mechanisms favoring pluripotency under these conditions are unknown.

The experiments described in this paper were undertaken to address four main questions relating to the effects of O₂ on hESC pluripotency. The first question we asked was whether there are consistent differences in gene expression associated with culture under 20% versus 4% O₂. As the appearance of differentiated cell populations in hESC

cultures under 20% O₂ does not occur until about day 10 if the cells were cultured under physiological O₂ in previous passage, we expected that any changes in gene expression between cells cultured under the two conditions at 7 days post passage would likely be subtle and not of major magnitude. This hypothesis was born out. Only 149 genes consistently exhibited altered expression at high versus low O₂ conditions, and the changes observed were generally quite small. Importantly, several genes invariably associated with a pluripotent phenotype, including *OCT4*, *NANOG*, *SOX2*, did not display changes in expression. Such an outcome was not unexpected, as these stem cell lines have been maintained under atmospheric O₂ for at least 10 years without loss of pluripotency, emphasizing the robustness of the transcriptional network that maintains stemness even under

TABLE 4. CONSISTENTLY DECREASED TRANSCRIPTS FOR ENZYMES OF THE GLYCOLYTIC AND PENTOSE PHOSPHATE PATHWAYS UNDER 20% O₂

<i>Probe Set ID</i>	<i>Accession</i>	<i>Unigene</i>	<i>Symbol</i>	<i>Descriptions</i>	<i>Fold</i>
Glycolysis					
201968_s_at	NM_002633	Hs.1869	PGM1	Phosphoglucomutase 1	-2.1
202022_at	NM_005165	Hs.155247	ALDOC	Aldolase C, fructose-bisphosphate	-2.0
200737_at	NM_000291	Hs.78771	PGK1	Phosphoglycerate kinase 1	-2.0
238996_x_at	NM_000034	Hs.513490	ALDOA	Aldolase A, fructose-bisphosphate	-1.8
200650_s_at	NM_005566	Hs.2795	LDHA	Lactate dehydrogenase A	-1.7
208308_s_at	NM_000175	Hs.466471	GPI	Glucose phosphate isomerase	-1.5
201251_at	NM_002654	Hs.534770	PKM2	Pyruvate kinase, muscle	-1.5
200822_x_at	NM_000365	Hs.524219	TPI1	Triosephosphate isomerase 1	-1.4
202934_at	NM_000189	Hs.198427	HK2	Hexokinase 2	-1.4
217294_s_at	U88968	Hs.517145	ENO1	Enolase 1, (alpha)	-1.3
200697_at	NM_000188	Hs.118625	HK1	Hexokinase 1	-1.2
Pentose phosphate pathway					
201968_s_at	NM_002633	Hs.1869	PGM1	Phosphoglucomutase 1	-2.1
238996_x_at	NM_000034	Hs.513490	ALDOA	Aldolase A, fructose-bisphosphate	-1.8
208308_s_at	NM_000175	Hs.466471	GPI	Glucose phosphate isomerase	-1.5

Fold increases shown are the average of three paired comparisons.

TABLE 5. HIF1A-REGULATED GENES SHOWING DIFFERENTIAL EXPRESSION BETWEEN 20% AND 4% O₂ CONDITIONS

Probe Set ID	Accession	Unigene	Symbol	Descriptions	Fold
201848_s_at	NM_004052	Hs.144873	BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3	-4.6
201010_s_at	NM_006472	Hs.533977	TXNIP	Thioredoxin interacting protein	-4.1
236180_at	W57613	Hs.419240	SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	-3.2
202718_at	NM_000597	Hs.162	IGFBP2	Insulin-like growth factor binding protein 2	-3.1
202887_s_at	NM_019058	Hs.523012	DDIT4	DNA-damage-inducible transcript 4	-3.0
207543_s_at	NM_000917	Hs.500047	P4HA1	Procollagen-proline, 2-oxoglutarate 4-dioxygenase, alpha polypeptide I	-2.2
201105_at	NM_002305	Hs.227751	LGALS1	Lectin, galactoside-binding, soluble, 1	-2.1
1562529_s_at	BC040965	Hs.569497	RORA	RAR-related orphan receptor A	-2.1
202022_at	NM_005165	Hs.155247	ALDOC	Aldolase C, fructose-bisphosphate	-2.0
200737_at	NM_000291	Hs.78771	PGK1	Phosphoglycerate kinase 1	-2.0
238996_x_at	NM_000034	Hs.513490	ALDOA	Aldolase A, fructose-bisphosphate	-1.8
200650_s_at	NM_005566	Hs.2795	LDHA	Lactate dehydrogenase A	-1.7
203234_at	NM_003364	Hs.488240	UPP1	Uridine phosphorylase 1	-1.6
210512_s_at	NM_003376	Hs.73793	VEGF	Vascular endothelial growth factor	-1.6
202627_s_at	NM_000602	Hs.414795	SERPINE1 (PAI-1)	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	-1.5
208308_s_at	NM_000175	Hs.466471	GPI	Glucose phosphate isomerase	-1.5
201251_at	NM_002654	Hs.534770	PKM2	Pyruvate kinase, muscle	-1.5
200822_x_at	NM_000365	Hs.524219	TPI1	Triosephosphate isomerase 1	-1.4
202934_at	NM_000189	Hs.198427	HK2	Hexokinase 2	-1.4
217294_s_at	U88968	Hs.517145	ENO1	Enolase 1, (alpha)	-1.3
209773_s_at	BC001886	Hs.75319	RRM2	Ribonucleotide reductase M2 polypeptide	1.4

Genes in the top panel are positively-regulated by HIF1A and exhibit decreased expression under 20% O₂. The gene in the bottom panel, RRM2, has been shown to be repressed HIF1A and are up-regulated in human embryonic stem cells cultured under 20% O₂. Fold changes shown are the average of three paired comparisons.

FIG. 2. HIF1A and SSEA-4 (A–F) and HIF2A and OCT4 (G–L) expression in human embryonic stem cells (hESCs) cultured under either 20% (A–C and G–I) or 4% (D–F and J–L) O₂. H1 cells (p33) were cultured for 10 days under either 20% or 4% O₂ conditions. Immunofluorescent detection of HIF1A (red) and SSEA-4 (green) expression in colonies cultured under 20% (A and B) or 4% (D and E) O₂. Spontaneous differentiation of H1 cells (*d*) under 20% O₂ can be seen in the upper right (A–C) or bottom right corner (G–I) of the figure. Expression of HIF2A (green) and OCT4 (red) as observed by immunofluorescence in hESC colonies under 20% (G and H) or 4% (J and K) O₂. Nuclei are stained blue using TO-PRO-3 (C, F, I, and L). All images were captured by confocal microscopy using a 20× objective. Bar, 0.1 mm.

conditions that are nonphysiological. OCT4 concentrations, in particular, must be tightly controlled, as partial down-regulation of its expression can lead to the emergence of trophectoderm and its up-regulation to endoderm [67,68]. In addition, OCT4, NANOG, and SOX2 appear to regulate each other and to function together to repress genes involved in lineage specification [33,34].

The second question we addressed was whether transcriptional profiles were more consistent in cells under 4% than 20% O₂. As expected, they were. Whereas profiles observed across cell lines and at different passage number were remarkably alike under low O₂, the greater heterogeneity under 20% O₂ suggested that cryptic differentiation might have already begun despite the lack of morphological changes in the colonies, thereby allowing us to address our third question relating to whether signs of lineage specification were evident in day 7 cultures under 20% O₂. Indeed, some genes regulated by the OCT4, NANOG, and SOX2 triad of transcription factors showed significantly reduced expression at the higher O₂ concentration (Table 2), while other genes, including ones associated with lineage-specific differentiation, for example, *HAND1*, *EOMES*, *MEIS2*, *GATA3* & -6, *MSX2*, *TFAP2A* [69], and *H19* [27] exhibited increased expression in at least one of the samples cultured under 20% O₂ (Supplemental Table 4). As the latter cells were indistinguishable from cells grown under 4% O₂ and harvested prior to any visible signs of differentiation (Fig. 1B), our results suggest that morphological cues are poor guides when selecting hESCs for passage. Thus, culture under 20% O₂ is likely to provide a mixed group of cells, including subpopulations already programmed to differentiate.

Perhaps the most interesting regulated gene was *LEFTY2*, which displayed a 2- to 3-fold higher expression under 4% as compared to 20% O₂. *LEFTY2*, the human ortholog of murine *Lefty A*, is regulated by SOX2 and OCT4, and is able to prevent differentiation initiated by Nodal signaling by blocking the formation of an active Nodal/Activin receptor complex [70–72]. Conceivably, *LEFTY2* is a key gene in preventing the spontaneous differentiation of hESCs along the mesoderm and endoderm lineages. Its down-regulation as O₂ concentrations rise may render the pluripotent gene network less stable and more responsive to signals that drive differentiation, a hypothesis that can be readily tested by knockdown of *LEFTY2* expression.

The final question we attempted to address was whether there is involvement of the HIF transcription factors in maintaining the pluripotency of hESCs, particularly when the cells are cultured under physiological O₂. Mammalian cells rely on HIFs to ensure accommodation to low O₂ levels, primarily through the action of HIF1A and HIF2A and their common binding partner, ARNT [12,73,74] and an extensive list of HIF target genes have been identified. The higher expression of genes encoding glycolytic enzymes and other known HIF targets such as *BNIP3*, *DDIT4*, *IGFBP2*, and *VEGF* in hESCs cultured under 4% O₂ (Table 5) is entirely consistent with the view that the HIFAs are most active under low O₂ environments where they have a major role in maintaining cellular homeostasis. The gene list in Table 5 provides few clues as to any potential involvement in controlling pluripotency. While some workers have argued that HIF1A

and HIF2A display redundancy regarding HIF targets [75], others have indicated differences in gene preference [73,76], especially under acute hypoxia [77]. Although no exclusive HIF2A target gene has been identified [78], HIF2A may regulate *OCT4* expression specifically [21] and has been shown to activate *SERPINE1* (PAI-1) [79]. Such a link to *OCT4* expression would place HIF2A as a central player in the network of genes maintaining the pluripotent phenotype. Our observation that HIF2A was confined to the undifferentiated cells of day 10 colonies cultured under 20% O₂, while HIF1A was located throughout the colonies, including the overtly differentiated areas (Fig. 2), is consistent with this notion, as is the report that HIF2A is localized to the pluripotent ICM of bovine blastocysts, while HIF1A is absent from these cells but present in trophectoderm [13].

In conclusion, this report emphasizes the importance of employing physiological concentrations of O₂ when culturing hESCs. The transcript profiles of cultures under 20 % O₂ suggest that the cells are more poised to differentiate than when they are under the lower 4% O₂ conditions and that the down-regulation of *LEFTY2* under 20% O₂ may destabilize the network of genes maintaining ESC pluripotency. Finally, the association of HIF2A with undifferentiated but not differentiating cells is consistent with a particular role for that transcription factor in control of pluripotency.

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Address reprint requests to:

Dr. Toshihiko Ezashi

240h Christopher S. Bond Life Sciences Center

University of Missouri-Columbia

1201 E. Rollins Street

Columbia, MO 65211-7310

E-mail: ezashit@missouri.edu

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