A reduced oxygen tension (5%) is not beneficial for maintaining human embryonic stem cells in the undifferentiated state with short splitting intervals

Hsin-Fu Chen\textsuperscript{1,2}, Hung-Chih Kuo\textsuperscript{3}, Wannhsin Chen\textsuperscript{4}, Fang-Chun Wu\textsuperscript{1}, Yu-Shih Yang\textsuperscript{1}, and Hong-Nerng Ho\textsuperscript{1,5,6}

\textsuperscript{1}Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, College of Medicine and the Hospital, National Taiwan University, No. 7 Chung-Shan S. Rd, Taipei 100, Taiwan
\textsuperscript{2}Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan
\textsuperscript{3}Institute of Cellular and Organismic Biology and Genomics Research Center, Academia Sinica, Taipei, Taiwan
\textsuperscript{4}Biomedical Engineering Research Laboratories, Industrial Technology Research Institute, Taipei, Taiwan
\textsuperscript{5}Graduate Institute of Immunology, College of Medicine, National Taiwan University, Taipei, Taiwan
\textsuperscript{6}Correspondence address. Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, College of Medicine and the Hospital, National Taiwan University, No. 7 Chung-Shan S. Rd, Taipei 100, Taiwan. Tel: +886-2-23123456 ext. 5161; Fax: +886-2-23418557; E-mail: hnhao@ntu.edu.tw

\textbf{BACKGROUND:} Human embryos grow naturally \textit{in vivo} in lower oxygen (O\textsubscript{2}) tension environments than atmospheric O\textsubscript{2} tension. Therefore, human embryonic stem cells (hESC), a derivative of embryos, will likely grow more favorably in a reduced O\textsubscript{2} tension. This study aimed to compare the behavior of hESC under reduced O\textsubscript{2} tension (5%) versus normoxia (21%).

\textbf{METHODS:} hESC lines were cultured in different O\textsubscript{2} tensions and then examined for morphology, apoptosis and gene expression profiles.

\textbf{RESULTS:} hESC grown in 5% O\textsubscript{2} tension were not morphologically different from hESC grown in normoxia on day 7 of the first and fourth passages. However, after prolonged culture without splitting (10–14 days), hESC colonies were thinner and looked better morphologically in 5% O\textsubscript{2}, but the cells proliferated more slowly and their sizes were larger. At most time points, the gene expression profiles in both O\textsubscript{2} tensions showed no major difference in representative stemness genes (Oct-3/4, Nanog and Cripto), differentiation genes (Desmin, Nestin, α-fetoprotein and GDF-9) and hypoxia-related genes (HIF-1α and VEGF). A lower level of cyclin-D\textsubscript{1} mRNA (suggestive of less Wnt pathway signaling on day 7 of the fourth passage) and a higher level of Desmin (suggestive of more differentiation to mesoderm, at day 7 of the first passage) were detected in 5% O\textsubscript{2}.

\textbf{CONCLUSIONS:} This study suggests that for routine culture of hESC with a short splitting interval (7 days), a low O\textsubscript{2} tension (5% O\textsubscript{2}) probably does not provide significant advantages over the standard 21% O\textsubscript{2} tension for the maintenance of an undifferentiated state by the criteria used in this study.

\textbf{Key words:} embryonic stem cells / hypoxia / undifferentiated / oxygen / cell culture
Introduction

Human embryos normally develop before and after implantation in environments with relatively low oxygen (O$_2$) tension compared with the atmosphere. It has been demonstrated that the O$_2$ tension in the female reproductive tract where embryos grow and develop is much lower than that of the atmosphere (~21%). For example, the O$_2$ tension in the lumens of oviducts in hamsters, rabbits and rhesus monkeys has been estimated to be ~8% (Fischer and Bavister, 1993). The O$_2$ tension is even lower in the uterus, ranging from 5% in the rabbit to 1.5% in the rhesus monkey (Fischer and Bavister, 1993). A mean O$_2$ tension of 11.8% air saturation (i.e. 2.5% O$_2$ tension or a partial pressure of 18.9 mmHg) in the human uterine endometrial surface was reported recently (Ottosen et al., 2006). Previous reports have also suggested that culture in reduced O$_2$ tensions (5–7%) results in improved development of animal (Quinn and Harlow, 1978; Thompson et al., 1990; Harvey, 2007) and human embryos in vitro (Petersen et al., 2005). Due to the above observations, the culture of mammalian embryos (including human) under reduced O$_2$ tension has become more extensive in recent years. Many human infertility centers are also starting to use this culture environment in their in vitro fertilization programs. However, a low O$_2$ tension is known to induce angiogenesis and glycosylation for cell growth, and in extreme hypoxic states, growth arrest and apoptosis may occur (Koshiji et al., 2004). In a recent pilot study, the use of a reduced O$_2$ tension (5%) did not affect the fertilization rate and pregnancy rate in an in vitro fertilization program, although the morphologic scores of these human embryos appeared to be better (Kea et al., 2007). Such data are confusing and do not provide strong enough evidence to support the unanimous use of low O$_2$ tension in culturing human embryos.

Embryonic stem cells (ESC) are typically derived from the inner cell mass of blastocysts and therefore may be regarded in some aspects as extensions of embryos. Using such reasoning, if a low O$_2$ tension is good for embryo growth, mouse or human ESC (hESC) should also grow favorably in a reduced O$_2$ environment. Up to now, only some reports (Ezashi et al., 2005; D’Ippolito et al., 2006; Forsyth et al., 2006; Gibbons et al., 2006; Kurosawa et al., 2006; Peura et al., 2007) have explored this issue and they have provided some evidence for this assumption. Specifically, low O$_2$ tension has been shown to inhibit the differentiation of hESC (3–5% O$_2$) (Ezashi et al., 2005) and human MIAMI cells (3% O$_2$) (D’Ippolito et al., 2006), and to exert positive effects on the establishment and maintenance of mouse ESC (Gibbons et al., 2006). However, in striking contrast, hypoxia (1% O$_2$) has been shown to inhibit self-renewal and induce early differentiation of mouse ESC through the induction of HIF-1α and subsequent suppression of LIF-STAT3 signaling in vitro (Jeong et al., 2007). Interestingly, a higher O$_2$ tension (40%) than atmosphere retards the differentiation of ESC (Kurosawa et al., 2006). It is clear from these reports that 1% O$_2$ tension is probably very different physiologically from 3 to 5% O$_2$ tension. In addition, it is interesting to note that even after the publication of those positive reports about low O$_2$ tension, many labs still use a 20–21% O$_2$ tension as a standard for culturing hESC and human embryos. Accordingly, more data are needed to determine whether a low O$_2$ tension is beneficial in the culture of hESC. We planned to clarify this point by comparing the culture of hESC in atmospheric (21%) and low (5%) O$_2$ tensions. The proliferation, morphology, status of apoptosis and hypoxia and stemness and differentiation gene expression profiles of the hESC were examined. It is hoped that the resulting data will provide further information for reference in the future culturing of hESC.

Materials and Methods

Culture of hESC

The hESC used for this study were the passages 50–70 NTU1 and NTU3 hESC lines reported previously and have been routinely maintained in our lab (Chen et al., 2007). These hESC have been maintained on murine embryonic fibroblast (MEF) feeders using serum-free medium (ReproCELL primat and human ES Cell culture medium; ReproCELL, Japan, http://www.reprocell.com/en/). The cells were maintained and split using the protocols published previously but with some modifications (Chen et al., 2007). The hESC were split every 7 days by mechanical methods using a 30-gauge insulin needle or by enzymatic methods using dispase and collagenase type IV (Gibco). Beginning 48 h after splitting, the hESC colonies were observed and the medium was changed every day. For the study of the effect of reduced O$_2$ tension, the dissociated hESC pieces were plated evenly on gelatin-treated (0.1%) organ culture dishes (NUNC), 35-mm culture dishes (NUNC) or 10-cm culture dishes (BD Falcon), depending on the number of cells needed for experiments. These culture dishes had previously been plated with mitomycin-C-treated MEF feeder cells (6 × 10⁴ cells/cm²). The culture dishes were then divided and put into one of two types of culture conditions: (i) normoxic culture: 21% O$_2$ and 5% CO$_2$ or (ii) reduced O$_2$ culture: 5% O$_2$ and 5% CO$_2$. The starting number of hESC in each culture dish was the same for each group. A standard CO$_2$ incubator (RCO300T [capacity, 164 l]; Revco), which provided 5% CO$_2$ in humidified air, was used for the normoxic culture. A triple gas incubator (AFM-30 D [capacity, 30 l]; Astec), which provided a 5% CO$_2$ and 5% O$_2$ air mixture, was used for the reduced O$_2$ culture. This latter incubator controls O$_2$ and CO$_2$ tensions at desirable levels by the periodic and on-demand injection of CO$_2$ and N$_2$. The medium (ReproCELL medium) was changed daily and the morphology of the hESC colonies was observed, recorded and photographed. The parameters of morphology included the shape, size, area of differentiation and thickness of the colonies of hESC. The thickness of the colonies indicated the depth (number of cell layers) of the colonies and was subjectively evaluated by adjusting the fine adjustment knob of the microscope to identify the upper and lower margins of the colony. Individual stem cells were also visualized under high power of the light microscope for their size, nucleus and nucleus/cytoplasm ratio. The level of differentiation of the colony was scored as good (>3/4 of the colony was transferable), fair (<1/2 to 3/4 transferable) and poor (<1/2 transferable). The hESC were then collected periodically at pre-determined time points (days 3, 5, 7 and 14 after splitting) by mechanical methods. These harvested cells were fixed immediately for flow cytometry studies or were stored for further RNA extraction. For immunofluorescence (IF) studies, split hESC pieces were cultured on gelatin-treated, MEF (6 × 10⁴ cells/cm²) plated chamber slides (NUNC) and cultured for designated periods of time. At the end of culture, the cells were fixed and analyzed immediately. For each comparison, experiments were repeated at least three to five times.

Since the hESC were maintained in a 21% O$_2$ environment before this study and these cells needed time to adapt to the reduced O$_2$ conditions, we also compared the differences of hESC at the end of four consecutive passages in different O$_2$ tensions. Therefore, for this experiment, the hESC were split and allocated in parallel again into two culture conditions (21% and 5% O$_2$). Subsequently, each group of hESC was maintained and split...
every 7 days for a consecutive four passages without a change of culture conditions (i.e. persistently 21% O₂ or 5% O₂), and the hESC were collected at the end (day 7) of the 4th passage. The hESC were then collected for PCR, flow cytometry and IF studies. As controls for the gene expression study, the split hESC pieces were cultured in suspension in Petri dishes for 5 days according to the previous protocol (Chen et al., 2007) for embryoid body (EB) formation. Some EBs were collected for direct examination at this point and others were put into a differentiating culture for 21–28 days using conditioned medium obtained from the CHO-K1 cell line, a Chinese hamster ovary cell line (BCRC 60 006; original source: aTCC CCL-61) and collected for study.

### IF study and alkaline phosphatase staining

After culture, the hESC on chamber slides were fixed and examined by IF according to the protocols provided by the manufacturer (Chemicon) and a previous report (Chen et al., 2007). Briefly, the hESC were fixed in 4% paraformaldehyde and then subjected to the following procedures: permeabilization (0.1% Triton X-100), blocking (4% normal goat serum), primary antibody treatment for 1 h at room temperature (RT), three cycles of washing, fluorescence-labeled secondary antibody treatment for 1 h at RT, three cycles of washing, cover slipping and mounting with anti-fade mounting solution. Anti-β-catenin (mouse IgG1) (BD Biosciences) was used in this study and the cells were counterstained with Hoechst 33342 for nuclear staining. The stained slides were observed and photographed using a fluorescence microscope. The hESC colonies were also stained with alkaline phosphatase (AP) (Alkaline phosphatase substrate kit III, SK-5300, Vector) to determine its stem cell nature.

### Flow cytometric analysis

The apoptosis status and the expression of stemness genes (Oct-3/4 and TRA-1-60) of hESC after culture in different O₂ tensions were compared using flow cytometric analysis. Briefly, the hESC colonies were treated with 0.25% trypsin—EDTA for 5 min and dissociated into single cells. The hESC were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 at RT for 30 min. The single cells were collected at the end (day 7) of the 4th passage. The hESC were then collected and re-suspended in phosphate-buffered saline (PBS) with 0.1% Triton X-100 at RT for 30 min. The single cells were collected at the end (day 7) of the 4th passage. The hESC were then collected and re-suspended in propidium iodide (PI) in binding buffer. The cells were then subjected to flow cytometric analysis immediately.

### Statistical analysis

A Student’s t-test, paired t-test and one-way ANOVA were used where appropriate for the statistical analysis in this study. SPSS statistical package (SYSTAT Software Inc.) was used to analyze these tests. A P-value <0.05 was considered statistically significant.

**Table I** TaqMan primers and probe assay ID of the genes studied by real-time PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Assay ID (ABI)</th>
<th>Gene description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct-3/4</td>
<td>Hs00999632_gj</td>
<td>POU class 5 homeobox</td>
</tr>
<tr>
<td>Nanog</td>
<td>Hs02387400_gj</td>
<td>Nanog homeobox</td>
</tr>
<tr>
<td>Cripto</td>
<td>Hs02339499_gj</td>
<td>Teratocarcinoma-derived growth factor I</td>
</tr>
<tr>
<td>Cyclin-D1</td>
<td>Hs00227039_ml</td>
<td>Cyclin-D1</td>
</tr>
<tr>
<td>α-FP</td>
<td>Hs00173490_ml</td>
<td>α-Fetoprotein</td>
</tr>
<tr>
<td>Desmin</td>
<td>Hs00157258_ml</td>
<td>Desmin</td>
</tr>
<tr>
<td>Nestin</td>
<td>Hs00707120_sl</td>
<td>Nestin</td>
</tr>
<tr>
<td>GDF-9</td>
<td>Hs00193364_ml</td>
<td>Growth differentiation factor 9</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hs00936368_ml</td>
<td>Hypoxia-inducible factor I, α subunit (basic helix–loop–helix transcription factor)</td>
</tr>
<tr>
<td>VEGF</td>
<td>Hs00900054_ml</td>
<td>Vascular endothelial growth factor A</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Hs99999905_ml</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
</tbody>
</table>
Results

Morphology, proliferation and AP activity of hESC

After culture for 7 days of the first passage of NTU1 hESC (the first passage after last splitting), the only detectable morphologic difference was that the hESC colonies in a reduced O2 culture were slightly thinner, more transparent under dark field, more fragile and could be more easily dissociated during manual or enzymatic splitting (Figs 1, 2A and I). At the end (day 7) of the fourth consecutive passage, the difference between the two O2 tensions was similar to the findings on day 7 of the first passage (data not shown). The above observations, such as the thinner colonies in 5% O2, became more consistent and apparent at the end of a prolonged culture for 14 days (Fig. 2B and J). Comparable to the findings of thinner colonies, the AP staining was also less intense in a reduced O2 culture on days 7 and 14 of culture in 5% O2 tension (Table III). The percentages of good colonies were significantly higher on days 10 and 14 of the first passage (Fig. 2G and O), possibly suggesting more cells in a very early stage of differentiation. Similar findings were demonstrated in the NTU3 hESC line (data not shown). Taken together, these observations revealed that when hESC (both NTU1 and NTU3 lines) were split at a routine 7-day interval, it was difficult to identify morphologic differences, except the thickness of colonies, between the two gaseous tensions. In a longer-term culture (10–14 days) than routine however, the difference became significant but the comparative levels of differentiation could not be definitely determined by morphologic criteria alone. In addition, using the same starting cell numbers of the NTU1 hESC line, it was found that the cell numbers increased exponentially in both O2 tensions (Fig. 2H). Although the cell numbers were not different on days 3, 5 and 7, they were significantly lower in 5% O2 than in 21% O2 after a consecutive 14 days of culture (P = 0.019) (Fig. 2H). Similar findings were also revealed in the NTU3 hESC line (data not shown).

Apoptosis and expression of TRA-1-60 and Oct-3/4

For the NTU1 hESC line, there were low levels of apoptosis in both O2 tensions (Fig. 3A–E). Although a trend towards higher levels of apoptosis was noted in 5% O2 culture, especially after 14 days, the difference was not significant (P = 0.207 on day 14 of culture) (Fig. 3A–E). The percentages of NTU1 hESC expressing the stem cell markers, TRA-1-60 and Oct-3/4, were not significantly different between the two O2 tensions on days 7 and 14 of the first passage and day 7 of the fourth passage (Fig. 3F–J). In addition, when the product levels of each gene itself (TRA-1-60 or Oct-3/4) were compared at different time points of the same O2 tension, there was no difference (Fig. 3). The latter result indicates that even after 14 days of culture without splitting, the percentage of cells expressing TRA-1-60 and Oct-3/4 remained favorable in the present culture condition at both O2 tensions. Again no difference of apoptosis and stem cell markers (TRA-1-60 and Oct-3/4) was found between the two O2 tensions for the NTU3 hESC line (data not shown).

Expression of stemness, differentiation, Wnt pathway-related and hypoxia-related genes

It was found that the expression of stemness genes (Oct-3/4, Nanog and Cripto) and the differentiation genes (Nestin, Desmin, α-FP and

Figure 1  Representative photos of NTU1 hESC line colonies cultured on MEFs feeder in a reduced O2 culture (5% O2) or normoxic culture (21% O2), as described in Materials and Methods. (A–F) A reduced O2 culture. (G–L) Normoxic culture. (A, G) Day 3 of culture. (B, H) Day 5. (C, I) Day 7. (D, J) Day 10. (E, K) Day 12. (F, L) Day 14. Scale bar = 100 μm.
GDF-9) were not different at most time points (with one exception) for the NTU1 hESC line at different O2 tensions (Fig. 4A–C and E–H). There was a borderline increase in Desmin expression on day 7 of the first passage in 5% O2 culture ($P = 0.042$) (Fig. 4F).

The stemness genes were highly expressed in undifferentiated hESC, and as expected were barely detectable in differentiated hESC (Fig. 4A–C). When the mRNA levels of each gene itself were compared at different time points of undifferentiated cultures at the same O2 tension, there were no differences (Fig. 4A–C and E–H). This latter result suggests that no major difference in gene expression patterns occurs when hESC are cultured for up to 14 days without splitting. According to the cyclin-D1 data, the Wnt pathway signaling was probably less activated in the reduced O2 tension than in normoxia on day 7 of the fourth passage ($P = 0.012$) (Fig. 4D), but the differences at other time points were not significant. In addition, no increase of hypoxia-related gene (HIF-1α and VEGF) expression was detected in the reduced O2 culture than in normoxic culture (Fig. 4I and J). The EBs and differentiated hESC as expected expressed significantly higher levels of VEGF than undifferentiated hESC (Fig. 4J).

By IF, β-catenin was localized in the cytoplasm near the cell membrane (Fig. 4K–O). However, only few cells with nuclear accumulation of β-catenin (an indication of Wnt pathway activation status) (Moon et al., 2002) were noted (Fig. 4K–N), and thus no difference was identified between the two O2 tensions. After the data about the NTU1 hESC line became available, the real-time PCR study was also performed on the NTU3 line for the expression of

**Figure 2** Representative photos of NTU1 hESC colonies cultured on MEFs feeder in a reduced O2 culture (5% O2) or normoxic culture (21% O2), and observed by light microscopy, AP staining, immunofluorescence staining or flow cytometry, as described in Materials and Methods. (A–G) A reduced O2 culture; (I–O) normoxic culture. (A, I) Day 7 of the first passage, dark field; (B, J) day 14 of the first passage, dark field; (C, K) day 7 of the first passage, AP staining (blue); (D, L) day 14 of the first passage, AP staining (blue); (E, M) day 14 of the first passage, Hoechst 33342 staining (blue); (F, N) day 14 of the first passage, high power of light microscopy; (G, O) day 14 of the first passage, flow cytometry; (H) comparison of hESC numbers in the two O2 tensions. The data represents mean ± SEM. $P = 0.016$ at day 14 of culture. $P > 0.05$ at all other time points. Scale bar = 100 μm.
reduced O2 tension was shown to be unfavorable with respect to the prolonged culture (14 days) without splitting was attempted, a gross differentiation of hESC colonies was found, showing a more homogeneous appearance with a reduced area of differentiation between the two O2 tensions for the NTU3 hESC line (data not shown).

The data from this study in some aspects are not consistent with previous reports (Ezashi et al., 2005; Forsyth et al., 2006; Gibbons et al., 2006; King and Miller, 2007; Peura et al., 2007), which showed that reduced O2 tension always improves the undifferentiated state of hESC in vitro (Ezashi et al., 2005) and enhances the establishment of mouse ESC (Gibbons et al., 2006). The study by Forsyth et al. (2006) suggested that H1 or H9 hESC cultured in a reduced O2 tension (2%) are smaller and less granular. The present study, to the contrary, consistently identified that at day 14 of culture, the NTU1 and NTU3 hESC in 5% O2 tension presented with larger individual cell sizes, more complexity and thinner but grossly less differentiated colonies. It is unclear that how this discrepancy occurred, although different O2 levels (2 versus 5%) are likely a factor. In addition, it is unknown at present whether the larger individual cell size and the increased complexity are indications of subtle or early differentiation, which could not be detected by the other experimental methods used in this and other studies. The study by Ezashi et al. (2005) showed morphologic, biochemical and immunofluorescent

**Table II** Colony sizes of NTU1 and NTU3 hESC cultured under different O2 tensions

<table>
<thead>
<tr>
<th>O2 tension (%)</th>
<th>hESC line</th>
<th>Number of colonies examined</th>
<th>Surface area of hESC colony (μm²) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 7 of first passage</td>
</tr>
<tr>
<td>5%</td>
<td>NTU1</td>
<td>50</td>
<td>1719.1 ± 282.0</td>
</tr>
<tr>
<td></td>
<td>NTU3</td>
<td>50</td>
<td>1664.5 ± 342.7</td>
</tr>
<tr>
<td>21%</td>
<td>NTU1</td>
<td>50</td>
<td>1855.8 ± 427.2</td>
</tr>
<tr>
<td></td>
<td>NTU3</td>
<td>50</td>
<td>1799.2 ± 576.3</td>
</tr>
</tbody>
</table>

P-value (5 versus 21%)

<table>
<thead>
<tr>
<th>hESC line</th>
<th>NTU1</th>
<th>NTU3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.754</td>
<td>0.323</td>
<td></td>
</tr>
<tr>
<td>0.645</td>
<td>0.201</td>
<td></td>
</tr>
</tbody>
</table>

* A good colony indicates a colony with more than 3/4 of the area is transferable.

**Table III** The percentages (mean ± SD) of good* NTU1 and NTU3 hESC colonies cultured under different O2 tensions

<table>
<thead>
<tr>
<th>O2 tension (%)</th>
<th>hESC line</th>
<th>Day 7 (%)</th>
<th>Day 10 (%)</th>
<th>Day 14 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>NTU1</td>
<td>99 ± 1</td>
<td>88 ± 5</td>
<td>81 ± 8</td>
</tr>
<tr>
<td></td>
<td>NTU3</td>
<td>99 ± 2</td>
<td>86 ± 6</td>
<td>77 ± 9</td>
</tr>
<tr>
<td>21%</td>
<td>NTU1</td>
<td>99 ± 2</td>
<td>78 ± 4</td>
<td>59 ± 7</td>
</tr>
<tr>
<td></td>
<td>NTU3</td>
<td>98 ± 2</td>
<td>77 ± 5</td>
<td>64 ± 8</td>
</tr>
</tbody>
</table>

P-value (5 versus 21%)

<table>
<thead>
<tr>
<th>hESC line</th>
<th>NTU1</th>
<th>NTU3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.51</td>
<td>0.01</td>
<td>0.002</td>
</tr>
<tr>
<td>0.43</td>
<td>0.01</td>
<td>0.004</td>
</tr>
</tbody>
</table>

This study examined the gene expression patterns of hESC and confirmed that with only two exceptions, there were no significantly different expressions of representative genes of stemness, three germ layers and germ cells, hypoxia and the Wnt pathway in the O2 tensions. The selection of the genes for the study was purely empirical in some cases but was based on the following considerations in others. Physiologically under hypoxia, animal and human tissues respond to low O2 tensions with the expression of hypoxic genes, including HIF-1α, VEGF, GLUT-1 and ALDA, and switching from aerobic to anaerobic metabolism and neovascularization follow closely (Maltepe et al., 1997; Nanka et al., 2006). This study therefore aimed to analyze this issue but did not detect increased expression of HIF-1α and VEGF in 5% O2, though the effect of an even lower level (e.g. 1–3%) O2 remains unknown. This result suggested that a 5% O2 tension was not truly hypoxic to NTU1 and NTU3 hESC in the present culture conditions. At present, we do not know exactly the molecular pathways underlying pluripotency of hESC, but the potential candidates include inhibitors of FGF, TGFβ/BMP and the Wnt pathways (Sato et al., 2003). A previous study confirmed that the activation of Wnt signaling by the GSK-3-specific inhibitor maintained the pluripotency of hESC, but the potential candidates include inhibitors of FGF, TGFβ/BMP and the Wnt pathways (Sato et al., 2003). A previous study confirmed that the activation of Wnt signaling by the GSK-3-specific inhibitor maintained the pluripotency of hESC in vitro (Sato et al., 2004). We therefore compared the activation status of the Wnt pathway through the analysis of the intermediate and end products of the Wnt pathway (Sato et al., 2004), including β-catenin (Moon et al., 2002) and cyclin-D1 (a Wnt target gene) (Tetsu and McCormick, 1999). It was hypothesized, though without existing evidence or report, that if the O2 tension could affect the pluripotency of hESC, the Wnt signaling pathway might be one of the targets through which different levels of O2 would exert their action. This study identified a lower activation level of the Wnt pathway (by cyclin-D1 expression) at the end of the fourth but not the first passage in a reduced O2 culture, suggesting that comparatively decreased activation might be observed only after several passages. However, this issue could not be confirmed at present, because the nuclear levels of β-catenin were low in both O2 tensions. According to these results, a reduced O2 tension (5%) cannot be regarded as a more favorable environment for maintaining undifferentiated NTU1 and NTU3 hESC lines. However, this study cannot exclude the possibility that O2 may use other signaling pathways (e.g. TGFβ/BMP) to regulate hESC self-renewal.

Discussion

The differentiation of hESC can be identified based on the morphologic changes, or on the down- or up-regulation of stem cell markers and markers related to differentiation. This study analyzed the differences in hESC growth, morphology, apoptosis and gene expression patterns under different O2 tensions. It was found that in the routine transfer of hESC at an interval of 7 days, hESC culture under a reduced O2 tension (5%) did not provide significant advantages for maintenance of an undifferentiated state over the 21% O2. On the contrary, when prolonged culture (14 days) without splitting was attempted, a reduced O2 tension was shown to be unfavorable with respect to the cell proliferation rate, but a comparatively improved morphology showing a more homogeneous appearance with a reduced area of gross differentiation of hESC colonies was found.
evidence of decreased differentiation in hypoxic cultures (Ezashi et al., 2005). The present study, comparable with the report of Ezashi et al. (2005), showed that after prolonged culture (after 10 days of culture), the hESC colonies were morphologically better with less gross differentiation in 5% O2 tension. However, since hESC colonies in the same culture dish may differ greatly in their levels of differentiation and gene expression, a quantitative method (i.e. flow cytometry and real-time PCR used in this study) might provide more information with respect to the general behaviors of all hESC. Our data did not support any major difference in representative markers for differentiation and undifferentiation between normoxia and reduced O2 tension. A previous study did not support the use of low O2 tensions by showing that hypoxia (1% O2) inhibited the self-renewal of mouse ESC and induced early differentiation in vitro, at least in part through the suppression of the LIF-STAT3 pathway (Jeong et al., 2007). At present, it is unclear that what reason(s) lead to the discrepancies.

Figure 3 Representative photos and comparison of apoptosis status (by Annexin V/PI method) and products of OCT-3/4 and TRA-1-60 genes in NTU1 human embryonic stem cells (hESC) by a reduced O2 culture (5% O2) or normoxic culture (21% O2), as described in Materials and Methods. (A–E) apoptosis. (F–J) Oct-3/4 and TRA-1-60 gene products. (A) Day 7 of the first passage in 5% O2. (B) Day 7 of the first passage in 21% O2. (C) Day 7 of the fourth passage in 5% O2. (D) Day 7 of the fourth passage in 21% O2. (E) Comparison of apoptosis status in the two O2 tensions. *P* > 0.05 at all the time points examined. (F) Oct-3/4, 5% O2. (G) Oct-3/4, normoxia. (H) TRA-1-60, 5% O2. (I) TRA-1-60, normoxia. (J) Comparison of the two O2 tensions about OCT-3/4 and TRA-1-60 expression. *P* > 0.05 at all the time points examined. P4 day 7: day 7 of the fourth passage. Day 7: day 7 of the first passage. Day 14: day 14 of the first passage.
Comparison of the expression of stemness genes (Oct-3/4, Nanog and Cripto), related gene for Wnt pathway (cyclin-D1), differentiated genes (α-fetoprotein, Desmin, Nestin and GDF-9), and hypoxia genes (HIF-1α and VEGF) by real-time PCR and IF study of β-catenin between the reduced O₂ culture (5% O₂) and normoxic culture (21% O₂) in NTU1 hESC, as described in Materials and Methods. (A–J) Data for real-time PCR. (A) Oct-3/4. (B) Nanog. (C) Cripto. (D) Cyclin-D1. *P = 0.012 at day 7 of the fourth passage. (E) α-Fetoprotein. (F) Desmin. *P = 0.042 at day 7 of the first passage. (G) Nestin. (H) GDF-9. (I) HIF-1α. (J) VEGF. The P-values between 5% O₂ and 21% O₂ were more than 0.05 for all genes at all time points except for the two indicated above. D7: day 7 of the first passage. D14: day 14 of the first passage. P4D7: day 7 of the fourth passage. (K–O) Immunofluorescence of β-catenin (FITC, green) and counterstaining with DAPI (blue). (K) Day 7 of the fourth passage in 5% O₂. (L) Day 7 of the fourth passage in 21% O₂. (M) Day 14 of the first passage in 5% O₂. (N) Day 14 of the first passage in 21% O₂. (O) Negative control. Scale bars = 100 μm.
between all these studies, but the followings are the presumptive mechanisms. Taking the study by Ezashi et al. (2005) as an example, there were differences in a number of culture conditions between the study by Ezashi et al. (2005) and the present study. For example, the hESC lines (NTU1 and NTU3 lines in our study versus the H1 line in the Ezashi’s) and the media (ReproCELL medium versus DMEM/F-12 plus KO serum replacement) were different (Ezashi et al., 2005; Chen et al., 2007). Each culture medium likely needs different gaseous tensions (O2 and CO2) for optimal function. In addition, we used different incubators for different O2 tensions, but in the Ezashi et al.’s (2005) study, tissue culture plates kept in sealed chambers with a humidified gas mixture were used. Furthermore, since real-time PCR did not detect an enhanced expression of hypoxia-related genes, it was speculated that a 5% O2 tension in the present culture setup did not provide a practically effective hypoxic environment. These above reasons singly or combined probably can partially explain the divergent results, especially when the potential effects of low O2 tensions are inherently small. Therefore, this study does not provide evidence to support that a low O2 tension (5%) is the optimal or more beneficial culture for hESC maintenance with short splitting intervals. This conclusion however cannot exclude the possibility that for specific culture environment and cell lines or for specific purposes, reduced O2 tensions may be better. For example, this study did not explore the hypoxic effect on hESC behavior after they are intended to differentiate in vitro. Our data did not address whether an even longer period of culture (e.g. continuous culture for more than 4 passages) would show any difference in hESC behavior. With respect to the difference in cell lines, a comparison between NTU1 or NTU3 hESC lines with reference lines such as H1 or H9 was not done in the current study. However, a recent experiment with different O2 tensions (5 versus 21%) on hESC culture has been done by one of the authors (W.C.) at the Industrial Technology Research Institute (ITRI) in Taiwan using two hESC lines, an in-house line TW1 derived by the ITRI and the H9 line, both using a medium composed of 85% DMEM/F-12 plus KO serum replacement. The preliminary findings, which were comparable and consistent in both cell lines (TW1 and H9), were that when grown at 5% O2 tension, the hESC colonies were smaller, thinner and expressed weaker AP activity (personal communication). This observation is comparable with that using NTU1 and NTU3 lines in ReproCELL medium. Although no further study was done on the stemness or hypoxia gene expression profiles, these data using H9 line probably could support the observations in this study and suggest that the findings are not limited only to the cell lines we used. In addition, since many labs study hESC using cell lines other than H1 or H9, and commercial media such as ReproCELL are becoming more popular, the present report should provide useful information and evidence to support the need to explore the potential differences between cell lines and culture media. For example, ReproCELL medium has recently been used successfully in the derivation and maintenance of human induced pluripotent stem cells (Takahashi et al., 2007) and this medium deserves further investigation.

The opinion that reduced O2 tensions may not be optimal or necessary for hESC cultures can be further supported circumstantially by the fact that although hypoxic culture setups have long been available, many recent reports have not used hypoxic cultures for hESC (Clark et al., 2004; Sato et al., 2004; Kim et al., 2005; Klimanskaya et al., 2005, 2006; Mateiel et al., 2006; Watanabe et al., 2007), but have still obtained favorable culture results. It is therefore argued that reduced O2 tensions may not be absolutely necessary for the maintenance of full pluripotency. However, it is presently unknown whether reduced O2 tensions provides any advantages/disadvantages in culturing hESC for other subtle or undetected parameters such as oxidative stress damage or activation of a specific signaling transduction pathway for self-renewal. These issues therefore need further study. It addition, it needs to be considered how far the low O2 culture system might affect the daily practice of hESC culture in the lab. The lower O2 tension needs to be maintained through the use of N2, which in a small-sized triple-gas incubator (with a capacity of 30 l) which exhausts about 4800 l of N2 every 2–4 weeks. This will significantly increase the maintenance cost and workload in the lab. It thus needs to be clarified whether a reduced O2 tension should be used in all, or in which specific situations, for the culture of hESC. Since the present study identified that NTU1 and NTU3 hESC colonies cultured in a reduced O2 tension are thinner and proliferate more slowly, the data probably can be extrapolated into a reduced undifferentiated cell numbers after prolonged culture. However, for the derivation of new hESC lines, which will sometimes take a longer period before first splitting, a 5% O2 may be beneficial due to the significantly better morphology at days 10–14 of continuous culture. The next consideration is that although a reduced O2 tension probably represents a physiologic stimulus for the up-regulation of a set of genes needed for embryo development in vivo, there are actually significant differences between the growing embryos in vivo and the undifferentiated hESC in vitro. Naturally in vivo, it is the default for the inner cell mass to undergo differentiation into all cells in the body, and therefore, the maintenance of hESC (a derivative of the inner cell mass) in an undifferentiation state can be regarded as non-physiologic. Taken together, it may be interesting to advance the hypothesis that a low O2 culture (physiologic) may be optimal when one plans to differentiate the hESC (a physiologic condition mimicking embryo growth in vivo), whereas normoxic culture (a non-physiologic condition) may be better for the maintenance of hESC in undifferentiated state (a non-physiologic condition), since hESC naturally tend to differentiate.

In conclusion, this study determined that culture of hESC in a reduced O2 tension (5%) in short splitting intervals (every 7 days) would not significantly alter the proliferation and the stemness gene expression profiles, although after a longer duration of continuous culture (14 days), the proliferation rate is comparatively slower but there is possibly decreased differentiation. However, the present conclusions are based only on the experimental criteria we used to analyze the cells, and the potential impact of low O2 tensions on other cellular attributes such as telomerase activity can be clarified only through further studies. Taken together from the present study with the previous reports (Ezashi et al., 2005; Forsyth et al., 2006; Gibbons et al., 2006; King and Miller, 2007; Peura et al., 2007), it is suggested that different constitutents of culture, duration of culture and purposes of hESC maintenance probably need different O2 tensions for optimal performance. Since these issues are not settled completely, further exploration will be needed before the implementation of reduced O2 cultures for hESC can be universally recommended.
Funding

This work was supported by grants from the National Science Council of the Republic of China (NSC94-2314-B-002-199 and NSC95-2314-B-002-037), grants from Industrial Technology Research Institute (ITRI) (Grant 455-1) and a research fund from National Taiwan University Hospital (MG 237, the Stem Cell Research Fund, donated by Mr Ted Wen).

References


