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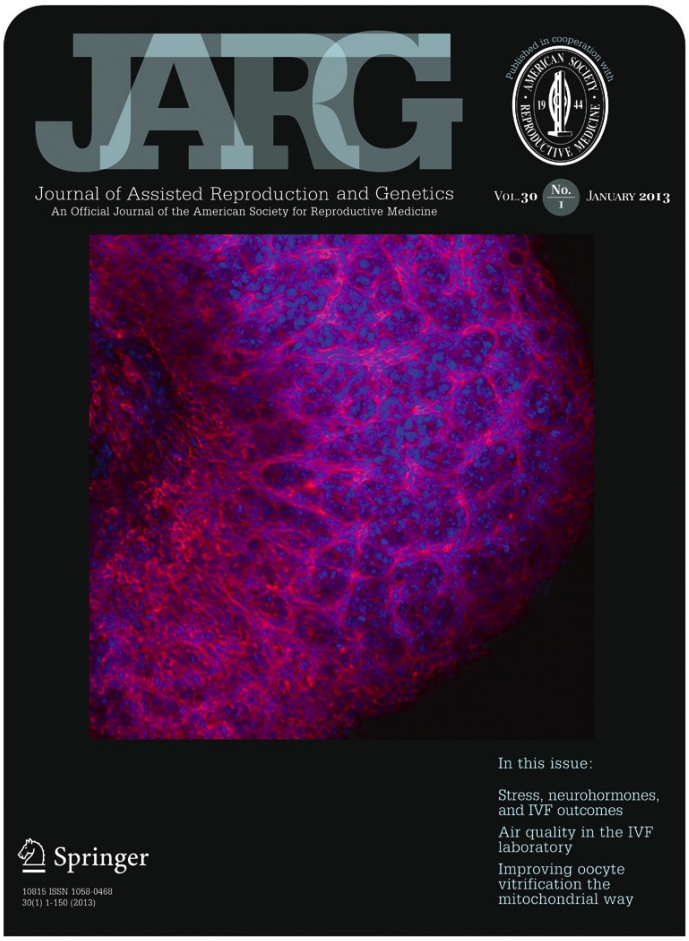


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EMBRYO BIOLOGY

# Effects of oxygen tension and IGF-I on HIF-1α protein expression in mouse blastocysts

**Jeong Yoon · Kyoung-Mi Juhn · Jin-Kyung Ko · San-Hyun Yoon · Yong Ko · Chul-Young Lee · Jin-Ho Lim**

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**Abstract**
*Purpose* Hypoxia inducible factors (HIFs) are key regulators of oxygen homeostasis in response to reduced oxygenation in somatic cells. In addition, HIF-1α protein can be also induced by insulin-like growth factor I (IGF-I) treatment in various cell lines under normoxic condition. However, the expression and function of HIF-1α in embryogenesis are still unclear. Therefore, the objectives of this study were to examine the expression of HIF-1α in mouse blastocysts cultured under hypoxic and normoxic conditions, and to determine whether oxygen tension and IGF-I influence embryonic development through stimulation of HIF-1α expression.
*Methods* Mouse embryos were cultured from the 1-cell to blastocyst stage under 5 % or 20 % $O_2$ in both the absence and presence of IGF-I.
*Results* The embryonic development rates to the blastocyst stage were not affected by oxygen tension or IGF-I treatment. HIF-1α protein was localized to the cytoplasm of blastocysts, and its levels were independent of oxygen concentration or IGF-I treatment. Blastocysts cultured under 5 % $O_2$ exhibited significantly higher total cell numbers (83.4±18.1) and lower apoptotic index (3.7±1.5) than those cultured under 20 % $O_2$ (67.4±15.6) (6.9±3.5) ($P< 0.05$). IGF-I reduced the apoptotic index in both oxygen conditions, but a significant decrease was detected in the 20 % $O_2$ group.
*Conclusions* HIF-1α may not be a major mediator that responds to change in oxygen tension within blastocysts, inconsistent with that of somatic cells. Supplementation of culture media with IGF-I has been shown to promote embryo development by an anti-apoptotic effect, instead of increasing HIF-1α protein expression.

**Keywords** HIF-1 · IGF-I · Embryo development · Low oxygen tension

*Capsule* Reduced oxygen tension along with IGF-I supplementation can increase the developmental potential of mouse blastocysts, and this may be attributed to the suppression of apoptosis, but not to an influence of HIF-1α expression.

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## Introduction

Oxygen concentration is important for cellular metabolism so that most mammalian cells respond to changes in oxygen conditions. It has been reported that adaptive responses to acute hypoxic exposure induced within a few hours occurs mainly as a result of alterations of pre-existing proteins,

whereas those to chronic exposure up to several days occurs predominantly as a result of alterations in gene expression [1, 2]. Hypoxia-inducible factor (HIF) is a member of the basic helix-loop-helix PAS protein family. HIF consists of two subunits HIF-1α and HIF-1β, forming a heterodimer. HIF has been reported to be an essential mediator of transcriptional and/or metabolic responses to both acute and chronic hypoxia in various cell types [3–6]. In most IVF clinics, embryos are routinely cultured under 5 % oxygen concentration for 3–6 days, which represents the state of chronic hypoxia. There were several reports that expressions of oxygen-regulated genes had important roles in the regulation of pre-implantation embryonic metabolism, and that low oxygen tension increased embryo development to the blastocyst stage [7–9]. However, although these results suggest the possibility that HIF-1α is a regulator of embryonic developmental processes, the expression and function of HIF-1α in embryogenesis is still unclear.

On the other hand, HIF-1α protein expression can also be induced by a variety of stimuli other than hypoxia, including hormones, cytokines, and growth factors [4]. In vascular smooth muscle cells, HIF-1α protein levels are strongly increased under normoxic conditions when cells are stimulated with angiotensin II (Ang II), thrombin, and platelet derived growth factor (PDGF) [10]. Similarly, IGF-I can induce HIF-1α protein synthesis and function in several cell lines under normoxic conditions [3, 11]. This occurs independently of oxygen tension, but by regulatory mechanisms that involve mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt signaling pathways [12, 13]. In embryogenesis, several reports indicated that IGF-I can promote pre-implantation embryo development and influence cell numbers [14–17]. Moreover, a number of studies have demonstrated that IGF-I is a powerful inhibitor of apoptosis through the activation of its receptor [14, 17–19]. However, the role of IGF-I in HIF-1α expression in embryogenesis has not yet been studied.

Therefore, the objectives of this study were to examine the expression of HIF-1α in mouse blastocysts cultured under chronic hypoxic condition commonly used for embryo culture in clinical laboratory, and to determine whether oxygen tension and IGF-I influence embryonic development through stimulation of HIF-1α protein expression.

## Materials and methods

### Mouse embryo production and development

B6D2 F1 female mice injected with 5 IU pregnant mare's serum gonadotropin (PMSG), followed by 5 IU human chorionic gonadotropin (hCG) 48 h later, were paired with B6D2 F1 males. Coitus was evaluated by the presence of a vaginal plug 16 h after hCG injection. One-cell embryos were collected and cultured in 20 μl MTF medium containing 4 mg/ml human serum albumin (HSA) in one of four groups: 5 % $O_2$, 5 % $O_2$ and 100 ng/ml IGF-I (Invitrogen, USA), 20 % $O_2$, and 20 % $O_2$ and 100 ng/ml IGF-I, under a layer of mineral oil in a 6 % $CO_2$ incubator at 37°C. Embryo morphology and development were daily monitored until day 4 of the blastocyst stage.

### Immunofluorescence of HIF-1α protein

Blastocysts were fixed in 3.7 % paraformaldehyde in PBS for 20 min at room temperature (RT) and washed twice in PBS containing BSA (BPBS). Embryos were permeabilized with 0.2 % Triton X-100 in PBS for 30 min at RT, then washed twice in BPBS. Embryos were incubated in blocking solution (0.1 % Tween 20 in BPBS) for 1 h at RT and then incubated with primary antibody (HIF-1α monoclonal, 1:100 dilution; Novus Biologicals NB100-105) overnight at 4°C. On the following morning, embryos were rinsed three times in BPBS and exposed to a secondary antibody (Fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse IgG for HIF-1α, 1:400 dilution; Novus Biologicals NB720-F) for 1 h at RT in the dark. After washing in BPBS, embryos were finally exposed to propidium iodide (PI) (25 μg/ml) and RNase A (200 μg/ml) in BPBS to counterstain nuclei for 1 h at RT. The stained embryos were mounted in an anti-bleaching solution (Vector labs) and observed in confocal laser-scanning microscope (Carl-Zeiss, Germany) for detecting fluorescence of FITC and PI. Negative control embryos were processed as described above, but the primary antibody was omitted. The HeLa cells treated with cobalt chloride ($CoCl_2$; a chemical inducer of HIF-1α) were used as a positive control [20].

### Differential staining

Differential staining of inner cell mass (ICM) and trophectoderm (TE) cell was carried out as described previously [21]. Briefly, zona intact, or partially hatching blastocysts were incubated in 1 ml MOPS-buffered MTF medium with 1 % Triton X-100 and 100 μg/ml PI for up to 10 sec. Then blastocysts were immediately transferred into 1 ml of fixative solution of 100 % ethanol with 25 μg/ml bisbenzimide (Hoechst 33258) and stored at 4°C overnight. Blastocysts were then mounted onto a glass microscope slide in a drop of glycerol, gently flattened with a coverslip, and visualized for cell counting. Cell counts were determined from digital photographs of images obtained on an inverted microscope (Nikon, Japan) with an ultraviolet lamp.

### Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

DNA fragmentations of blastocysts were analyzed using the TUNEL assay as described previously [22]. Blastocysts

were fixed in 3.7 % paraformaldehyde in PBS for 1 h at RT. After fixation, the embryos were washed three times in PBS containing polyvinylpyrolidone (PVP, 1 mg/ml; PBS/PVP) and permeabilized in 0.5 % (*v*/*v*) Triton X-100 for 1 h at RT. The embryos were then washed twice in PBS/PVP, and incubated with fluorescein-conjugated dUTP and the terminal deoxynucleotidyl transferase enzyme (in situ cell detection system; Roche, USA) in the dark for 1 h at 37°C. After counterstaining with 50 μg/ml PI for 1 h at RT, the embryos were washed extensively and mounted with slight coverslip compression in an anti-bleaching solution (Vector labs). Embryos were examined under a fluorescence microscope (Carl-Zeiss, Germany).

### Statistical analyses

Statistical analyses were conducted using one-way analysis of variance (ANOVA) to compare total cell numbers, ICM and TE cell numbers, and apoptotic index of the four groups. Data were analyzed using SPSS, and a P value less than 0.05 was considered statistically significant.

## Results

The embryonic development rates to the blastocyst stage were not affected by either oxygen tension or IGF treatment (Table 1). The hatching rates were significantly increased in blastocysts cultured under 5 % $O_2$ (64.7 %) compared to those cultured under 20 % $O_2$ (40.3 %) ($P< 0.05$). However, the addition of IGF-I to the culture medium did not significantly influence hatching rates in either group (Table 1).

The presence of HIF-1α protein was determined by immunofluorescence staining. To validate the HIF-1α antibody used in this study, a HeLa cell line was used as a positive control after treatment with cobalt chloride, a well-known chemical inducer of HIF-1α. HIF-1α was readily detected in HeLa cells, and was localized to the nucleus (Fig. 1e). In contrast, HIF-1α protein was predominantly localized to the cytoplasm of blastocysts, and its levels were independent of oxygen concentration or IGF-I treatment (Fig. 1 h, k, n, q).

Blastocysts cultured under 5 % $O_2$ showed significantly higher total cell numbers (83.4±18.1) than those cultured under 20 % $O_2$ (67.4±15.6) ($P< 0.05$). IGF-I treatment tended to increase total cell number in both groups, but there was no significant difference (Table 2). On the other hand, blastocysts produced under 5 % $O_2$ were observed to have significantly more ICM cells and TE cells (24.4±7.4 and 59.0±13.2) than those produced under 20 % $O_2$ (18.4± 6.3 and 49.0±11.0) ($P< 0.05$). Moreover, IGF-I treatment of the culture medium significantly increased ICM cells cultured under 20 % $O_2$, but not those cultured under 5 % $O_2$ (Table 2). The ratio of TE per ICM was not influenced by oxygen tension or IGF-I treatment (Table 2).

The proportion of nuclei showing features of apoptosis was significantly lower in the 5 % $O_2$ group (3.7±1.5) than in the 20 % $O_2$ group (6.9±3.5) ($P< 0.05$). IGF-I reduced the apoptotic index under both oxygen conditions, but a significant decrease was detected in the 20 % $O_2$ group ($P< 0.05$) (Table 2 and Fig. 2).

## Discussion

Oxygen tension has been known to influence embryo development and cell numbers in a number of species, and improvements in embryo development have been observed under lower oxygen tensions (5 % or 7 %) rather than under an atmospheric oxygen condition (20 %) [23–26]. Several reports suggested that mouse and bovine embryos have the capacity to detect and respond to low oxygen environment with changes in the expression of oxygen-regulated genes such as glucose transporter-1 (GLUT-1), GLUT-3, and vascular endothelial growth factor (VEGF) [7–9, 27].

In somatic cells, oxygen levels have a significant influence on gene expression patterns that are mediated by the heterodimeric transcription factor HIF-1 consisting of two subunits α and β [13, 28]. The α-subunit is hydroxylated at conserved prolyl and asparaginyl residues and targeted for degradation by the von Hippel-Lindau (VHL) ubiquitin E3 ligase complex under normal oxygen conditions. In response to low levels of oxygen, HIF-1α becomes activated and dimerizes with a constitutively expressed HIF-1β, and

**Table 1** Blastocyst formation and hatching rates in mouse embryos cultured under 5 % or 20 % $O_2$ in the absence or presence of IGF-I

| Oxygen tension | IGF-I concentration (ng/ml) | No. of zygotes examined | % day4 embryos developed to | | | |
|---|---|---|---|---|---|---|
| | | | Total blastocyst | > Hatching blastocyst | Expanding blastocyst | Degenerated embryo |
| 5 % | 0 | 307 | 95.5±3.4 | 64.7±17.6[a] | 30.8±14.8 [a] | 4.5±3.4 |
| 5 % | 100 | 304 | 92.9±6.3 | 66.2±18.0[a] | 26.7±14.8 [a] | 7.1±6.3 |
| 20 % | 0 | 304 | 91.4±6.4 | 40.3±17.7[b] | 51.1±14.2 [b] | 8.6±6.4 |
| 20 % | 100 | 293 | 92.4±4.0 | 44.1±12.1[b] | 48.4±9.4 [b] | 7.6±4.0 |

[a, b] indicate significant differences in the same column ($P<0.05$ in ANOVA)

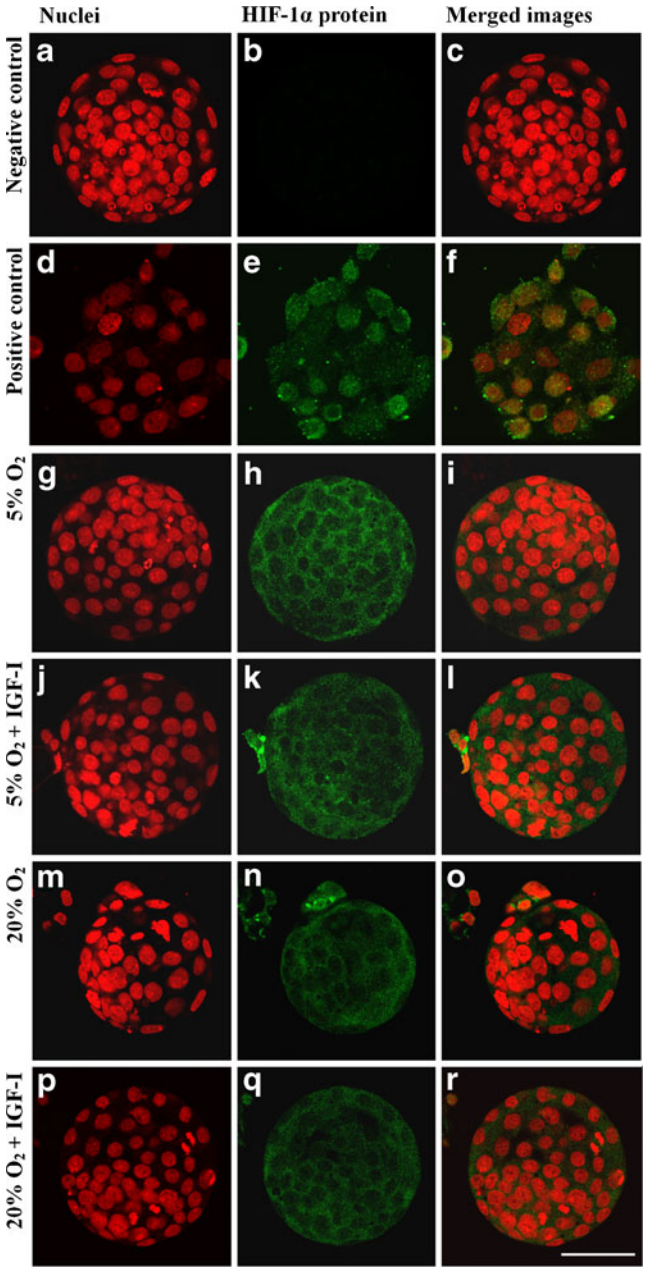


**Fig. 1** Immunofluorescence analysis of HIF-1α protein expression with PI staining of nuclei (**a**, **d**, **g**, **j**, **m**, **p**), FITC-labeled HIF-1α protein (**b**, **e**, **h**, **k**, **n**, **q**), and merged images (**c**, **f**, **i**, **l**, **o**, **r**). Negative control staining by omission of the primary antibody in the mouse blastocyst (**a-c**) and positive control staining in the HeLa cell (**d-f**). Localization of HIF-1α protein expression in mouse blastocysts culture under 5 % $O_2$ (**g-i**), 5 % $O_2$ and IGF-I (**j-l**), 20 % $O_2$ (**m-o**), and 20 % $O_2$ and IGF-I (**p-r**). Scale bar=50 μm

the complex binds to cis-acting hypoxia response elements in the promoter of target genes, which eventually regulates many cellular activities involved in oxygen homeostasis [3, 4, 11, 29–31]. Consistent with this, Harvey [7, 32] suggested that HIFs may be involved in molecular mechanisms which respond to changes in oxygen status during embryo development by expressing genes of HIF-1α, -2α, and -1β subunits in mouse and bovine blastocysts. Therefore, this study was conducted in order to determine whether the 5 % oxygen concentration has an influence on embryonic development through stimulation of HIF-1α expression. However, this study did not determine its stage-specific expression because embryo culture in most IVF clinics has been commonly performed under a chronic hypoxic condition. Therefore, the expression of HIF-1α in mouse blastocysts under a chronic hypoxic condition would be more informative for practical purposes in many IVF clinics than an acute hypoxic condition.

In the present study, HIF-1α protein detected by immunofluorescence staining was localized only in the cytoplasm of blastocysts, and was not affected by oxygen tension, although the hatching rates and cell numbers were significantly increased in blastocysts cultured under 5 % oxygen compared to those cultured under 20 % oxygen (Table 1 and 2). Furthermore, expressions of HIF-1α protein did not differ between two groups (Fig. 1), and even in more reduced oxygen tension (2 %) (data not shown). In order for HIF-1α to act as a transcription factor, it should be observed in the nucleus. HIF-1α is translocated into the nucleus and dimerized with HIF-1β, and then the HIF-1 complexes bind to DNA regulatory sequences of HIF-1 target genes [29]. However, in this study HIF-1α protein was determined to be localized only in the cytoplasm, which is consistent with the previous study conducted by Thompson et al. [33] that HIF-1α was detected in the cytoplasm of mouse blastocysts when cultured in low (2 %) oxygen concentration during compaction and blastulation. But, an acute exposure (2 h) to 2 % oxygen concentration resulted in increased nuclear localization of HIF-1α. Collectively, these suggest that HIF-1α may not be a major modulator for oxygen regulation in response to chronic hypoxia, but might be a major mediator in response to acute hypoxia. Moreover, HIF-1α protein is usually undetectable in somatic cells because the von Hippel-Lindau tumor suppressor protein recognizes and degrades it under normoxic conditions [34], whereas it was detected in mouse blastocyst culture under 20 % oxygen in this study. In addition, Harvey et al. [7] detected HIF-1α protein in the cytoplasm of bovine cumulus cells matured for 24 h under 20 % oxygen. These results indicated that HIF-1α protein can exist in the mouse blastocyst and bovine cumulus cells regardless of oxygen tension. They also describe a cell type-specific difference in the expression and function of HIF-1α protein.

Harvey et al. [7] reported that HIF-1α protein was undetectable in bovine blastocysts following culture under 2 %, 7 %, and 20 % oxygen, whereas nuclear localization of HIF-2α protein was detected with increasing intensity following post-compaction culture under 2 % oxygen. Therefore, they concluded that HIF-2α is a possible mediator of responses of the bovine pre-implantation embryo to environmental changes

**Table 2** Number of cells and apoptotic index of day 4 blastocysts cultured under 5 % or 20 % $O_2$ in the absence or presence of IGF-I

| Oxygen tension | IGF-I concentration (ng/ml) | Cell number ($n$=50) | | | | Apoptotic index ($n$=50) |
|---|---|---|---|---|---|---|
| | | Total cells | ICM cells | TE cells | ICM:TE ratio | |
| 5 % | 0 | 83.4±18.1[a] | 24.4±7.4[a] | 59.0±13.2[a] | 1 : 2.42 | 3.7±1.5[a] |
| 5 % | 100 | 88.6±23.6[a] | 27.1±9.9[a] | 61.5±15.9[a] | 1 : 2.27 | 3.2±1.2[a] |
| 20 % | 0 | 67.4±15.6[b] | 18.4±6.3[b] | 49.0±11.0[b] | 1 : 2.66 | 6.9±3.5[b] |
| 20 % | 100 | 72.8±14.5[ab] | 22.5±6.5[a] | 50.4±10.1[b] | 1 : 2.24 | 4.1±2.3[a] |

[a, b]indicate significant differences in the same column ($P$<0.05 in ANOVA)

in oxygen concentration, and there was a species-specific difference in the expression of HIF-1α protein. Wenger [34] reported that in vitro functional similarities between HIF-1α and HIF-2α exist in genomic organization, modular protein structure, hypoxic protein stabilization, heterodimerization, DNA binding, and the transactivation function of reporter genes. Furthermore, Pringle et al. [4] reviewed that HIF-2α protein levels are increased in a number of endothelial cell lines under mild hypoxia (5 % oxygen tension) and continue to increase with time, thereby playing an important role in mediating effects on gene expression under conditions of prolonged exposure to low oxygen. Therefore, additional studies should be conducted to clarify HIF-2α protein expression as an oxygen mediator in response to chronic hypoxia.

Although HIF-1α protein levels were independent of oxygen concentration in mouse blastocyst cultured in vitro, oxygen tension in uterine environment, especially during early mammalian fetal development, should be crucial for placental development accelerated with trophoblast growth, invasion, differentiation, and stimulation of maternal and fetal blood vessel growth [35]. During this period, HIF may be a primary sensor used by trophoblasts and the developing embryo to respond to low oxygen tension. In those regards, Cowden Dahl et al. [36] reported that *Hif-1α*$^{-/-}$ *Hif-2α*$^{-/-}$ mice displayed a 17 % reduction in trophoblast invasion compared with wild type placenta and that several pro- and anti-invasive factors expressed by either the trophoblasts or the maternal decidua were HIF target genes. Moreover, Fryer and Simon [35] found that both Arnt$^{-/-}$ and *Hif-1α*$^{-/-}$ *Hif-2α*$^{-/-}$ mouse embryos were not able to survive past day E10.5, due to severe placental defects including shallow placental invasion of the decidua and altered formation of trophoblast tissues. These studies suggest that HIFs appear to act as key mediators in regulation of placental differentiation, growth and function during early pregnancy and furthermore of maintenance of normal pregnancy.

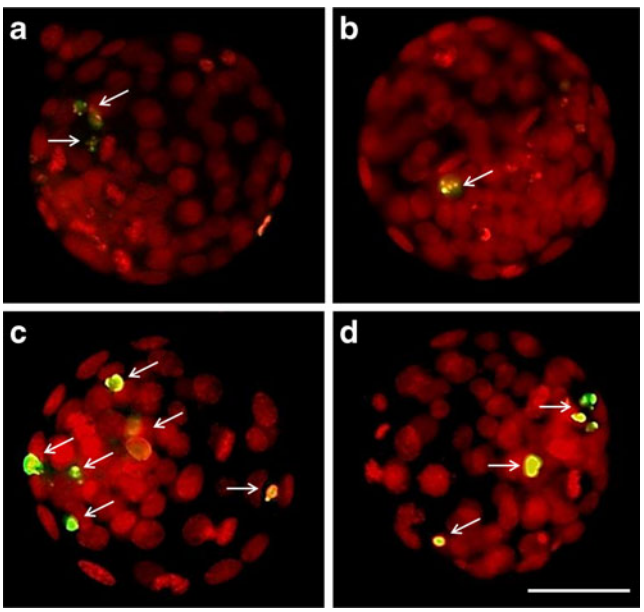


**Fig. 2** Detection of apoptotic nuclei in the mouse blastocysts cultured under (**a**) 5 % $O_2$, (**b**) 5 % $O_2$ and IGF-I, (**c**) 20 % $O_2$, and (**d**) 20 % $O_2$ and IGF-I. Scale bar=50 μm

IGF-I treatment induces HIF-1α protein expression, HIF-1 DNA binding activity, and transactivation of target genes in various cell lines under normoxic conditions. Fukuda et al. [13] suggested that IGF-I treatment increased the expression of HIF-1α in HCT116 human colon carcinoma cells, and Slomiany and Rosenzweig [37] demonstrated that IGF-I stimulates HIF-1α expression in human retinal pigment epithelial cell lines. Similarly, IGF-I has been reported to induce HIF-1α expression in human retinal epithelial cells [38] and MCF-7 breast carcinoma cell lines [3]. Therefore, to investigate the role of IGF-I in HIF-1α expression in mouse blastocysts, this study analyzed HIF-1α protein expression and localization following treatment with IGF-I.

In the present study, HIF-1α protein levels were found to be independent of IGF-I treatment in blastocysts, contrary to that of somatic cells (Fig. 1). A number of studies have demonstrated that IGF-I is a powerful inhibitor of apoptosis through activation of its receptor in many cell types [14, 17–19, 39, 40]. Indeed, Herrler et al. [16] suggested that IGF-I in preimplantation rabbit embryos was capable of reducing apoptosis and increasing cell proliferation. Also, several studies reported that IGF-I promotes pre-implantation embryo development and cell numbers in many species, including mouse [15], cow [18], and human [17]. The anti-apoptotic effects of IGF-I generally act through a phosphatidylinositol 3-kinase (PI3K)/Akt signal pathway which subsequently inactivates pro-apoptotic proteins such as Bad and

caspase-9 and stimulates anti-apoptotic proteins, including Bcl-2, a number of transcription factors and translational regulatory proteins [41, 42]. Therefore, IGF-I may promote embryonic development by an anti-apoptotic effect instead of increasing HIF-1α expression.

Embryos cultured in vitro under low (5 %) oxygen tension have been reported to show higher developmental rates than those cultured under 20 % oxygen in mice [43], cattle [44], and human [23]. These reports indicated that a high oxygen tension during in vitro culture was found to be toxic to mammalian embryos, probably due to the formation of reactive oxygen species (ROS) [45, 46]. ROS, such as hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^-$), can damage cell membranes and DNA and might play a role in apoptosis [47]. In this regard, Yang et al. [48] reported a direct relationship between increasing concentrations of $H_2O_2$ production and elevated numbers of fragmented embryos, suggesting that ROS may also induce apoptosis in human embryos. In the present study, the proportion of blastocysts that showed DNA fragmentation was significantly lower in the 5 % $O_2$ group (3.7±1.5) than in the 20 % $O_2$ group (6.9±3.5) ($P<0.05$), similar to results reported previously for mouse embryos [49]. Moreover, additions of IGF-I to the culture medium reduced the apoptotic index under both oxygen conditions, but a significant decrease was detected in the 20 % $O_2$ group (Table 2 and Fig. 2), although IGF-I did not significantly influence embryo development (Table 1). These results were consistent with a previous study [19], which suggested that the addition of IGF-I to the culture medium decreased the percentage of apoptotic cells in the presence of an apoptosis inductor, but that IGF-I addition had no significant influence on embryonic development.

In conclusion, HIF-1α may not be a major mediator that responds to changes in oxygen tension within blastocysts, which is inconsistent with previous reports for that of somatic cells. Thus, supplementation of culture medium with IGF-I failed to increase HIF-1α expression in mouse blastocysts. However, IGF-I plays an important role in increasing ICM and decreasing apoptosis during embryonic development at 20 % oxygen tension. Therefore, reduced oxygen tension along with IGF-I supplementation can increase the developmental potential of mouse blastocysts, and this may be attributed to the suppression of apoptosis, but not to an influence of HIF-1α expression.

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