Original Article

# Lipocalin2 enhances the matrix metalloproteinase-9 activity and invasion of extravillous trophoblasts under hypoxia

**Running title**: Lipocalin2 in early placenta

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**Abstract**

【Objectives】 The invasion of extravillous trophoblasts (EVTs) to the decidua and spiral arteries in early pregnancy is a crucial step for a successful pregnancy; however, its mechanisms are not fully understood. Lipocalin2 (LCN2), a multifunctional secretory protein known as neutrophil gelatinase-associated lipocalin (NGAL), reportedly enhanced invasiveness via the activation of matrix metalloproteinase-9 (MMP-9) in several cancer cells. In this study, the expression and function of LCN2 in early placenta were analyzed.

【Methods】 Early placental tissues between 7 and 10 weeks of gestation were obtained from normal pregnant women who underwent elective termination. The expression of LCN2 was examined using immunostaining and RT-PCR. EVTs isolated from these placental tissues and a choriocarcinoma cell line (JAR) were used to investigate the effects of LCN2 on proliferation, invasion potential, and MMP-9 activity under hypoxia using a WST-1 assay, Matrigel invasion assay, and gelatin gel zymography, respectively.

【Results】 The immunohistochemical expression of LCN2 was observed in the cytoplasm of EVTs, cytotrophoblasts and the decidua, but not in syncytiotrophoblasts. The addition of recombinant LCN2 did not affect proliferation, but enhanced the invasiveness (500ng/mL, $p<0.01$) and MMP-9 activity of primary cultured EVTs and JAR in a dose-dependent manner. Silencing LCN2 using shRNA reduced the invasiveness ($p<0.01$) and MMP-9 activity of JAR.

In addition, the hypoxic condition (2 % O2) increased LCN2 expression ($p<0.01$), MMP-9 activity, and invasive ability ($p<0.01$).

【Conclusions】 LCN2 was involved in the invasiveness of EVTs, especially under hypoxia, via increased MMP-9 activity.

**Keywords:**

Lipocalin2, matrix metalloproteinase-9, extravillous trophoblasts, early placentation, invasion, oxygen concentration

## Introduction

Normal placental development is characterized by a uterine spiral artery remodeling process through the invasion of extravillous trophoblasts (EVTs) to the uterine spiral arteries [1]. EVTs originate at the tips of the anchoring villi, from where they extend as cytotrophoblast (CT) cell columns and invade the uterine decidua and inner myometrium. EVT invasion to the uterine decidua and inner myometrium is thought to be a critical step for the establishment of a successful pregnancy [2]. Reduced EVT invasion and inadequate uterine spiral artery remodeling may be involved in the complications associated with pregnancy such as preeclampsia or fetal growth restriction (FGR) [3]. This pathological state often manifests as the shallow implantation of the placenta [2]. Therefore, the mechanisms of EVT invasion need to be elucidated.

The invasion of EVTs was shown to be regulated by several factors including matrix metalloproteinases (MMPs) [4]. MMPs belong to the neutral protease family, which degrade the extracellular matrix. The invasion process of EVTs requires the degradation and remodeling of the extracellular matrix, which is mainly exerted by MMPs, especially members of the gelatinase family such as MMP-2 and MMP-9 [5]. Therefore, the aberrant function of these proteins on EVTs results in the shallow invasion of these cells [6]. The ambient oxygen partial pressure ($PO_2$) is also thought to regulate EVT invasion [7]. The $PO_2$ in the uterine cavity is

relatively low in early pregnancy and gradually decreases towards the embryo from the decidua. However, the mechanism of the effect of O2 on EVT invasion has not been clarified.

Lipocalin2 (LCN2), known as neutrophil gelatinase-associated lipocalin (NGAL), is a 25-kDa secretory glycoprotein that belongs to the lipocalin family [8]. LCN2 was initially isolated from human neutrophils as a factor that forms complexes with MMP-9 through disulfide bonds [8]. Studies have revealed the various functions of LCN2 including a transport shuttle for small compounds such as retinol, arachidonic acids, and iron [9, 10] and a regulator of innate immunity to bacterial infection [11, 12]. In addition, the binding of LCN2 to MMP-9 was shown to protect MMP-9 from autodegradation, resulting in enhanced invasion [2]. The over-expression of LCN2 has been shown in several carcinoma cells [13, 14]. We also reported that LCN2 was up-regulated in endometrial carcinoma, and enhanced the invasion of tumor cells [15]. Regarding the expression of LCN2 in the placenta, only one previous study reported the expression of LCN2 in term placenta [16], while that in early placenta remains undetermined. The aim of this study was to investigate the expression and function of LCN2 in early placental tissues, particularly EVTs. In addition, we examined the effect of oxygen concentration on the expression and function of LCN2.

## Materials & Methods

***Sample collection***

Early placental tissues between 7 and 10 weeks of gestation were obtained from normal pregnant women who underwent elective surgical termination according to their request. Gestational ages were determined according to the last menstrual period with a correction by the detection of fetal heart movement or measurement of crown-rump length using transvaginal ultrasonography. These placental tissues were used for immunohistochemistry and isolation of EVTs. Each tissue sample was used with the approval of the Ethics Committee of Shinshu University, after obtaining written consent.

***Immunohistochemistry***

Indirect immunohistochemical staining for 15 cases of formalin-fixed early placental tissue was performed by the streptavidin-biotin-peroxidase complex method using a Histofine MAX-PO detector kit (Nichirei, Tokyo, Japan) as previously described [17]. The rat-monoclonal anti-human LCN2 antibody (clone number 220310, 25 μg/ml for working concentration; R&D Systems, Minneapolis, MN) was used as a primary antibody. Immunostaining against cytokeratin7 (CK7) (clone number OV-TL 12/30, 20 μg/ml; Life Technologies, Gaithersburg, MD) was used to refer CTs. Keratinocytes were used as a positive control of LCN2 immunostaining [18]. Staining with non-immune rat antibody (Rat IgG2a, kappa monoclonal

antibody, clone number RTK2758, 20 μg/ml; Cambridge, MA) was used as a negative control.

***Cell culture of human EVT and JAR***

EVT was isolated from early placental chorionic villi using a previously described method [19, 20]. In brief, placental chorionic villi were dissected into mince and digested in 0.25 % trypsin (Life Technologies). Supernatants were also collected and spread onto a 5 % step-layer Percoll gradient (GE Healthcare, Piscataway, NJ). The layer containing EVTs (35 %-45 % Percoll) was collected. More than 95 % cells were positive for the markers of EVT (CK7 and HLA-G). These isolated EVTs were seeded on a plate coated with growth factor-reduced Matrigel (Becton Dickinson, East Rutherford, NJ) and cultured in DMEM/F12 medium containing 10 % fetal bovine serum (FBS) and 1 % Antibiotic-Antimycotic (Life Technologies). The human choriocarcinoma cell line, JAR, used as the model for EVTs [21], was purchased from the American Type Culture Collection (Manassas, VA). JAR was maintained in RPMI1640 medium containing 10 % FBS. In several experiments, recombinant LCN2 (rLCN2) (Gene Tex, San Antonio, TX) was added to the medium. The plasma concentration of LCN2 in pregnant women was reported to be around 50 ng/ml [22]. The LCN2 concentration in microenvironment around the cells secreting LCN2 could be higher than serum. Therefore, we applied 50 to 500 ng/ml of rLCN2 concentration in our experiments corresponding to normal state of EVT. In

hypoxic experiments, these cells were cultured under various oxygen conditions (21 %, 5 % or 2 % $O_2$ with 5 % $CO_2$) for 24 hours.

***Immunofluorescence staining***

Immunofluorescent staining for LCN2 in cultured EVT or JAR cells was performed as described previously [23], using a rat-monoclonal anti-human LCN2 (working concentration; 25 μg/ml) and fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG antibody (Sigma-Aldrich, Saint Louis, MO). Nuclear counterstaining was performed using DAPI-Fluoromount-G (ready to use; CosmoBio, Tokyo, Japan). All specimens were observed using a BX60 fluorescence microscope (Olympus, Tokyo, Japan).

***Western blotting***

Proteins extracted from cultures of EVT or JAR cells were subjected to a Western blot analysis as described previously [15], using antibodies against human LCN2 (rat-monoclonal, 3 μg/ml for working concentration) or β-actin (mouse monoclonal, Clone number AC-15, 1 μg/ml; BioMakor, Rehovot, Israel) as primary antibody. The membranes were blotted with primary antibody at 4°C overnight and then incubated with a peroxidase-conjugated secondary antibody. Bound antibodies were visualized

using the ECL Western blot detection reagent (Amersham, Piscataway, NJ).

***Reverse transcriptase polymerase chain reaction (RT-PCR) and real-time quantitative PCR***

Total RNA was extracted by the TRIzol reagent (Life Technologies) according to the manufacturer's instructions, and reverse-transcribed to cDNA for PCR using the PrimeScript® RT-PCR Kit (Takara Bio, Shiga, Japan). Sequences of the specific primer sets and PCR conditions were listed in Supplementary Table 1 [15, 24]. Real-time quantitative PCR was performed using LightCycler® 480 DNA SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) in LightCycler® 480 system II (Roche Diagnostics GmbH) according to the manufacturer's instructions. The amplified efficiency of LCN2 primers used for real-time RT-PCR was 1.970. The expression of LCN2 mRNA was quantitated using β-2 microglobulin as an internal control gene. Each experiment of real-time RT-PCR was independently repeated 7 times with 3 replicates.

***Establishment of LCN2-silenced JAR***

LCN2-silenced JAR (JAR-sh) was established by transfection of the pGFP-V-RS vector (OriGene Technologies, Rockville, MD) stably producing LCN2-specific short-hairpin RNA (shRNA). Control-JAR (JAR-cont) was also established by transfection of the same vector

109 producing non-effective scrambled shRNA. Semi-quantitative RT-PCR showed the reduced
110 expression of LCN2 in JAR-sh (Figure 3c). In addition, real-time RT-PCR revealed that the
111 expression of LCN2 in JAR-sh was 93.6 % lower than that in JAR-cont. These cells were used
112 for the subsequent assays.
113
114 ***WST-1 assay***
115 Cell proliferation was assessed using the WST-1 reagent (Roche Diagnostics GmbH) according
116 to the manufacturer's instructions. On the first day, $2 \times 10^4$ (EVT) or $5 \times 10^3$ (JAR)
117 cells/well were plated onto 96-well plates. From the next day, cells were cultured under each
118 condition for 24 hours. The WST-1 reagent was then added to the medium. After 2.5 hours,
119 A450 wavelength light was measured using the microplate reader, Multiskan JX (Thermo
120 Bioanalysis, Tokyo, Japan). Each result was obtained from 6 independent experiments with 8
121 replicates.
122
123 ***Matrigel invasion assay***
124 Cell invasive ability was analyzed using the Matrigel invasion assay (BD Biosciences, Bedford,
125 MA) according to the manufacturer's instructions. After seeding cells on the matrigel and
126 adding human rLCN2, these cells were cultured for 24 hours. The number of migratory cells to

the lower surface was counted in five fields arbitrarily selected by a microscope with 200x magnification. Each result was obtained from 3 independent experiments with 5 replicates.

***Gelatin gel zymography***

To determine the activity of secreted MMP-9, gelatin gel zymographic analysis was performed according to the manufacturer's instructions. Samples of 50 μg total protein extracted from the cells cultured under each condition for 24 hours were electrophoresed on Novex® Zymogram gels (Life Technologies). The specific bands detected by staining with Coomassie Brilliant Blue (Life Technologies) were analyzed by densitometry using CS Analyzer 3.0 (ATTO, Tokyo, Japan). Each experiment was independently repeated 3 times.

***Statistical analysis***

Statistical analysis was conducted with the Scheffe's test or Mann-Whitney U test.

## Results

***Immunohistochemical expression of LCN2 protein in early placental tissues***

Immunohistochemical staining for LCN2 in early placental tissues demonstrated the strong expression of LCN2 protein in the cytoplasm of EVTs, CTs and the decidua, but not in

145 syncytiotrophoblasts (STs) (Figures 1a - d). The EVT cell column was also strongly stained
146 (Figures 1a - d).
147
148 ***The expression of LCN2 mRNA and protein in EVT and JAR***
149 The expression of LCN2 mRNA and protein was observed in primary cultured EVTs and JAR
150 cells using RT-PCR, western blotting, and immunocytochemistry. The specific band at 298 base
151 pairs for LCN2 was indicated in EVTs and JAR (Figures 2a, b). In addition, LCN2 protein
152 expression was also observed in EVTs and JAR (Figure 2c). Immunocytochemistry (Figures 2d,
153 e) revealed the expression of the LCN2 protein in cytoplasm in EVTs and JAR.
154
155 ***The effect of LCN2 on the cell proliferation of EVTs and JAR***
156 We then examined the effect of LCN2 on the proliferation of EVTs and JAR according to a
157 previous study [15]. The WST-1 assay revealed that the addition of human rLCN2 up to
158 500ng/ml had no significant effect on the proliferation of EVT and JAR cells (Figures 3a, b).
159 No significant difference in proliferation was observed between LCN2-silenced JAR (JAR-sh)
160 and JAR-cont (Figure 3d).
161
162 ***The effect of LCN2 on the cell invasion of EVTs and JAR***

The effect of LCN2 on the cell invasive ability of EVTs and JAR was investigated using the Matrigel invasion assay. The addition of human rLCN2 significantly up-regulated the invasion indexes of both cells in a dose-dependent manner ($p<0.01$ in both cells, Figures 4a, b). The invasive ability of JAR-sh was significantly decreased from that of JAR-cont, and this reduction was partially recovered by the addition of rLCN2 (Figure 4c).

***The effect of LCN2 on the enzymatic activity of MMP-9***

Because LCN2 was shown to stabilize MMP-9 [25] via binding to MMP-9, we then analyzed the effect of LCN2 on MMP-9 activity in EVTs and JAR. Gelatin gel zymography revealed that the rLCN2 treatment increased the enzymatic activity of MMP-9 in both cells, in a dose dependent manner (Figure 4d). Moreover, the MMP-9 activity of JAR-sh was lower than that of JAR-cont (Figure 4d), which indicated that MMP-9 activity was increased by LCN2.

***The effect of the hypoxic condition on the cell proliferation of EVTs and JAR***

The effect of oxygen concentration on the cell proliferation in EVT, JAR, JAR-cont and JAR-sh was examined. The WST-1 assay revealed that a lower oxygen concentration increased cell proliferation in all cells (Supplementary Figure 1).

***The effect of the hypoxic condition on the expression and function of LCN2 in EVTs and JAR***

The effect of oxygen concentration on the expression and function of LCN2 in EVTs and JAR was examined. Real-time RT-PCR revealed that a lower oxygen concentration induced the elevated expression of LCN2 in both cells (Figures 5a, b). Gelatine gel zymography also demonstrated that MMP-9 activity was inversely correlated with oxygen concentrations in both cells (Figures 5c, d). Furthermore, the Matrigel invasion assay revealed that the hypoxic condition significantly enhanced the invasive ability of EVTs (Figure 5e) and JAR (Figure 5f). The invasive ability of JAR-sh was not increased under the hypoxic condition, but was increased by the addition of rLCN2 (Figure 5f).

## Discussion

The present study demonstrated the expression of LCN2 in early placental tissues, especially in CTs and EVTs, but not in STs. Regarding the topological distribution of LCN2-positive cells in the placenta, Tadesse et al. reported that the immunohistochemical expression of LCN2 in term placenta was observed in CTs, EVTs, but not in STs or the decidua [16]. The results obtained in the present study were largely consistent with their report. Interestingly, we found the strong expression of LCN2 in EVTs, especially those composing the cell column of the anchoring villi,

where EVTs vigorously invade the decidua to establish the early step of the feto-maternal interface [1]. The expression of LCN2 in term placenta was reported to be increased in women with chorioamnionitis [16]. In addition, inflammatory cytokines such as IL-1b and TNF $\alpha$ induced the expression of LCN2 in term CTs in vitro, which suggests that the expression of LCN2 is controlled by local cytokines [16]. In early placenta, the elevated expression of IL-1, as well as TNF$\alpha$ was demonstrated in the trophoblasts [26-28]. Trophoblastic IL-1 induced the expression of endometrial prostaglandins and integrins [26]. TNF$\alpha$ in the trophoblasts is considered to regulate invasiveness through the degradation of ECM [27, 28]. These local cytokines involved in the function of EVT may enhance the expression of LCN2 in EVTs of early placenta.

Several studies have shown pregnancy-related change in the plasma levels of LCN2; however, its source and significance were not fully elucidated. Cesur et al. showed that plasma levels of LCN2 were higher than that of control non-pregnant women [22], and that plasma levels of LCN2 were higher in obese pregnant women and those with insulin resistance, which suggests that LCN2 is produced from adipose tissue. D'Anna et al. demonstrated that plasma LCN2 levels were elevated especially with preeclampsia [29, 30], and suggested that increased LCN2 may be linked to the macrophage-related systemic inflammatory response. In contrast, Cemgil et al. reported that LCN2 levels were decreased in preeclamptic women [31]. Tadesse et

al. suggested that the increased expression of LCN2 in term trophoblasts may stimulate parturition because of the pro-inflammatory activity of LCN2 and subsequent onset of labor [16]. Our present data suggested that the EVT, CT and decidua was one of the source of elevated plasma LCN2 in pregnant women.

The most important role of EVT was invasion to decidua and inner myometrium [2]. In this regard, our in vitro study using EVTs isolated from placental tissue and JAR clearly demonstrated that LCN2 was directly involved in the invasive ability of those cells, in association with the elevated enzymatic activity of MMP-9. MMP-9 is well known to play a critical role in cell invasion [32]. Yan et al. demonstrated using Western blotting and Gelatin gel zymography that LCN2 binding to urinary MMP-9 enhanced its activity by protecting it from autodegradation [25]. LCN2 enhanced the invasive ability and increased MMP-9 activity in tumor cells such as cholangiocarcinoma [33], esophageal carcinoma [34], and breast carcinoma cells [35]; however, this is the first report of LCN2 contributing invasiveness via MMP-9 in trophoblastic cells. On the other hand, our study indicated that the reduction of invasive ability by LCN2 down-regulation (JAR-sh) (Figure 4c) was much larger compared from that of MMP-9 activity (Figure 4d). These finding suggested that other factors in addition to MMP-9 might be involved in LCN2-induced EVT invasion. Yang et al. reported that LCN2 enhanced invasiveness of breast cancer cells through inducing epithelial mesenchymal transition [36].

235 Oxygen density is considered to be the most important factor in the process of EVT
236 invasion [7, 37]. Interestingly, the present study revealed that the hypoxic condition (2 % $O_2$
237 concentration) increased the expression of LCN2, MMP-9 activity, and invasive ability in EVTs
238 and JAR. The hypoxic condition also increased cell proliferation. These hypoxia-induced
239 proliferation and invasion seemed to be important roles of EVT for placental development. Once the
240 EVT could get enough oxygen, it might reduce invasion and proliferation. Luo et al.
241 demonstrated that the hypoxic condition induced by the addition of cobalt chloride enhanced the
242 expression of MMP-9 mRNA and invasion ability of TEV-1, the human EVT cell line [38].
243 Hypoxia was shown to induce the over-expression of LCN2 in mouse renal tubular cells [39].
244 Jiang et al. reported that an intravenous injection of cobalt chloride, a chemical inducer of
245 HIF-1α stabilization that mimics a low oxygen state, markedly up-regulated the transcription of
246 LCN2 in the livers of mice [40]. Our study revealed that the down-regulation of LCN2 using
247 LCN2 shRNA significantly decreased the hypoxia-induced invasion of JAR cells, whereas
248 rLCN2 addition restored JAR invasion. These findings strongly suggested that LCN2 was a
249 crucial factor controlling hypoxia-induced invasion of EVTs and JAR.

250 In conclusion, we identified the expression of LCN2 in early placental tissues,
251 especially in CTs, EVTs and the decidual cells. In vitro experiments revealed that LCN2
252 enhanced EVT invasion by increasing MMP-9 activity, and that LCN2 was involved in

hypoxia-induced EVT invasion. These findings suggest that LCN2 plays an important role in controlling EVT invasion in early placentation. Furthermore, a dysfunction of LCN2 might be involved in the pathogenesis of shallow implantation of the placenta.

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**Conflict of Interest Statement**

The authors declare that there are no conflicts of interest.

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**Figure legends**

**Figure 1**

Immunostaining in the serial sections of early placental tissue. **a;** Hematoxylin and Eosin staining**. b**; EVTs (**) and CTs(arrows) were visualized by immunostaining for CK7. Decidual cells were indicating CK7-negative (*). **c**, **d**; Immunostaining for LCN2. The strong expression of LCN2 was observed in decidual cells (*), EVTs, especially at cell column (**), and CTs (arrows), but not in STs (arrowheads). **e**; Keratinocytes in the mature cystic teratoma of the ovary were used as a positive control in immunostaining for LCN2. **f**; Staining for early placental tissues with non-immune rat antibody was used as a negative control.

**Figure 2**

RT-PCR, Western blotting and immunofluorescence staining for LCN2 in EVTs and JAR. The specific band of LCN2 mRNA and protein was observed in EVTs (**a, c**) and JAR (**b, c**). The green fluorescence of the LCN2 protein was observed in EVTs (**d**) and JAR (**e**). The blue color indicates nuclear counterstaining by DAPI.

**Figure 3**

**a, b**; The cell proliferation assay (WST-1 assay) with the addition of rLCN2. The addition of

rLCN2 had no additional effect on cell proliferation in EVTs (**a**) and JAR (**b**). **c**; RT-PCR for LCN2 in JAR-cont (transfection of the vector producing scrambled shRNA) and JAR-sh (transfection the vector producing LCN2 shRNA). Density of the specific band for LCN2 was low in JAR-sh. d; The cell proliferation assay (WST-1 assay) in JAR-cont and JAR-sh. The reduced expression of LCN2 (JAR-sh) had no additional effect on cell proliferation relative to that with JAR-cont.

The error bars indicated standard deviation. n.s.: no significant difference.

**Figure 4**

Effects of LCN2 on the invasion and MMP-9 activity of EVTs and JAR. **a**: The Matrigel invasion assay for EVT. The addition of rLCN2 significantly increased the number of invading cell in a dose- dependent manner. **b**: The Matrigel invasion assay for JAR. The addition of rLCN2 significantly increased the number of invading cells, dose-dependently. **c**: The Matrigel invasion assay for JAR-cont and JAR-sh. The reduced expression of LCN2 (JAR-sh) significantly decreased the number of invading cells. However, the addition of rLCN2 could restore that invasive ability. **d**; Gelatin zymography for EVTs, JAR, JAR-cont, and JAR-sh. These photographs indicate the specific band of the MMP-9 active form at 84 kDa. The numeric values under each photograph were the ratios of band densities calculated by a densitometer.

The addition of rLCN2 increased the enzymatic activity of MMP-9, and the reduced expression of LCN2 (JAR-sh) decreased its activity.

rLCN2; recombinant LCN2, *: P<0.05, **: P<0.01 by Scheffe's test. The error bars indicated the standard deviation.

**Figure 5**

Effects of oxygen concentration on the expression and function of LCN2 in EVTs and JAR. **a, b**; Real-time RT-PCR for LCN2 mRNA. A low oxygen concentration increased the expression of LCN2 mRNA in EVTs (**a**) and JAR-cont (**b**). **c, d**; Gelatin zymography. These photographs indicate the specific band of the MMP-9 active form at 84 kDa. The numeric values under each photograph were the ratios of band densities calculated by a densitometer. A low oxygen concentration also increased the enzymatic activity of MMP-9 in EVTs (**c**) and JAR-cont (**d**). **e, f**; The Matrigel invasion assay under various oxygen concentrations. A low oxygen concentration significantly increased the number of invading cells in EVTs (**e**) and JAR-cont (**f**). However, the reduced expression of LCN2 (JAR-sh) significantly decreased the number of invading cells regardless of the oxygen concentration (**f**) (P<0.01). This reduction in invading cells was restored by the addition of rLCN2 (**f**).

rLCN2; recombinant LCN2, O2; O2 concentration, *: P<0.05, **: P<0.01 by Scheffe's test. The

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**Supplementary Figure 1**

Effect of different oxygen concentrations on cell viability and proliferation. The graphs of EVT (**a**), JAR (**b**), JAR-cont (**c**) and JAR-sh (**d**) indicated relative cell viabilities under various oxygen concentrations. Low oxygen concentration increased cell viability of these cells.

*; significantly different from 21% $O_2$ ($P<0.05$), **; significantly different from 5% $O_2$ ($P<0.05$)

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