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The Protective Effects of FGF-4 on Hypoxia-mediated Apoptosis of Trophoblast Stem Cells

Hansol Lee[1], Hyewon Kim[2], Jaymin Oh

Department of Anatomy, School of Medicine, Wonkwang University, Iksan Korea
[1]*Department of Orthopaedic Surgery, School of Medicine, Wonkwang University, Iksan Korea*
[2]*Department of Diagnostic Radiology, School of Medicine, Wonkwang University, Iksan Korea*

Preeclampsia and fetal growth restriction are conditions associated with placental hypoperfusion and villous hypoxia. The villous response to this environment includes elevated apoptosis. Recently, trophoblast stem (TS) cells had been successfully derived. FGF-4 locates in the inner cell mass (ICM) of blastocyst and TS cells have fibroblast growth factor receptor-2 (FGFR-2). To identify whether FGF-4 protects hypoxia-induced apoptosis in TS cells, this study was carried out. TS cells were cultured up to 48 h in standard (PO_2 = 20%) or hypoxic (PO_2 = 3%) conditions. TS cells were very vulnerable against exposure to hypoxia for 48 h but embryonic stem (ES) cells were very resistant to hypoxia-mediated apoptosis. Death of TS cells bears the typical hallmarks of apoptosis as determined by DNA laddering. FGF-4 and epidermal growth factor (EGF) protected the hypoxia-mediated cell death of trophoblast but granulocyte-macrophage colony stimulating factor (GMSF) and transforming growth factor-beta (TGF-beta) did not protect. In conclusion, we speculate that the effects of FGF-4 on apoptosis in trophoblasts may play an important role in protecting the placenta from hypoxic injury in pregnancy related with placental hypoperfusion.

Key words : FGF-4, Trophoblast stem cells, Hypoxia, Apoptosis

Introduction

Clinical conditions such as preeclampsia, anemia, smoking, and living in a high altitude can lead to placental underperfusion and villous hypoxia, characterized by diminished mainly undifferentiated trophoblasts (Benirschke et al. 1995). Apoptosis is a process of normal development and differentiation in many tissues. This type of cell death may be enhanced by deleterious stimuli such as hypoxia (Muschel et al. 1995) and distorting the balance of cellular physiology including proliferation, differentiation, and death, thereby impairing placental function. Indeed, a higher degree of apoptosis is found in placenta of pregnancy complicated by fetal growth restriction (Smith et al. 1997). Similarly, apoptosis is more prevalent in trophoblasts from pregnancies complicated by preeclampsia, than those of which obtained from uncomplicated pregnancies (DiFederico et al. 1999). And preeclampsia and fetal growth restriction are associated with placental hypoperfusion and villous hypoxia. Hypoxia induces diminished trophoblast differentiation and enhanced apoptosis. The enhancement of apoptosis is associated with an increased expression of proapototic proteins p53 and Bax and with decreased expression of antiapoptotic protein Bcl-2 family. In the placenta, p53 and Bax are primarily expressed in undifferentiated trophoblasts and in trophoblasts of gestational tro-

* This study was supported by a Wonkwang University grant 2003.
correspondence to : Dr. Jaymin Oh (Department of Anatomy, School of Medicine, Wonkwang University)
e-mail : jmoh@wonkwang.ac.kr

phoblastic disease, a neoplastic condition with prominent apoptosis (Qiao et al. 1998). In contrast, Bcl−2 is expressed at higher levels in the fully differentiated trophoblast (Sakuragi et al. 1994).

Two distinct cell lineages, trophectoderm (TE) and inner cell mass (ICM) are already present in embryo. The peripheral TE consists of a single layer of epithelioid cells that differentiate into trophoblasts and contribute to the formation of placenta. The ICM, in contrast, is an inner cluster of totipotent stem cells that continue to diversify into a variety of cell types to genernate the different fetal tissues and organs during organogenesis. Studies have shown that ICM cells and TE cells have many different cellular, biochemical and molecular characteristics, including their susceptibility to embryotoxic environments and agents (Pampfer et al. 2000). Although little is known about the nature of interactions between ICM and TE cells, several kinds of evidence suggest that close cellular contact is required between the two cell linages to ensure the proper development of implanting blastocyst (Pampfer et al. 2000). Studies of ICM−TE interaction indicate that potent regulatory signals such as fibroblast growth factors (FGFs), could be produced by ICM cells and participate in the regulation of TE growth by paracrine or juxtacrine mechanisms (Rappolee et al. 1994). FGF −4 is thought to be one of these major candidate signals because it has been shown to be preferentially produced in ICM cells of mouse blastocyst and help maintaining mouse TE cells in undifferentiated state and proliferative model. But the function of FGF−4 and the effect of differentiation of trophoblast on hypoxic induced damages are not still known very well.

In present study, the protective effects of FGF−4 were studied on trophoblast damaged by hypoxia stress.

Materials and Methods

1. Cell culture

A medium for TS cell culture (TS medium) and an embryonic fibroblast−conditioned medium (EMFI−CM) was prepared as previously described (Tanaka et al. 1998). At day 3.5 after mating, blastocysts were released from the ampulla into media by rupturing the oviduct with the aid of a 25−gauge needle and then TS cells were derived by culturing blastocysts on embryonic fibroblasts with medium consisting of 70% EMFI−CM, 30% TS cell medium, FGF−4 of 25 ng/ml (Sigma, St. Louis, MO, USA), and heparin of 1 μg/ml (Sigma). TS cells were plated in EMFI−CM with FGF −4 under 5% CO_2 −95% air at 37°C. Undifferentiated ES cells were kindly provided by Dr Jurisicova, Toronto Medical University, Toronto, Canada) and the cells were cultured with complete medium Dulbecco's modified Eagle's medium containing 10% fetal calf serum, leukemia inhibitory factor (LIF), and beta−mercaptoethanol. Non−adherent cells were removed after 24 hr by washing three times with medium. Then, 20 mM HEPES (Sigma, St. Louis, Missouri, USA) was added into EMFI−CM and adherent cells were transferred to hypoxic incubator. Hypoxic condition was made by hypoxic incubator (Sanyo, Osaka, Japan), define as 3% oxygen.

2. Trypan blue exclusion

Cells were incubated for 2∼5 min in a solution of 0.2% trypan blue in phosphate buffered saline. To assess cell viability, aliquots of cells were mixed with trypan blue (1 : 1) and loaded onto a hemocytometer, and the percentage of dead cells per sample was calculated. Values were calculated as the percentage of nonviable cells (stained cells) and results represent the mean ± S.E.

3. DAPI staining and FACS analysis

TS cells were washed with 1 × PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, and washed again 1 × PBS. Cells were treated with DAPI (1 μg/ml) (Sigma, St. Louis, Missouri, USA) for

15 min, and washed with 1 × PBS for 5 min. DAPI staining of cells was observed by fluorescence microscopy (Leica Co). Adherent and detached cells were combined and fixed overnight in 70% ethanol in wash buffer (PBS containing 5 mM EDTA) at 4˚C. After centrifugation at 3000 rpm for 1 min, cell pellets were incubated for 30 min with 500 µl wash buffer and 50 µl RNAse A (10 mg/ml). Cells were then stained in 500 µl PBS containing 100 µg/ml propidium iodide. Cells were filtered with 50 µm nylon mesh and analysed by flow cytometer (FACS-vantage: Becton Dickinson Biosciences, San Jose, CA, USA).

4. Agarose gel electrophoresis for DNA fragmentation

Cells (4×10^5) were lysed in 200 µl of lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.5% Triton X-100) followed by incubation with 40 µg of RNase A (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 1 h at 37˚C and 100 µg of proteinase K (Roche) for 1 h at 37˚C, and only fragmented DNA was extracted. The pellet was resuspended in TE buffer (10 mM Tris-HCl, pH 7.4), 1 mM EDTA) and treated with DNase-free RNase (Roche) for 1 h at 37 ˚C. DNA was ethanol-precipitated and finally resuspended in distilled water. The fragmented DNA was electrophoretically fractionated on 1.5% agarose gel and stained with ethidium bromide.

5. Electron microscopy

Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 hours, washed in the same buffer, and post-fixed for 4 hours at room temperature in 2% osmium tetroxide in distilled water to which a few drops of 2% aqueous potassium-ferrocyanate were added. The cells were embedded in 2% agar in 0.1 mol/L sodium cacodylate buffer. The tip of the agar blocks containing the cell pellet was cut off, dehydrated in a graded series of ethanol, and embedded in eponate resin (Ted Pella Inc, Redding, CA, USA). The resultant samples were directly dehydrated and embedded in resin without agar embedding. Thin sections were cut with a diamond knife on an LKB Nova ultramicrotome (LKB, Bromma, Sweden) and collected on parlodion-coated 200-mesh copper grids (Ted Pella, Inc). The sections were stained with uranyl

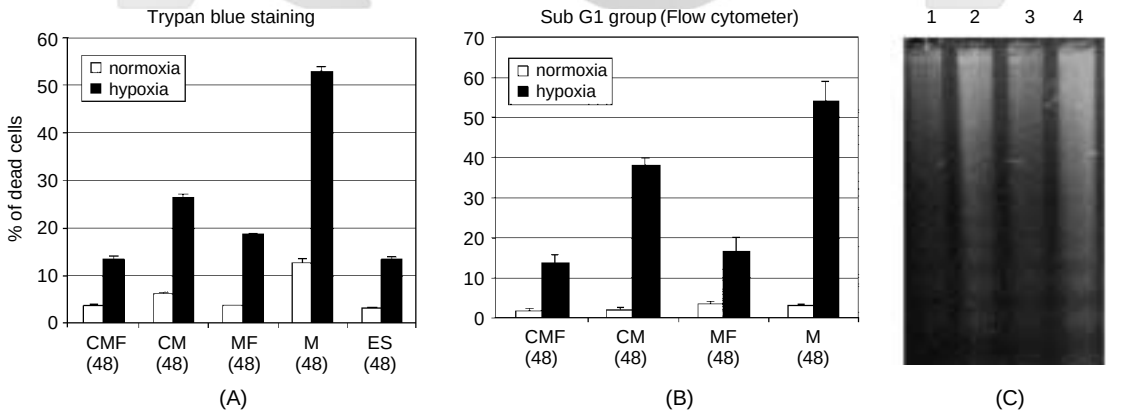

Fig. 1. Trophoblast stem (TS) cells and embryonic stem (ES) cells were cultured under hypoxia for 48 h with several conditions (CMF: conditioned media with FGF-4, CM: conditioned media alone, MF: media with FGF-4, M: Media alone). And then, the cells were assayed for trypan blue exclusion at 48 h after hypoxia (A). Total cell population was assayed for DNA content by flow cytometry at 48h after hypoxia (B). Cells were assayed for DNA ladder formation on 1% agarose gel electrophoresis (lane 1: CMF, lane 2: CM, lane 3: MF, lane 4: M) (C). Results represent the mean ± S.E.

acetate and lead citrate and examined with electron microscope (× 5000).

Results

1. The protective effects of FGF−4 on hypoxia−meidated apoptosis in TS cells

The effects of hypoxia−mediated cell damages on TS cells were determined. With the use of trypan blue exclusion method, we found that hypoxia for 48 hr caused a marked increase in damage of TS cells. FGF −4 protected hypoxia−mediated cell damage. Interestingly, ES cells were less susceptible to hypoxia compared to TS cell (Fig. 1A). Several growth factors such as FGF−4, EGF, GMSF, and TGF−beta were treated for checking the protective effects during hypoxic stress mediated−cell damages. FGF−4 and EGF of 25 ng/ml concentration effectively protected cell damages of TS cells but other growth factors of same concentration did not protect (Fig. 4).

To assess whether hypoxia induces apoptosis in TS cells, we monitored the appearance of PI stained cells with sub−G_1 DNA content and checked DNA laddering with 1.5% agarose gel electrophoresis. TS cells of media alone group was associated with 53% of cells with sub−G_1 DNA and FGF−4 suppressed sub−G_1 DNA to 17% (Fig. 1B) and FGF−4 prevented hypoxia mediated DNA laddering (Fig. 1C). In addition, FGF−4 suppressed hypoxia−induced DNA fragmentation and apoptotic body formation in TS cells (Figs. 2, 3). Therefore, these data suggest that FGF−4 protects hypoxia mediated−apoptosis on TS cells.

2. FGF−4 dose−dependently prevented DNA ladder fragmentation

The above result was confirmed by the DNA ladder assay (Fig. 5), increasing FGF−4 concentration from 2.5 ng/ml to 50 ng/ml produced a dose−dependent decrease in the laddering of DNA as compared with control group. These data demonstrated that FGF−4 protects hypoxia−mediated apoptosis in TS cells.

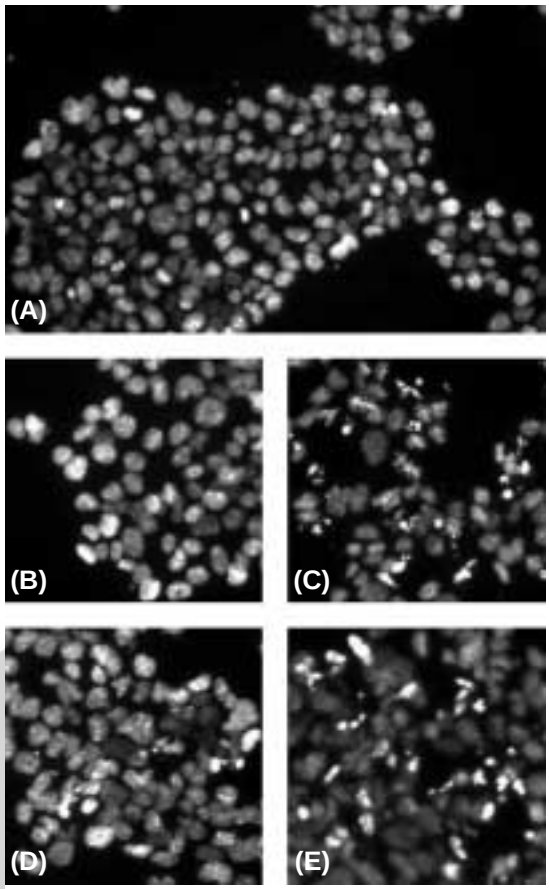

Fig. 2. TS cells were cultured with/without FGF−4 under normoxic or hypoxic condition for 48 h. Cells were washed with ice−cold PBS, followed by fixation in 2% paraformaldehyde/PBS for 30 min and stained with DAPI. a: CMF (normoxia, 48 hr), b: CM (hypoxia, 48 hr), c: CM (hypoxia, 48 hr), d: MF (hypoxia, 48 hr), e: M (hypoxia, 48 hr)

Discussion

This study demonstrates that hypoxia enhances the apoptosis of cultured TS cells. FGF−4 diminishes the

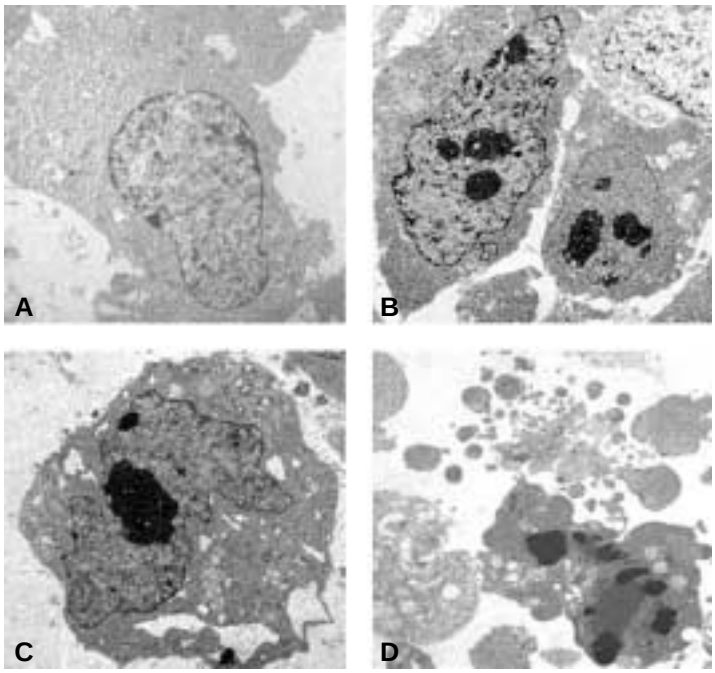

Fig. 3. TS cells were cultured in the presence or absence of FGF-4 under hypoxic condition for 48 hr. Cells were washed with ice-cold PBS and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, washed in the same buffer, and post-fixed for 4 hr at room temperature in 2% osmium tetroxide. And the sections were stained with uranyl acetate and lead citrate and examined with electron microscope (× 5000). a: CMF (normoxia, 48 hr), b: CM (normoxia, 48 hr), c: CMF (hypoxia, 48 hr), d: CM (hypoxia, 48 hr)

degree of apoptosis induced by hypoxia in cultured TS cells, so we speculate that the effect of FGF-4 on apoptosis in trophoblasts may play an important role in protecting the placenta from hypoxic injury in pregnancies complicated by placental hypoperfusion. Hypoxia triggers apoptosis in a number of cell systems (Muschel et al. 1995, Graber et al. 1996, Banasiak et al. 1998). The mechanism by which hypoxia induces apoptosis is postulated to involve mitochondrial pathways, as opposed to ligand-receptor stimuli mediated by cytokines, such as TNF-α or Fas ligand. In the former pathway, stimulation of death signals occurs through modulation of the expression of specific genes, such as p53 and members of the Bcl-2 family (Dragovich et al, 1998). The p53 protein plays a pivotal role in the cellular response to DNA damage, specifically

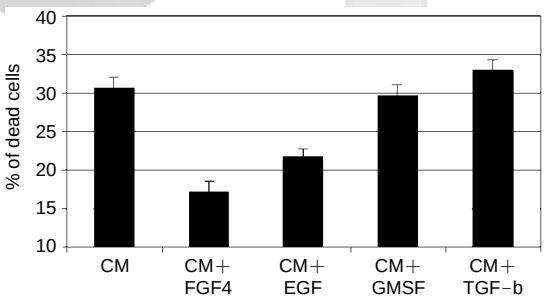

Fig. 4. Trophoblast stem (TS) cells were cultured under hypoxia for 48 h with/without FGF-4, EGF, GMSF, and TGF-beta of 25 ng/ml concentration. And then, the cells were assayed with trypan blue exclusion at 48 h after hypoxia. Results represent the mean ± S.E.

halting the cell cycle and allowing repair of DNA. If repair is not possible, p53 promotes apoptosis (Lane et

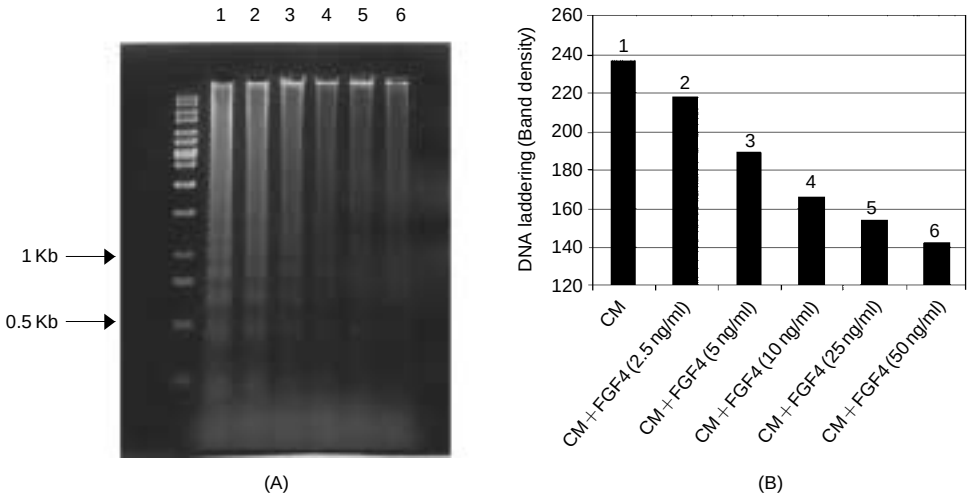

Fig. 5. TS cells were treated with various concentrations of FGF−4 and the cells were incubated in hypoxic incubator for 48 h. Then, DNA fragmentation assay was carried out. The formation of DNA ladder was examined in 1% agarose gel electrophoresis (A). And the degree of DNA laddering was displayed by graph through analysis of each bands of 500 bp using Gel−Pro analyzer, image analysis program (B). The following concentrations of FGF−4 were used: lane 1, control; lane 2, 2.5 ng/ml; lane 3, 5 ng/ml; lane 4, 10 ng/ml; lane 5, 25 ng/ml; lane 6, 50 ng/ml.

al. 1992). The p53 is an unstable protein with a short half−life, but exogenous stimuli such as hypoxia and oxidative stress stabilize the p53 protein (An et al. 1998). The stable p53 protein plays a role in hypoxia−induced cell death in neurons (Banasiak et al. 1998), cardiomyocytes (Long et al. 1997), and endothelial cells (Stempien et al. 1999). The Bcl−2 family of proteins plays a major role in the regulation of the apoptotic processes (Korsmeyer et al. 1999). Hetero− and homodimers of the proapoptotic Bax and the antiapoptotic Bcl−2 determine cell survival or death by affecting the permeability of the mitochondrial membrane (Oltvai et al. 1993). Bax homodimers likely play a major role in the apoptotic process in trophoblasts. Others have found that Bax is translocated from the cytosol to the mitochondria during hypoxia (Saikumar et al. 1998). Additional experiments in which p53 and Bax are overexpressed or inhibited are necessary to further clarify the role of these proteins in mediating hypoxia−induced apoptosis in trophoblasts. In this study, FGF−4 blocked dose dependently hypoxia−mediated DNA laddering, DNA fragmentation. In future study, we need more experiments to check that FGF−4 regulates the expression of apoptosis related−gene under hypoxia. Collectively, these data suggest an important role for FGF−4 in protecting the integrity of the placenta against endogenous and exogenous stress. Finally, we speculate that the effect of FGF−4 on apoptosis in trophoblasts may play an important role in protecting the placenta from hypoxic injury in pregnancies complicated by placental hypoperfusion.

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Trophoblastic stem (TS) cell에서 저산소증에 의해 유도된 apoptosis에 대한 FGF-4의 방어효과

이 한 솔[1], 김 혜 원[2], 오 재 민
원광대학교 의과대학 해부학교실,
[1]원광대학교 의과대학 정형외과학교실
[2]원광대학교 의과대학 진단방사선과학교실

간추림 : Preeclampsia와 fetal growth restriction은 태반이나 융모의 저산소증과 밀접한 관계가 있다. 저산소증은 태반이나 융모에서 apoptosis를 일으키게 되는데 과거에는 in vivo model을 이용하여 그 기전을 연구했었다. 그러나 최근에 trophoblast stem (TS) cell을 blastocyst에서 성공적으로 분리하여 in vitro model에서 apoptosis를 일으키는 기전을 연구할 수 있게 되었다.

FGF-4는 blastocyst의 inner cell mass에 존재하며 paracrine 방법으로 TS의 fibroblast growth factor receptor-2 (FGFR-2)에 작용한다. 그래서 저산소증에 의해 유발되는 세포독성 및 apoptosis를 조절하는데 FGF-4가 효과가 있는지를 조사하였다. 저산소증은 3% 산소농도를 유지하는 5% 이산화탄소 배양기에서 48시간동안 세포를 배양함으로써 유도하였다. TS cell은 저산소환경에서 매우 쉽게 손상을 받았으나 embryonic stem (ES) cell은 저산소환경에서 유도된 세포독성에 비교적 쉽게 손상받지 않았다. FGF-4를 비롯한 여러 growth factor들의 효과를 조사하였는데, FGF-4와 EGF는 TS cell이 저산소증에서 유도된 세포독성으로부터 TS cell을 보호하였으나 GMSF나 TGF-beta는 저산소증에 대하여 세포보호 효과가 없었다. 저산소증은 TS cell에서 DNA laddering을 증가시켰고, DNA의 subG1 phase에서의 축적을 유도하였다. FGF-4는 이러한 apoptotic 세포손상으로부터 TS cell을 보호하였다. 결론적으로 저산소증으로부터 유도되는 임신과 관련된 질환에서 FGF-4는 태반을 보호하는데 중요한 역할을 하리라고 생각한다.

찾아보기 낱말 : FGF-4, 저산소증, 태반, apoptosis

교신저자 : 오재민 (원광대학교 의과대학 해부학교실)
전자우편 : jmoh@wonkwang.ac.kr