대 한 체 질 인 류 학 회 지
Korean J Phys Anthrop
16(3): 197 ~ 204, 2003

The Roles of S1P on Placental Development

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Sphingosine-1-phosphate (S1P), a bioactive sphingolipid metabolite, has various functions to affect many signalling pathways leading to cellular proliferation and differentiation and to regulate of cell migration, invasion, and angiogenesis. However, there are little reports about the relation between trophoblast stem cells and S1P. Thus, the physiologic effects of S1P on trophoblast stem (TS) cells were investigated in this study. S1P was involved in early stage development of trophoblast via upregulation of Eomesodermine mRNA expression and suppressed differentiation of TS cells through activation of extracellular-signal regulated kinase (ERK) activation. Other actions of S1P were the activation of p38 and the induction of Dlx-3 mRNA expression for angiogenesis in TS cells. Interestingly, TS cells cultured with S1P for 4 days in thrombin-fibrinogen gel culture system, specific culture system for endothelial cells, showed good healthy appearance, but TS cells cultured without S1P got severe damages. Taken together, we suggest that S1P has very important roles on placenta such as development of early stage trophoblast, suppression of differentiation, and angiogenesis on placenta.

Key words : S1P, trophoblast stem cell, Eomesodermine, Erk, p38, Dlx-3

Introduction

Trophoblast stem (TS) cells can be derived from blastocysts by culture in the presence of fibroblast growth factor (FGF)-4 plus heparin and primary embryo fibroblast-conditioned medium. TS cells only contribute to the trophoblast lineages of the placenta and not to the fetus itself. They behave as restricted stem cells, capable of recapitulating the entire differentiation pathway of the trophoblast lineages involving labyrinth trophoblast, spongiotrophoblast, and giant cells (Donald et al. 1998, Tanaka et al. 1998, Janet et al. 2001). So, TS cell is a very good material for studying the mechanism of placentation involing implantation.

Sphingosine-1-phosphate (S1P), a bioactive sphingolipid metabolite formed by sphingosine kinase, showing versatile ability. It is stored in platelets, released upon their activation, and is present in an albumin-bound form at physiologically relevant concentrations in serum. Like many other sphingolipids, S1P was thought to be mainly a degradative metabolite of sphingolipids formed during turnover of eukaryotic cell membranes (Shawn et al. 2002). It is now well accepted that extracellular S1P is an important mediator of many physiological processes. The platelet-derived growth factor (PDGF) activated sphingosine kinase, thereby incresing intracellular S1P levels,

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implicated a role for intracellular S1P in cell survival, proliferation, angiogenesis and the inhibition of apoptosis (Susan et al. 2000). But there is no report about the relation between S1P and trophoblast.

The mammalian embryo is critically dependent on the establishment of a chorioallantoic placenta, which functions as the primary means of nutrient and gas exchange. Dlx-3 is a homeodomain transcription factor and a member of the vertebrate Distal-less family. Dlx-3 is expressed in the ectoplacental cone cells and chorionic plate, and later in the labyrinthine layer of murine animal placenta. Targeted deletion of the mouse Dlx3 gene results in embryonic death between day 9.5 and day 10 because of placental defects that alter the development of the labyrinthine layer (Maria et al. 1999).

Although p38 was originally identified through its role in stress responses, p38 isoform also plays an essential role in normal embryonic development. A null mutation in p38 results in embryonic lethality at midgestation stages, most likely as a consequence of defective placental development. In particular, there are distinct defects in the placenta, corresponding to a severe reduction in the spongiotrophoblast layer, as well as a near absence of the labyrinth layer because of the failure of vascularization by endothelial cells from the underlying chorionic plate (John et al. 2000).

The Erk have been implicated in the control of cell proliferation, differentiation and survival. Previous study showed that disruption of the Erk2 locus leads to embryonic lethality early in mouse development after the implantation stage. In addition, Erk mutant embryos fail to form the ectoplacental cone and extra-embryonic ectoderm, which give rise to mature trophoblast derivatives in the fetus (Saba-El-Leil et al. 2003). However, the specific role of Erk for regulating the proliferation and the differentiation of polar trophectoderm cells has not been known well. In this study, we checked roles of S1P on development of placenta.

Materials and Methods

1. Cell culture

Medium for TS cell culture (TS medium) and embryonic fibroblast conditioned medium (EMFI-CM) were prepared as previously described (Andreas et al. 2000). 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.

2. Western blot analysis

Cells were collected by centrifugation, washed with PBS, and resuspended in suitable volume of 1× sample buffer. The sample solutions were cleared by centrifugation at 12,000 rpm at 4°C for 20 min. After centrifugation, the supernatant was boiled at 98°C for 5 min, and then each 10 μg proteins separated on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transfered onto polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) in a Bio-Rad Trans-Blot electrophoresis apparatus using Towbin's buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 20% (v/v) methanol). The membrane was incubated in 5% skim milk at room temperature for 1 h, rinsed, and reacted with anti-Erk and anti-p38 (Santa Cruz, CA, USA) in Tris-buffered saline with 0.05% Tween-20 (TBS-T) at 4°C for overnight. The membrane was washed four times in TBS-T and reacted with secondary antibody conjugated with horse radish peroxidase for 1 hr. After four washes with TBS-T, the immunoreactive bands were visualized by enhanced chemiluminescence

(ECL) kit (Amersham Corp., England) and exposed to X-ray film.

3. RT-PCR and dot blot analysis

Total RNA was extracted from 10^5 cells using Trizol reagent (Life Technologies, Inc., Rockville, MD, USA) following the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed into first-strand cDNA using Superscript II (Life Technologies, Inc., Rockville, MD, USA) in a 20 μl reaction. The cDNA was subsequently diluted 5 folds and PCR was then performed in a reaction mixture containing 1 μl of cDNA, 25 ng of each primer, 0.1 mM dNTPs (containing dATP, dCTP, dGTP, and TTP), 2 μl of 10× reaction buffer (Roche Molecular Biochemicals, Indianapolis, IN), 0.5 units of platinum *Taq* (Life Technologies, Inc., Rockville, MD, USA), and sterile water to 20 μl. Two to 2.5 ml of the resulting cDNA was used in PCR amplifications using pairs of the following primers:

(sense) 5′-AAGTGGGTGCAGTGTGGAAAGG-3′; (antisense) 5′-CGCGCCTCCTCTTAGAGTCTCC-3′ for Eomesodermin. (sense) 5′-AGACAAGAGGAGGCCTAGAACTC-3′; (antisense) 5′-GAGAAGGCTGGGGAGG-3′ for Dlx-3. The cycling conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 62~68°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 7 min. Two microliter of the PCR mixture was put on nitrocellulose membrane for DNA blot, the results were analyzed through dot blot hybridization with probe generated from from clone related to the Dlx-3 and Eomesodermin using specific above primers. PCR products served as templates to generate ^{32}P-labeled anti-sense probes for dot blots with random primer.

4. Cell culture on the thrombin-fibrinogen gels

This Thrombin-fibrinogen gel was prepared as described by previous study (Montesano et al. 1987). Briefly, bovine fibinogen (Sigma) was dissolved in PBS at a concentration of 2.5 mg/ml. The 0.5 ml fibriongen solution was transferred to each well of 24 well plate. Clotting was induced by addition of thrombin (0.625 U/ml, Sigma). Before seeding of TS cells, TS cells were cultured with/without S1P for 2 days in the medium only. After gel clotting, each TS cells were cultured on the plate for 30 min and then added another 0.5 ml fibrinogen solution with thrombin (0.625 U/ml) on the cells. TS cells were cultured for another 4 days.

Results

1. S1P involved in development of early stageplacenta

The T-box gene Eomesodermine performs essential functions in early stage development of placenta. So to identify that S1P is involved in development of early stage placenta, we checked the expression of Eomesodermine mRNA. After treatment with S1P for 48 hr, the expression of Eomesodermine mRNA by

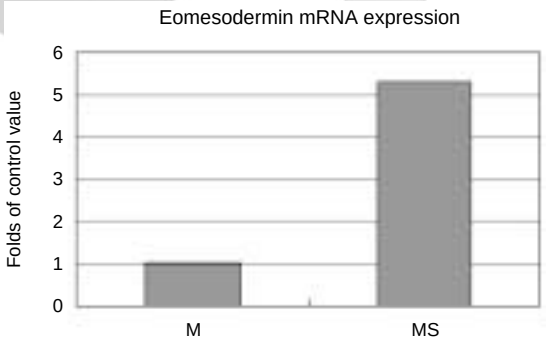

Fig. 1. S1P enhanced the expression of Eomesodermin mRNA in TS cells. Cells were treated with 20 μM S1P for 48 hr and then total RNAs were extracted with Trizol and prepared for RT-PCR and dot blotting. Results are shown representative of experiments performed at least 3 times. The result represented as fold changes of mRNA expression compare to the Eomesodermin mRNA expression of medium alone group. (M: medium alone, MS: medium +S1P)

S1P is markedly increased to 5.3 folds compare to medium alone group (Fig. 1). This data means that S1P plays important roles in development of early stage placenta.

2. S1P involved in development of placental circulatory system through the increase of p38 activity and the induction of Dlx-3 mRNA expression

Cells were treated with 20 μM S1P for 0, 5, 15, 30 and 60 min and lysate was used to measure the expression of p38 and phospho-p38 proteins by western blot analysis. S1P increased p38 activity in TS cells within 5 min and increased p38 activity continued during 30 min (Fig. 2A, 2B). In this study, we also examined the expression of Dlx-3 mRNA induced by S1P on TS cells. The expression of Dlx-3 mRNA on TS cells was increased to 2.8 folds compared to medium alone group by S1P (Fig. 3). In addition TS cells were cultured with/without S1P for 4 days in thrombin-fibrinogen gel culture system, specific culture system for endothelial cells. TS cells cultured with S1P were healthy (Fig. 4A) but TS cells cultured without S1P got severe damages (Fig. 4B). These data indicate that S1P may be a very important molecule for angiogenesis of placenta and circulatory connection between mother and fetus.

3. S1P maintained proliferative state of TS cells through induction of Erk activation

S1P induced the increase of Erk activity in TS cells for 5, 15 and 30 min (Fig. 5). To check the roles of Erk on suppression of TS cell differentiation, we treated with PD 98059, Erk inhibitor. PD 98059 blocked the action of S1P on maintaining proliferative state of TS cells (Fig. 6). This data means that S1P make to main-

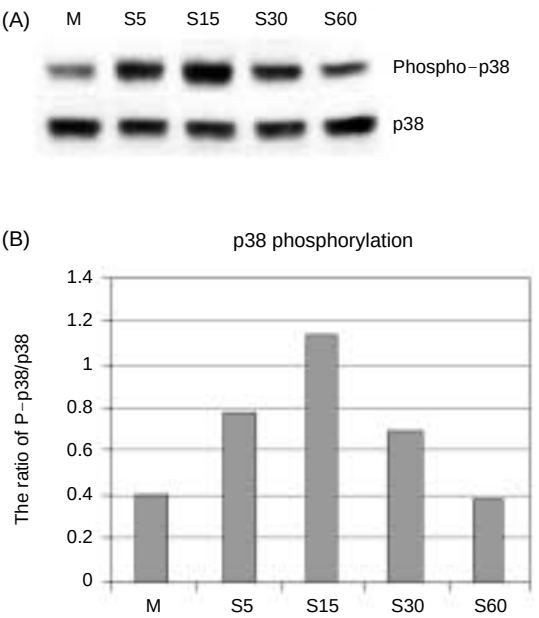

Fig. 2. S1P induced increase of p38 activity. Cells were treated with 20 μM S1P for 0, 5, 15, 30 and 60 min. Then cells were used to measure the activation of p38. 10 μg of each protein was analyzed by Western blotting with anti-p38 and anti-phospho-p38 antibodies. The immunoreactive bands were then visualized by ECL kit (A). The data was demonstrated a ratio of dimension of phosphorylation of p38 band to p38 one by a graph (B).

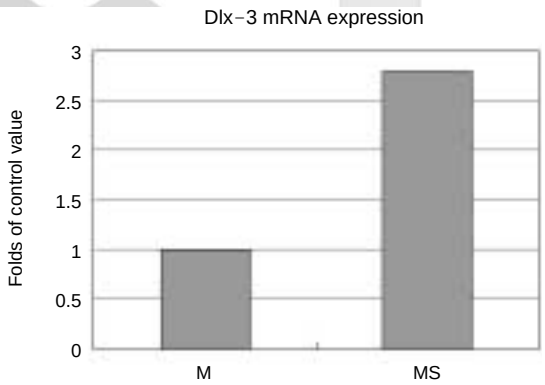

Fig. 3. S1P enhanced the expression of Dlx-3 mRNA in TS cells. TS cells were cultured with/without S1P for 48 hr and then total RNAs were extracted with Trizol and prepared for RT-PCR and dot blotting. Results are shown representation of experiments performed at least 3 times. The result represented as fold changes of mRNA expression compare to the Dlx-3 mRNA expression of medium alone group. (M: medium alone, MS: medium +S1P)

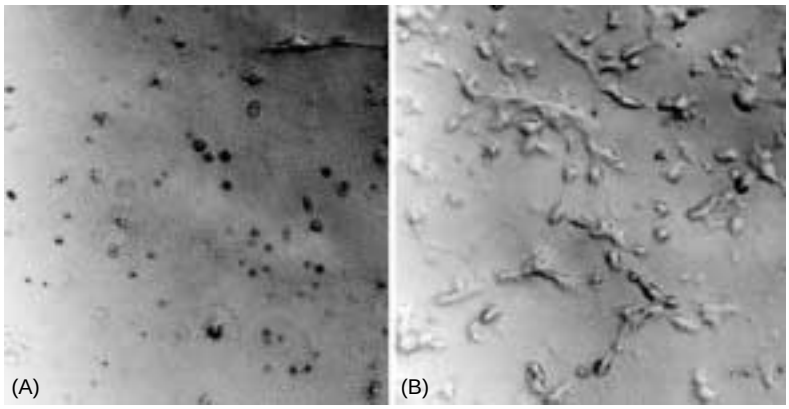

Fig. 4. S1P made to survive TS cells on the fibriongen−thrombin gel. TS cells were cultured with medium alone (A) and medium with 20 μM S1P (B) for 2 days. TS cells were cultured for another 4 days after transfer of cells to gel and then observed with phase contrast microscope (× 100).

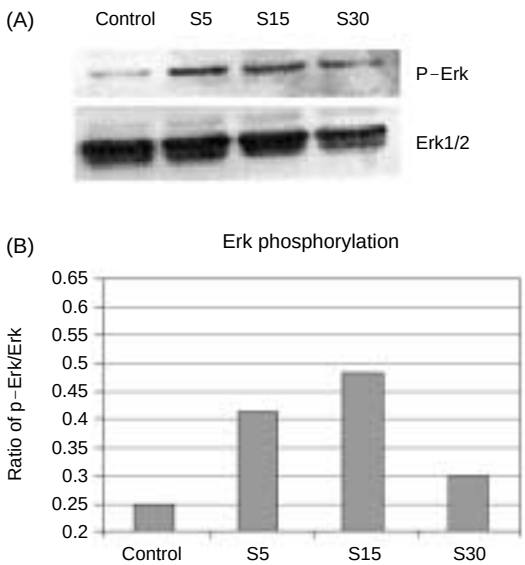

Fig. 5. S1P induced increase of Erk activity. Cells were treated with 20 μM S1P for 0, 5, 15, and 30 min. 10 μg of each protein was analyzed by Western blotting with anti−Erk and anti−phospho−Erk antibodies. The immunoreactive bands were then visualized by ECL kit. The data was demonstrated a ratio of dimension of phosphorylation of p38 band to p38 one by a graph (B).

tain proliferative state of TS cells through ERK activation.

Discussion

Sphingosine−1−phosphate (S1P) is a polar sphingolipid metabolite that functions as both an extracellular and intracellular messenger to regulate diverse cell signalling pathway, including cell growth, differentiation, survival, motility, apoptosis and calcium homeostasis (Shawn et al. 2002). Extracellular effects of S1P are mediated via a newly identified family G−protein coupled cell surface receptors (GPCRs), the endothelial differentiation gene−1 (EDG−1) family (Herve et al. 2002). In this study the physiologic or pathologic effect of S1P on TS cells was investigated.

The T−box gene Eomesodermine performs essential functions in trophoblast development. So, mouse embryos lacking Eomesodermine arrest at the blastocyst stage (Andreas et al. 2000) Indeed, Eomesodermine defines a conserved molecular pathway controlling the morphogenetic movements of germ layer formation and has acquired a new function in mammals in the differentiation of trophoblast (Andreas et al. 2000). In this study, S1P enhanced the expression of Eomesodermine compared to control group of trophoblast. This data suggests S1P can be involved in

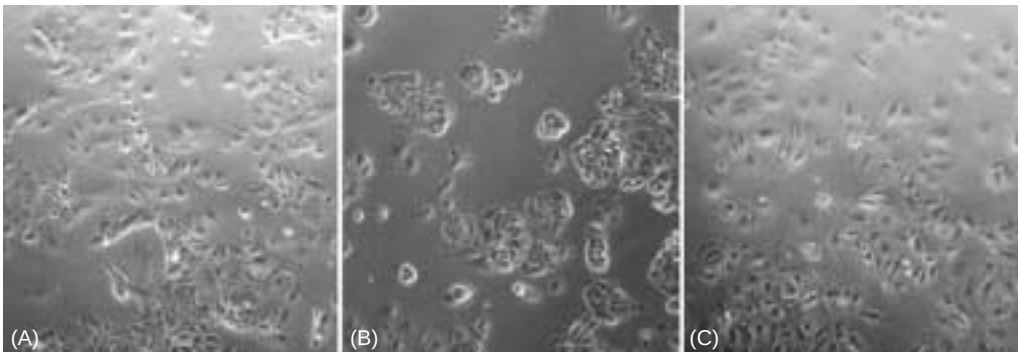

Fig. 6. S1P made to maintain proliferative state of TS cells. 10 μM of PD 98059 was added at 30 min before 20 μM S1P treatment. S1P suppressed differentiation of TS cells, and PD 98059 inhibited this action of S1P. Cells were observed with phase contrast microscope (× 200). A: medium alone, B: medium alone with S1P, C: medium alone with S1P and PD98059.

early stage process of trophoblast development by upregulation of Eomesodermine expression.

VEGF signal transduction bifurcates both upstream and downstream of Ras, with different Ras-dependent signals as p38 signaling pathways controlling endothelial cell proliferation and migration, essential components of the angiogenic response (Peters et al. 2000, Meadows et al. 2001). Actually, K/O mouse of p38 lose almost labyrinth layer and decreased spongiotrophoblast (John et al. 2000). In this study, S1P induced increased phospholyation of p38 in TS cells. This data means that S1P plays a role in angiogenesis through activation of p38.

Dlx-3 is a homeodomain transcription factor. Targeted deletion of the mouse Dlx-3 gene results in embryonic death between 9.5 day and 10 day because of placental defects that alter the development of the labyrinthine layer (Maria et al. 1999). Thus, Dlx-3 is essential for embryonic survival (Maria et al. 1999). In this study, agree with previous study, S1P enhanced expression of Dlx-3 gene on TS cells. These data indicate that S1P may be a very important molecule for angiogenesis of placenta and circulatory connection between mother and fetus.

Another role of S1P on TS cells was to maintain proliferative state of TS cells through induction of increased Erk activity (Figs. 5, 6). This action of Erk has not been known very well. This data indicate that S1P play a role on suppression of differentiation of TS cells.

This data means that S1P is very important on development of labyrinthine trophoblast, the suppression of differentiation of TS cells and angiogenesis in placenta.

References

Andreas PR, Slgrid W, William HC : Eomesoderm is required for mouse trophoblast development and mesoderm formation. Nature 404 : 95-99, 2000.

Tanaka S, Kunath T, Hadjantonakis AK, Nagy A, Rossant J : Promotion of trophoblast stem cell proliferation by FGF4. Science 282 : 2072-2075, 1998.

Donald W, Morrish JD, Hongshi L : Functional regulation of human trophoblast differentiation. J Reprod Immunol 39 : 179-195, 1998.

Herve LS, Courtney P, Hong L, Sheldon M, Sarah A : Sphingosine-1-phosphate and lipid phosphohydrelases. Biochimica et Biophysica Acta 1582 : 8-17, 2002.

Janet R : Stem cells from the mammalian blastocyst. Stem cells 19 : 477-482, 2001.

John S, Mudgett JD, Lucia GS, Nicole A, Chartrain LY, Shobhna G, Michael MS : Essential role for p38 mitogen-

activated protein kinase in placental angiogenesis. PNAS. 97 : 10454−10459, 2000.

Maria IM, Alexander G, Gertraud R, Thomas DS, Kathleen AM : Placental failure in mice lacking the homeobox gene Dlx3. PNAS 96 : 162−167, 1999.

Meadows KN, Bryant P, Pumiglia K : Vascular endothelial growth factor induction of the angiogenic phenotype requires Ras activation. J Biol Chem 28; 276(52) : 49289−49298, 2001.

Montesano R, Pepper MS, Vassalli JD, Orci L : Phorbol ester induces cultured endothelial cells to invade a fibrin matrix in the presence of fibrinolytic inhibitors. J Cell Physiol 132 : 509−516, 1987.

Peters TJ, Champman BM, Wolfe MW, Soares MJ : Placental lactogen−I gene activation in differentiating trophoblast cells: extrinsic and intrinsic regulation involving mitogen−activated protein kinase signalling pathways. Endocrinology 165 : 443−456, 2000.

Saba−El−Leil MK, Vella FD, Vernay B, Voisin L, Chen L, Labrecque N, Ang SL, Meoche S : An essential function of mitogen−activated protein kinase Erk2 in mouse trophoblast development. EMBO (Epub ahead of print) 2003.

Shawn GP, Sheldon M, Sarah S : Sphingosine 1−phosphate: dual messenger functions. FEBS. Letters 531 : 54−57, 2002.

Susan P, Nigel P : Sphingosine 1−phosphate signalling via the endothelial differentiation gene family of G−protein−coupled receptors. Pharmacol. Thera. 88 : 115−131, 2000.

Korean J Phys Anthrop
16(3): 197 ~ 204, 2003

Trophoblastic stem cell에서의 Sphingosine-1 phosphate (S1P)의 역할

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간추림 : S1P는 sphingolipid의 대사산물로써 세포의 proliferation, differentiation, migration, invasion, angiogenesis, 및 여러 세포에서 apoptosis를 포함한 stress에 대하여 세포를 보호하는 기능 등의 많은 작용을 가지고 있다. 그러나 S1P가 이렇게 많은 기능을 가지고 있음에도 불구하고 태반이나 trophoblast에 대하여 연구된 보고는 거의 없는 실정이다. 따라서 본 실험은 S1P가 태반의 발달과정이나 trophoblast에 미치는 영향을 알아보기 위하여 시행하였다. Trophoblastic stem (TS) cell에 처리된 S1P는 48시간 후에 태반의 조기발달을 유도하는 Eomesodermine의 mRNA발현을 증가시켰고 태반발달과정 중에 태반의 angiogenesis에 중요한 역할을 하는 p38을 활성화시켰으며 모체와 태아의 혈액순환을 매개하는 labyrinthtrophoblast의 중요한 유전자인 Dlx-3의 발현역시 2.8배 증가시켰다. 더욱이 S1P는 내피세포만 특이적으로 자랄 수 있는 firbinogen-thrombin gel에서 TS cell의 손상을 억제하였다. 또한 Erk를 활성화시킴으로써 TS cell의 differentiation을 억제하고 TS cell을 proliferative state로 유지하였다. 이러한 결과들을 종합하여 볼 때, S1P는 태반의 초기발달과정과 angiogenesis에 중요한 역할을 하리라고 생각된다.

찾아보기 낱말 : S1P, trophoblast stem cell, Eomesodermine, p38, Erk, Dlx-3

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