A THESIS FOR THE DEGREE OF MASTER

Protective effect of netrin-1 on hypoxia-induced mitochondrial apoptosis through HSP27 expression in human umbilical cord blood derived mesenchymal stem cells

저산소 유도 인간 제대혈 중간엽 줄기세포의 미토콘드리아성 세포자멸사에 대한 netrin-1의 HSP27 발현에 의한 방어 효과

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Abstract

Protective effect of netrin-1 on hypoxia-induced mitochondrial apoptosis through HSP27 expression in human umbilical cord blood derived mesenchymal stem cells

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Netrin (Ntn) has the potential to provide an anti-apoptotic agent with a high affinity for tissue, for therapeutic strategies of human umbilical cord blood derived mesenchymal stem cell (hUCB-MSC), although the mechanism by which Ntn-1 protects hypoxic injury has yet to be identified. Therefore, the present study examined the effect of Ntn-1 on hypoxia-induced hUCB-MSC apoptosis, as well as the potential underlying mechanisms. Hypoxia (72 h) induced a decrease in the rate

of MTT reduction and [^{3}H]-thymidine incorporation, which were inhibited by Ntn-1 (10 ng/ml). Also, Ntn-1 decreased the increase of hypoxia-induced Bax, cleaved caspase-9, and cleaved caspase-3, but blocked the decrease of hypoxia-reduced Bcl-2. Next, in order to examine the Ntn-1-related signaling cascade in protection of hypoxic injury, six Ntn receptors in hUCB-MSC were analyzed. DCC and integrin (IN) α6β4 except UNC5A-C, and neogenin were existed in hUCB-MSC. Among them, IN α6β4 only was detected in lipid raft fractions. In addition, Ntn-1 induced the dissociation of DCC and APPL-1 complex thereby stimulating the formation of APPL-1 and Akt2 complex. Ntn-1 also reversed the hypoxia-induced decrease of Akt and GSK-3β phosphorylation, which is involved in HSF-1 expression. Ntn-1-induced phospho-Akt and -GSK-3β was inhibited by DCC and IN α6β4 function-blocking antibody, and Akt inhibitor. Hypoxia and/or Ntn-1 stimulated HSP27 expression, which was blocked by *HSF-1*-specific siRNA. Furthermore, *HSP27*-specific siRNA reversed the Ntn-1-induced increase of phospho-Akt. Additionally, *HSP27*-specific siRNA attenuated the Ntn-1-reduced loss of mitochondrial membrane injury via inhibition of cytochrome c (cyt c) release and formation of cyt c and HSP27 complex. Moreover, the inhibition of each signaling proteins attenuated Ntn-1-induced blockage of apoptosis. In conclusion, Ntn-1-induced HSP27 protected hypoxic injury-related hUCB-MSC apoptosis through DCC- and IN α6β4-dependent Akt, GSK-3β, and HSF-1 signaling pathways.

Keywords: human umbilical cord blood derived mesenchymal stem cell; netrin-1; hypoxic injury; apoptosis; cytoprotection; heat shock protein.

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List of Abbreviations

Ab	Antibody
APPL	An adaptor protein containing a pleckstrin homology domain, a PTB domain and a leucine zipper motif
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Cav	Caveolin
cyt c	Cytochrome c
DCC	Deleted in colorectal cancer
FBS	Fetal bovine serum
GSK-3β	Glycogen synthase kinase 3β
HSF	Heat shock factor
HSP	Heat shock protein
hUCB-MSC	Human umbilical cord blood derived mesenchymal stem cell
IN	Integrin
IP	Immunoprecipitation
MTT	3-(4,5,-dimethyl thiazolyl-2)-2,5-Diphenyl tetrazolium bromide
NAC	*N*-Acetylcysteine
Ntn	Netrin
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol 3-kinases
PVDF	Polyvinylidene fluoride

ROD	Relative optical density
RT-PCR	Reverse transcription-polymerase chain reaction
SC	Stem cell
SE	Standard errors
siRNA	Small interfering ribonucleic acid
TBST	Tris-buffer solution-tween20
TCA	Trichloroacetic acid
UNC5	Uncoordinated family member 5

Introduction

Human mesenchymal stem cells (hMSCs) have distinct characteristics, with a self-renewal capacity and the ability to generate multiple differentiated cell types (Qiao et al., 2008). Hence, hMSCs have been targeted in the field of developmental biology and have been used in a vast number of therapeutic applications (Sensebé et al., 2010). Although several studies involving clinical applications have detailed MSC transplantation and/or therapeutic methods (Wakitani et al., 2007), the serious problem of hypoxic injury may arise during the transplantation and cell therapy (Zhu et al., 2006). Interestingly, alteration of duration of hypoxic conditions [oxygen concentrations lower than 2 %] has been shown to play a contrasting role in stem cells (SCs). In a short period of time (under 48 h), hypoxia induced proliferation, migration, and differentiation of various SCs, including MSCs (Lee et al., 2011; Prado-Lopez et al., 2010). Additionally, over a longer period of time (more than 72 h), hypoxia can reduce SC proliferation and/or lead to programmed cell death (Lee et al., 2012). In respect of the SC therapy or transplantation, although short-term hypoxic condition stimulated SC viability, injected or transplanted SCs were underwent under long-term hypoxic condition and subsequently long-term hypoxic condition was the leading cause of cell death. Thus, it is important to illuminate tissue affinitive protective factors of hypoxic injury to apply MSC for regeneration strategies and its mechanisms controlling hypoxia-mediated MSC apoptosis must be understood in detail.

Netrin (Ntn), an evolutional conserved family of laminin-related protein, is a multifunctional guidance molecule that serves as a regulatory factor of cell apoptosis, morphogenesis, and invasion (Lai Wing Sun et al., 2011). With respect to apoptosis, Ntn-1 has been introduced as an anti-apoptotic agent against hypoxic injury of the brain tissue (Bayat et al., 2012; Wu et al., 2008). Therefore, it is possible that Ntn-1 in the extracellular plasma environment may be involved in a protective effect against hypoxia-induced hUCB-MSC apoptosis. Recent findings have implicated Ntn in the regulation of adult stem cell migration, tumor cell survival, and embryonic development, suggesting potentially novel means of promoting recovery from cellular injury and achieving improvements in tissue regeneration (Bradford et al., 2009; Fitamant et al., 2008; Petit et al., 2007). In addition, Ntn is crucial to maintain the survival of Ntn receptor expressed cell during tissue development (Xie et al., 2011). In this regard, Ntn, Ntn receptors, and the associated downstream signaling mechanisms involved are promising targets for the prevention for extracellular damage. However, there are no previous reports related to anti-apoptotic effects of tissue affinitive Ntn-1 in hUCB-MSCs for regeneration strategies. I assume that exogenous and tissue affinitive Ntn-1 can protect cell death from extracellular damage and improve subsequent survival. If so, Ntn, Ntn receptors, and the downstream signaling mechanisms involved are promising targets for the prevention for extracellular damages. Thus, determining how Ntn receptors and signal transduction proteins function as an ensemble in regulating apoptosis remains a major challenge for current studies. Therefore, the present study examined the effect of Ntn-1 on hypoxia-induced hUCB-MSC apoptosis and its related signal pathways.

Materials and Methods

1. Materials

hUCB-MSCs were obtained from the Obstetrics of College medicine, Chosun National University (Gwangju, Korea). Fetal bovine serum (FBS) was purchased from BioWhittaker Inc. (Walkersville, MO, USA). Ascorbic Acid (Vitamin C), Lithium Chloride, and N-acetylcysteine (NAC) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Anti-Akt2, APPL-1, Bax (6A7), Bcl-2, β-actin, caveolin-1, caveolin-2, caspase-3, caspase-9, cyt c, flotillin-2, GSK-3β, HIF-1α, HSF-1, HSP27, HSP60, HSP70, HSP90, IgG, integrin α6, integrin β4, lamin A/C, pan-cadherin, phospho-Akt^{thr308}, phospho-Akt^{ser473}, phospho-GSK-3β, and total-Akt antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Akt inhibitor and DCC antibody were purchased from Calbiochem (La Jolla, CA, USA). Cox IV antibody was purchased from Abcam (Cambridge, UK). Recombinant human Ntn-1 was purchased from R&D Systems (Minneapolis, MN, USA). [^{3}H]-thymidine was obtained from Dupont/NEN (Boston, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit IgG were purchased from Jackson Immunoresearch (West Grove, PA, USA). Liquiscint was obtained from National Diagnostics (Parsippany, NJ, USA). All other reagents were of the highest purity commercially available and were used as received.

2. Isolation and characterization of human UCB-MSCs

The isolation and characterization of human UCB-MSC were performed by cell surface marker analysis and multilineage differentiation in previous report (Yun et al., 2009). After the initial 31 days of primary culture, hUCB-MSCs adhered to a plastic surface and presented a small population of single cells with a spindle shape. On days 7-10 after the initial plating, cells had the appearance of long, spindle-shaped fibroblastic cells, began to form colonies, and became confluent. After being replated, the fibroblast-like cells were polygonal or spindly with a long process. hUCB-MSC passages 4-12 were observed under a microscope. Cells appeared normal on the basis of typical morphology. hUCB-MSCs were positive for CD29, CD44, CD73, CD90, CD105, and HLA-ABC, but negative for CD14, CD34, CD45, CD74, CD106, and HLA-DR. With osteogenic supplementation, differentiation was apparent after 1 week of incubation. By the end of the second week, a portion of hUCB-MSC became von Kossa positive. Similarly, the portion of cells that was induced with adipogenic medium contained numerous oil red-O positive lipid droplets. With neuronal supplementation, differentiation was apparent after 1 week of incubation. After 1 week, media were observed under a microscope to check for axon formation and enlargement of nuclei (all data not shown).

3. Culture of hUCB-MSCs

hUCB-MSCs were cultured without a feeder layer in phenol-red free Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) supplemented with 3.7 g/L sodium bicarbonate, 1 % penicillin and streptomycin, 1.7 mM L-glutamine, 0.1 mM b-mercaptoethanol, and 10 % FBS. For each experiment, cells were grown in wells of 6- and 12-well plates, and in 35, 60, or 100 mm diameter culture dishes in an incubator maintained at 37 °C with 5 % CO_2. The medium was replaced with serum-free DMEM at least 24 h before exposure to hypoxia. Following incubation, the cells were washed twice with phosphate-buffered saline (PBS) and then maintained in a serum-free DMEM including all supplements and indicated agents.

4. Hypoxic treatment of hUCB-MSCs

hUCB-MSCs were washed twice with PBS, and the medium was exchanged with fresh DMEM. Experiments were performed in a modular incubator chamber (Billups-Rotheberg, Del Mar, CA) at 37 °C for 30 min under normoxic (92.3 % air and 5.5 % CO_2) or hypoxic (2.2 % O_2, 5.5 % CO_2, and 92.3 % N_2) conditions at a flow rate of 20 l/min. The chamber was purged with gas, sealed, and placed in a conventional incubator at 37 °C.

5. [^{3}H]-thymidine incorporation

[^{3}H]-thymidine incorporation experiments were carried out as previously described (Brett et al., 1993). In this study, the cells were cultured in a single well until they reached 70 % confluence. They were then washed twice with PBS and maintained in serum-free DMEM including all supplements. After 24 h incubation, the cells were washed twice with PBS and incubated with fresh serum-free DMEM containing all supplements and the indicated agents. After the indicated incubation period, 1 mCi of [methyl-^{3}H]-thymidine was added to the cultures. Incubation with [^{3}H]-thymidine continued for 1 h at 37 °C. The cells were washed twice with PBS, fixed in 10 % trichloroacetic acid (TCA) at 23 °C for 30 min, and then washed twice with 5 % TCA. The acid-insoluble material was dissolved in 2 N NaOH for 12 h at 23 °C. Aliquots were removed to measure the radioactivity using a liquid scintillation counter. All values are reported as the mean ± SE of triplicate experiments. The values were converted from absolute counts to a percentage of the control in order to allow comparison between experiments.

6. 3-(4,5,-dimethyl thiazolyl-2)-2,5-Diphenyl tetrazolium bromide (MTT)

hUCB-MSCs were harvested after hypoxic condition for various periods (0-72 h). 300 μl MTT reagent (Sigma) was added to each well 2 h prior to harvest. The supernatant was then removed and incubated with 400 μl dimethylsulphoxide

(Sigma) for 10 min. Absorbance at 540 nm was recorded using an enzyme-linked immunosorbent assay plate reader.

7. Gene silencing with small interfering (si)RNA transfection

hUCB-MSCs were grown until 75 % of the surface of the plate was covered after which they were transfected for 24 h with either a siRNA specific for *HSF-1* (100 nmol/L; Santa Cruz Biotechnology, CA, USA; sc-35611), *HSP27* (100 nmol/L; Santa Cruz Biotechnology, CA, USA; sc-29350), *HSP70* (100 nmol/L; Dharmacon, Lafayette, CO, USA; L-065710-00) or a *non-targeting* siRNA as a negative control (100 nmol/L; Dharmacon, Lafayette, CO, USA; D-001206-13-20) with Hyperfectamine (QIAGEN, Valencia, CA) according to the manufacturer's instructions.

8. Cell Fractionation

For fractionation of cells into cytosolic membrane and nuclear fractions, a digitonin fractionation method was used as previously described (Sørensen et al., 2008; Wiedłocha et al., 2005). hUCB-MSCs were serum starved for 24 h, and then pretreated with or without Ntn-1 (10 ng/ml) for various periods (0-72 h) under hypoxic condition. The cells were then washed with PBS, and 20 μg/ml digitonin (Sigma) was added to permeabilize the cells. The cells were kept at 25 °C for 5 min and then on ice for an additional 30 min to allow the cytosol to diffuse into the

buffer. The buffer was recovered and designated the cytosolic fraction. The remainder of the cells were lysed with lysis buffer, scraped from the plastic, and centrifuged at 15,800 x g for 15 min. The supernatant was designated the membrane fraction, and the pellet was designated the nuclear fraction. The nuclear fraction was resuspended in PBS, sonicated, and centrifuged to remove undissolved material.

9. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from hUCB-MSCs using STAT-60, which is a monophasic solution of phenol and guanidine isothiocyanate purchased from Tel-Test, Inc. (Friendswood, TX, USA). Reverse transcription (RT) was carried out using 3 μg of RNA using a RT system kit (Bioneer, Daejeon, Korea) with oligo(dT)18 primers. A PCR kit (Bioneer) was used to amplify 5 μl of the RT product under the following conditions: denaturation at 94 °C for 5 min, 30 cycles at 94 °C for 45 sec, Annealing Tm. (Table 1) for 30 sec, and 72 °C for 30 sec, followed by 5 min of extension at 72 °C. Amplifications of *DCC, UNC5A, UNC5B, UNC5C*, and *Neogenin* cDNA were performed in hUCB-MSCs using the primers described in Table 1. PCR of *β-actin* was also performed as a control for RNA quantity.

Table 1. Primers used for polymerase chain reaction

Gene	Identification	Primer sequence, 5'–3'	Annealing Tm. (℃)	Size (bp)	Ref.
DCC	Sense	CAAGTGCCCCGCCTCAGAACG	55	434	Shekarabi and Kennedy, 2002
	Antisense	GCTCCCAACGCCATAACCGATAAT			
UNC5A	Sense	GCACAAGCCGGAAGACGTGAG	56	440	Rodrigues et al., 2007
	Antisense	GCATCGTGGGTGTCATGCAGG			
UNC5B	Sense	CAAGGCAGAAAGTACCCTCCCGCT	56	420	Rodrigues et al., 2007
	Antisense	CAGCACCTCCTTCAGTGCTAC			
UNC5C	Sense	GGCAGCAAGACAAGATCTGC	56	460	Rodrigues et al., 2007
	Antisense	ATGGGTGGCCTCATAGTTTC			
Neogenin	Sense	CGCTACCTTTGAATTAGTTCCT	60	380	Wang et al., 2009
	Antisense	GATGATGTAACCTGTAATCTTGCC			
β-actin	Sense	AGCCATGTACGTAGCCATCC	55	350	Jang et al., 2012
	Antisense	CTCTCAGCTGTGGTGGTGAA			

Abbreviations: DCC, Deleted in colon cancer; *UNC5A,* Uncoordinated-5-A; *UNC5B,* Uncoordinated-5-B; *UNC5C*, Uncoordinated-5-C; Ref, reference.

10. Immunoprecipitation

Immunoprecipitation lysates were incubated with appropriate antibody and protein A-sepharose beads with gentle shaking overnight. Samples were washed thrice with lysis buffer and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Bands were visualized with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK) and quantified with TINA 2.09 software.

11. Western blot analysis

Cells were harvested, washed twice with PBS, and lysed with buffer (20 mM Tris [pH 7.5], 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 1 mg/ml aprotinin, and 1 mM phenylmethylsulfonylfluoride [PMSF]) for 30 min on ice. The lysates were then cleared by centrifugation (22,250 x g at 4 ℃ for 10 min). Protein concentration was determined by the Bradford method (Bradford, 1976). Equal amounts of protein (20 μg) were resolved by 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. After the blots were washed with TBST (tris-buffer solution-tween20) (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, and 0.05 % Tween-20), the membranes were blocked with 5 % skim milk for 1 h and incubated with an appropriate primary antibody at the dilution recommended by the supplier. The membrane was then washed and primary antibodies were detected with a horseradish peroxidase-conjugated secondary antibody. The bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech Inc.). Densitometric analysis was performed using the TINA version 2.09 program package. The ratios between each treated- and control samples were calculated for each individual experiment and expressed as a percentage of controls.

12. Detergent-free purification of caveolin-rich membrane fraction

Caveolin-enriched membrane fractions were prepared as described previously (Song et al., 1996). Cells were washed twice with ice-cold PBS, scraped into 2 ml of 500 mM sodium carbonate (pH 11.0), transferred to a plastic tube, and homogenized with a Sonicator 250 apparatus (Branson Ultrasonic, CT, USA) using three 20-sec bursts. The homogenate was adjusted to 45 % sucrose by the addition of 2 ml 90 % sucrose prepared in 2-(*N*-morpholino) ethanesulfonic acid (MES)-buffered solution consisting of 25 mM MES-buffer solution (pH 6.5) and 0.15 M NaCl and placed at the bottom of an ultracentrifuge tube. A 5–35 % discontinuous sucrose gradient was formed (4 ml each of 5 % and 35 % sucrose, both in MES-buffer solution containing 250 mM sodium carbonate) and centrifuged at 40,000 rpm for 20 h in a SW 41 Rotor (Beckman Coulter, CA, USA). Twelve fractions (1 ml each) were collected and analyzed by 10-12 % SDS-PAGE.

13. Detection of mitochondrial membrane potential

Loss of the mitochondrial membrane potential ($\Delta\psi$) was assessed using the fluorescent probe JC-1 (Molecular Probes, OR, USA), which exists predominantly in the monomeric form in cells with depolarized mitochondria, which fluorescence green at 490 nm. Cells with polarized mitochondria predominantly contain JC-1 in an aggregated form and fluoresce reddish-orange. Cells were incubated with 5 μM

JC-1 for 10 min at 37 ℃ then washed and placed on a warmed microscope stage at 37 °C. Cells were viewed at x 600 magnification with a FluoView 300 confocal microscope. Fluorescence was excited at 488 nm, and emitted light was observed at 515-540 nm. To quantify the mitochondria membrane potential, JC-1-treated cells were rinsed twice in ice-cold PBS and scraped off the surface. A 100 μl cell suspension was loaded into wells of a 96-well plate and examined with a Victor3 luminometer (Perkin-Elmer) and a fluorescent plate reader, with excitation and emission wavelengths of 485 and 535 nm, respectively. The ratio of J-aggregate intensity to JC-1 monomer intensity for each region was calculated. A decrease in this ratio was interpreted as loss of $\Delta\psi m$, whereas an increase was interpreted as gain in $\Delta\psi m$.

14. Isolation of mitochondria

hUCB-MSCs were serum starved for 24 h, and then pretreated with or without Ntn-1 (10 ng/ml) for various periods (0-72 h) under hypoxic condition. The cells were then extracted using the PIERCE Mitochondria Isolation Kit for Cultured Cells (Thermo Fisher Scientific, IL, USA) according to the manufacturer's instructions.

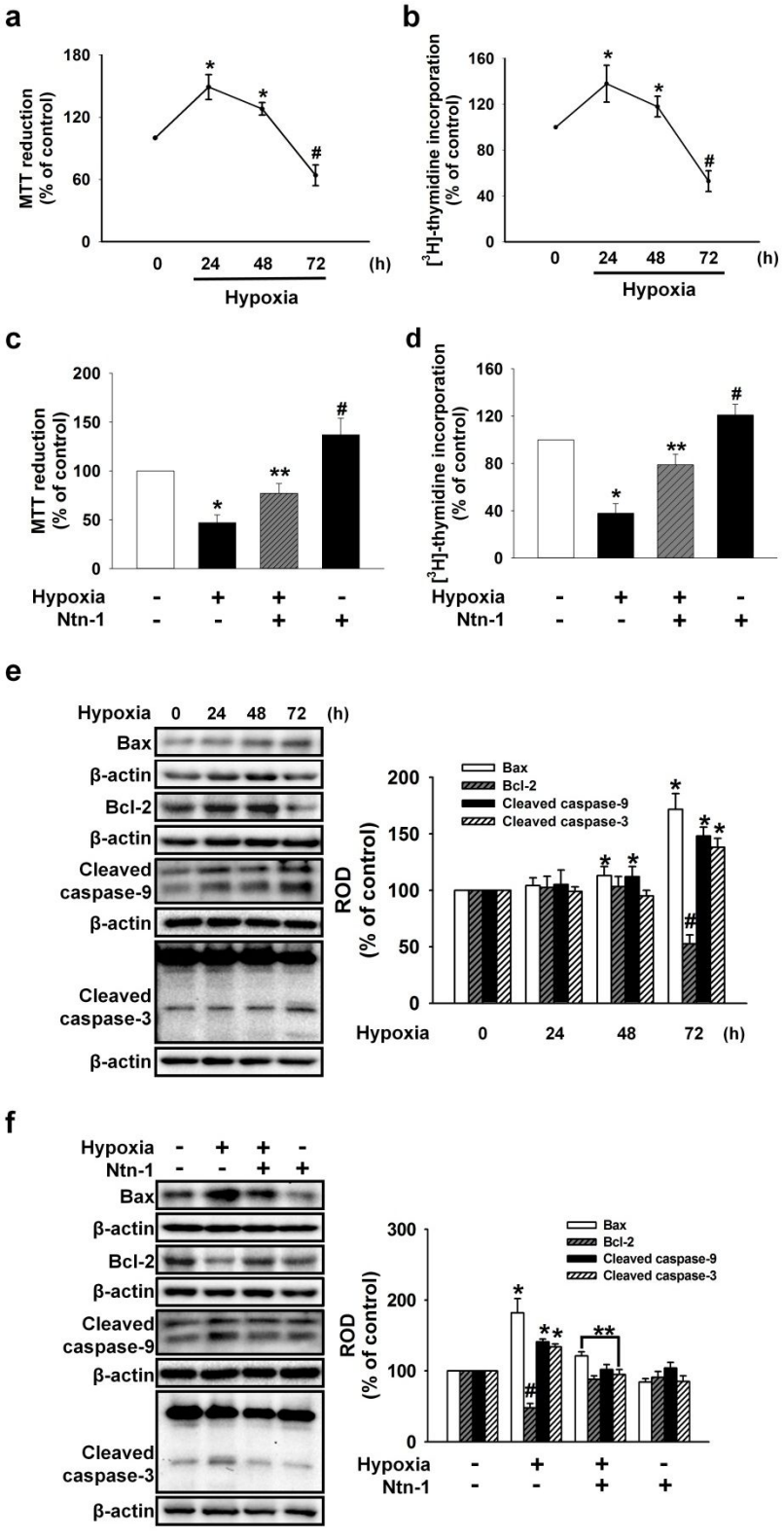
15. Statistical analysis

Results are expressed as means ± standard errors (SE). All experiments were analyzed by ANOVA, followed in some cases by a comparison of treatment means with the control using the Bonferroni-Dunn test. Differences were considered statistically significant at $P < 0.05$.

Results

1. Effect of Ntn-1 on hypoxia-induced hUCB-MSC apoptosis via lipid raft independent DCC and lipid raft dependent IN α6β4

To ensure that the hypoxic condition (2.2 % O_2, 5.5 % CO_2, and 92.3 % N_2) used in this study induces typical cell apoptosis in hUCB-MSCs, the cells were exposed to hypoxic conditions for various periods (0-72 h). MTT reduction levels and [^{3}H]-thymidine incorporation levels were increased until 48 h under hypoxic conditions, but significantly decreased after 48 h (Figures 1a and b). To investigate the protective effect of Ntn-1 on hypoxic injury, cells were pretreated with Ntn-1 (10 ng/ml) for 72 h. Pretreatment of Ntn-1 reversed hypoxia injury markers (Figures 1c and d). Consistent with these results, hypoxia increased Bcl-2-associated X protein (Bax), cleaved caspase-9, and cleaved caspase-3, but decreased B-cell lymphoma 2 (Bcl-2) expression which were reversed by Ntn-1 (Figures 1e and f). In experiments to compare efficacy of anti-apoptotic effect, Ntn-1, *N*-Acetylcysteine (NAC, 1 mM), and ascorbic acid (1 μM) have similar effect (Figure 2).


a
MTT reduction (% of control)
180
120
60
0
0 24 48 72 (h)
Hypoxia
b
[3H]-thymidine incorporation (% of control)
160
120
80
40
0
0 24 48 72 (h)
Hypoxia
c
MTT reduction (% of control)
200
150
100
50
0
Hypoxia - + + -
Ntn-1 - - + +
d
[3H]-thymidine incorporation (% of control)
160
120
80
40
0
Hypoxia - + + -
Ntn-1 - - + +
e
Hypoxia 0 24 48 72 (h)
Bax
β-actin
Bcl-2
β-actin
Cleaved caspase-9
β-actin
Cleaved caspase-3
β-actin
Bax
Bcl-2
Cleaved caspase-9
Cleaved caspase-3
ROD (% of control)
200
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Hypoxia 0 24 48 72 (h)
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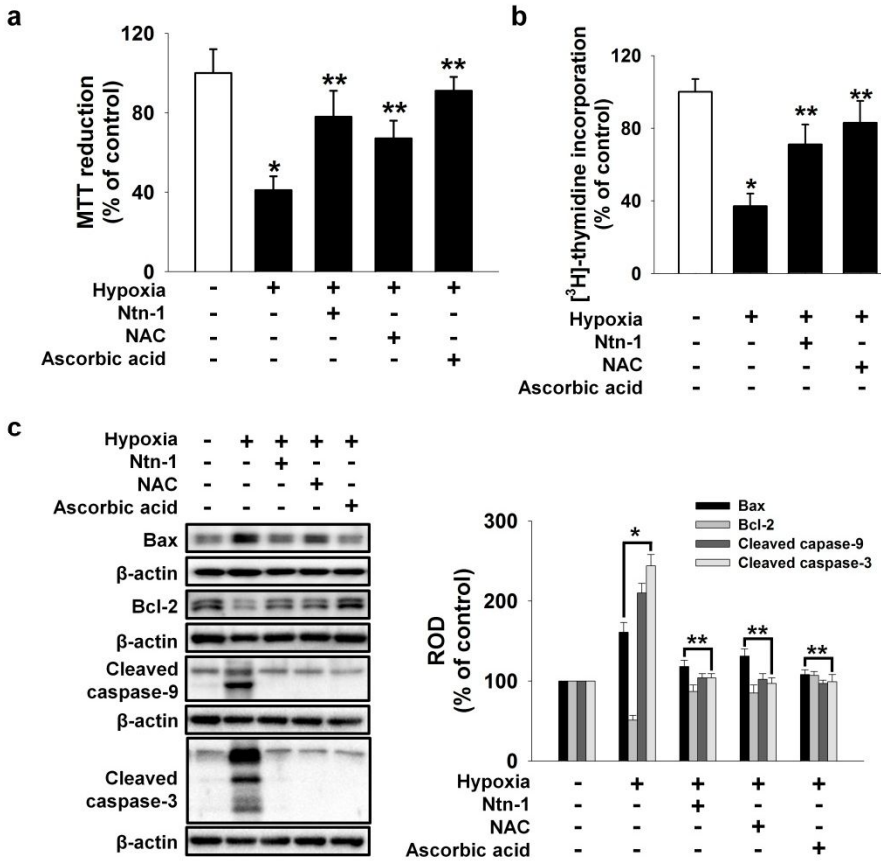
Hypoxia - + + -
Ntn-1 - - + +
Bax
β-actin
Bcl-2
β-actin
Cleaved caspase-9
β-actin
Cleaved caspase-3
β-actin
Bax
Bcl-2
Cleaved caspase-9
Cleaved caspase-3
ROD (% of control)
300
200
100
0
Hypoxia - + + -
Ntn-1 - - + +

Figure 1. Effect of Ntn-1 on hypoxia-induced hUCB-MSC apoptosis.

(**a**) Cells were exposed to hypoxia for 0-72 h and analyzed for their viability by MTT assay. The values are reported as a mean ± SE of three independent experiments with triplicate dishes. $*^{\#}P < 0.05$ vs. control. (**b**) Cells were exposed to hypoxia for 0-72 h and pulsed with 1μCi of [^{3}H]-thymidine for 1 h prior to counting. The values are reported as a mean ± SE of three independent experiments with triplicate dishes. $*^{\#}P < 0.05$ vs. control. (**c**) Cells were pretreated with Ntn-1 (10 ng/ml) for 30 min before being exposed to hypoxia for 72 h and analyzed for their viability by MTT assay. The values are reported as a mean ± SE of three independent experiments with triplicate dishes. $*^{,\#}P < 0.05$ vs. control**,** $**P < 0.05$ vs. hypoxia alone. (**d**) Cells were pretreated with Ntn-1 for 30 min before being exposed to hypoxia for 72 h and then pulsed with 1μCi of [^{3}H]-thymidine for 1 h prior to counting. The values are reported as a mean ± SE of three independent experiments with triplicate dishes. $*^{,\#}P < 0.05$ vs. control**,** $**P < 0.05$ vs. hypoxia alone. (**e**) Cells were exposed to hypoxia for 0-72 h and Bax, Bcl-2, caspase-9, and caspase-3 were detected by Western blot. Each example shown is representative of five experiments. (**f**) Cells were pretreated with Ntn-1 for 30 min before being exposed to hypoxia for 72 h and Bax, Bcl-2, caspase-9 and caspase-3 were detected by Western blot. Each example shown is representative of five experiments. The right part (**e**, **f**) depicting the bars denotes the mean ± SE of five independent experiments for each condition determined from densitometry relative to β-actin. $*^{,\#}P < 0.05$ vs. control**,** $**P < 0.05$ vs. hypoxia alone. ROD, relative optical density.


a
MTT reduction (% of control)
120
80
40
0
Hypoxia - + + + +
Ntn-1 - - + - -
NAC - - - + -
Ascorbic acid - - - - +
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[3H]-thymidine incorporation (% of control)
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Ascorbic acid - - - - +
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Hypoxia - + + + +
Ntn-1 - - + - -
NAC - - - + -
Ascorbic acid - - - - +
Bax
β-actin
Bcl-2
β-actin
Cleaved caspase-9
β-actin
Cleaved caspase-3
β-actin
Bax
Bcl-2
Cleaved capase-9
Cleaved caspase-3
ROD (% of control)
300
200
100
0
Hypoxia - + + + +
Ntn-1 - - + - -
NAC - - - + -
Ascorbic acid - - - - +

Figure 2. Effect of Ntn-1, NAC, and ascorbic acid on hypoxia-induced hUCB-MSC apoptosis.

(**a**) Cells were pretreated with Ntn-1 (10 ng/ml), NAC (1 mM), or ascorbic acid (1 μM) for 30 min before being exposed to hypoxia for 72 h and analyzed for their viability by MTT assay. The values are reported as a mean ± SE of three independent experiments with triplicate dishes. *$P < 0.05$ vs. control**,** **$P < 0.05$ vs. hypoxia alone. (**b**) Cells were pretreated with Ntn-1 (10 ng/ml), NAC (1 mM), or ascorbic acid (1 μM) for 30 min before being exposed to hypoxia for 72 h and then pulsed with 1μCi of [^{3}H]-thymidine for 1 h prior to counting. The values are reported as a mean ± SE of three independent experiments with triplicate dishes. *$P < 0.05$ vs. control**,** **$P < 0.05$ vs. hypoxia alone. (**c**) Cells were pretreated with Ntn-1 (10 ng/ml), NAC (1 mM), or ascorbic acid (1 μM) for 30 min before being exposed to hypoxia for 72 h and Bax, Bcl-2, caspase-9 and caspase-3 were detected by Western blot. Each example shown is representative of five experiments. The right part (**c**) depicting the bars denotes the mean ± SE of five independent experiments for each condition determined from densitometry relative to β-actin. *$P < 0.05$ vs. control**,** **$P < 0.05$ vs. hypoxia alone. ROD, relative optical density.

In order to examine the mechanism of Ntn-1 effects, the existence of Ntn receptors such as those deleted in colorectal cancer (DCC), Un-coordinated family member (UNC) 5, neogenin, and integrin (IN) α6β4, and their function on hypoxia-induced hUCB-MSC apoptosis were determined. As shown in Figures 3a and b, DCC and IN α6β4 were detected but not UNC5A, UNC5B, UNC5C, and neogenin. Also, DCC and IN α6β4 expression were not changed by Ntn-1 or hypoxia. To further elucidate the localization of DCC and IN α6β4, DCC and IN α6β4 were assessed using immunoprecipitation and discontinuous sucrose density gradient centrifugation. DCC did not co-localize with IN α6β4, and IN α6β4 localized in the lipid raft fraction, but not DCC (Figures 3c and d). In addition, pretreatment of DCC or IN α6β4 function-blocking antibody inhibited an Ntn1-induced decrease of Bax and an increase of Bcl-2 (Figures 3e and f). These results suggest that the protective effect of Ntn-1 on hypoxia-induced hUCB-MSC apoptosis is mediated by lipid raft independent DCC and lipid raft dependent IN α6β4.

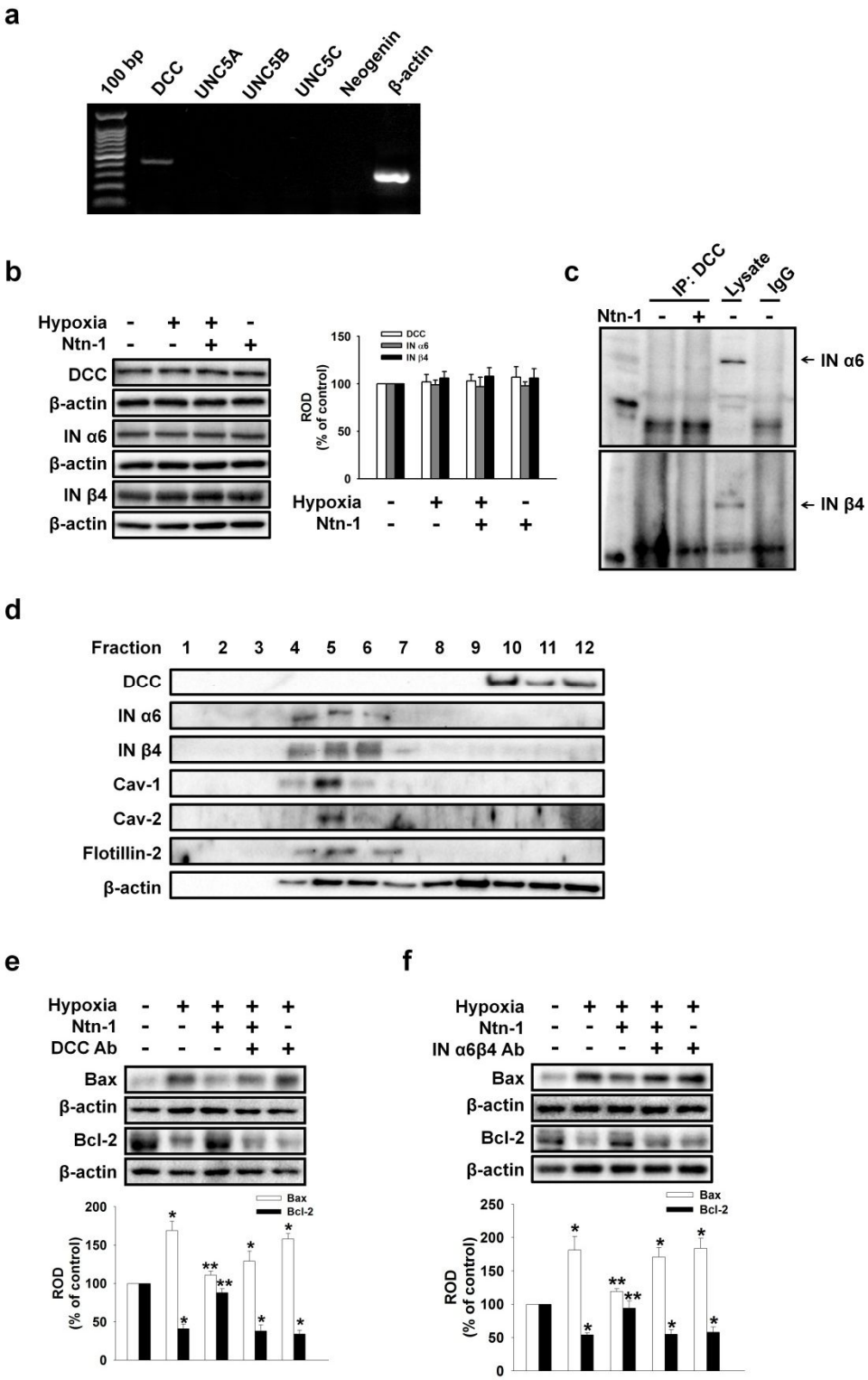

a
100 bp
DCC
UNC5A
UNC5B
UNC5C
Neogenin
β-actin
b
Hypoxia - + + -
Ntn-1 - - + +
DCC
β-actin
IN α6
β-actin
IN β4
β-actin
DCC
IN α6
IN β4
ROD (% of control)
150
100
50
0
Hypoxia - + + -
Ntn-1 - - + +
c
IP: DCC
Lysate
IgG
Ntn-1 - + - -
← IN α6
← IN β4
d
Fraction 1 2 3 4 5 6 7 8 9 10 11 12
DCC
IN α6
IN β4
Cav-1
Cav-2
Flotillin-2
β-actin
e
Hypoxia - + + + +
Ntn-1 - - + + -
DCC Ab - - - + +
Bax
β-actin
Bcl-2
β-actin
Bax
Bcl-2
ROD (% of control)
200
150
100
50
0
f
Hypoxia - + + + +
Ntn-1 - - + + -
IN α6β4 Ab - - - + +
Bax
β-actin
Bcl-2
β-actin
Bax
Bcl-2
ROD (% of control)
250
200
150
100
50
0

Figure 3. Protective effect of Ntn-1 on hypoxia-induced hUCB-MSC apoptosis via DCC and IN α6β4.

(**a**) Total RNA from hUCB-MSC was reverse transcribed, and *DCC*, *UNC5A*, *UNC5B*, *UNC5C*, and *neogenin* cDNA were amplified by PCR as described in *Materials and Methods*. Each example shown is representative of five independent experiments. (**b**) Cells were pretreated with Ntn-1 (10 ng/ml) for 30 min before being exposed to hypoxia for 72 h and DCC, IN α6 and IN β4 were detected by Western blot. (**c**) Cells were incubated in the presence of Ntn-1 for 24 h and then harvested. Cell lysates were analyzed by Western blotting with antibodies to IN α6 and IN β4. Anti-DCC immunoprecipitation was analyzed by Western blotting with IN α6 and IN β4 antibodies. (**d**) Control lysate was subjected to discontinuous sucrose density gradient fractionation, after which DCC, IN α6, IN β4, caveolin (Cav)-1, Cav-2, flotillin-2, and β-actin were detected. Each fraction was assessed by Western blot. (**e**, **f**) Cells were pretreated with DCC function-blocking antibody (2.5 μl/ml) or combination of IN α6 and IN β4 function-blocking antibodies (2.5 μl/ml) for 30 min prior to hypoxia with Ntn-1 exposure for 72 h and Bax and Bcl-2 were detected by Western blot. (**b - f**) Each example shown is representative of five experiments. The lower or right part (**b**, **e**, **f**) depicting the bars denotes the mean ± SE of five independent experiments for each condition determined from densitometry relative to β-actin. **P* < 0.05 vs. control**,** ***P* < 0.05 vs. hypoxia alone. ROD, relative optical density.

2. Effect of Ntn-1 on DCC-related anti-apoptotic protein complex, Akt, GSK-3β, and HSF-1

A cytoprotective effect of Ntn-1 is thought to occur through various signal pathways. Thus, whether or not Ntn-1 plays a role in regulating DCC dependent signal pathways including those of DCC/caspase-3, DCC/APPL-1, and APPL-1/Akt2 complex was examined. As shown in Figure 4a, the DCC/caspase-3 complex existed in normal hUCB-MSCs, but was not changed by hypoxic conditions with or without pretreatment with Ntn-1. However, Ntn-1 increased the dissociation of DCC/APPL-1 complex, which was blocked by DCC function-blocking antibody (Figure 4b). Also, the formation of APPL-1/Akt2 complex was increased by Ntn-1 (Figure 4c). Next, Akt phosphorylation which is generally well known as a key molecule in the survival pathway was examined. The phosphorylation of Akt^{thr308} and Akt^{ser473} was decreased after 72 h incubation under hypoxic conditions, whereby Ntn-1 reversed Akt^{thr308} and Akt^{ser473} phosphorylation, which was blocked by DCC and IN α6β4 function-blocking antibody (Figures 5a and b).

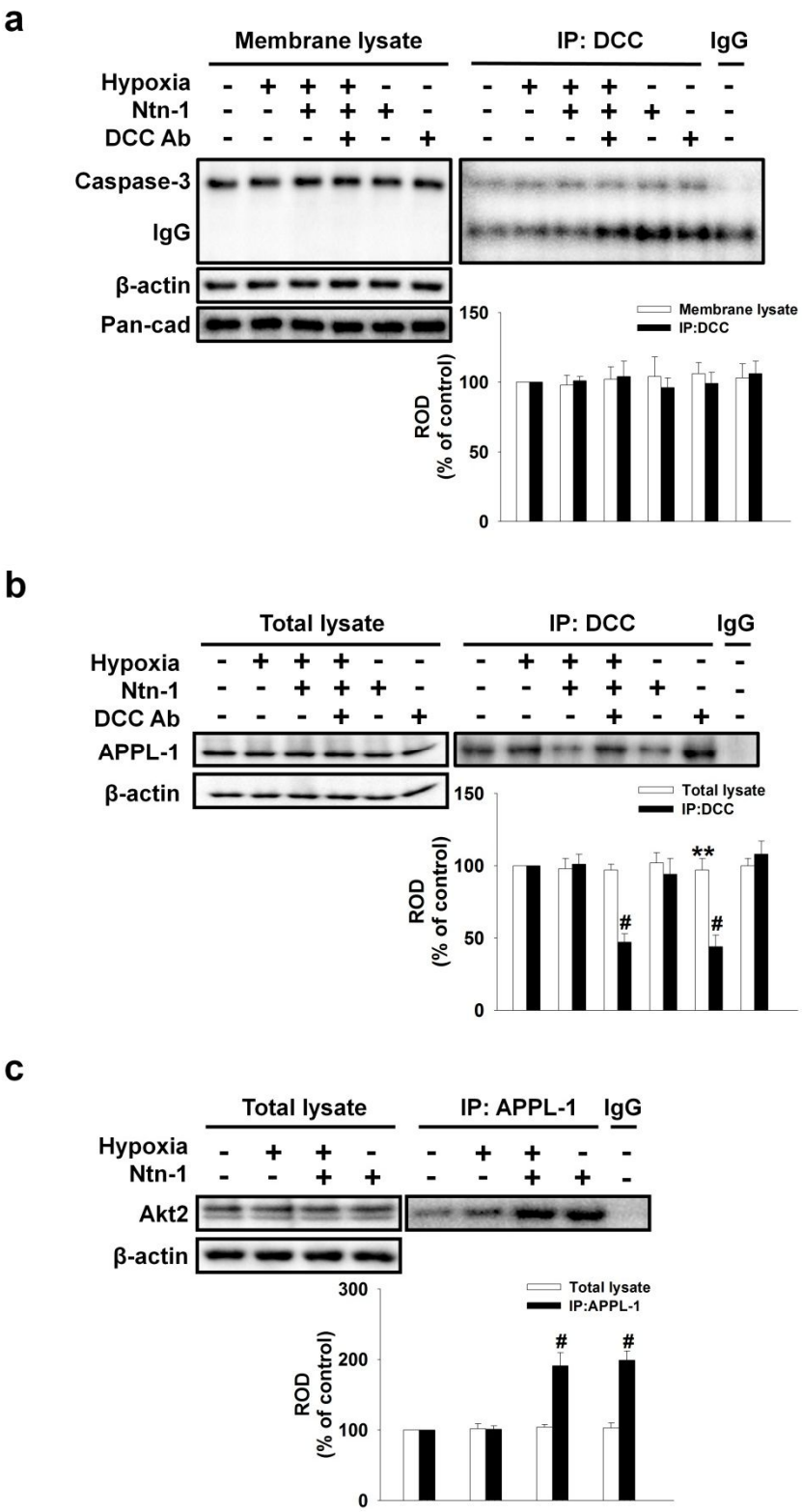
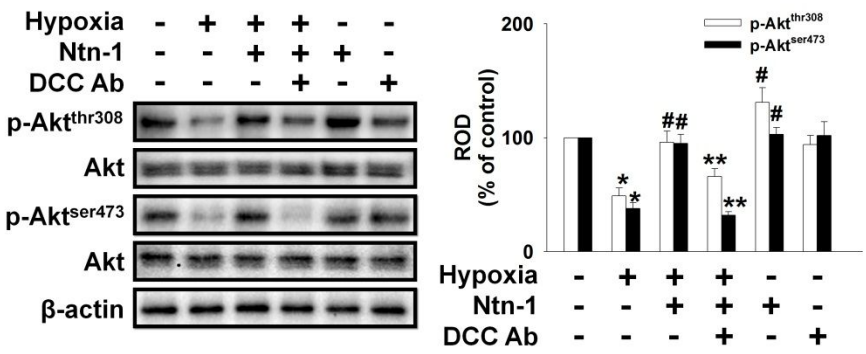

a
Membrane lysate
IP: DCC
IgG
Hypoxia
Ntn-1
DCC Ab
Caspase-3
IgG
β-actin
Pan-cad
Membrane lysate
IP:DCC
ROD
(% of control)
150
100
50
0
b
Total lysate
IP: DCC
IgG
Hypoxia
Ntn-1
DCC Ab
APPL-1
β-actin
Total lysate
IP:DCC
ROD
(% of control)
150
100
50
0
**
#
#
c
Total lysate
IP: APPL-1
IgG
Hypoxia
Ntn-1
Akt2
β-actin
Total lysate
IP:APPL-1
ROD
(% of control)
300
200
100
0
#
#

Figure 4. Involvement of DCC/caspase-3 and APPL-1/Akt2 complex.

(**a**, **b**) Cells were pretreated with Ntn-1 (10 ng/ml) or DCC function-blocking antibody (2.5 μl/ml) for 30 min prior to 72 h incubation in hypoxic condition. Cell lysates were analyzed by Western blotting with antibodies that recognize caspase-3 or APPL-1. Immunoprecipitation of anti-DCC was analyzed by Western blotting with antibodies that recognize caspase-3 or APPL-1. (**c**) Cells were pretreated with Ntn-1 (10 ng/ml) for 30 min prior to 72 h incubation in hypoxic condition. Cell lysates were analyzed by Western blotting with antibodies that recognize Akt2. Immunoprecipitation of anti-APPL-1 was analyzed by Western blotting with antibodies that recognize Akt2. (**a** - **c**) Each of the examples is representative of four independent experiments. The lower part (**a** - **c**) depicting the bars denotes the mean ± SE of four independent experiments for each condition determined from densitometry relative to β-actin. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. hypoxia alone, $^{**}P < 0.05$ vs. combination of hypoxia and Ntn-1. ROD, relative optical density.

a


Hypoxia - + + + - -
Ntn-1 - - + + + -
DCC Ab - - - + - +
p-Akt^thr308
Akt
p-Akt^ser473
Akt
β-actin
p-Akt^thr308
p-Akt^ser473
ROD (% of control)
200
100
0
Hypoxia - + + + - -
Ntn-1 - - + + + -
DCC Ab - - - + - +

b

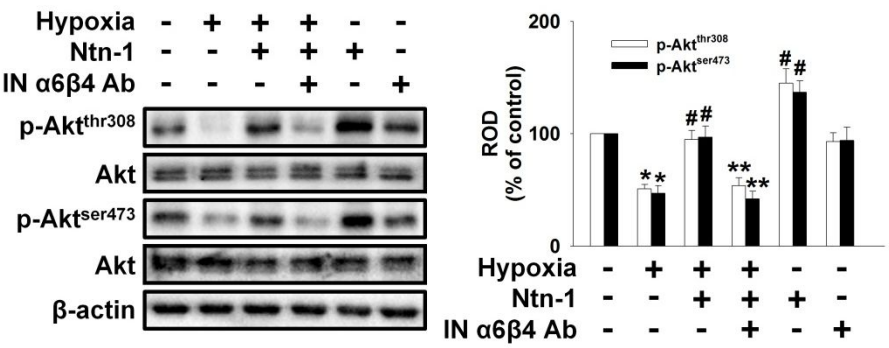

Hypoxia - + + + - -
Ntn-1 - - + + + -
IN α6β4 Ab - - - + - +
p-Akt^thr308
Akt
p-Akt^ser473
Akt
β-actin
p-Akt^thr308
p-Akt^ser473
ROD (% of control)
200
100
0
Hypoxia - + + + - -
Ntn-1 - - + + + -
IN α6β4 Ab - - - + - +

Figure 5. Involvement of Akt phosphorylation.

(**a**, **b**) Cells were pretreated with DCC function-blocking antibody (2.5 μl/ml), or combination of IN α6 and β4 (2.5 μl/ml) for 30 min prior to a 30 min Ntn-1 (10 ng/ml) treatment. And then, the cell incubated prior to 72 h in hypoxic condition. Total protein was extracted and blotted with phospho-Akt^{thr308}, phospho-Akt^{ser473}, Akt antibody. Each of the examples is representative of four independent experiments. The right part depicting the bars denotes the mean ± SE of four independent experiments for each condition determined from densitometry relative to total Akt. *$P < 0.05$ vs. control**,** $^{\#}P < 0.05$ vs. hypoxia alone, **$P < 0.05$ vs. combination of hypoxia and Ntn-1. ROD, relative optical density.

To examine whether or not Ntn-1 plays a role in the regulation of Akt related glycogen synthase kinase-3β (GSK-3β) phosphorylation, and heat shock factor-1 (HSF-1) expression, whether Ntn-1 induces GSK-3β phosphorylation and HSF-1 expression was assessed. As shown in Figures 6a and b, Ntn-1 reversed the hypoxia-induced decrease of GSK-3β phosphorylation, which was blocked by Akt inhibitor. In addition, GSK-3β inhibitor lithium chloride (LiCl) and Ntn-1 significantly increased HSF-1 levels in both the non-nuclear and nuclear fractions, confirming that the increase of HSF-1 results from GSK-3β phosphorylation (Figure 6c).

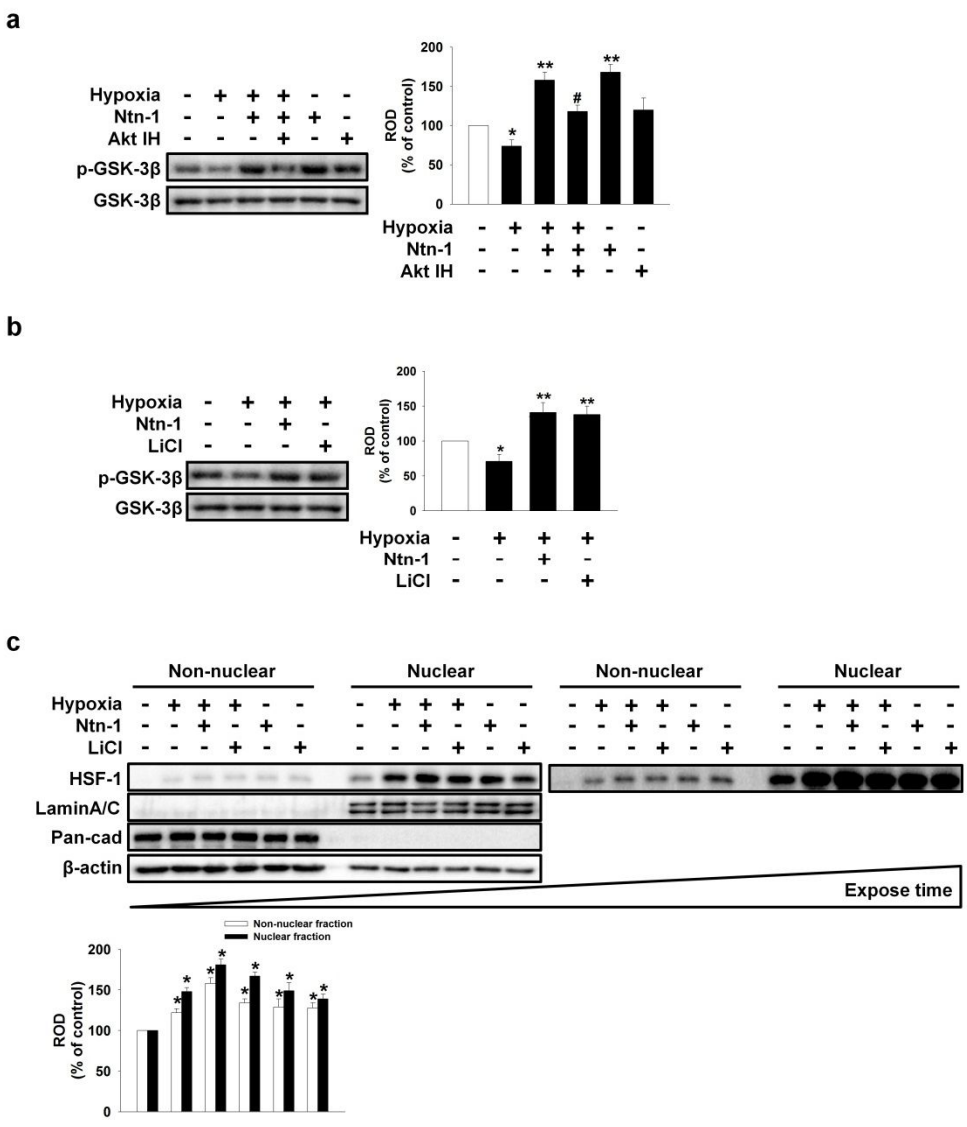

a
Hypoxia - + + + - -
Ntn-1 - - + + + -
Akt IH - - - + - +
p-GSK-3β
GSK-3β
ROD (% of control)
200
150
100
50
0
*
**
#
**
Hypoxia - + + + - -
Ntn-1 - - + + + -
Akt IH - - - + - +
b
Hypoxia - + + +
Ntn-1 - - + -
LiCl - - - +
p-GSK-3β
GSK-3β
ROD (% of control)
200
150
100
50
0
*
**
**
Hypoxia - + + +
Ntn-1 - - + -
LiCl - - - +
c
Non-nuclear
Nuclear
Non-nuclear
Nuclear
Hypoxia - + + + - - - + + + - - - + + + - - - + + + - -
Ntn-1 - - + - + - - - + - + - - - + - + - - - + - + -
LiCl - - - + - + - - - + - + - - - + - + - - - + - +
HSF-1
LaminA/C
Pan-cad
β-actin
Expose time
Non-nuclear fraction
Nuclear fraction
ROD (% of control)
200
150
100
50
0

Figure 6. Involvement of GSK-3β/HSF-1 dependent HSP expression.

(**a**) Cells were pretreated with Ntn-1 (10 ng/ml) and/or Akt inhibitor (10 μM) for 30 min before being exposed to hypoxia for 72 h and phospho-GSK-3β and GSK-3β were detected by Western blot. (**b**) Cells were pretreated with Ntn-1 or LiCl (10 mM) for 30 min before being exposed to hypoxia for 72 h and phospho-GSK-3β and GSK-3β were detected by Western blot. (**c**) Cells were pretreated with Ntn-1 or LiCl (10 mM) for 30 min before being exposed to hypoxia, and HSF-1, lamin A/C, pan-cadherin, and β-actin in the nuclear and non-nuclear fraction were detected by Western blot. Each of the examples (**a - c**) is representative of four independent experiments. The right or lower part (**a - c**) depicting the bars denotes the mean ± SE of five independent experiments for each condition determined from densitometry relative to β-actin or total GSK-3β. $^{*}P < 0.05$ vs. control**,** $^{**}P < 0.05$ vs. hypoxia alone, $^{\#}P < 0.05$ vs. combination of hypoxia and Ntn-1. ROD, relative optical density.

3. Role of heat shock protein 27 (HSP27) and Ntn-1 related signaling proteins in Ntn-1-induced cytoprotective effects

The role of HSP isoforms in Ntn-1-induced cytoprotective effects was determined. In order to determine the HSP isoform expression by hypoxia and/or Ntn-1, variations in HSP27, HSP60, HSP70, and HSP90 expression under treatment conditions were determined. As shown in Figure 7a, HSP27 expression was increased by hypoxia and/or Ntn-1, but not HSP60, HSP70, and HSP90. In addition, pretreatment of *HSF-1*-specific siRNA inhibited Ntn-1-indcued HSP27 expression (Figure 7b). Effect of basal level and efficacy of *HSF-1*-specific siRNA used in this study were determined as shown in Figure 7c. As shown in Figure 8a, Ntn-1 reversed hypoxia-induced decrease of Akt^{thr308} and Akt^{ser473} phosphorylation, which were blocked by *HSP27*-specific siRNA. Effect of basal level and efficacy of *HSP27*-specific siRNA used in this study were determined as shown in Figure 8c. The Ntn-1-induced decrease of Bax was blocked by Akt inhibitor in the cytosolic and mitochondrial fractions (Figure 8b). Moreover, Ntn-1 reversed hypoxia-induced mitochondrial dysfunction and increase of cytochrome complex (cyt c) release, which was inhibited by *HSP27*-specific siRNA (Figures 9a and b). To further determine the specificity of the HSP27 and cyt c complex formation, cell lysates were immunoprecipitated with anti-cyt c antibody, and then immunoblotted with an anti-HSP27 or an anti-HSP70 antibody. These experiments confirmed that cyt c could interact with HSP27, but not HSP70 (Figure 9c). Also, *HSP27*-specific siRNA inhibited the Ntn-1 induced increase of HSP27 and cyt c complex, but did not change cyt c expression in cell lysate (Figure 9d).

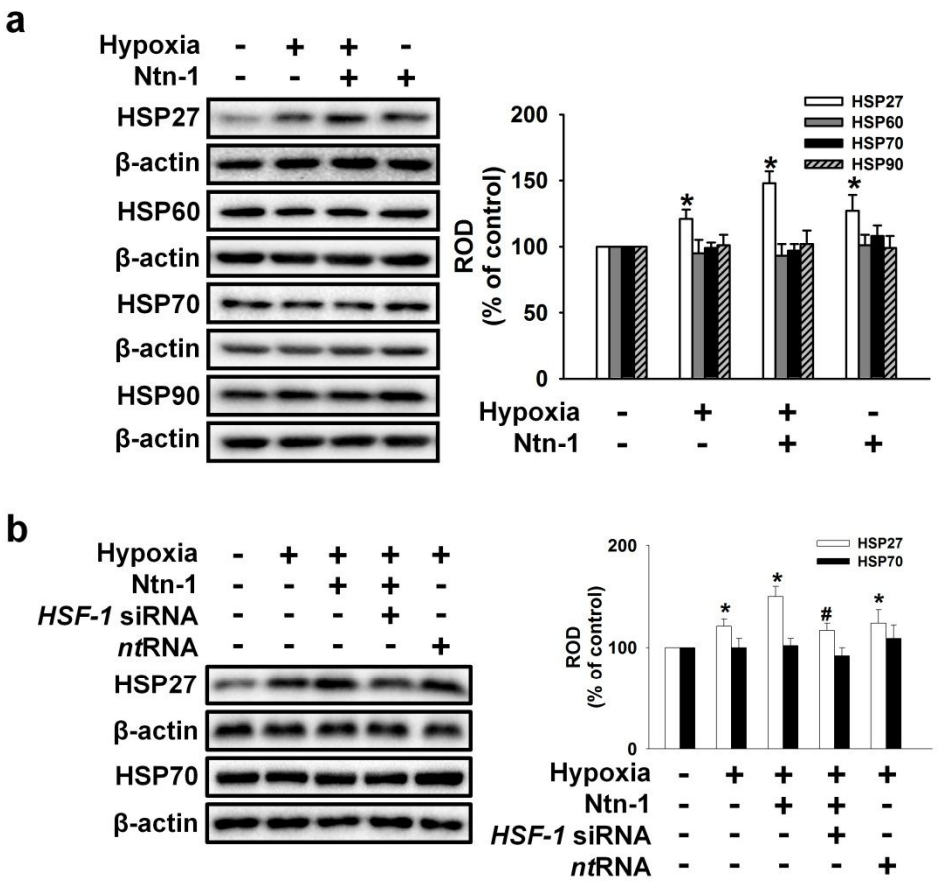
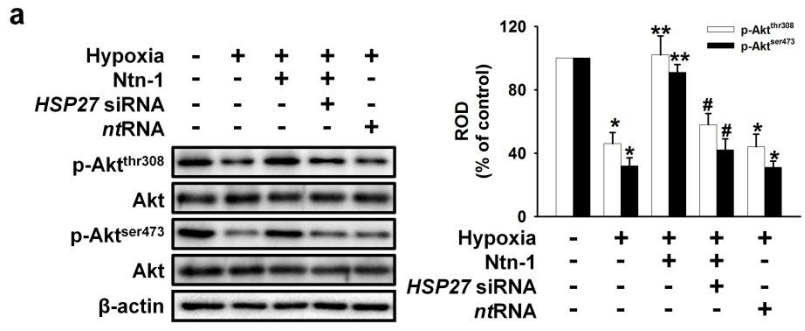
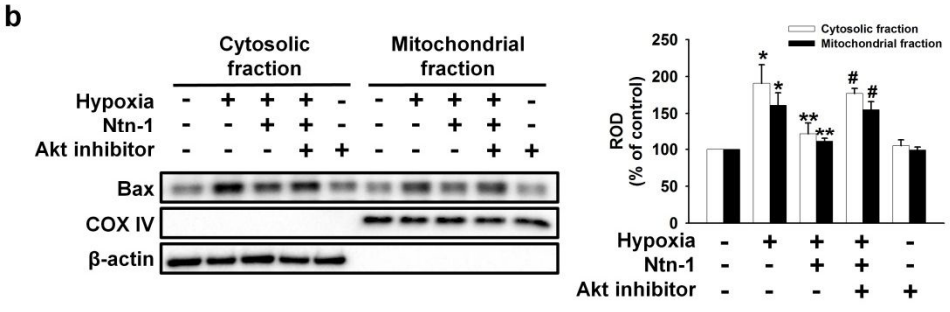

a
Hypoxia - + + -
Ntn-1 - - + +
HSP27
β-actin
HSP60
β-actin
HSP70
β-actin
HSP90
β-actin
HSP27
HSP60
HSP70
HSP90
ROD (% of control)
200
150
100
50
0
Hypoxia - + + -
Ntn-1 - - + +
b
Hypoxia - + + + +
Ntn-1 - - + + -
HSF-1 siRNA - - - + -
ntRNA - - - - +
HSP27
β-actin
HSP70
β-actin
HSP27
HSP70
ROD (% of control)
200
100
0
Hypoxia - + + + +
Ntn-1 - - + + -
HSF-1 siRNA - - - + -
ntRNA - - - - +
c
HSF-1 siRNA - + -
ntRNA - - +
HSF-1
β-actin
24 (h)
ROD (% of control)
150
100
50
0

Figure 7. Involvement of HSP27 expression.

(**a**) Cells were treated with Ntn-1 for 30 min before hypoxia exposure for 72 h and then HSP27, HSP60, HSP70, and HSP90 were detected by Western blot. (**b**) Cells were transfected for 24 h with either *HSF-1*-specific siRNA (100 nmol/l) or *nontargeting* control siRNA (100 nmol/l) using Hyperfectaine prior to hypoxia with Ntn-1 exposure for 72 h. HSP27 and HSP70 expression was analyzed using Western blot. (**c**) Cells were transfected for 24 h with either *HSF-1*-specific siRNA (100 nmol/L) or *non-targeting* control siRNA (100 nmol/L) using Hyperfectamine. HSF-1 expression was analyzed using Western blot. Each of the examples (**a** - **c**) is representative of five independent experiments. The right or lower part (**a** - **c**) depicting the bars denotes the mean ± SE of five independent experiments for each condition determined from densitometry relative to β-actin. $*P < 0.05$ vs. control**,** $**P < 0.05$ vs. hypoxia alone, $^{\#}P < 0.05$ vs. combination of hypoxia and Ntn-1. ROD, relative optical density.


a
Hypoxia - + + + +
Ntn-1 - - + + -
HSP27 siRNA - - - + -
ntRNA - - - - +
p-Akt thr308
Akt
p-Akt ser473
Akt
β-actin
ROD (% of control)
120
80
40
0
p-Akt thr308
p-Akt ser473
Hypoxia - + + + +
Ntn-1 - - + + -
HSP27 siRNA - - - + -
ntRNA - - - - +


b
Cytosolic fraction
Mitochondrial fraction
Hypoxia - + + + - - + + + -
Ntn-1 - - + + - - - + + -
Akt inhibitor - - - + + - - - + +
Bax
COX IV
β-actin
Cytosolic fraction
Mitochondrial fraction
ROD (% of control)
250
200
150
100
50
0
Hypoxia - + + + -
Ntn-1 - - + + -
Akt inhibitor - - - + +

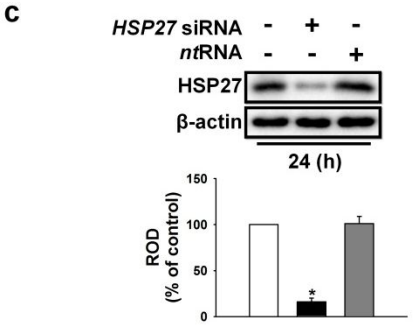

c
HSP27 siRNA - + -
ntRNA - - +
HSP27
β-actin
24 (h)
ROD (% of control)
150
100
50
0

Figure 8. Involvement of Akt phosphorylation and Bax expression.

(**a**) Cells were transfected for 24 h with either *HSP27*-specific siRNA (100 nmol/l) or *nontargeting* control siRNA (100 nmol/l) using Hyperfectaine prior to hypoxia with Ntn-1 exposure for 72 h. phospho-Akt^{thr308}, phospho-Akt^{ser473}, and Akt expression was analyzed using Western blot. (**b**) Cells were pretreated with Ntn-1 (10 ng/ml) and/or Akt inhibitor (10 μM) for 30 min before being exposed to hypoxia, and Bax, COX IV, and β-actin in the cytosolic and mitochondrial fraction were detected by Western blot. (**c**) Cells were transfected for 24 h with *HSP27*-specific siRNA (100 nmol/L) or *non-targeting* control siRNA (100 nmol/L) using Hyperfectamine. HSP27 expression was analyzed using Western blot. Each of the examples (**a** - **c**) is representative of five independent experiments. The right or lower part (**a** - **c**) depicting the bars denotes the mean ± SE of five independent experiments for each condition determined from densitometry relative to β-actin or total Akt. *$P < 0.05$ vs. control**,** **$P < 0.05$ vs. hypoxia alone, $^{\#}P < 0.05$ vs. combination of hypoxia and Ntn-1. ROD, relative optical density.

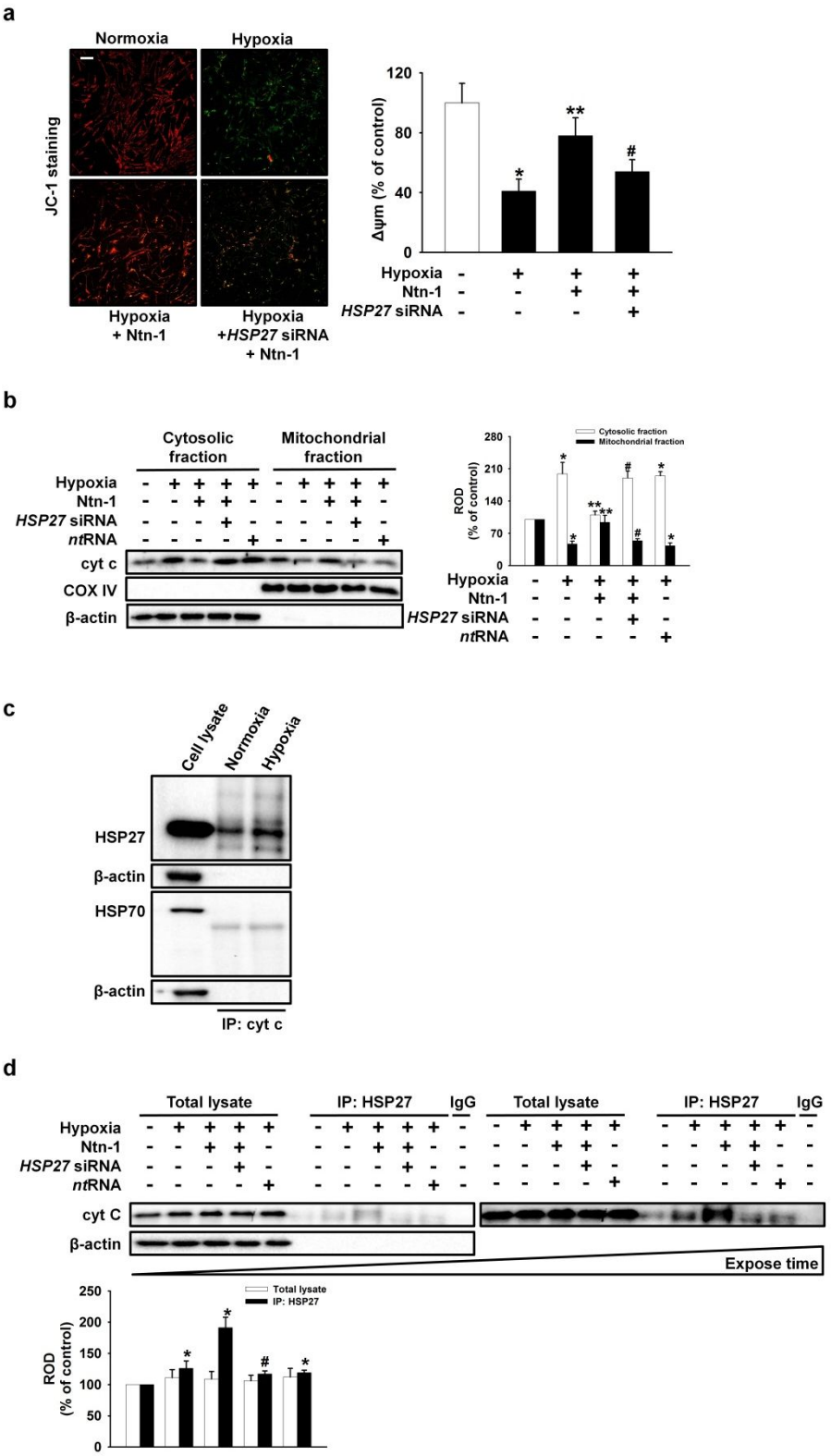

a
Normoxia
Hypoxia
JC-1 staining
Hypoxia + Ntn-1
Hypoxia +HSP27 siRNA + Ntn-1
Δψm (% of control)
120
80
40
0
Hypoxia
Ntn-1
HSP27 siRNA
b
Cytosolic fraction
Mitochondrial fraction
Hypoxia
Ntn-1
HSP27 siRNA
ntRNA
cyt c
COX IV
β-actin
ROD (% of control)
280
210
140
70
0
c
Cell lysate
Normoxia
Hypoxia
HSP27
β-actin
HSP70
β-actin
IP: cyt c
d
Total lysate
IP: HSP27
IgG
Hypoxia
Ntn-1
HSP27 siRNA
ntRNA
cyt C
β-actin
Expose time
ROD (% of control)
250
200
150
100
50
0
Total lysate
IP: HSP27

Figure 9. Effect of HSP27 on mitochondrial injury.

(**a**) Acquisition of JC-1 fluorescence images of mitochondria was performed using confocal microscopy. Quantification of mitochondria membrane potential is expressed as a ratio of J-aggregate to JC-1 monomer fluorescence intensity. Values are expressed as the mean ± SE of four independent experiments with triplicate measurement in one sample. *$P < 0.05$ vs. control, **$P < 0.05$ vs. hypoxia alone, $^{\#}P < 0.05$ vs. combination of hypoxia and Ntn-1. (**b**) Cells were pretreated with *HSP27*-specific siRNA or *nontargeting* control siRNA for 24 h prior to hypoxia with Ntn-1 exposure for 72 h and then harvested. Cyt c, COX IV, and β-actin in the cytosolic and mitochondrial fraction were detected by Western blot (**c**) Cells were exposed to hypoxia for 72 h and then immunoprecipitation of anti-cyt c was analyzed by Western blotting with antibodies that recognize HSP27 or HSP70. (**d**) Cells were pretreated with *HSP27*-specific siRNA or *nontargeting* control siRNA for 24 h prior to hypoxia with Ntn-1 exposure for 72 h and then harvested. Cell lysates were analyzed by Western blotting with antibody to cyt c. Anti-HSP27 immunoprecipitation was analyzed by Western blotting with cyt c antibody. Each of the examples (**b - d**) is representative of five independent experiments. The right or lower part (**b, d**) depicting the bars denotes the mean ± SE of five independent experiments for each condition determined from densitometry relative to β-actin. *$P < 0.05$ vs. control**,** **$P < 0.05$ vs. hypoxia alone, $^{\#}P < 0.05$ vs. combination of hypoxia and Ntn-1. ROD, relative optical density.

To investigate the involvement of DCC, IN α6β4, Akt, HSF-1, HSP27, and HSP70 on the protection of hypoxic injury by Ntn-1, hUCB-MSCs were pretreated with DCC function-blocking antibody, IN α6β4 function-blocking antibody, a combination of DCC and IN α6β4 function-blocking antibody, Akt inhibitor, *HSF-1-, HSP27-, HSP70-*, and *nontarget*-specific siRNA, prior to incubation for 72 h in hypoxic conditions both with/without Ntn-1. As shown in Figures 10a and b, DCC function-blocking antibody, IN α6β4 function-blocking antibody, the combination of DCC and IN α6β4 function-blocking antibody, Akt inhibitor, *HSF-1-,* and *HSP27*-specific siRNA decreased the Ntn-1-induced increase of MTT reduction level and [^{3}H]-thymidine incorporation level, while *HSP70*-specific siRNA did not.

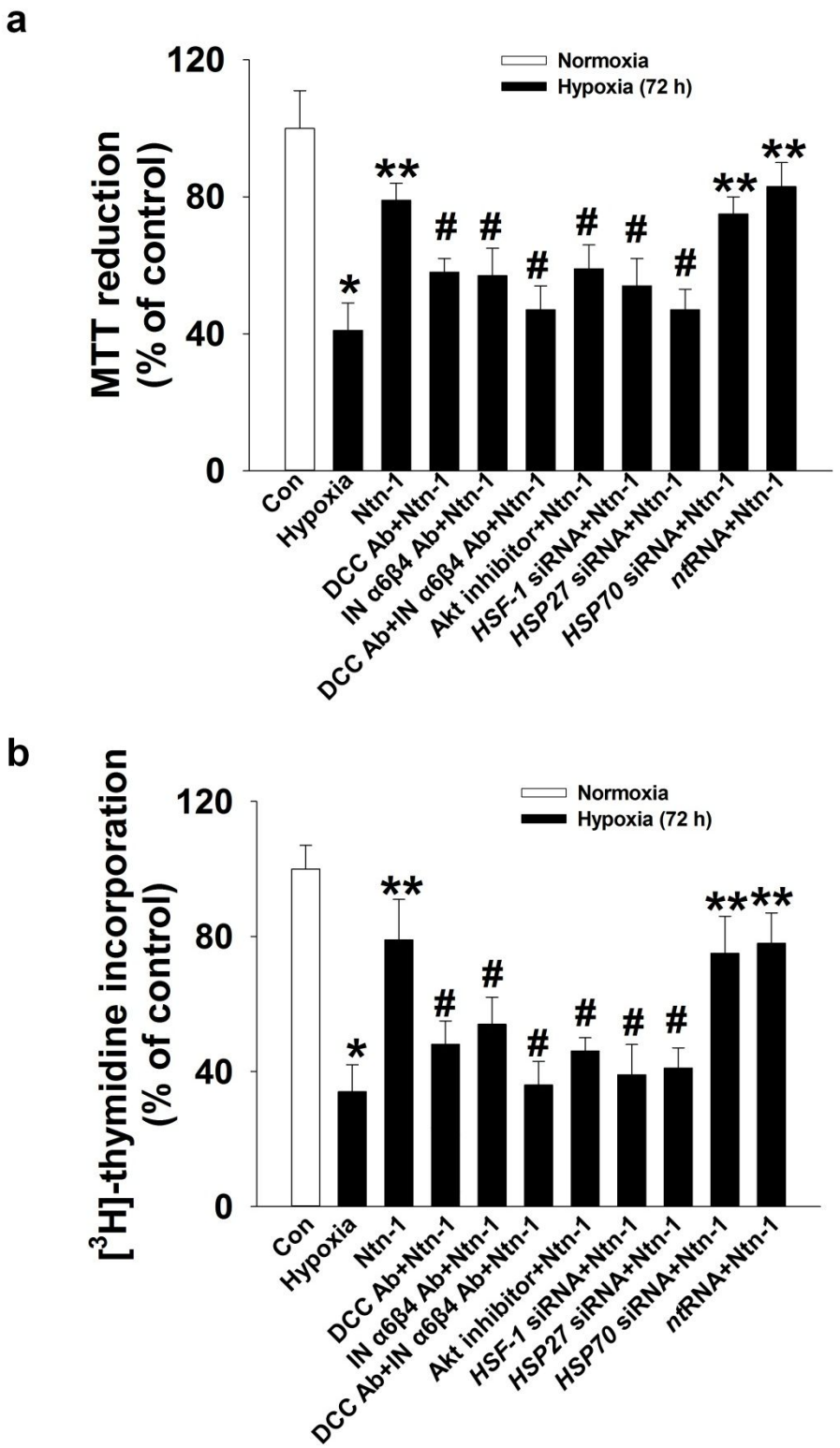

a
MTT reduction
(% of control)
120
80
40
0
Normoxia
Hypoxia (72 h)
Con
Hypoxia
Ntn-1
DCC Ab+Ntn-1
IN α6β4 Ab+Ntn-1
DCC Ab+IN α6β4 Ab+Ntn-1
Akt inhibitor+Ntn-1
HSF-1 siRNA+Ntn-1
HSP27 siRNA+Ntn-1
HSP70 siRNA+Ntn-1
ntRNA+Ntn-1
b
[3H]-thymidine incorporation
(% of control)
120
80
40
0
Normoxia
Hypoxia (72 h)
Con
Hypoxia
Ntn-1
DCC Ab+Ntn-1
IN α6β4 Ab+Ntn-1
DCC Ab+IN α6β4 Ab+Ntn-1
Akt inhibitor+Ntn-1
HSF-1 siRNA+Ntn-1
HSP27 siRNA+Ntn-1
HSP70 siRNA+Ntn-1
ntRNA+Ntn-1

Figure 10. Involvement of DCC, IN α6β4, Akt, GSK-3β, HSF-1, HSP27, and HSP70 on Ntn-1-induced protection of apoptosis in hypoxic condition.

Cells were pretreated with DCC function-blocking antibody, IN α6β4 function-blocking antibodies, combination of DCC, and IN α6β4 function-blocking antibodies, Akt inhibitor, *HSF-1*-, *HSP27*-, *HSP70*-specific siRNA, and *nontargeting* control siRNA for 30 min or 24 h prior to hypoxia with Ntn-1 exposure for 72 h. (**a**) Cells were analyzed for their viability by MTT assay. The values are reported as a mean ± SE of three independent experiments with triplicate dishes. $*P < 0.05$ vs. control, $**P < 0.05$ vs. hypoxia alone, $^{\#}P < 0.05$ vs. combination of hypoxia and Ntn-1. (**b**) Cells were incubated with 1 μCi of [^{3}H]-thymidine for 1 h prior to counting. The values are reported as a mean ± SE of three independent experiments with triplicate dishes. $*P < 0.05$ vs. control, $**P < 0.05$ vs. hypoxia alone, $^{\#}P < 0.05$ vs. combination of hypoxia and Ntn-1. ROD, relative optical density.

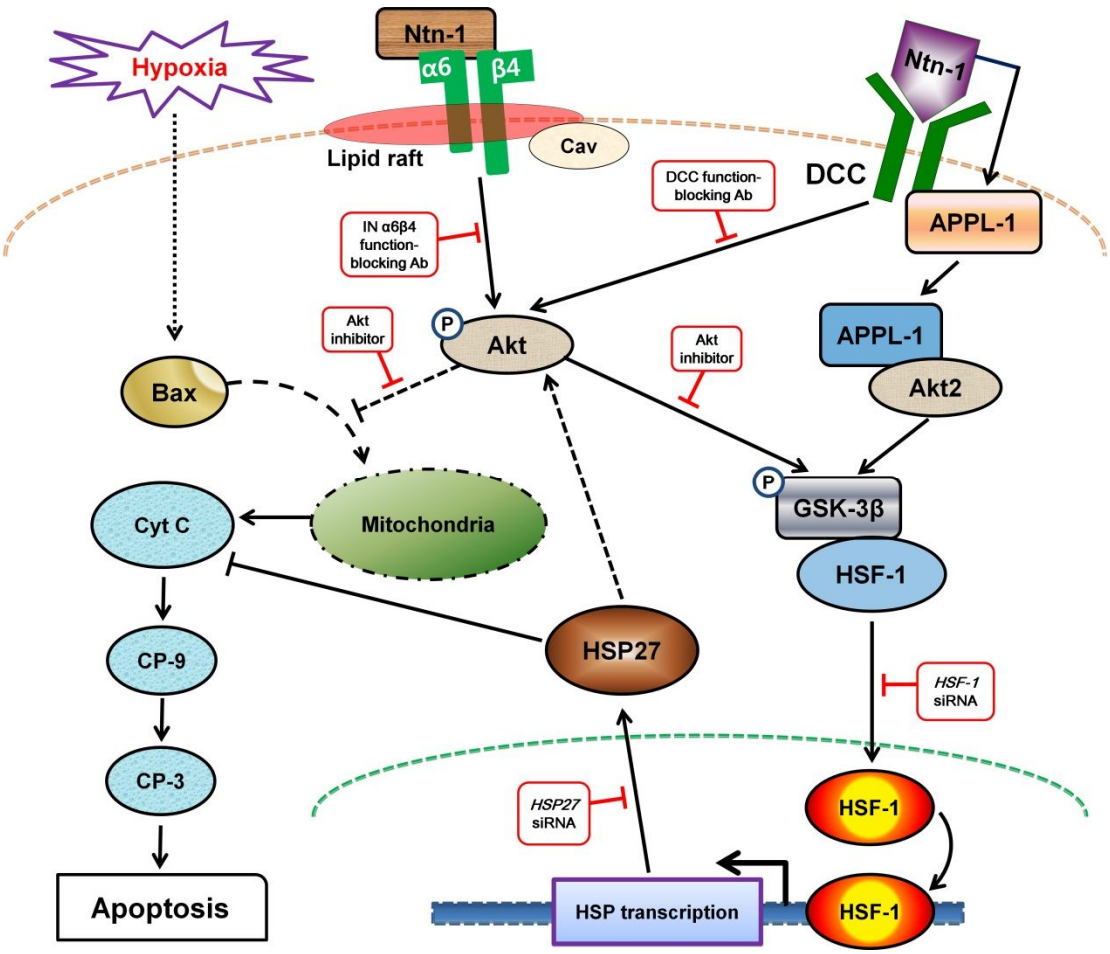

Hypoxia
Ntn-1
α6
β4
Lipid raft
Cav
Ntn-1
DCC
APPL-1
DCC function-blocking Ab
IN α6β4 function-blocking Ab
Akt inhibitor
P
Akt
Akt inhibitor
APPL-1
Akt2
Bax
Cyt C
Mitochondria
P
GSK-3β
HSF-1
CP-9
HSP27
HSF-1 siRNA
CP-3
HSP27 siRNA
HSF-1
Apoptosis
HSP transcription
HSF-1

Figure 11. Hypothetical model of Ntn-1-related anti-apoptotic effect.

Ntn-1 increased APPL-1/Akt2 complex formation and Akt phosphorylation via lipid raft-independent DCC and lipid raft-dependent IN α6β4. This signal increased GSK-3β phosphorylation and HSF-1 expression and subsequently increased HSP27 expression. Finally, Ntn-1-induced HSP27 inhibited Bax expression and cyt c release expression thereby contributing to the protecting of hypoxia-induced hUCB-MSC apoptosis. DCC function-blocking antibody, IN α6β4 function-blocking antibody, the combination of DCC and IN α6β4 function-blocking antibody, Akt inhibitor, *HSF-1-,* and *HSP27*-specific siRNA inhibited protective effect of Ntn-1 on hypoxia-induced hUCB-MSC apoptosis

Abbreviations: Ab, antibody; APPL, an adaptor protein containing a pleckstrin homology domain, a PTB domain and a leucine zipper motif; Bax, Bcl-2-associated X protein; Cav, caveolin; CP, caspase; Cyt C, cytochrome c; DCC, deleted in colorectal cancer; GSK-3β, glycogen synthase kinase 3β; HSF, heat shock factor; HSP, heat shock protein; IN, integrin; Ntn, netrin; siRNA, small interfering ribonucleic acid.

Discussion

The results of the present study demonstrate that Ntn-1 effectively protected hUCB-MSCs from hypoxia injury through inhibition of mitochondrial dysfunction via HSP27 expression through lipid raft-independent DCC-, lipid raft-dependent IN α6β4-mediated Akt/GSK-3β/HSF-1. Thus, these results strongly suggested that Ntn-1 becomes a good candidate protective agent of hypoxic injury during cell transplantation and cell therapy. Ntn-1 has broad functions and regulates many biological processes including cell proliferation, differentiation, and the determination of cell fate (Li et al., 2011; Petit et al., 2007; Xu et al., 2010), although Ntn-1 was originally thought to participate in the formation of axon (Lai Wing Sun et al., 2011). These effect of Ntn-1 is dependent on the specificity of their interaction with different types of receptors, such as DCC, UNC5, neogenin, and integrins, and the cell types in which they are differentially expressed (Bernet and Fitamant, 2008; Nikolopoulos and Giancotti, 2005; Yebra et al., 2003). Moreover, recent studies have demonstrated that the expression of these receptors takes place in embryo and SCs, suggesting the influence of Ntn on morphogenesis, development, and proliferation of SCs (Bernadskaya et al., 2012; Krishna et al., 2011; Sampath et al., 2008). In this study, DCC and IN α6β4 were existed in hUCB-MSC, but not UNC5 and neogenin. Moreover, DCC and IN α6β4 are required in lipid rafts for netrin-1 function, and may initiate various anti-apoptotic signaling transduction and regulates early embryonic development (Chen et al., 2009; Herincs et al., 2005; Nasarre et al., 2010; Whitsett and Kalinichenko, 2011).

However, in this study, IN α6β4 was located in lipid raft but not DCC in hUCB-MSCs. Also, Ntn-1 effectively protected hUCB-MSC from hypoxia-induced apoptosis, which was inhibited by DCC- and IN α6β4-function blocking antibodies. Although lipid raft dependent DCC and IN α6β4 is important to protect cell death, the results in this study strongly suggest that lipid raft independent DCC as well as lipid raft dependent IN α6β4, is a key mediator in Ntn-1-induced cytoprotection in hypoxia-induced hUCB-MSC apoptosis.

A critical matter with respect to the diverse functions of Ntn-1 is its signaling pathway involvement in cytoprotective effects, as mediated by activation of survival signaling molecules via alteration of Ntn receptor dependent molecule complex (Arakawa, 2004). Based on the results in this study, Ntn-1 upregulated APPL-1/Akt2 complex formation. These results are supported by previous studies in which DCC-dependent signaling stimulated APPL-1/Akt2 complex formation (Cirulli and Yebra, 2007; Mitsuuchi et al., 1999), and therefore might be able to activate AKT2, thereby partially contributing to the anti-apoptotic effect (Mitsuuchi et al., 1999). However, Ntn-1 did not affect the DCC/caspase-3 complex in the plasma membrane of hUCB-MSCs. Although the precise mechanisms that lead to Ntn-1-related anti-apoptotic protein complex dissociation or association remain obscure, the results in this study suggest that DCC dependent APPL-1/Ak2 complex signaling is required for Ntn-1-stimulated anti-apoptotic effects in hUCB-MSCs. However, this DCC-dependent APPL-1/Akt2 complex is simply not sufficient to fully explain cytoprotection, and the identification of other anti-apoptotic molecules is still required. Recent studies have shown that Ntn-

1/Ntn receptor interaction especially enhances signaling, regulating levels of phosphorylated Akt, which regulates various cell functions, including proliferation, motility, development, and survival (Wang et al., 2009). Many studies have implicated the INs-mediated PI3K/Akt pathway in cell protection under various stresses (Faghiri and Bazan, 2010; Miyamoto et al., 2009) and in negatively regulating Bax (Gardai et al., 2004). Thus, I assumed that Ntn receptor dependent Akt phosphorylation is also important for cytoprotection in hUCB-MSCs. The results in this study show that Ntn-1 stimulated Akt phosphorylation, which was inhibited by DCC and IN α6β4 function-blocking antibodies. The present findings suggest that DCC and IN α6β4 dependent APPL-1/Ak2 complex and Akt signaling play an important role in Ntn-1-mediated cytoprotection, although DCC and/or integrins dependent other signals such as Erk and NF-kB pathways have an effect on cell survival and protection (Donthamsetty et al., 2011; Forcet et al., 2002).

GSK-3β exists downstream of Akt and regulates many related transcription factors including HSF in several cell types (Jope and Johnson, 2004). Consistent with previous findings reported for human erythroid progenitors and muscle progenitor cells (Somervaille et al., 2001; Wang et al., 2011), anti-apoptotic protein levels were significantly increased and pro-apoptotic protein levels were decreased when GSK-3β was inactivated by Ntn. In addition, GSK-3β plays a key role in increasing levels of the HSF family, which enhances its cytoprotective effect (Jones et al., 2011). In this context, I investigated the role of GSK-3β/HSF-1 on Ntn-1 effects in hypoxia-induced hUCB-MSC apoptosis. Akt inhibitor reduced Ntn-1-induced GSK-3β phosphorylation and LiCl up-regulated Ntn-1-induced

HSF-1 expression. These results are supported by previous studies that GSK-3β-dependent signaling stimulates HSF-1 expression and translocation in the nucleus (Jones et al., 2011). Based upon these results, I suggest the possibility that Ntn-1-induced GSK-3β phosphorylation up-regulates the HSF-1-dependent signal.

Previous studies have shown that HSP isoforms, which are regulated by HSF, are implicated in maintaining cellular homeostasis and providing cellular defense by inhibition of various apoptotic signaling or by maintenance of survival signaling (Sreedhar and Csermely, 2004). Among HSP isoforms, it is well known that HSP27, HSP60, HSP70, and HSP90 are the key factors responsible for protection of cellular damage (Sreedhar and Csermely, 2004). Interestingly, in human endothelial cells, HSP isoform mRNA and protein expression were up-regulated by hypoxia or ischemic injury (Kang et al., 2008). In this study, hypoxia and Ntn-1 promoted HSP27 expression, but not HSP60, HSP70, or HSP90 expression. In addition, it was observed that *HSF-1*-specific siRNA only prevented an Ntn-1-induced increase of HSP27 expression, which suggests that Ntn-1-induced increase of HSP27 expression are HSF-1 dependent in hUCB-MSCs. Although HSF-1 regulated other HSP isoforms (Sreedhar and Csermely, 2004), this discrepancy of results might due to differences in species, cell types, or experimental conditions. Moreover, in this context, I investigated the role of Ntn-1-induced HSP27 on apoptotic signal transduction pathways. *HSP27*-specific siRNA attenuated Ntn-1-induced Akt phosphorylation, and Akt inhibitor prevented Ntn-1-reduced Bax expression. These results are supported by previous studies that HSP27 inhibited Bax expression via PI3K-dependent mechanism in human embryonic kidney cells

(Havasi et al., 2008). In addition, Ntn-1 significantly inhibited hypoxia-induced loss of mitochondrial membrane potential and cyt c release in mitochondria. Also, Ntn-1 increased cyt c and HSP27 complex formation which was inhibited by *HSP27*-specific siRNA. These results are consistent with previous studies which suggested that HSP27 may inhibit cyt c release by specifically interacting with mitochondria or interfering with apoptosome formation (Bruey et al., 2000; Samali et al., 2001). The present result demonstrated that Ntn-1-related HSP27 is a key player involved in the inhibition of hypoxia-induced hUCB-MSC apoptosis through blockage of cyt c release in mitochondria and/or inhibition of Bax expression. Taken together, the results in this study show that within the broader range of Ntn-1 receptor, intracellular signaling to regulate pro-, anti-apoptotic protein through the HSF-1 and HSP27 cascade is important to generate Ntn-1-dependent protective strategies of hypoxia-induced hUCB-MSC apoptosis (Figure 7). Therefore, identifying the mechanistic basis of Ntn receptor-mediated HSP27 by Ntn-1 may offer important insights to better understand the role of Ntn-1 in hypoxic injury, and then might be a powerful tool or a potential therapeutic candidate for modulating hUCB-MSC functions and future tissue regenerative strategies. In conclusion, Ntn-1 stimulates HSP27 expression through DCC- and IN α6β4-dependent APPL-1, Akt, GSK-3β, and HSF-1 signaling pathways, thereby partially contributing to the protection of hypoxia-induced hUCB-MSC apoptosis via inhibition of mitochondrial dysfunction.

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국 문 초 록

저산소 유도 인간 제대혈 중간엽 줄기세포의 미토콘드리아성 세포자멸사에 대한 netrin-1의 HSP27 발현에 의한 방어 효과

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네트린은 제대혈 유래 중간엽 줄기세포를 이용한 치료에 있어 높은 조직친화성을 지닌 세포자멸 억제 인자로 이용될 수 있다. 하지만 네트린의 저산소성 손상에 대한 보호 효과의 기전에 대해서는 아직 밝혀진 바가 없다. 따라서 이 연구에서는 저산소에 의한 인간 제대혈 유래 중간엽 줄기세포의 세포자멸에 대한 네트린-1의 효과와 그 기전에

대해 조사했다. 72시간 동안의 저산소 상태에서 감소된 MTT reduction과 [^{3}H]-thymidine incorporation은 네트린-1 (10 ng/ml)에 의해 증가되었다. 또한, 네트린-1은 저산소에 의해 유도된 Bax, cleaved caspase-9, cleaved caspase-3의 발현을 감소시키고, 저산소에 의해 감소되는 Bcl-2의 발현을 증가시켰다. 저산소성 손상에 대한 네트린-1의 보호효과와 관련된 신호전달체계를 조사하기 위해 네트린 수용체를 분석한 결과 UNC5A-C를 제외한 DCC와 integrin (IN) α6β4만이 존재하였으며, 이 중 IN α6β4만이 지질 래프트(lipid raft)에 존재했다. 그리고 네트린-1은 DCC와 APPL-1 복합체의 분리를 유도해서 APPL-1과 Akt2 복합체의 형성을 촉진시켰고, 저산소에 의해 감소된 Akt와 GSK-3β의 인산화를 증가시켰다. Ntn-1에 의해 유도된 Akt와 GSK-3β의 인산화는 DCC와 IN α6β4의 기능 차단 항체와 Akt 억제제에 의해 억제되었다. 저산소 또는 네트린-1의 처리 시 HSP27의 발현이 증가하였고, 이는 *HSF-1* 특이적인 siRNA에 의해 차단되었다. 더불어 *HSP27* 특이적인 siRNA는 네트린-1에 의해 증가된 Akt의 인산화를 감소시켰다. 네트린-1은 cytochrome c (cyt c) 방출 및 cyt c와 HSP27 복합체의 형성을 억제함으로써 미토콘드리아 막의 손상을 보호했고, 이는 *HSP27* 특이적인 siRNA에 의해 차단되었다. 저산소 상태에서 네트린-1의 세포보호 효과와 관련된 신호 단백질들을 각각의 siRNA, 기능 차단 항체, 또는 억제제로 차단한 결과 네트린-1에 의한 세포자멸 억제 효과가 감소되었다. 결론적으로 이 연구에서는 네트린-1이 DCC와 IN α6β4

의존적인 Akt, GSK-3β, HSF-1 신호전달경로를 통해 HSP27의 발현을 증가시켜 저산소성 손상과 관련된 제대혈 유래 중간엽 줄기세포의 세포자멸을 보호하는 것으로 밝혀졌다.

주요어: 인간 제대혈 유래 중간엽 줄기세포; 네트린-1; 저산소성 손상; 세포자멸; 세포보호; 열충격단백질.

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