Netrin-1 protects hypoxia-induced mitochondrial apoptosis through HSP27 expression via DCC- and integrin α6β4-dependent Akt, GSK-3β, and HSF-1 in mesenchymal stem cells

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Netrin (Ntn) has the potential to be successfully applied as an anti-apoptotic agent with a high affinity for tissue, for therapeutic strategies of umbilical cord blood-derived mesenchymal stem cells (UCB-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 UCB-MSC apoptosis, as well as the potential underlying mechanisms of its protective effect. Hypoxia (72 h) reduced cell viability (MTT reduction, and [3H]-thymidine incorporation) and cell number, and induced apoptosis (annexin and/or PI positive), which were reversed by Ntn-1 (10 ng/ml). Moreover, Ntn-1 decreased the increase of hypoxia-induced Bax, cleaved caspase-9, and -3, but blocked the decrease of hypoxia-reduced Bcl-2. Next, in order to examine the Ntn-1-related signaling cascade in the protection of hypoxic injury, we analyzed six Ntn receptors in UCB-MSC. We identified deleted in colorectal cancer (DCC) and integrin (IN) α6β4, except uncoordinated family member (UNC) 5A–C, and neogenin. 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 glycogen synthase kinase 3β (GSK-3β) phosphorylation, which is involved in heat shock factor-1 (HSF-1)-expression. Ntn-1-induced phospho-Akt and -GSK-3β were inhibited by DCC function-blocking antibody, IN α6β4 function-blocking antibody, and the Akt inhibitor. Hypoxia and/or Ntn-1 stimulated heat shock protein (HSP)27 expression, which was blocked by HSF-1-specific small interfering RNA (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 the inhibition of cytochrome c (cyt c) release and formation of cyt c and HSP27 complex. Moreover, the inhibition of each signaling protein attenuated Ntn-1-induced blockage of apoptosis. In conclusion, Ntn-1-induced HSP27 protected hypoxic injury-related UCB-MSC apoptosis through DCC- and IN α6β4-dependent Akt, GSK-3β, and HSF-1 signaling pathways.

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Human mesenchymal stem cells (hMSCs) have distinct characteristics, with a self-renewal capacity and the ability to generate multiple differentiated cell types. Therefore, hMSCs have been targeted in the field of developmental biology and used in a vast number of therapeutic applications. Although several studies involving clinical applications have generated multiple differentiated cell types. Therefore, hMSCs have a self-renewal capacity and the ability to generate multiple differentiated cell types. Therefore, hMSCs have been targeted in the field of developmental biology and used in a vast number of therapeutic applications.

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Abbreviations: Ab, antibody; APPL, an adapter 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; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; GSK-3β, glycogen synthase kinase 3β; HSP, heat shock factor; HSP, heat shock protein; 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; PI, propidium iodide; 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-Tween-20; TCA, trichloroacetic acid; UCB-MSC, umbilical cord blood-derived mesenchymal stem cell; UNC, uncoordinated family member
transplantation, although short-term hypoxic conditions stimulated SC viability, injected or transplanted SCs underwent long-term hypoxic conditions, and subsequent long-term hypoxic conditions were the leading causes of cell death. Thus, it is important to illuminate tissue-affinitive protective factors of hypoxic injury in order to apply MSC to regeneration strategies, and therefore, its mechanisms of 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.9 With respect to apoptosis, Ntn-1 has been introduced as an anti-apoptotic agent against hypoxic injury of the brain tissue.9,10 Therefore, it is possible that Ntn-1 in the extracellular plasma environment may be involved in a protective capacity against hypoxia-induced UCB-MSC (umbilical cord blood-derived mesenchymal stem cell) apoptosis. Recent findings have implicated Ntn in the regulation of adult SC migration, tumor cell survival, and embryonic development, suggesting potentially novel means of promoting recovery from cellular injury and achieving improvements in tissue regeneration.11–13 In addition, Ntn is crucial to maintain the survival of Ntn-receptor-expressed cells during tissue regeneration.14 In this regard, Ntn, Ntn receptors, and the associated downstream signaling mechanisms involved are promising targets for the prevention of extracellular damage. However, there are no previous reports related to anti-apoptotic effects of tissue-affinitive Ntn-1 in UCB-MSCs for regeneration strategies. We assume that exogenous and tissue-affinitive Ntn-1 can prevent 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 UCB-MSC apoptosis and its related signal pathways.

Results

Effect of Ntn-1 on hypoxia-induced UCB-MSC apoptosis via lipid raft-independent DCC and -dependent IN x6β/4.

To examine whether or not Ntn-1 has a role in regulating DCC-dependent signal pathways, including those of DCC/caspase-3, DCC/APPL-1, and APPL-1/Akt complexes. As shown in Figure 3a, the DCC/caspase-3 complex existed in normal UCB-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 3b). Moreover, the formation of APPL-1/Akt2 complex was increased by Ntn-1 (Figure 3c). Next, we examined Akt phosphorylation, which is generally well known as a key molecule in the survival pathway. The phosphorylation of AktThr308 and AktSer473 was decreased after 72 h incubation under hypoxic conditions, whereby Ntn-1 reversed AktThr308 and AktSer473 phosphorylation, which was blocked by DCC and IN x6β/4 function-blocking antibody (Figures 3d and e).

To examine whether or not Ntn-1 has a role in the regulation of Akt-related glycogen synthase kinase-3β (GSK-3β) phosphorylation and heat shock factor-1 (HSF-1) expression, we assessed whether Ntn-1 induces GSK-3β phosphorylation and HSF-1 expression. As shown in Figures 4a and b, Ntn-1 reverses 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 4c).
Role of heat shock protein 27 (HSP27) and Ntn-1-related signaling proteins in Ntn-1-induced cytoprotective effects. Heat shock protein (HSP) isoforms in Ntn-1-induced cytoprotective effects were determined. In order to determine the HSP isoform expression by hypoxia and/or Ntn-1, we determined variations in HSP27, HSP60, HSP70, and...
HSP90 expression under treatment conditions. As shown in Figure 5a, HSP27 expression was increased by hypoxia and/or Ntn-1, but not HSP60, HSP70, and HSP90. In addition, pretreatment of HSF-1-specific small interfering RNA (siRNA) inhibited Ntn-1-induced HSP27 expression (Figure 5b). As shown in Figure 5c, Ntn-1 reversed the hypoxia-induced decrease of Akt thr308 and Akt ser473 phosphorylation, which were blocked by HSP27-specific siRNA. The Ntn-1-induced decrease of Bax was blocked by Akt inhibitor in the cytosolic and mitochondrial fractions (Figure 5d). Moreover, Ntn-1 reversed hypoxia-induced mitochondrial dysfunction and increase of cytochrome complex (cyt c) release, which were inhibited by HSP27-specific siRNA (Figures 5e and f). To further determine the specificity of the HSP27 and cyt c complex formation, cell lysates were immunoprecipitated with the anti-cyt c antibody, and were 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 5g). Moreover, HSP27-specific siRNA inhibited the Ntn-1-induced increase of HSP27 and cyt c complex, but did not change cyt c expression in cell lysates (Figure 5h).

To investigate the involvement of DCC, IN α6/4, Akt, HSF-1, HSP-27, and HSP70 in the protection of hypoxic injury by Ntn-1, UCB-MSCs were pretreated with DCC and IN α6/4 function-blocking antibodies, a combination of DCC and IN α6/4 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 6a and b, pretreatment of DCC and IN α6/4 function-blocking antibodies, the combination of DCC and IN α6/4 function-blocking antibody, Akt inhibitor, HSF-1, and HSP27-specific siRNA decreased the Ntn-1-induced increase of MTT reduction level, and [3H]-thymidine incorporation level. In addition, these treatments blocked Ntn-1-induced reduction of annexin V and/or PI-positive cells, whereas HSP70-specific siRNA did not (Figure 6c).

Discussion

The results of the present study demonstrate that Ntn-1 effectively protects UCB-MSCs from hypoxia injury through the inhibition of mitochondrial dysfunction via HSP27 expression through lipid raft-independent DCC- and lipid raft-dependent IN α6/4-mediated Akt/GSK-3β/HSF-1. Thus, our findings strongly suggested that Ntn-1 is 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. Although Ntn-1 was originally thought to participate in the formation of axon, these effects of Ntn-1 are dependent on the specificity of their interaction with different types of receptors, such as DCC, UNC5, neogenin, and INs, and the cell types in which they are differentially expressed. Moreover, recent studies have demonstrated that the expression of these receptors takes place in embryos and SCs, suggesting the influence of Ntn on morphogenesis, development, and proliferation of SCs. In this study, we found that DCC and IN α6/β4 existed in UCB-MSC, but UNC5 and neogenin were not found. Moreover, DCC and IN α6/β4 are required in lipid rafts for Ntn-1 function, and may initiate various anti-apoptotic signaling transduction, and regulate early embryonic development. However, according to our data, IN α6/β4 was located in the lipid raft, but not in the DCC in UCB-MSCs. We also found that Ntn-1 effectively protects cells from hypoxia-induced UCB-MSC apoptosis, which was inhibited by DCC- and IN α6/β4-function-blocking antibodies. Although lipid raft-dependent DCC and IN α6/β4 are important to prevent cell death, our results strongly suggest that lipid raft-independent DCC, as well as lipid raft-independent IN α6/β4 are the key mediators in Ntn-1-induced cytoprotection in hypoxia-induced UCB-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 the activation of survival signaling molecules via the alteration of Ntn receptor-dependent molecule complex. Based on our results, 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, and therefore, might be able to activate Akt2, thereby partially contributing to the anti-apoptotic effect. However, Ntn-1 did not affect the DCC/caspase-3 complex in the plasma membrane of UCB-MSCs. Although the precise mechanisms that lead to Ntn-1-related anti-apoptotic protein complex

Figure 3: Involvement of DCC/caspase-3, APPL-1/Akt2 complexes and Akt phosphorylation. (a and b) Cells were pretreated with Ntn-1 (10 ng/ml) or DCC function-blocking antibody (2.5 μg/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. (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 antibody that recognize Akt2. (d and e) Cells were pretreated with DCC function-blocking antibody (2.5 μg/ml), or combination of INs α6 and β4 (2.5 μg/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<sup>th73</sup>, phospho-Akt<sup>ser473</sup>, or Akt antibody. (a–e) Each of the examples is representative of four independent experiments. The right or lower part (a–e) depicting the bars denotes the mean ± S.E. of four independent experiments for each condition determined from densitometry relative to β-actin or total Akt. *P<0.05 versus control, **P<0.05 versus combination of hypoxia and Ntn-1. ROD, relative optical density.
dissociation or association remain obscure, our results suggest that DCC-dependent APPL-1/Akt2 complex signaling is required for Ntn-1-stimulated anti-apoptotic effects in UCB-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 the levels of phosphorylated Akt, which regulates various cell functions, including proliferation, motility, development, and survival. Many studies have implicated the INs-mediated PI3K/Akt pathway in cell protection under various stresses and in negatively regulating Bax. Thus, we assumed that Ntn receptor-dependent Akt phosphorylation is also important for cytoprotection in UCB-MSCs. Our results show that Ntn-1 stimulated Akt phosphorylation, which was inhibited by DCC- and IN x6/4 function-blocking antibodies. The present findings suggest that DCC- and IN x6/4-dependent APPL-1/Akt2 complex and Akt signaling have important roles in Ntn-1-mediated cytoprotection, although DCC and/or INs are dependent on other signals, such as Erk and NF-κB pathways, which have an effect on cell survival and protection.

**Figure 4** 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; 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; 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 part (a–c) depicting the bars denotes the mean ± S.E. of four independent experiments for each condition determined from densitometry relative to β-actin or total GSK-3β. *P < 0.05 versus control, **P < 0.05 versus hypoxia alone, ***P < 0.05 versus combination of hypoxia and Ntn-1. ROD, relative optical density.

DCC- and INs are dependent in UCB-MSCs. Although HSF-1 regulated other HSP isoforms, the discrepancy of these results might due to differences in species, cell types, or experimental conditions. Moreover, in this context, we investigated the role of Ntn-1-induced increase of HSP27 expression, which suggests that the Ntn-1-induced increase of HSP27 expression is HSF-1-dependent in UCB-MSCs. Although HSF-1 regulated other HSP isoforms, this discrepancy of these results might due to differences in species, cell types, or experimental conditions. Moreover, in this context, we investigated the role of Ntn-1-induced HSP27 expression, which suggests that the Ntn-1-induced increase of HSP27 expression is HSF-1-dependent in UCB-MSCs. Although HSF-1 regulated other HSP isoforms, this discrepancy of these results might due to differences in species, cell types, or experimental conditions. Moreover, in this context, we investigated the role of Ntn-1-induced increase of HSP27 expression, which suggests that the Ntn-1-induced increase of HSP27 expression is HSF-1-dependent in UCB-MSCs.
In addition, we found that Ntn-1 significantly inhibited the hypoxia-induced loss of mitochondrial membrane potential and cyt c release in mitochondria. Moreover, Ntn-1 increases cyt c and HSP27 complex formation, which is inhibited by HSP27-specific siRNA. The results are consistent with previous studies, which have suggested that HSP27 might inhibit cyt c release by specifically interacting with mitochondria or interfering with apoptosis formation.

Figure 5  Role of HSP27 in Ntn-1-induced cytoprotective effects. (a) Cells were treated with Ntn-1 (10 ng/ml) for 30 min before hypoxia exposure for 72 h, and then HSP27, HSP80, HSP70, and HSP90 were detected by western blot. (b and c) Cells were transfected for 24 h with either HSF-1- or HSP27-specific siRNA (100 nmol/l) or non-targeting control siRNA (100 nmol/l) using Hyperfectamine prior to hypoxia with Ntn-1 exposure for 72 h. HSP27, HSP70, phospho-Akt ( Thr308), phospho-Akt (Ser473), and Akt expressions were analyzed using western blot. (d) 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. (e) Acquisition of JC-1 fluorescence images of mitochondria was performed using confocal microscopy. Quantification of mitochondrial membrane potential is expressed as a ratio of J-aggregate to JC-1 monomer fluorescence intensity. Values are expressed as the mean ± S.E. of four independent experiments with triplicate measurement in one sample. *P < 0.05 versus control, **P < 0.05 versus hypoxia alone, #P < 0.05 versus combination of hypoxia and Ntn-1. ROD: Relative Optical Density.
inhibition of hypoxia-induced UCB-MSC apoptosis through the blockage of cyt c release in mitochondria and/or inhibition of Bax expression. Taken together, our results show that within the broader range of Ntn-1 receptor, intracellular signaling to regulate pro- and anti-apoptotic proteins through the HSF-1 and HSP27 cascade is important to generate Ntn-1-dependent protective strategies of hypoxia-induced UCB-MSC apoptosis (Figure 7). Therefore, identifying the mechanistic basis of Ntn receptor-mediated HSP27 by Ntn-1 may offer important insights into better understand the role of Ntn-1 in hypoxic injury, and therefore, might be a powerful tool or a potential therapeutic candidate for modulating UCB-MSC functions, as well as 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 UCB-MSC apoptosis via the inhibition of mitochondrial dysfunction.

Materials and Methods

Materials. UCB-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 NAC were obtained from Sigma Chemical Company (St. Louis, MO, USA). Anti-Akt2, APPL-1, Bax, β-actin, caveolin-1, caveolin-2, caspase-3, caspase-9, cyt c, flotillin-2, GSK-3β, HIF-1α, HSF-1, HSP27, HSP60, HSP70, IgG, integrin α6, integrin β4, lamin A/C, pan-cadherin, phospho-Akt ( thr308, 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). [3H]-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 (Parisippany, NJ, USA). FITC Annexin V Apoptosis Detection Kit I was purchased from BD Pharmingen (Franklin Lakes, NJ, USA). All other reagents were of the highest purity commercially available and were used as received.
Isolation and characterization of human UCB-MSCs. The isolation and characterization of human UCB-MSCs were performed by cell surface marker analysis and multilineage differentiation in our previous report. After the initial 31 days of primary culture, UCB-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 subcultured, the structure of fibroblast-like cells appeared polygonal or spindly with a long process (appearance and growth of fibroblast-like hMSC colonies on the 31st day of culture as well as hMSCs at passages 4 and 12 of culture before multilineage differentiation). UCB-MSC passages 4–12 were observed under a microscope. Cells appeared normal on the basis of typical morphology. UCB-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 UCB-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).

Culture of UCB-MSCs. UCB-MSCs were cultured without a feeder layer in phenol-red-free Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) supplemented with sodium bicarbonate, penicillin and streptomycin, L-glutamine, 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% CO2. 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.

Hypoxic treatment of UCB-MSCs. UCB-MSCs were washed twice with PBS, and the medium was exchanged with fresh DMEM. Experiments were performed in a modular incubator chamber at 37 °C for 30 min under normoxic (92.5% air and 5.5% CO2) or hypoxic (2.2% O2, 5.5% CO2, and 92.5% N2) 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. In this study, Ntn-1 did not affect HIF-1α protein expression level (Supplementary Figure 2). We determined hypoxic efficacy using HIF-1α antibody (Supplementary Figure 2).

[^H]-thymidine incorporation. [^H]-thymidine incorporation experiments were carried out as previously described. 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 the supplements and indicated agents. After the indicated incubation period, 1 μCi of [methyl-[^H]]-thymidine was added to the cultures. Incubation with [^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 ± S.E. of triplicate experiments. The values were converted from absolute counts to a percentage of the control in order to allow the comparison between experiments.

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Cell number and viability assay. The cells were pretreated with Ntn-1 (10 ng/ml) for 30 min before being exposed to hypoxia for 72 h and washed twice with PBS. The cells were then dissociated from the culture dishes utilizing a 0.05% trypsin and 0.5 mM EDTA solution, and the detachment was quenched with Soybean trypsin inhibitor (0.05 mg/ml). Subsequently, 0.4% (w/v) trypsin blue solution (500 μl) was added to the cell suspension, and the dead, live, and total cells were counted using Countess automated cell counter (Invitrogen, Carlsbad, CA, USA).

Fluorescence-activated cell sorter (FACS) analysis. The cells were pretreated with Ntn-1 (10 ng/ml) for 30 min before being exposed to hypoxia for 72 h. They were then dissociated in trypsin/EDTA pelleted by centrifugation. The cells were resuspended in PBS containing 0.1% bovine serum albumin (BSA). Next, they were resuspended in 1× Binding Buffer, and then stained with 5 μl of FITC Annexin V and 5 μl of PI, which were included in the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen). Then the cells were kept at 25 °C for 15 min in the dark. The stained cells were analyzed by flow cytometry (Beckman Coulter, Fullerton, CA, USA). At least 10^6 events were recorded for each sample. The samples were analyzed using CXP software (Beckman Coulter).

Gene silencing with siRNA. UCB-MSCs were grown until 75% of the surface of the plate was covered, after which they were transfected for 24 h with either an siRNA specific for HSF-1 (100 nM; Santa Cruz Biotechnology; sc-35611), HSP27 (100 nM; Santa Cruz Biotechnology; sc-23350), HSP70 (100 nM; Dharmacco, Lafayette, CO, USA; L-056710-00) or a non-targeting siRNA as a negative control (100 nM; Dharmacco; D-001206-13-20) with Hyperfectamine (qiangen, Valencia, CA, USA), according to the manufacturer’s instructions. In this study, we determined each siRNA efficacy and effect of basal level, respectively (Supplementary Figure 3).

Cell fractionation. For fractionation of cells into cytosolic, membrane and nuclear fractions, we used a detergent fractionation method that was described previously.47 UCB-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 as the cytosolic fraction. The remainder of the cells was lysed with lysis buffer, scraped from the plastic, and centrifuged at 15,800 × g for 15 min. The supernatant was designated as the membrane fraction, and the pellet as the nuclear fraction. The nuclear fraction was resuspended in PBS, sonicated, and centrifuged to remove undissolved material.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from UCB-MSCs using STAT-60, which is a monophosphoric 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(17) 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 s, annealing temperature (Table 1) for 30 s, and 72 °C for 30 s, followed by 5 min of extension at 72 °C. Amplifications of DCC, UNC5A, UNC5B, UNC5C, and Neogenin cDNA were performed in UCB-MSCs, using the primers described in Table 1. PCR of β-actin was also performed as a control for RNA quantity.

Immunoprecipitation. Immunoprecipitation lysates were incubated with appropriate antibody and protein A-agarose beads with gentle shaking overnight. Samples were washed thrice with lysis buffer and collected 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 (DesignSoft, Budapest, Hungary).

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 (22,000 × g at 4 °C for 10 min). Protein concentration was determined by the Bradford method.49 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–TWEEN-20) (10 mM Tris-HCI (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.

Detergent-free purification of caveolin-rich membrane fraction. Caveolin-enriched membrane fractions were prepared as described previously.50 Cells were washed twice with ice-cold PBS, scraped into 2 ml of 50 mM sodium carbonate (pH 11.0), transferred into a plastic tube, and homogenized with a Sonicator 250 apparatus (Branson Ultrasonic, Danbury, CT, USA) using three 20-s bursts. The homogenate was adjusted to 45% sucrose by the addition of 2 ml of 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 30,000 rpm, for 20 h in a SW 41 Rotor (Beckman Coulter). Twelve fractions (1 ml each) were collected and analyzed by 10–12% SDS-PAGE.

Detection of mitochondrial membrane potential. Loss of the mitochondrial membrane potential (ΔΨm) was assessed by using the fluorescent probe JC-1 (Molecular Probes, Eugene, OR, USA), which exists predominantly in the monomeric form in cells with depolarized mitochondria and is fluorescent

| Gene     | Identification | Primer sequence, 5′–3′ | Annealing Temperature (°C) | Size (bp) | Ref. |
|----------|----------------|------------------------|----------------------------|-----------|------|
| DCC      | Sense          | CAAGTGCCCCCCTCGAAGCG   | 55                         | 434       | 51   |
|          | Antisense      | GCTCCCAAGCCATACGGGATAT | 56                         | 440       | 52   |
| UNC5A    | Sense          | GCACAAGCGGAAGACATGAG   | 56                         | 420       | 52   |
| UNC5B    | Sense          | CAAAGAGCAAGGTACCTGGGCTCCTTCCCGGT | 56 | 440 | 52 |
| UNC5C    | Sense          | GGACACAGGACAAGATGTCG   | 56                         | 420       | 52   |
| Neogenin | Sense          | ATGGGCGGCGCTTGGTCTTTC  | 60                         | 380       | 30   |
| β-Actin  | Sense          | AGCCATCTGGACGAGCCATTTCTC | 55 | 350 | 53 |

Abbreviations: DCC, deleted in colon cancer; Ref, references; UNC5A, uncoordinated-5-A; UNC5B, uncoordinated-5-B; UNC5C, uncoordinated-5-C.
green at 490 nm. Cells with polarized mitochondria predominantly contain JC-1 in an aggregated form and are fluoresce reddish-orange. Cells were incubated with 5 μM JC-1 for 10 min at 37 °C, 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 mitochondrial 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 Inc., Waltham, MA, USA) 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 Δψm.

Isolation of mitochondria. UCB-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, Rockford, IL, USA) according to the manufacturer’s instructions.

Statistical analysis. Results are expressed as means ± S.E. 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.

Conflict of Interest

The authors declare no conflict of interest.

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