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Role of DARPP-32 in the Proliferation and Survival of Human Neural Stem Cells

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To investigate the role of dopamine- and cAMP-regulated neuronal phosphoprotein 32 kDa (DARPP-32), also known as protein phosphatase 1 regulatory subunit 1B (PPP1R1B), in cell proliferation and survival of human neural stem cells (hiNSCs) induced from human adipose-derived stem cells, DARPP-32 expression was knocked down by a lentiviral vector. Cell proliferation was relatively slower and doubling time was longer in the DARPP-32-knockdown hNSCs than in the scrambled control. Oxidative stress testing showed that DARPP-32 knockdown caused more cell death in hiNSCs after hydrogen peroxide (H_2O_2) treatment than in the scrambled control. The expressions of *CTNNB1, CXCR4,* and *VCAM-1*, which are related to cell proliferation, were lower in the DARPP-32-knockdown hiNSCs than in the scrambled control. In addition, DARPP-32 knockdown decreased the expression of *Bcl-2* and increased the expression of *Bax* in response to H_2O_2 treatment. We also confirmed reduced expression of phosphorylated Akt (p-AKT) in DARPP-32-knockdown hiNSCs. In conclusion, these findings demonstrate the mechanisms by which DARPP-32 positively affects cell proliferation and survival in hiNSCs.

Keywords: Cell proliferation, Cell survival, DARPP-32, Neural stem cell, Oxidative stress

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Introduction

Adult stem cells (ASCs) possess a great potential for stem cell therapy, especially in the central nervous system (CNS)[1]. ASCs can overcome the limitations of embryonic stem cells (ESCs) associated with their ethical problems, and induced pluripotent stem cells (iPSCs) associated with their cancer-prone tendencies. This study used human adipose derived stem cells (hADSCs), a type of ASC, that were differentiated into human induced neural stem cells (hiNSCs) without genetic modification.

Since the majority of the cells in the CNS were originated from neural stem cells (NSCs), NSCs have been used in studies on neurogenesis and various neurological disorders. NSCs have two characteristics: self-renewal and multi-potentiality[2]. NSCs have the ability to differentiate into neurological cells, such as neurons and glial cells. Thus, the underlying mechanisms of their differentiation into neurons or glial cells are an important aspect of the study of neural development and the treatment of brain diseases.

The function of dopamine- and cAMP-regulated neuronal phosphoprotein 32kDa (DARPP-32), also known as protein phosphatase 1 regulatory subunit 1B (PPP1R1B), has been studied a lot in cancer cells, brain tissues, and neurons. There are two forms of DARPP-32: full length DARPP-32 (f-DARPP-32) containing four phosphorylation (P) sites [threonine 34 (T34), threonine 75 (T75), serine 102 (S102), and serine 137 (S137)], and truncated DARPP-32 (t-DARPP-32) which lacks the T34 phosphorylation site of DARPP-32[3]. Thus, t-DARPP-32 cannot control the physiological activities of several key proteins using the potent inhibition of protein phosphatase 1 (PP1)[3].

Dopaminergic and glutamatergic receptor stimulation regulates phosphorylation of DARPP-32[4]. For example, DARPP-32 plays various important roles in dopaminergic neurons through the dopaminergic signaling pathways. Many studies suggest that dopaminergic dysfunction is associated with Parkinson's disease and Huntington's disease[4]. Impairment of dopaminergic neurotransmission is associated with schizophrenia[4]. These findings indicate that DARPP-32 may serve as a potential therapeutic target for the treatment of neurologic and psychiatric diseases.

However, there are very few studies related to the function of DARPP-32 in hNSCs. NSCs grow quickly during the proliferative stage of brain, and differentiate into neurons and glia[5]. Thus, the number of neurons is decided on the proliferative potential of NSCs in the brain[6]. The number of neurons generated during development is important for circuit formation and brain size[6]. Therefore, regulation in the proliferation of NSCs is important for normal development of the brain[6].

In this study, we sought to identify the function of DARPP-32 related to cell proliferation and cell survival in hNSCs. We observed that cell proliferation rate was reduced and doubling time was increased in DARPP-32-knockdown hiNSCs compared to the scrambled control. The cell viability rate of the DARPP-32-knockdown hiNSCs was also reduced compared to the scrambled control with or without oxidative stress. Taken together, our study suggests a novel mechanism by which DARPP-32 positively regulates proliferation and survival of hNSCs.

Materials and methods

Culture and Induction of hADSCs into iNSCs

Isolated hADSCs were maintained in culture medium consisting of alpha-minimum essential medium (α-MEM; HyClone) supplemented with 10% fetal bovine serum (Gibco) and 100 μg/mL penicillin and streptomycin (Gibco). Cells were subcultured every 3-4 days at a density of 1,400 cells/cm^2 and maintained at 37°C with 5% CO_2. hADSCs were induced into iNSCs as previously reported[7]. Briefly, hADSCs (passages 4-5) were seeded on gelatin-coated dishes (10,000 cells/cm^2) in NSC induction medium, consisting of Dulbecco's modified Eagle's medium with F12 (DMEM/F12; Gibco) supplemented with 3% knockout serum replacement (KOSR; Gibco), 1% glutamax (Gibco), 1% nonessential amino acids (Gibco) and 4 ng/mL basic fibroblast growth factor (bFGF; Peprotech), for 8 days with three small molecules: 10 μM SB431542 (Sigma), 100 ng/ml noggin (R&D), and 0.5 μM LDN193289 (Stemgent) (STEP1). Next, cells were cultured for 5 days in neurobasal medium (Gibco):DMEM/F12 (1:1) supplemented with 2% B27 (Gibco), 1% N_2 (Gibco), 1% Glutamax (Gibco) and 200 μM ascorbic acid (Sigma) (STEP2). The medium was replaced with the aforementioned medium supplemented with 20 ng/mL epidermal growth factor (EGF; Peprotech) and 20 ng/mL bFGF, and cells were cultured for another 7 days (STEP3).

Transduction (Infection) of Scramble and DARPP-32 shRNA

The hiNSCs were seeded on poly-l-ornithine (PLO; Sigma, 10 ㎍/ml)/ fibronectin (FN; Sigma, 1 ㎍/ml) coated plates (25,000 cells/cm^2) and incubated at 37℃ for 24 hours. After being washed in 1X phosphate-buffered saline (PBS), hiNSCs were infected with a mixture of complete medium (scrambled control and DARPP-32 lentivirus: B27-free STEP2 medium = 9 : 1 ratio, polybrene 8 ㎍/ml). Because hiNSCs had a low infection efficiency, they were stabilized for at least 3 days before being re-infected. Thus, the infection of hiNSCs was carried out over two steps, increasing the infection efficiency of hiNSCs. After 72 hours, GFP expression of infected cells was observed under a digital inverted fluorescence microscope (Nikon). The medium was replaced with fresh growth medium containing puromycin (0.1 μg/ml) at 96 hours. Then, RNA or protein sample preparation was performed, and the stable cell lines were analyzed at DIV10.

Cell proliferation assay

hiNSCs were seeded on PLO (10 ㎍/ml)/ FN (1 ㎍/ml) coated plates (10,000 cells/cm^2). Absorbance was measured at day 0 (3 hours), day 2 (48 hours), and day 4 (96 hours). Cell proliferation rate was analyzed using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The doubling-time [duration x log(2)/ log(final concentration)−log(initial concentration] was calculated.

Oxidative stress testing

After hiNSCs were treated with hydrogen peroxide (H_2O_2, 0.1 mM), they were incubated at 37℃ for 10 minutes. The control consisted of only DMEM/F12 medium. After being washed twice in DMEM/F12, STEP3 medium was added to each well and the hiNSCs were incubated at 37℃ for 24 hours. Cell

viability after H_2O_2 treatment was analyzed using an MTT assay.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA samples were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). The reverse transcription from RNA to cDNA was carried out using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) at 42℃ for 1 hours. cDNA (2 ㎍) was used as the template in the PCR reactions (total volume= 20 ㎕), 10X buffer (Intron BioTechnology, Seongnam, Korea) and dNTPs (Intron BioTechnology), primers (10 pM, Table 1), and Taq DNA polymerase (Takara Bio, Tokyo, Japan). The samples were visualized by electrophoresis using 1% agarose gel. The data were normalized to the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and quantified using the Image J program (NIH, Bethsesda, MD, USA).

Table 1. List and information of primers

Gene	Primer	Sequences	Product size (base pairs)	Annealing temperature (℃)	Cycle number	Gene bank Accession number
Full length DARPP-32 (PPP1R1B)	Forward	5' ACCGCAAGAAGATCCAGTTC 3'	198	55	34	NM_032192.4
	Reverse	5' GGTTGGGTCTCTTCGACTTG 3'				
Truncated DARPP-32 (PPP1R1B)	Forward	5' CATGCTGTTCCGGCTCTC 3'	104	55	34	NM_181505.3
	Reverse	5' GGTTGGGTCTCTTCGACTTG 3'				
Nestin	Forward	5’ CACCTGTGCCAGCCTTTCTTA 3’	170	60	40	NM_006617
	Reverse	5’ TTTCCTCCCACCCTGTGTCT 3’				
Pax6	Forward	5’ AGGTATTACGAGACTGGCTCC 3’	104	60	40	NM_001604
	Reverse	5’ TCCCGCTTATACTGGGCTATTT 3’				
Musashi1	Forward	5’ TTCGGGTTTGTCACGTTTGAG 3’	250	60	40	NM_002442
	Reverse	5’ GGCCTGTATAACTCCGGCTG 3’				
CTNNB1	Forward	5' CACAAGCAGAGTGCTGAAGGTG 3'	146	55	34	NM_001330729.2
	Reverse	5' GATTCCTGAGAGTCCAAAGACAG 3'				
CXCR4	Forward	5' CCCTCCTGCTGACTATTCCC 3'	135	55	34	NM_003467.3
	Reverse	5' TAAGGCCAACCATGATGTGC 3'				
VCAM-1	Forward	5' GAGGGTCTACCAGCTCCAGA 3'	199	55	34	NM_080682.3
	Reverse	5' GATCTCTAGGGAATGCTTGAACA 3'				
Bcl-2	Forward	5' AGGCTGGGATGCCTTTGTGGAA 3'	108	55	34	NM_000633.2
	Reverse	5' CAAGCTCCCACCAGGGCCAAA 3'				
Bax	Forward	5' GCCGGGTTGTCGCCCTTTTC 3'	159	55	34	NM_001291428.1
	Reverse	5' GCAGCCCCCAACCACCCTG 3'				
GAPDH	Forward	5’ GTCAGTGGTGGACCTGACCT 3’	256	59	30	NM_001256799
	Reverse	5’ CACCACCCTGTTGCTGTAGC 3’				

Western blotting

Proteins (20 ㎍/well) were loaded on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and blotted with anti-DARPP-32 (Santa Cruz Biotechnology, CA, USA), anti-Akt (Cell Signaling, Beverly, MA, USA), and anti-p-Akt (Cell Signaling) at 1:1000 dilution. Anti-α-tubulin (Sigma-Aldrich, St Louis, MO, USA) at 1:10000 dilution was used as the protein loading control on the same membrane. Primary antibodies were detected with anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma-Aldrich, St Louis, MO, USA) at 1:5000 dilution. Image J program was used for quantification of western blotting.

Statistical analysis

Results were analyzed using analysis of variance (ANOVA) and Student's t-test. All data were expressed as means ± standard error of the mean (SEM) with each sample being measured with at least 3 (N≥3) independent experiments. P values less than 0.05 (P<0.05) were considered statistically significant.

Results

DARPP-32-knockdown in hiNSCs

To investigate the role of DARPP-32 in hNSCs, the DARPP-32 gene was knocked down using lentiviruses containing green fluorescent protein (GFP) labled-DARPP-32-shRNA plasmid. GFP-scrambled-shRNA was used as a control. Lentiviral infection was confirmed by GFP expression (Fig. 1A). We observed the knockdown of both *f-DARPP-32* (***P<0.01) and *t-DARPP-32* (*P<0.05) at the RNA level in the DARPP-32-knockdown hiNSCs compared to the scrambled control (Fig. 1B, C).

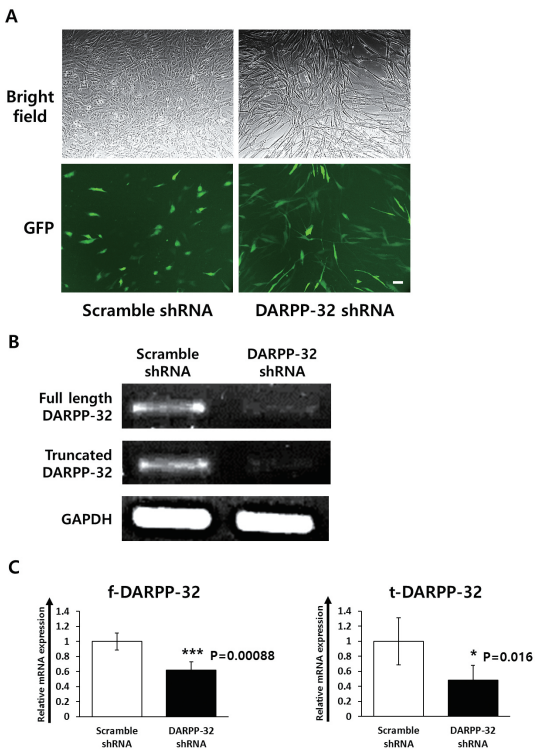
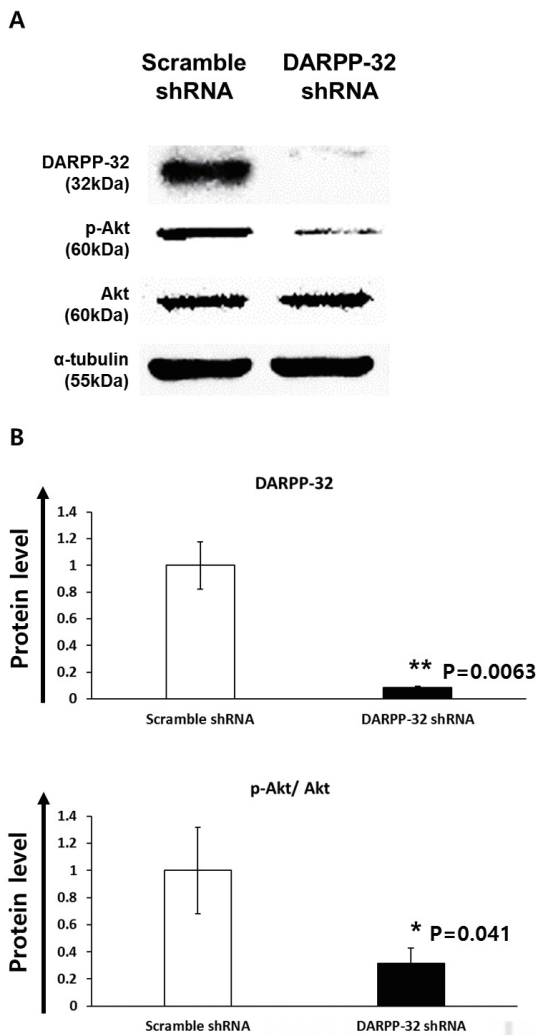


Figure 1. DARPP-32-knockdown in hiNSCs. (A) Bright-field and fluorescent images of lentiviral infected (scrambled and DARPP-32 shRNA) hiNSCs. Scale bar: 100㎛. (B) RT-PCR bands of *f-DARPP-32, t-DARPP-32,* and *GAPDH* expression in the scrambled control and the DARPP-32 (knockdown) shRNA-infected hiNSCs. (C) Quantitative analysis of RT-PCR bands. *GAPDH* was used to normalize the gene expression. The data were analyzed using Student's t-test. Error bars represent mean ± standard error of the mean (S.E.M.), n=6. * P<0.05, ***P<0.001 compared to scrambled shRNA-infected hiNSCs.

DARPP-32-knockdown reduces the expression of p-Akt in hiNSCs

Next, we examined the protein expressions of DARPP-32, p-Akt, and Akt in the scrambled control and the DARPP-32-knockdown hiNSCs (Fig. 2). We observed that both the expressions of DARPP-32 (**P<0.01) and p-Akt (*P<0.05) were reduced in the DARPP-32-knockdown hiNSCs compared to the scrambled control (Fig. 2).



DARPP-32-knockdown inhibits cell proliferation in hiNSCs

The cell proliferation assay showed that proliferation rate was reduced at day 2 and day 4 (*P<0.05 and **P<0.01, respectively, Fig. 3A) and doubling time was increased (Fig. 3B) in DARPP-32-knockdown hiNSCs compared to the scrambled control. Although the p-value for doubling time (P=0.051) was not statistically significant, increasing the number of samples would likely produce statistical significance. This result indicates that DARPP-32-knockdown caused a suppression of proliferation in hiNSCs.

Figure 2. Reduced expression of p-Akt by DARPP-32-knockdown in hiNSCs. (A) Western blot bands of DARPP-32, p-Akt, Akt, and α-tubulin expression of scrambled (control) and DARPP-32 (knockdown) shRNA-infected hiNSCs. α-tubulin was used as loading control to normalize the levels of proteins. (B) Quantitative analysis of western blot bands. The data were analyzed using Student's t-test. Error bars represent mean ± S.E.M., n=6. *P<0.05, **P<0.01 compared to scrambled shRNA-infected hiNSCs.

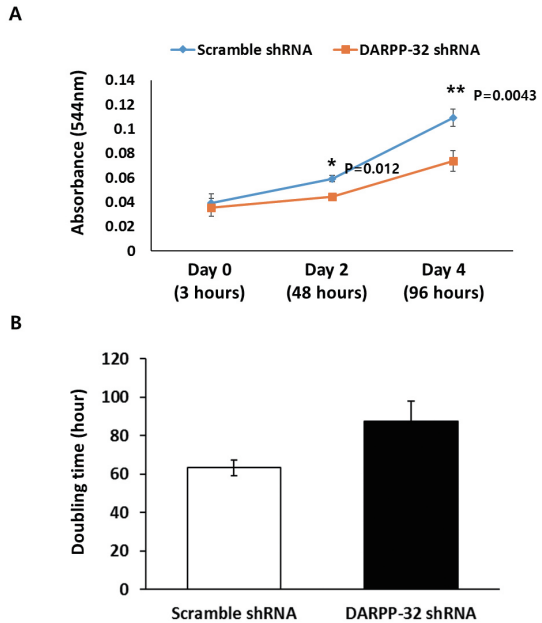


Figure 3. Cell proliferation assay in hiNSCs. (A) MTT assay in hiNSCs. Absorbance was recorded on day 0 (3 hours), day 2 (48 hours), and day 4 (96 hours). (B) Doubling time (hour) of MTT assay in hiNSCs. The data were analyzed using Student's t-test. Error bars represent mean ± S.E.M., n=4. All the experiments were performed in triplicate. *P<0.05, **P<0.01 compared to scrambled shRNA-infected hiNSCs.

DARPP-32-knockdown inhibits cell survival in response to oxidative stress in hiNSCs

Next, we sought to examine the role of DARPP-32 in iNSC survival in response to oxidative stress. After the scrambled control and the DARPP-32 shRNA-infected hiNSCs were treated with H_2O_2 (0.1 mM), we observed that the cell viability of DARPP-32-knockdown hiNSCs was lower than in the scrambled control, indicating that DARPP-32-knockdown inhibits cell survival in hiNSCs with or without oxidative stress (Fig. 4A).

We further examined the expression of genes related to proliferation and survival in the control and the DARPP-32-knockdown hiNSCs before and after H_2O_2 treatment using RT-PCR analysis (Fig. 4B). The mRNA expression of *CTNNB1, CXCR4,* and *VCAM-1* was lower in the DARPP-32-knockdown hiNSCs after treatment with H_2O_2 (0.1 mM) than in the scrambled control (Fig. 4C). In addition, a decreased *Bcl-2* expression and an increased *Bax* expression indicate increased apoptosis and decreased cell survival in the DARPP-32-knockdown hiNSCs compared to the scrambled control (Fig. 4C). In particular, after the DARPP-32-knockdown hiNSCs were treated with H_2O_2 (0.1 mM), *Bcl-2* expression was at its lowest, and *Bax* expression was at its highest, confirming increased cell death in the hiNSCs compared to the scrambled control (Fig. 4C).

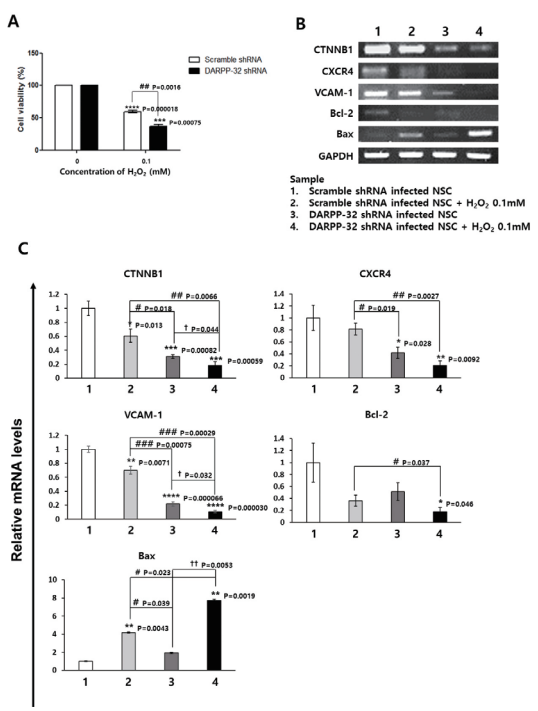


Figure 4. Analysis of gene expression related to DARPP-32 signaling, cell proliferation, and cell survival after oxidative stress in hiNSCs. (A) Cell viability

(y axis) according to concentrations of H_2O_2 (x axis). The data were analyzed by two-way ANOVA with turkey post-hoc. Error bars represent mean ± S.E.M., n=4. All the experiments were performed in triplicate. ***P<0.001, ****P<0.0001 compared to scrambled and DARPP-32 shRNA-infected hiNSCs + 0 mM H_2O_2 (control) respectively; ##P<0.01 compared to scrambled shRNA-infected hiNSC + 0.1 mM H_2O_2. (B) RT-PCR bands of *CTNNB1, CXCR4, VCAM-1, Bcl-2, Bax,* and *GAPDH* expression in the scrambled control and the DARPP-32 (knockdown) shRNA-infected hiNSCs before and after H_2O_2 treatment. (C) Quantitative analysis of RT-PCR bands. The data were analyzed by one-way ANOVA with Turkey post-hoc. Error bars represent mean ± S.E.M., n=3. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared to scrambled shRNA-infected hiNSCs; #P<0.05, ##P<0.01, ###P<0.001 compared to scrambled shRNA-infected hiNSCs + H_2O_2 (0.1 mM); †P<0.05, ††P<0.01 compared to DARPP-32 shRNA-infected hiNSCs.

Taken together, our results suggest that down-regulation of DARPP-32 might increase apoptosis and decrease cell survival through the regulation of several downstream genes in hNSCs (Fig. 5).

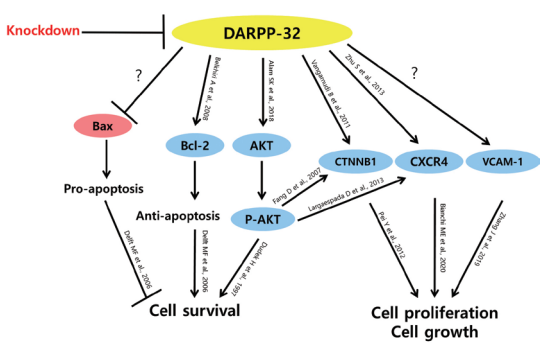


Figure 5. Summary of the role of DARPP-32 in the proliferation and survival of hiNSCs. The schematic diagram shows the interaction of DARPP-32 with genes related to proliferation and survival. DARPP-32-knockdown inhibits the signaling of cell proliferation and cell survival in hiNSCs. Activation is depicted by arrows, while inhibition is depicted by T-lines.

Discussion

Many studies have reported that DARPP-32 plays important roles in human mature neurons, such as regulating striatal synaptic plasticity[8]. However, there are very few studies about the role of DARPP-32 in hNSCs. In this study, we report that DARPP-32 may play an important role in cell proliferation and cell survival in hNSCs.

Neuronal viability is maintained via interacting networks of signaling pathways and can be disturbed in response to many cellular stresses and shifts in signaling pathways[9]. Apoptosis is a programmed cell death that occurs in multicellular organisms[10]. Neuronal apoptosis in the brain is inhibited by pro-survival proteins such as B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-xL), whereas pro-apoptotic proteins such as Bcl-2-associated X (Bax) and Bcl-2 homologous antagonist killer (Bak) promote neuronal death[11]. In this study, the mRNA expression of *Bcl-2* was reduced while the mRNA expression of *Bax* was increased in response to H_2O_2 treatment in DARPP-32-knockdown hiNSCs compared to the scrambled control. Our results indicate that downregulation of DARPP-32 caused an increase in the expression of *Bax* and a decrease in the expression of *Bcl-2* in hiNSCs. Moreover, the expression of p-Akt was reduced in the DARPP-32-knockdown hiNSCs. Akt is known to activate CTNNB1[12] and CXCR4[13], therefore, the decreased expression of p-Akt might be related to the decreased expression of *CTNNB1* and *CXCR4*. In addition, the expression of genes associated with cell proliferation (*CTNNB1, CXCR4,* and *VCAM-1*) was reduced in the DARPP-32-knockdown hiNSCs.

C-X-C chemokine receptor type 4 (CXCR4, also known as fusin or CD184) is an alpha-chemokine receptor specific for stromal-derived-factor-1 (SDF-1, also called CXCL12)[14]. CXCR4 plays a role in neuronal guidance and migration[15]. In addition, DARPP-32 promotes the invasion of gastric cancer cells interacting with CXCR4[16].

Catenin beta-1 (CTNNB1, also known as β-catenin) is a protein involved in controlling the proliferation[17] and balance[18] of NSCs through Wnt signaling[17,18]. Phosphorylation of β-catenin by Akt promotes β-catenin transcriptional activity, tumor cell invasion and development[12]. Wnt signaling is activated by crosstalk with CXCR4 signaling, downstream of Akt[13]. Malignant peripheral nerve sheath tumor cells secrete CXCL12 ligands that activate CXCR4 receptors, and β-catenin is activated via Akt[13]. β-catenin increased the proliferation of NSCs in the mouse cerebellar ventricular zone[19]. β-catenin is regulated by t-DARPP-32 in upper gastrointestinal cancer cells[20].

Vascular cell adhesion molecule 1 (VCAM-1) or cluster of differentiation 106 (CD106) functions as a cell adhesion molecule[21]. It is involved in transforming growth factor-beta 1 (TGF-β1)-mediated proliferation, migration, and invasion of endometriotic cyst stromal cells[22]. In this paper, the expression of genes associated with cell proliferation such as *CXCR4*, *CTNNB1,* and *VCAM-1* was reduced in DARPP-32-knockdown hiNSCs compared to the scrambled control. Therefore, these three genes may be related to DARPP-32-mediated signaling pathways and may play important roles in cell survival and proliferation of hNSCs.

In summary, our study suggests a novel mechanism by which DARPP-32 positively regulates cell proliferation and survival of hNSCs.

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한글초록

DARPP-32의 인간 신경줄기세포 증식과 생존에서의 역할 규명

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본 연구에서는, 도파민과 cAMP 조절 뉴런 인단백질 32kDa (dopamine- and cAMP-regulated neuronal phosphoprotein 32kDa, DARPP-32) 또는 단백질 탈인산화 효소 1 조절 아단위 1B (protein phosphatase 1 regulatory subunit 1B, PPP1R1B)가 사람 지방줄기세포에서 분화 유도된 인간 유도 신경줄기세포 (human induced neural stem cell, hiNSC)에서의 세포 증식 및 세포 생존에 관여하는 기전을 규명하기 위해 렌티바이러스를 이용하여 hiNSC에서 DARPP-32 발현을 감소 (knockdown)시켰다. DARPP-32 감소된 hiNSC에서 대조군 (scramble) hiNSC 보다 세포 증식이 상대적으로 더 느렸고, 배가 시간은 더 길었다. 산화 스트레스 실험을 통해, 과산화수소 (hydrogen peroxide, H_2O_2) 처리 후 DARPP-32 감소된 hiNSC에서 대조군 hiNSC 보다 더 많은 세포 사멸이 발생함을 관찰하였다. 세포 증식 및 DARPP-32 신호전달과 관련된 CTNNB1, CXCR4, 그리고 VCAM-1의 발현이 대조군 hiNSC 보다 DARPP-32 감소된 hiNSC에서 더 낮았다. 그리고, DARPP-32 감소된 hiNSC 에 H_2O_2를 처리했을 때 이 유전자들의 발현이 가장 낮았다. 또한, Bcl-2 (항-세포사멸 마커)의 발현은 H_2O_2 처리하지 않은 대조군 hiNSC에서 가장 높았고, H_2O_2 처리한 DARPP-32 감소된 hiNSC에서 가장 낮았다. 반면에, Bax (세포사멸 증진 마커)의 발현은 H_2O_2 처리하지 않은 대조군 hiNSC에서 가장 낮았고, H_2O_2 처리한 DARPP-32 감소된 hiNSC에서 가장 높았다. 그리고, 단백질 수준에서는 DARPP-32 감소된 hiNSC에서 인산화된 Akt (p-Akt)의 발현 감소가 확인되었다. 결론적으로, 이러한 결과들은 DARPP-32가 여러 유전자들, 인자들과 상호 작용함으로써 DARPP-32 하위 신호전달경로의 조절을 통해 인간 신경줄기세포에서 세포 증식과 세포 생존에 긍정적인 영향을 미치는 기작들을 제시한다.

주제어: 세포 증식, 세포 생존, DARPP-32, 신경줄기세포, 산화스트레스