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Research Article
Protective Role of Neuronal PAS Domain Protein 3 NPAS3 Gene in the Survival of Human Brain Stem Cell

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Abstract

Background and Objective: Neuronal PAS domain protein 3 (NPAS3) is one of the basic helix-loop-helix (Bhlh) transcription factor superfamily members. It is mainly expressed in human brain. Its disruption can result in the occurrence of many diseases, such as schizophrenia. However, the function of NPAS3 is still unknown in human brain stem cell. In this study, the role of NPAS3 was evaluated in cell viability, proliferation, apoptosis, cell cycle regulation and cell differentiation. **Materials and Methods:** Using trypan blue, methylene blue, MTS and BrdU methods, the experiments of cell viability and cell proliferation were performed respectively. Using SubG1 and Annexin V/Propidium iodide FAC analysis, cell apoptosis and cell cycle were investigated. Eventually, using immunofluorescent cytochemistry, cell differentiation was evaluated. **Results:** It was shown that a lack of NPAS3 expression significantly decreased the viability and the proliferation and induced more cells to undergo apoptosis, compared to the control cell line. Furthermore, a knockdown of NPAS3 expression induced a decrease of cells in the S phase of cell cycle. Finally, it was discovered that the capacity of cell differentiation was declined in a lack of NPAS3 expression, when compared to the control cell line. **Conclusion:** With the current findings, it is concluded that human brain stem cell is sensitive to the level of NPAS3 expression and hence only an optimal level is mandatory to mediate normal cellular process.

Key words: NPAS3, cell viability, human brain stem, helix loop helix, trypan blue, cell proliferation, cell apoptosis, cell differentiation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The members of the bHLH family are a group of related proteins that are involved in a number of biological and physiological processes such as the regulation of myogenesis[1], neurogenesis[2-3], toxin metabolism[4-5] and circadian rhythms[6-7]. These proteins contain a basic region that is involved in DNA binding and a helix-loop-helix region that is responsible for protein dimerization. A subset of the bHLH family includes a stretch of 200-300 amino acids, which are part of the PAS domain. One of the bHLH family members is NPAS3, which spans approximately 862 kb on chromosome 14 and is expressed principally within the central nervous system[8]. NPAS3 contains 14 human accelerated elements (HAEs), the largest cluster of non-coding accelerated elements in the human genome[9]. NPAS3 was initially identified by Kamnasaran and later by others as a gene that contributes to the cause of schizophrenia[10-18]. In addition, mice which lack NPAS3 have a defect in brain development and behavioral deficits similar to schizophrenia[19-20]. Furthermore, the functional abnormality of NPAS3 gene is associated with the neuropsychiatric disease[21-23]. Finally, NPAS3 was investigated in oncologic disease by Kamnasaran and with a role in astrocytomas[24].

More recently, it is indicated that NPAS3 is mainly expressed in the human fetal brain[25]. However, the function of NPAS3 is still unknown in human brain stem cell. Therefore, this study investigated the role of NPAS3 in human brain stem cell to understand its biological and disease roles.

MATERIALS AND METHODS

RenCell VM cell line transfection: The human brain stem cell line (ReNcell VM, Millipore) was purchased in 2013 and was used for the experiment about 3 months. It was cultured in RenCell Neural Stem Cell (NSC) maintenance medium (Millipore), containing 20 ng mL^{-1} epidermal growth factor and 20 ng mL^{-1} fibroblast growth factor. RenCell VM cells were washed once in NSC medium and resuspended in the specified electroporation buffer to a final concentration of 1.0×10^7 cells mL^{-1}. Two micrograms of plasmids (two shRNA plasmids, one over-expression plasmid and one PCDNA negative control plasmid) was mixed with 0.1 mL of cell suspension. The mixture was transferred to a 2.0 mm electroporation cuvette and nucleofected with an Amaxa Nucleofector TM apparatus (Amaxa, Cologne, Germany). After transfection, the cells were resuspended in NSC medium, containing 20 ng mL^{-1} epidermal growth factor and 20 ng mL^{-1} fibroblast growth factor and analyzed for cell viability, cell proliferation and cell apoptosis.

Cell viability analysis: After transfection, cells were cultured over 1, 3, 5 and 7 days, trypsinized prior to analysis and stained with 0.4% trypan blue. The cells were loaded in a haemocytometer and counted microscopically.

Methylene blue assay: Cell viability was also investigated following staining with 0.1% filtered methylene blue (Grading: basic blue 9) in 20% ethanol. The cells were incubated at room temperature for 2 min and washed gently with PBS, followed by colorimetric absorbance measurements taken at 650 nm.

MTS assay: The cell titre 96 well aqueous one cell proliferation assay kit-MTS (Promega) was used to evaluate cell cytotoxicity as described by manufacturer. Briefly, after transfection, 2000 cells were plated in each well of 96 well plates and incubated over 1, 3, 5 and 7 days. Prior to analysis, the MTS reagent was added and the cells were incubated at 37°C for 2 h, followed by colorimetric measurements taken at 490 nm.

BrdU assay: Cell proliferation was investigated using the BrdU ELISA kit (Roche) as described by manufacturer. Briefly, after transfection, 2000 cells were plated in 96 well plates and incubated over 1, 3, 5 and 7 days. Prior to analysis, cells were labelled with BrdU for 18 h, fixed onto the surface of the plate and then incubated with an anti-BrdU antibody, which binds to the BrdU incorporated into the DNA. After PBS washes, the substrate solution was added and colorimetric values were measured at 490 nm with an ELISA plate reader.

Cell cycle analysis: After transfection, 2×10^5 cells were grown in 6 well plates over 1, 3, 5 and 7 days. Prior to analysis, cells were stained with propidium iodide (FluoroPure™ Grade) and analyzed by flow cytometry.

Apoptotic assay: After transfection, 2×10^5 cells were grown in 6 well plates over 1, 3, 5 and 7 days. Cells were then trypsinized, washed with PBS, labelled with Annexin V-FITC and Propidium iodide and analyzed by flow cytometry.

Immunofluorescent cytochemistry: Cell differentiation was performed on poly D-lysine/laminin coated slides without 20 ng mL^{-1} epidermal growth factor and 20 ng mL^{-1} fibroblast growth factor for 14 days. After 14 days, cells were then fixed in -20°C ice cold methanol (ACS Grade) for 5 min, permeabilized with 0.5% Triton X100 (scintillation grade) and then blocked with 1% Rochestern blotting blocking reagent for 30 min. Fixed cells were hybridized with 1:200 dilution of primary antibody for 1 h at room temperature, washed with PBS, hybridized with 1:1000 dilution of protein G-FITC (Abcam) for another 1 h, mounted to vectashield medium with DAPI and positive cells were counted under immunofluorescence microscope.

Statistical analysis: All experiments were done in triplicate, with bar graphs representing values that are the Mean±SE. Data analysis were performed using the graph pad prism software. Multiple comparisons of data sets from treated and untreated samples were performed using Two-way ANOVA $p<0.05$ and Bonferroni *post hoc* tests.

RESULTS

Decrease of cell viability and cell proliferation and increase of cell apoptosis: Using trypan blue, methylene blue and MTS assays, it was shown that a knockdown of NPAS3 expression using two shRNA plasmids significantly decreased the viability of RenCell VM cell line over a 7 day period, compared to the control cell line (Fig. 1-3). Interestingly, over-expression NPAS3 also induced a similar effect in RenCell VM (Fig. 1-3). Since NPAS3 influences cell viability, the next step is to determine whether NPAS3 affects cell proliferation using BrDU assay. Both a knockdown and over-expression of NPAS3 decreased cell proliferation (Fig. 4). Since cell proliferation is also influenced by the cell cycle, it is important to evaluate the S phase in RenCell VM using propidium-iodide FAC assay. It was indicated that both a knockdown and over-expression of NPAS3 induced a decrease of cells in the S phase (Fig. 5). To determine whether NPAS3 effect on cell viability is also mediated via inducing apoptosis, it is necessary to undertake Sub-G1 FAC analysis and Annexin V/Propidium iodide assays. It was shown that a knockdown of NPAS3 expression also significantly induced the number of cells to undergo apoptosis, compared to the control cells (Fig. 6, 7a-d).

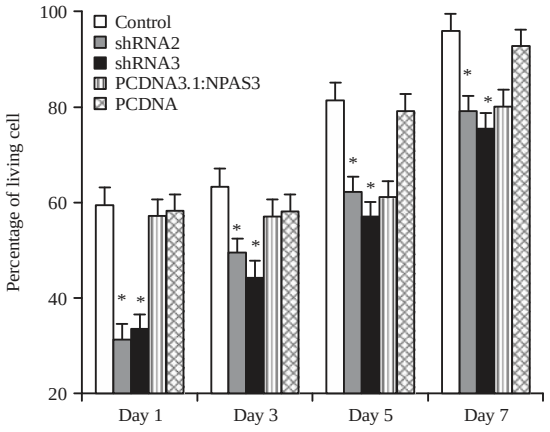

Fig. 1: Both knockdown and over-expression of NPAS3 induces more cell death over a 7 day period using trypan blue assay, comparing to the control cell line
PCDNA3.1: NPAS3 represents an over-expression plasmid and PCDNA represents a negative control, *p-values<0.05

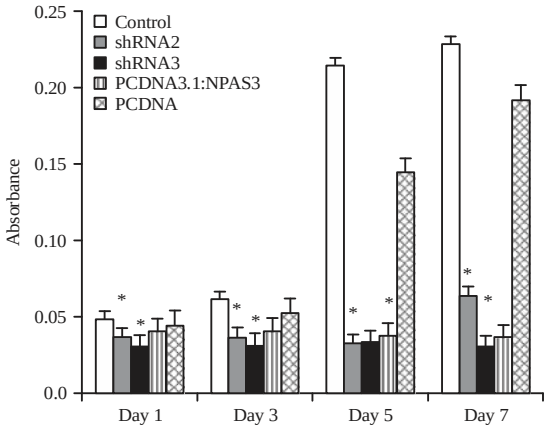

Fig. 2: Absorbance is more decreased in both knockdown and over-expression of NPAS3 over a 7 day period using methylene blue assay for detecting cell viability
PCDNA3.1: NPAS3 represents an over-expression plasmid and PCDNA represents a negative control, *p-values<0.05

Decline of cell differentiation: Finally, by using immunofluorescent cytochemistry, it discovered that within 14 days, a knockdown of NPAS3 decreased the number of glial cell population (astrocytes and oligodendrocytes) and neuronal cell population (β3-Tubulin) when compared to the parental cell line (Fig. 8, 9).

DISCUSSION

After initial cloning of NPAS3 gene, many research has been focused on the relationship of this gene and human

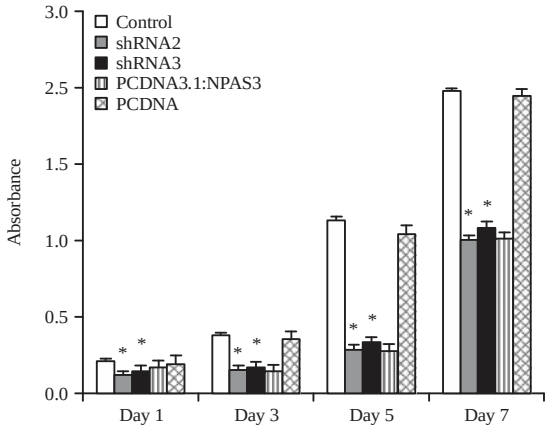

Fig. 3: Absorbance is more declined in both knockdown and over-expression of NPAS3 over a 7 day period using MTS assay for investigating cell viability

PCDNA3.1: NPAS3 represents an over-expression plasmid and PCDNA represents a negative control, *p-values<0.05

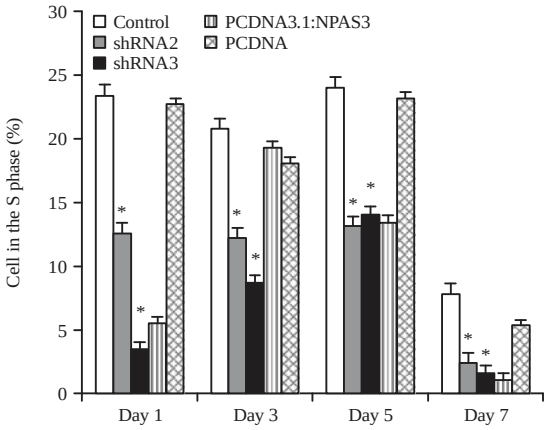

Fig. 5: Both knockdown and over-expression of NPAS3 induces a more significant decrease of cells in the S phase

PCDNA3.1: NPAS3 represents an over-expression plasmid and PCDNA represents a negative control, *p-values<0.05

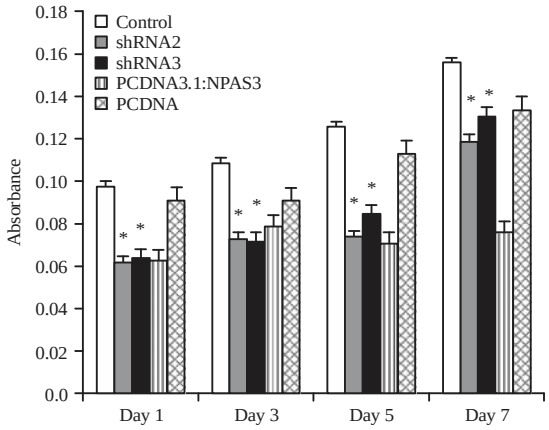

Fig. 4: Both knockdown and over-expression of NPAS3 inhibits more cell proliferation in over a 7 day period using BrdU assay

PCDNA3.1: NPAS3 represents an over-expression plasmid and PCDNA represents a negative control, *p-values<0.05

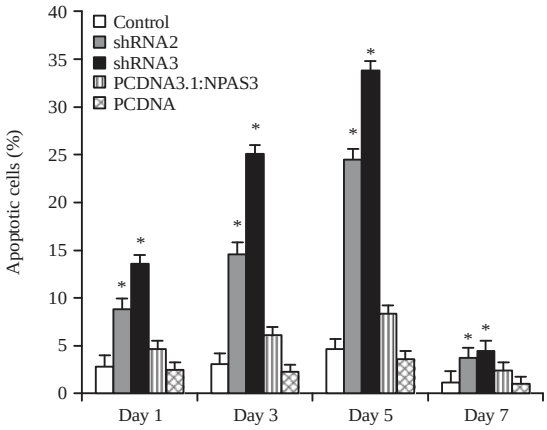

Fig. 6: SubG1 FAC analysis. Both knockdown and over-expression of NPAS3 induces more cell apoptosis over a 7 day period

PCDNA3.1: NPAS3 represents an over-expression plasmid and PCDNA represents a negative control, *p-values<0.05

diseases. Previous studies have suggested a potential role of NPAS3 in schizophrenia disease[10-17]. It has also been reported that NPAS3-deficient mice have neural behavioral abnormalities[19-20]. However, the function of NPAS3 is not known in human brain stem cell.

More recently, it has been demonstrated that NPAS3 is expressed in the human fetal brain[25]. Therefore, there is a question if a knockdown of NPAS3 gene has an effect on the viability of human brain stem cell. One recent report showed that Farnesoid X receptor could modulate the survival of renal medullary collecting duct cells. The deletion of this receptor could result in hypertonicity-induced cell viability decline[26]. Another study also indicated that EAAC1 is important for the survival of neurons. The deletion of this gene can reduce hippocampal neurogenesis[27]. Furthermore, the decrease of stem cell viability in human brain can result in some neurodegenerative diseases, such as Parkinson disease[28]. Therefore, it is not difficult to imagine that NPAS3 gene is functional in human brain stem cell. The loss of the cell viability associated with NPAS3 gene knockdown plays an important role in this case. In our study, it also found that a knockdown of NPAS3 expression significantly decreased the viability of RenCell VM cell line. Our research demonstrates once again the importance of this gene in human brain.

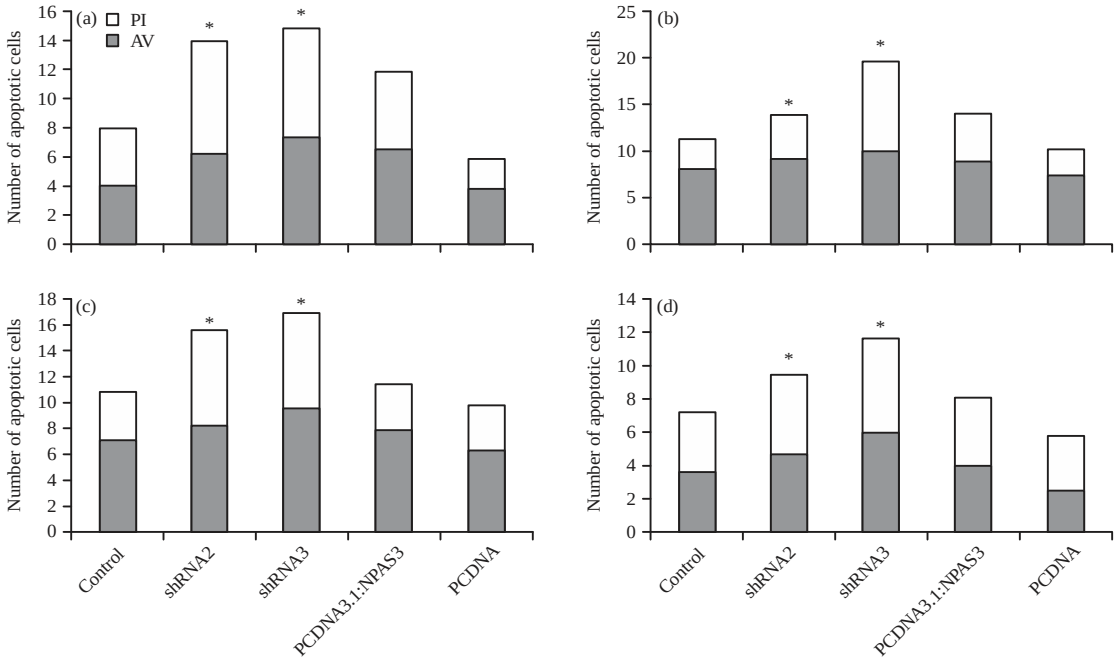

Fig. 7(a-d): Annexin V/Propidium iodide FAC analysis. Both knockdown and over-expression of NPAS3 undertakes more cell apoptosis over a 7 day period

PCDNA3.1: NPAS3 represents an over-expression plasmid and PCDNA represents a negative control, *p-values<0.05

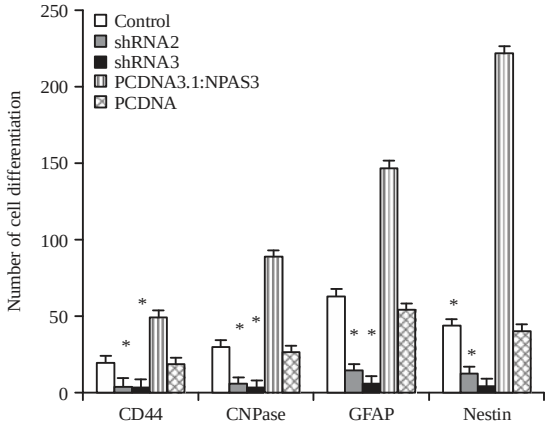

Fig. 8: Knockdown of NPAS3 induces more CD44, CNPase, GFAP and Nestin positive cell population decline over a 14 day period

PCDNA3.1: NPAS3 represents an over-expression plasmid and PCDNA represents a negative control, *p-values<0.05

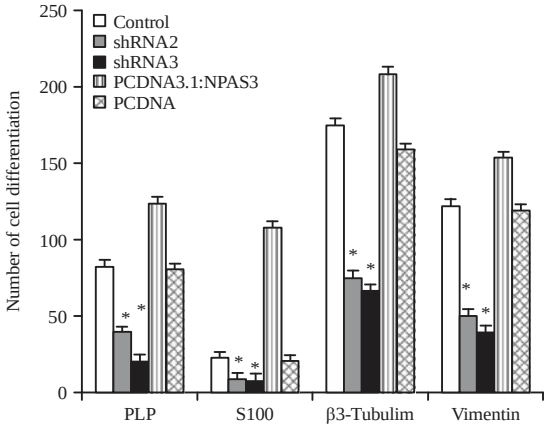

Fig. 9: Knockdown of NPAS3 induces more PLP, S100, β3-Tubulin and Vimentin positive cell population decrease over a 14 day period

PCDNA3.1: NPAS3 represents an over-expression plasmid and PCDNA represents a negative control, *p-values<0.05

Furthermore, it found that a knockdown of NPAS3 decreased cell proliferation and induced a decrease of cells in the S phase. It was reported that the mutations of grf9 org3 and GRF9ox org3 decreased cell proliferation, which could affect leaf growth[29]. The similar report indicated that neural precursor cell proliferation is significantly reduced in NPAS3 deficient mice compared to the wild-typelittermates[30]. Notably, a recent analysis of brain tissue from patients with schizophrenia has revealed that neural stem cell proliferation is significantly decreased in schizophrenia[31]. All studies mentioned above indicated the importance of cell proliferation and they are also consistent with our current findings.

To determine whether NPAS3 effect on cell viability is also mediated via inducing apoptosis, it undertake cell apoptotic assays. It found that a knockdown of NPAS3 expression also significantly induced the number of cells to undergo apoptosis, compared to the control cells. A recent study revealed that the mutation of DDHD2 gene induces apoptosis of motor neurons in the spinal cord[32]. The loss of *Bhihe*41 gene, which is one of the bHLH family members, can also induce cell death[33]. NPAS4 is another member of the bHLH transcription factor superfamily. It indicated that a knockdown of NPAS4 gene in cortical neurons lead to increased susceptibility to cell death[34]. The results of all these studies are also consistent with our current findings.

Human brain stem cells have the capacity to self-renew and produce three major cell types of the CNS. Therefore, it detected the effect of NPAS3 knockdown on RenCell VM differentiation. A knockdown of NPAS3 gene decreased the number of glial cell population (astrocytes and oligodendrocytes) and neuronal cell population (β3-Tubulin). A recent report indicated that the loss of tafazzin can influence myoblast differentiation and finally result in the occurrence of Barth syndrome[35]. Another study also showed that SPAG17 is important for male germ cell differentiation and the mutation of this gene is associated with the male infertility[36]. Furthermore, a knockdown of NPAS4 expression delays neural differentiation of embryonic stem cells[37]. These results demonstrate that NPAS3 gene is functional in the regulation of human brain stem cell differentiation.

CONCLUSION

NPAS3 is one of the bHLH transcription factor superfamily members. The deletion of NPAS3 gene is related to the decrease of cell viability, cell proliferation and cell differentiation and the increase of cell apoptosis. With the current findings, it is concluded that human brain stem cell is sensitive to the level of NPAS3 expression and hence only an optimal level is mandatory to mediate normal cellular process.

SIGNIFICANCE STATEMENT

This study discovers the importance of NPAS3 in human brain stem cell that can be beneficial for the treatment of disease of nervous system. This study will help the researcher to uncover the critical areas of neuroscience that many researchers were not able to explore. Thus a new theory on the function of NPAS3 gene may be arrived at.

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