Human Cell (2021) 34:847–861
https://doi.org/10.1007/s13577-021-00517-z

RESEARCH ARTICLE

# *Fndc5* knockdown significantly decreased the expression of neurotrophins and their respective receptors during neural differentiation of mouse embryonic stem cells

**Reihane Ebadi[1] · Farzaneh Rabiee[3] · DorMohammad Kordi-Tamandani[1] · Mohammad Hossein Nasr-Esfahani[3] · Kamran Ghaedi[2]**

Received: 27 September 2020 / Accepted: 5 March 2021 / Published online: 8 March 2021
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## Abstract

Fibronectin type III domain-containing-5 (Fndc5) is a trans-membrane protein which is involved in a variety of cellular events including neural differentiation of mouse embryonic stem cells (mESCs) as its knockdown and overexpression diminishes and facilitates this process, respectively. However, downstream targets of Fndc5 in neurogenesis are still unclear. Neurotrophins including NGF, BDNF, NT-3, and NT-4 are the primary regulators of neuronal survival, growth, differentiation, and repair. These biomolecules exert their actions through binding to two different receptor families, Trk and p75NTR. In this study, considering the fact that neurotrophins and their receptors play crucial roles in neural differentiation of ESCs, we sought to evaluate whether knockdown of *Fndc5* decreased neural differentiation of mESCs by affecting the neurotrophins and their receptors expression. Results showed that at neural progenitor stage, the mRNA and protein levels of BDNF, Trk, and p75NTR receptors decreased following the *Fndc5* knockdown. In mature neural cells, still, the expression of Trk and p75NTR receptors at mRNA and protein levels and BDNF and NGF expression only at protein levels showed a significant decrease in *Fndc5* knockdown cells compared to control groups. Taken together, our results suggest that decreased efficiency of neural differentiation following the reduction of *Fndc5* expression could be attributed to decreased levels of NGF and BDNF proteins in addition to their cognate receptors.

**Keywords** Neural differentiation · Fndc5 · Neurotrophins · Neurotrophin receptors

✉ Mohammad Hossein Nasr-Esfahani
mh.nasr-esfahani@royaninstitute.org

✉ Kamran Ghaedi
kamranghaedi@sci.ui.ac.ir

Reihane Ebadi
ebadi_reihane@pgs.usb.ac.ir

Farzaneh Rabiee
rabiee_farzaneh@yahoo.com

DorMohammad Kordi-Tamandani
dor_kordi@science.usb.ac.ir; dor_kordi@yahoo.com

[1] Department of Biology, University of Sistan and Baluchestan, Zahedan, Iran

[2] Department of Cell and Molecular Biology and Microbiology, Faculty of Biological Science and Technology, University of Isfahan, Hezar Jerib Ave, Azadi Square, 81746 Isfahan, Iran

[3] Department of Animal Biotechnology, Cell Science Research Center, Royan Institute for Biotechnology, ACECR, 816513-1378 Isfahan, Iran

## Abbreviations

| | |
|---|---|
| ANOVA | Two-way analysis of variance |
| ATP | Adenosine triphosphate |
| BDNF | Brain-derived neurotrophic factor |
| bHLH | Basic helix–loop–helix |
| BSA | Bovine serum albumin |
| cAMP | Cyclic adenosine monophosphate |
| CNS | Central nervous system |
| CREB | CAMP-response element binding protein |
| DAPI | 4, 6-Diamidino-2-phenylindole |
| DMEM/F12 | Dulbecco's modified Eagle's medium/Hams F12 medium |
| Dox | Doxycycline |
| EB | Embryoid body |
| ECL | Enhanced chemiluminescence |
| ERK | Extracellular signal-regulated kinase |
| ESC | Embryonic stem cell |
| ES-FBS | Embryonic stem cell qualified fetal bovine serum |
| FITC | Fluorescein isothiocyanate |

| | |
|---|---|
| Fndc5 | Fibronectin type III domain-containing-5 |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| HRP | Horse radish peroxidase |
| JNK | Jun N-terminal kinase |
| Ko-DMEM | Knock-out DMEM |
| LIF | Leukemia inhibitory factor |
| MAP2 | Microtubule-associated protein 2 |
| MAPK | Mitogen-activated protein kinases |
| mESC | Mouse embryonic stem cell |
| miRNA | MicroRNAs |
| mTOR | Mammalian target of rapamycin |
| NB | Neurobasal |
| NC | Neural cell |
| NGF | Nerve growth factor |
| NP | Neural progenitor |
| NT | Neurotrophin |
| NT-3 | Neurotrophin-3 |
| NT-4 | Neurotrophin-4 |
| p75NTR | P75 neurotrophin receptor |
| PBS | Phosphate-buffered saline |
| PeP | Proxisomal protein |
| PI3 | Phosphatidylinositol-3-kinase |
| PLC | Phospholipase C |
| PVDF | Polyvinylidenedifluoride |
| RA | Retinoic acid |
| RNAi | RNA interference |
| ROS | Reactive oxygen species |
| RT | Room temperature |
| RT-qPCR | Real-time quantitative polymerase chain reaction |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SEM | Standard error of the mean |
| shRNA | Small hairpin RNA |
| TRI | Total RNA isolation |
| Trk | Tropomyosin-related kinase |

## Introduction

Fibronectin type III domain-containing-5 (Fndc5), initially known as peroxisome protein (PeP), is a trans-membrane protein with 209 amino acid residues which was first identified in 2002 [1–5]. Fndc5 is identified as a crucial factor for neural, cardiac differentiation and a required factor for energy expenditure and lowering the insulin resistance in adipose tissue [6–10].

The importance of Fndc5 in the process of neural differentiation and protection has been shown in recent studies [6, 7, 11]. It is already well known that Fndc5 induces *Bdnf* expression in brain. Ectopic expression of *Fndc5*, could also induce *Bdnf* expression in the hippocampus [12]. However, except for BDNF, the downstream targets of Fndc5 involved in neurogenesis has not yet been determined.

Neurotrophins play critical roles in neurogenesis including growth, survival, differentiation, and repair of neurons in central and peripheral nervous systems. This family includes NGF, NT-3, NT-4, and BDNF in mammals. Brain-derived neurotrophic factor, also known as BDNF, is a member of the neurotrophin family of growth factors, which are found in the brain and the periphery tissues. Two different cell surface receptors mediate the neurotrophins functions. These receptors are the high–affinity Trk (tropomyosin-related kinase) family (TrkA, TrkB, and TrkC receptors) and the low-affinity p75 neurotrophin receptor (p75NTR), a member of tumor necrosis factor receptor family [13–15]. Binding of Trk family members to neurotrophins is specific, as TrkA binds NGF, TrkB binds BDNF, as well as NT-4 and TrkC have a unique ligand for NT-3 (TrkA and TrkB bind NT3 with a lower efficiency) [16]. Neurotrophins bind to Trk receptors and activates extracellular signal-regulated kinase (ERK), phosphatidyl inositol-3 (PI3)-kinase, and phospholipase C-γ1 signaling pathways which mediate cell survival and differentiation [17]. In contrast with Trk receptors, p75NTR binds all known NTs with similar affinity [16]. p75NTR facilitates cell survival through activation of Akt and NFκB pathways or result in neuronal death through activation of Jun N-terminal kinase (JNK) signaling cascade, in the absence of Trk receptors [15, 18].

The survival and development of neurons is based on the functional interaction between Trk and p75NTR signals, because these receptors are often co-expressed and can either augment or oppose each other's actions. p75NTR can enhance Trk receptors binding to cognate neurotrophins, while Trk receptor-mediated signaling may suppress the pro-apoptotic effects of p75NTR [15, 19–21]. Therefore, it is not supersizing to see that abundance of neurotrophins and their receptors are well documented in diseases of the nervous system [15].

Neural cells derived from embryonic stem cells (ESCs) have therapeutic potential for the treatment of neurodegenerative disease of CNS [22]. Due to established crucial roles of neurotrophins and their receptors in neural differentiation of stem cells [23–25], we investigated the role of *Fndc5* on their expression using a stably transduced *Fndc5* knockdown cell line. In this project, we sought to evaluate whether *Fndc5* knockdown affects the neurotrophins and their respective receptor expression or not.

## Materials and methods

### mESCs culture and neural differentiation

In this study, we used mouse ESCs (Royan B20 cells) derived from C57BL/6 strain which were obtained from

Royan Institute for Stem Cells and Developmental Biology (Tehran, Iran). mESCs were cultured on 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA) coated dishes containing serum-free medium consisted of DMEM/F12 (Invitrogen) and neurobasal (Invitrogen) at a 1:1 (v/v) ratio, 5 mg/mL bovine serum albumin (BSA; Sigma-Aldrich), 2 mM L-glutamine (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 1% penicillin–streptomycin (v/v) (Invitrogen), 2% B27 supplement (v/v) (Invitrogen), 1% N2 supplement (v/v) (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 1000 U/mL leukemia inhibitory factor (LIF; Chemicon, Temecula, CA, USA), 1 µM PD0325901 (Sigma-Aldrich), and 10 µM SB431542 (Sigma-Aldrich), an activin receptor-like kinase inhibitor, as described previously [26] at 37 °C and 5% CO2. Medium was changed daily and cells were passaged every 2 days for at least two times before the beginning of differentiation. mESCs' differentiation into neurons was performed according to the protocol previously described by Ostadsharif et al., using embryoid body (EB) [27]. For EBs formation, definite number of cells (~ $10^3$) were cultured for 2 days in 20 µl hanging drops in medium-containing knock-out DMEM (Ko-DMEM, Gibco) and 15% embryonic stem cell qualified fetal bovine serum (ES-FBS, GE Healthcare Hyclone, Illinois, CHI, USA) in the absence of LIF. For neural induction, EBs were suspended for 4 days in differentiation medium composed of Ko-DMEM, 10% ES-FBS, and 1 µM RA. Medium was refreshed every 2 days. Subsequently, for differentiation of NPs to mature neurons, EBs were plated on gelatinized 12-well plates (TPP, Zurich, Switzerland) containing neurobasal medium supplemented with 5% ES-FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1% penicillin/streptomycin, 0.1 mM β-mercaptoethanol, 2% B27, and 1% N2 for an additional 8 days to allow adherence and development of mature neural cells.

### *Fndc5* knockdown during neural differentiation

In the present study, we used *Fndc5* knockdown mESC line which was prepared previously by our laboratory [9]. Nazem et al. designed two shRNA sequences targeting *Fndc5* transcripts and one scramble shRNA (shCtrl) and then cloned these sequences in Doxycycline-controlled recombinant vector pLVPT-tTR-KRAB. Subsequently, human embryonic kidney cells were transfected with packaging vectors including recombinant inducible vector containing sh*Fndc5* or shCtrl using lipofectamine in order to produce lentiviral particles. Afterward, mESCs (Royan B20) were coinfected with these viral particles expressing two shRNA or shCtrl as a scramble. Measurement of transduction efficiency, selection of stable cell lines by antibiotic, verification of the presence of shRNA sequence in genomic DNA, confirmation of the *Fndc5* expression reduction, and evaluation of stemness features maintenance were performed [9]. In the current study, we treated sh*Fndc5* cell line with 1.5 µg/mL doxycycline (Dox, Clontech) to sh*Fndc5* expression induction and *Fndc5* knockdown from day 2 to day 14 during neural progenitors (NP) and differentiated neural cells formation. Also, untransduced (RB20) and scramble cell lines were treated with the same concentration of Dox as controls.

### RNA extraction and real-time quantitative PCR

Total RNA was extracted using total RNA isolation (TRI) reagent (Ambion, USA) protocol from mESCs (day 0), EB on day 6 (NPs) and mature neural cells on day 14. To eliminate residual genomic DNA, total RNA was treated with *DNase*I (Thermo Fisher Scientific). cDNA synthesis was performed with 1 µg of total RNA and random hexamer primer using revert Aid first strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was carried out with 25 ng of cDNA, 5 µL SYBR premix Ex*Taq*II (TaKaRa, Kusatsu, Japan), and 0.3 µM of each primer in a final volume of 10 µL in triplicate using a Thermal Cycler Rotor-Gene 6000 (Corbett, Australia). The expression levels of target genes were normalized to the level of housekeeping gene, *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) [28]. All measurements were carried out in triplicate from three separate cultures and data were calculated by the $\Delta\Delta C_t$ method [29, 30]. Primer sequences were designed by the Beacon designer software (Version 7.2, USA) and are presented in Table 1.

### Protein preparation and Western blot analysis

Cells and EBs (day 6 and day 14) were washed with phosphate-buffered saline (PBS), and then lysed by TRI reagent. Total protein was extracted according to the manufacturer's protocol (Ambion, USA). Bradford assay was used to evaluate protein concentration (Sigma-Aldrich) [31]. Equal amounts of protein from each sample (30 µg) were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidenedifluoride membrane (PVDF; Biorad, Hercules, CA USA). The membranes were blocked with 10% skim milk (Merck, Darmstadt, Germany) for an overnight, and then incubated at room temperature (RT) for 2 h with the particular primary antibodies: monoclonal anti-microtubule-associated protein 2 (MAP2) (1:2000 v/v; Sigma-Aldrich, M1406), rabbit anti-BDNF antibody (1:1000 v/v; Sigma-Aldrich, AV41970), mouse monoclonal anti-NGF antibody (1:500 v/v; Santa Cruz, SC-365944), mouse monoclonal anti-TrkB (1:25 v/v; Santa Cruz, SC-377218), and rabbit anti-p75 NGF receptor antibody (1:4000 v/v; Abcam, ab8874) and mouse anti-GAPDH antibody (1:5000 v/v; Sigma-Aldrich, A2228) as a loading control. Next step, membranes were washed with

**Table 1** Primers used for real-time PCR

| Gene | Primer sequence (5′→3′) | Annealing temperature (°C) | Product (bp) |
|---|---|---|---|
| *GAPDH* | F: TGCCGCCTGGAGAAACC<br>R: TGAAGTCGCAGGAGACAACC | 60 | 121 |
| *FNDC5* | F: TCATTGTTGTGGTCCTCTTC<br>R: GCTCGTTGTCCTTGATGATA | 60 | 81 |
| *PAX6* | F: TGAATGGGCGGAGTTATGAT<br>R: GGACGGGAACTGACACTC | 58 | 126 |
| *MAP2* | F: AAGTCACTGATGGAATAAGC<br>R: CTCTGCGAATTGGTTCTG | 54 | 174 |
| *TUJ-1* | F: GCCTCCTCTCACAAGTATG<br>R: CCTCCGTATAGTGCCCTT | 54 | 166 |
| *NGF* | F: AGTGTGTGGGTTGGAGATAAG<br>R: AAGGTGTGAGTCGTGGTG | 58 | 203 |
| *BDNF* | F: CCACTAAGATACATCATACC<br>R: CAGAACAGAACAGAACCA | 55 | 106 |
| *NT3* | F: CTTCGCAAACCTATGTCCGAG<br>R: AGGAAGTGTCTATTCGTATCCAGC | 63 | 81 |
| *NT4* | F: ATACCTCCCATCCAACAT<br>R: CAAAGTCCACCCTCCAAT | 57 | 104 |
| *TrkA* | F: AGTTGAGAAGCCTAACCATCG<br>R: AACAGGGCACAGGAACAATGCAG | 58 | 196 |
| *TrkB* | F: GACTAAATCCAGCCCCGACAC<br>R: TCACAGACTTTCCTTCCTCCAC | 60 | 152 |
| *TrkC* | F: AACAAGCCCACCCACTACAAC<br>R: CAGGCAAAGGCAGCAAGTCC | 61 | 233 |
| *P75NGF* | F: AAGCCTGTCTTGCCCCGTGA<br>R: GCCCCTGTTACCTTCTCTAGTGT | 58 | 100 |

*F and R* are forward and reverse primers

Tris-buffered saline (TBST; 50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH: 7.5) and then incubated at RT for 1 h with an appropriate secondary antibody: horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000 v/v; Dako, P0447) or goat anti-rabbit IgG (1:16,000 v/v; Santa Cruz, SC-2301). Subsequently, after washing membranes with TBST, protein bands were visualized using an Amersham enhanced chemiluminescence (ECL) advance western blotting detection kit (GE Healthcare, Dornstadt, Germany). All experiments were performed in triplicate and Image J processing software was used for quantification of blot intensity.

## Immunocytochemistry analysis

Suspended EBs, on day 6 were plated onto gelatinized coverslips in 12-well plates and allowed to attach for an additional 8 days. After washing differentiated cells on day 14 with PBS, they were fixed with 4% paraformaldehyde solution at 4 °C for 30 min and then permeabilized by 0.2% Triton-X100 in PBS for 20 min. For staining, cells were incubated overnight at 4 °C with the primary antibodies: anti-mouse antibody against MAP2 (1:200 v/v; Sigma-Aldrich, M1406) and anti-mouse antibody against β tubulin Isotype III (1:500 v/v; Abcam, ab7751). Subsequently, cells were washed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG as secondary antibody (1:200 v/v; Millipore, AP124F) for 1 h at 37 °C. BSA blocking buffer with 10 and 5 mg/mL concentration was used for dilution of primary and secondary antibodies, respectively. Finally, cells were treated with 4, 6-diamidino-2-phenylindole (DAPI, 0.1 mg/mL, Sigma-Aldrich, D8417) in PBS for 5 min for nuclear counterstaining. Stained cells on the coverslips were fixed on glass slides and analyzed using a fluorescent microscope (Olympus, Tokyo, Japan). An Olympus DP70 camera was used to capturing images and Image J software was used to analyzing the level of fluorescence [32].

## Statistical analysis

All results were reported as the mean value ± standard error of mean (SEM) from at least three independent replicates for each experiment. Statistical analysis was carried out by SPSS version 20. Independent *t* test was used to calculating statistical differences between the two independent groups and one-way ANOVA followed by Tukey's post hoc test was

used to comparing multiple groups of samples. Statistical significance was defined at the $p < 0.05$.

## Results

### Confirmation of *Fndc5* knockdown and stemness maintenance in sh*Fndc5* cell line

As mentioned in Materials and methods, in the current study, we used transduced (shCtrl and sh*Fndc5*) cell lines created previously by Nazem et al. To verification of *Fndc5* knockdown, we treated these cell lines with 1.5 µg/mL doxycycline for 24 h and assessed the expression level of *Fndc5*. Result showed a significant decrease of *Fndc5* transcript level in Dox-treated sh*Fndc5* cell line compared to untreated, scramble, and untransduced cell lines (Fig. 1a).

To evaluate the maintenance of stemness properties of transduced (shCtrl and sh*Fndc5*) mESCs, the expression levels of pluripotency markers (*Nanog*, *Rex1,* and *Oct4*) were assessed by RT-qPCR. Data indicated no significant differences in transcript levels of three aforementioned markers in these cell lines compared to untransduced cells (Fig. 1b).

### Generation and characterization of mESC-derived mature neural cells

To confirm appropriate occurrence of neural differentiation, we evaluated the expression level of stemness and mature neuronal markers during this process. Figure 2a represents the schematic depiction for neural differentiation procedure of mESCs and the morphology of cells at different stages. RT-qPCR data indicated significant downregulation of stemness markers (*Nanog* and *Oct4*) and upregulation of mature neuronal markers (*Map2* and *Tuj-1*) during the process of differentiation (Fig. 2b–e). Furthermore, immunofluorescence staining of mESC-derived cells on day 14 showed mature cells with positive expression of mature neuronal markers MAP2 and TUJ1 (Fig. 2f). Therefore, these results confirmed the accuracy

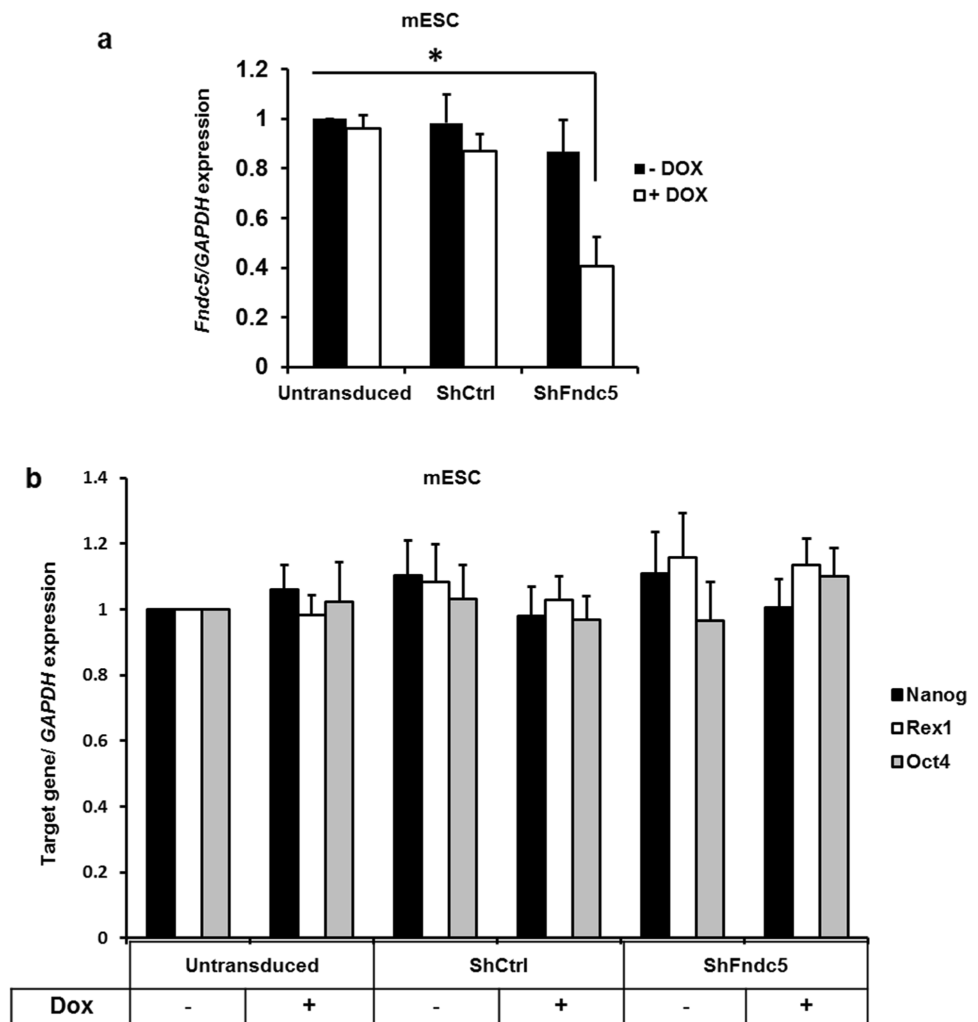
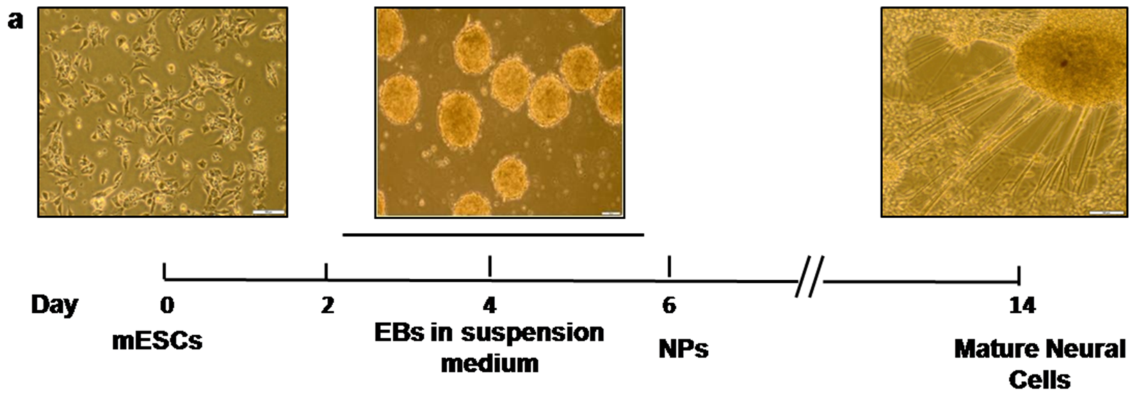
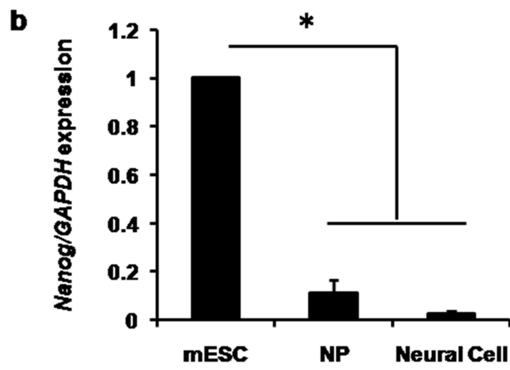
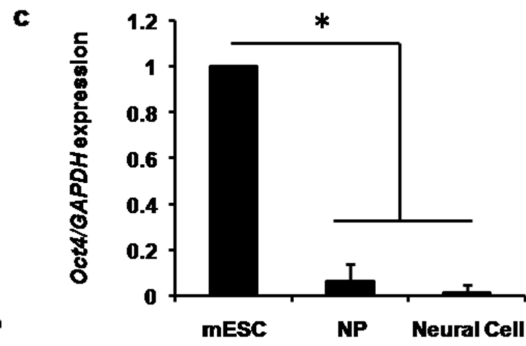
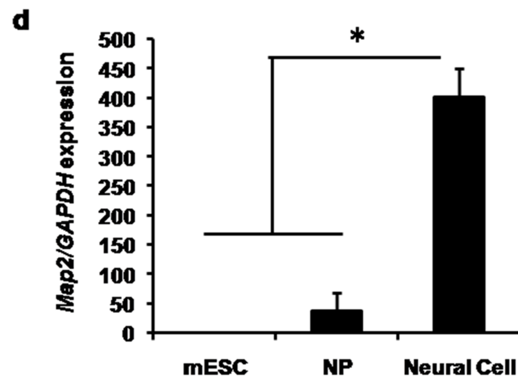


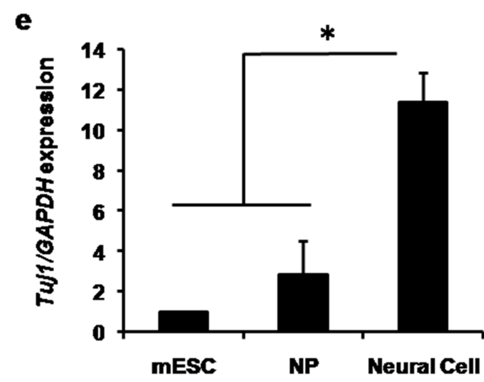

**Fig. 1** Confirmation of *Fndc5* knockdown and stemness characteristics' maintenance in sh*Fndc5* cell line. **a** RT-qPCR analysis of *Fndc5* expression in untransduced and transduced (shCtrl and sh*Fndc5*) cell lines in the presence and absence of Dox as an inducer of shRNA expression. **b** RT-qPCR analysis of stemness markers (*Nanog*, *Rex1*, and *Oct4*) expression in transduced (shCtrl and sh*Fndc5)* cell lines compared to untransduced cells in the presence and absence of Dox. Values are presented as mean ± SEM for three independent experiments. $*p < 0.05$

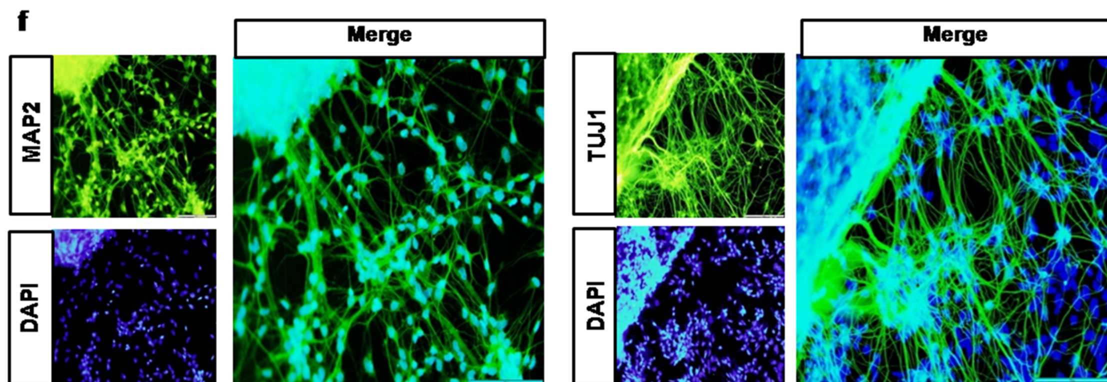

a
Day
0
2
4
6
14
mESCs
EBs in suspension medium
NPs
Mature Neural Cells


b
Nanog/GAPDH expression
1.2
1
0.8
0.6
0.4
0.2
0
*
mESC
NP
Neural Cell


c
Oct4/GAPDH expression
1.2
1
0.8
0.6
0.4
0.2
0
*
mESC
NP
Neural Cell


d
Map2/GAPDH expression
500
450
400
350
300
250
200
150
100
50
0
*
mESC
NP
Neural Cell


e
Tuj1/GAPDH expression
14
12
10
8
6
4
2
0
*
mESC
NP
Neural Cell


f
MAP2
DAPI
Merge
TUJ1
DAPI
Merge

◂**Fig. 2** Characterization of mESC-derived neural cells. **a** Schematic illustration of mESCs neural differentiation and morphological characterization of these cells during this process. Relative gene expression of the stemness markers (*Nanog* and *Oct4*) (**b**, **c**) and mature neuronal markers (*Map2 and Tuj-1*) (**d**, **e**) during the neural differentiation of mESCs. **f** Immunofluorescence staining of mESC-derived neural cells with anti-MAP2 and anti-TUJ1 antibodies. The nuclei were counterstained with DAPI. Scale bar is 100 µm. Values are presented as mean ± SEM for three independent experiments. $*p < 0.05$

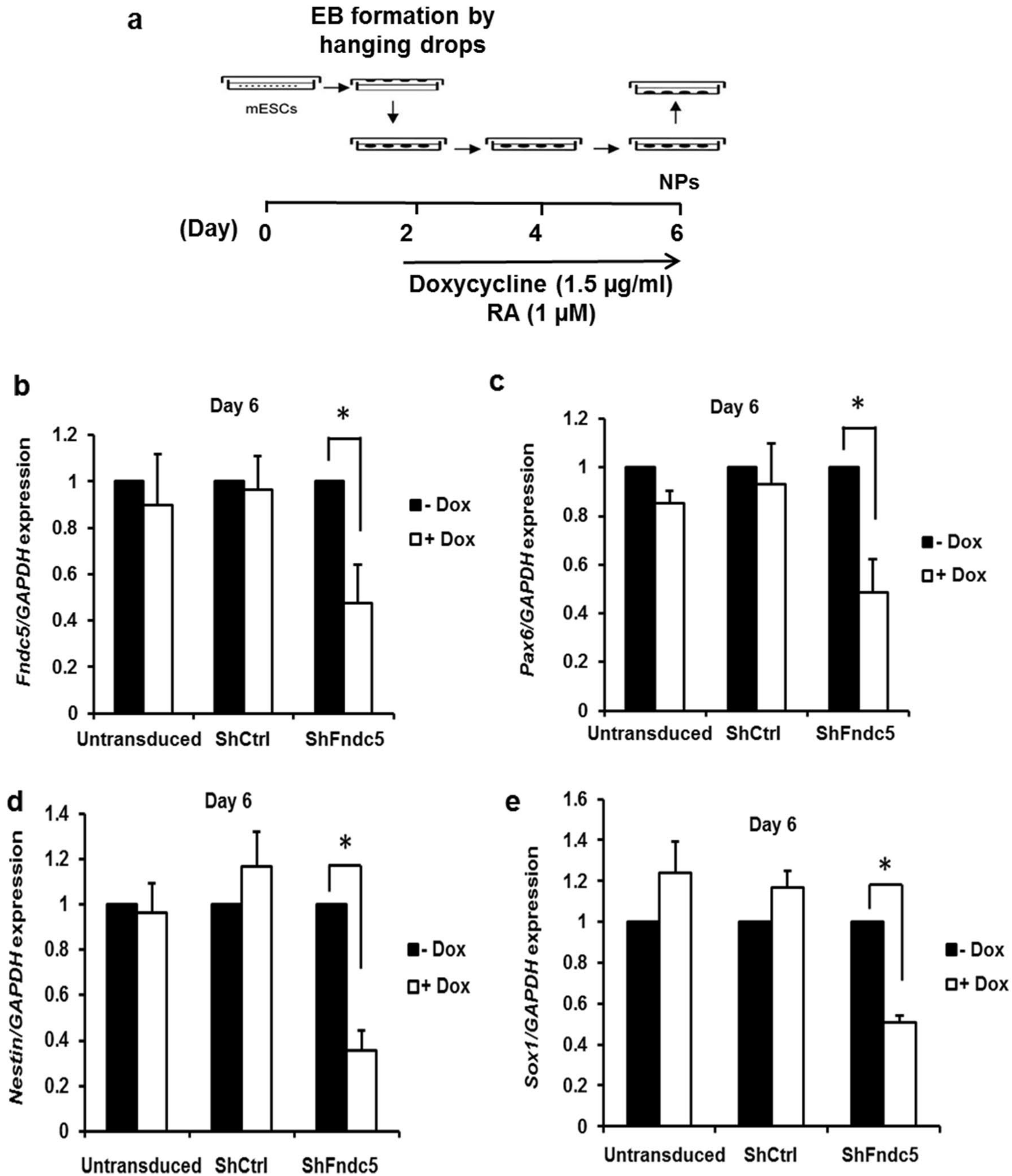
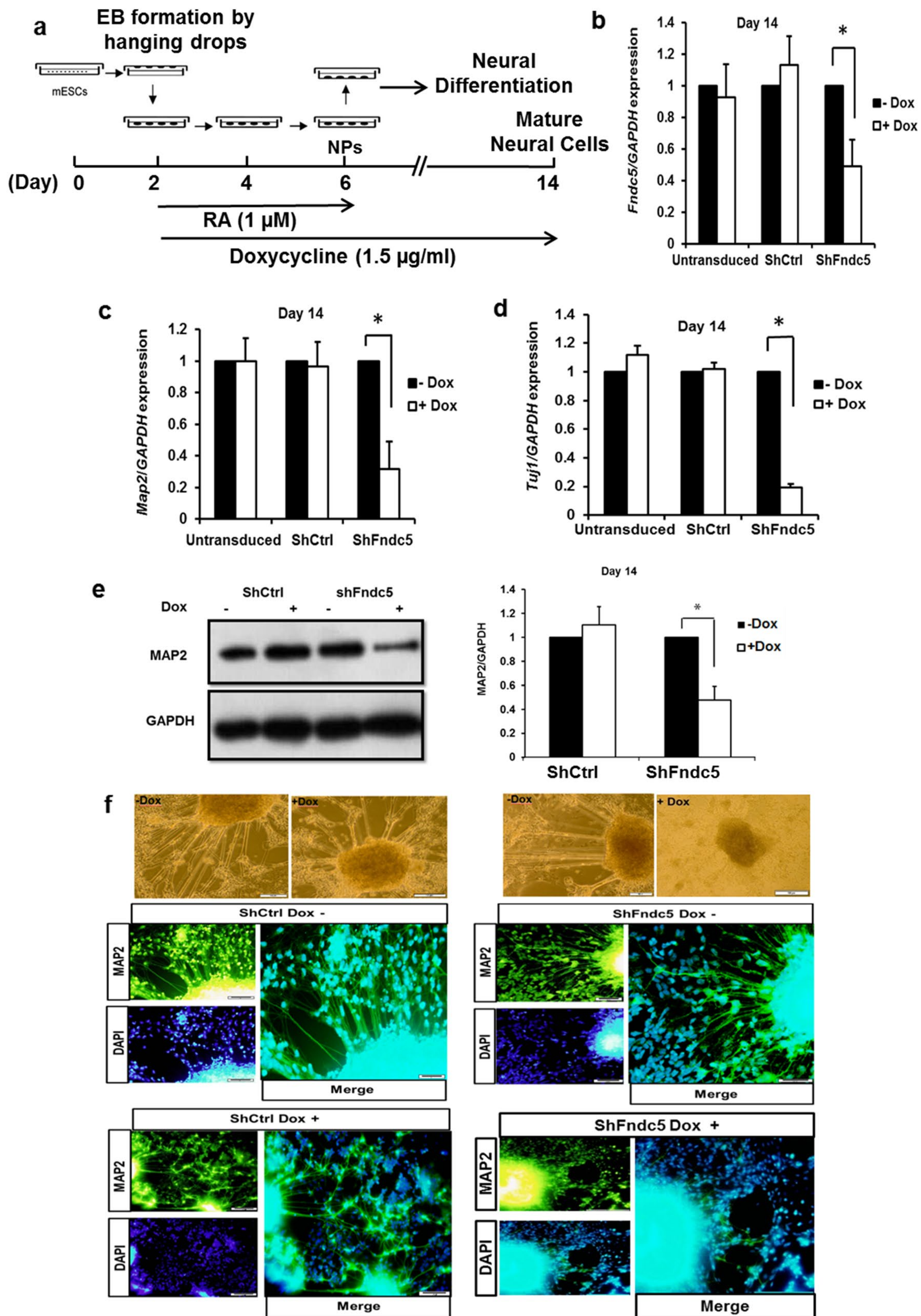



**Fig. 3** Confirmation of neural progenitor marker expression reduction in *Fndc5* knockdown cell line. **a** Schematic illustration of neural progenitors (NPs) formation. Early embryoid bodies (EBs; day 2) of transduced (shCtrl, sh*Fndc5*) and untransduced cell lines were suspended for 4 days in the presence of 1 µM RA and 1.5 µg/mL doxycycline (Dox). **b** RT-qPCR analysis of *Fndc5* expression in Dox-treated cell lines (untransduced, shCtrl and sh*Fndc5*) compared to untreated cells in neural progenitors (day 6). **c**–**e** RT-qPCR analysis of neural progenitor markers, *Pax6*, *Nestin*, and *Sox1* following induction of *Fndc5* knockdown by Dox in sh*Fndc5* cell line compared to untreated control. Represented values are mean of triplicate experiments ± SEM. $*p < 0.05$


a
EB formation by hanging drops
mESCs
Neural Differentiation
Mature Neural Cells
NPs
(Day) 0 2 4 6 14
RA (1 μM)
Doxycycline (1.5 μg/ml)
b
Day 14
Fndc5/GAPDH expression
Untransduced ShCtrl ShFndc5
- Dox
+ Dox
c
Day 14
Map2/GAPDH expression
Untransduced ShCtrl ShFndc5
- Dox
+ Dox
d
Day 14
Tuj1/GAPDH expression
Untransduced ShCtrl ShFndc5
- Dox
+ Dox
e
ShCtrl shFndc5
Dox - + - +
MAP2
GAPDH
Day 14
MAP2/GAPDH
ShCtrl ShFndc5
-Dox
+Dox
f
-Dox
+Dox
ShCtrl Dox -
MAP2
DAPI
Merge
ShFndc5 Dox -
ShCtrl Dox +
ShFndc5 Dox +

◂**Fig. 4** Confirmation of mature neuronal markers expression reduction in *Fndc5* knockdown cell line. **a** Illustrated protocol of mESCs neural differentiation. Transduced (shCtrl, sh*Fndc5*) and untransduced cell lines were treated with 1.5 µg/mL doxycycline (Dox) during and post-neural progenitor (NPs) formation (day 2–day 14). RT-qPCR analysis of *Fndc5* (**b**) and mature neuronal markers, *Map2* and *Tuj-1* (**c**, **d**) expression in Dox-treated cell lines (untransduced, shCtrl, and sh*Fndc5*) compared to untreated cells in mature neurons (day 14). **e** Western blot analysis for MAP2 in neural cells (day 14) relative to GAPDH in Dox-treated sh*Fndc5* cell line compared to untreated and shCtrl cell lines. **f** Upper panel: Phase contrast morphology of differentiated mature neural cells (day 14) from shCtrl and sh*Fndc5* cell lines in the presence and absence of Dox. Lower panel: immunofluorescence staining of Map2 in mature neural cells from shCtrl and sh*Fndc5* cell lines in the presence and absence of Dox. The nuclei were counterstained with DAPI. Scale bar is 100 µm. Values are presented as mean ± SEM for three independent experiments. $^*p < 0.05$

of this protocol for neural differentiation and production of mature neural cells.

## Confirmation of neural progenitor markers' expression reduction in sh*Fndc5* cell line

In 2013, Hashemi et al. showed that reduction of *Fndc5* expression by sh*Fndc5* cell line resulted in a significant decrease in expression levels of NP markers, *Sox1*, *Sox3*, *Pax6*, and *Nestin* [6]. Therefore, for this purpose, we assessed the transcript levels of *Fndc5* and NP markers (*Pax6*, *Nestin*, and *Sox1*) in neural progenitors (day 6) of transduced (shCtrl and sh*Fndc5*) cell lines obtained by Nazem et al., and untransduced cell line following Dox treatment compared to untreated groups. Transduced (shCtrl, sh*Fndc5*) and untransduced cell lines were differentiated to NPs with 1 µM RA and treated with 1.5 µg/mL Dox from day 2 to day 6 (Fig. 3a). As shown in Fig. 3b–e, in contrast to the untransduced and shCtrl cell lines, mRNA expression levels of *Fndc5*, *Pax6*, *Nestin*, and *Sox1* decreased significantly in neural progenitors of Dox-treated sh*Fndc5* cell line compared to untreated control.

It is important to note that as previously shown by Hashemi et al., [6], *Fndc5* knockdown only decreases the rate and efficiency of neural differentiation in mESCs and attenuates this process, but does not inhibit it completely, our results also confirmed their data as Dox-treated sh*Fndc5* cells in day 6 were also neural progenitors, because neural progenitor (*Pax6* and *Nestin*) and stemness (*Nanog* and *Oct4*) markers expression, respectively increased and decreased significantly in these cells compared to mESCs like other groups (Supplementary Fig. 1a–d). Furthermore, as Hashemi et al. had shown, the expression of mesodermal (*αSMA* and *αMHC*) and endodermal (*Sox17* and *Foxa2*) markers did not increase in Dox-treated sh*Fndc5* cell line compared to untreated control which proved neural differentiation reduction by *Fndc5* knockdown was independent of endodermal or mesodermal differentiation (Supplementary Fig. 1e).

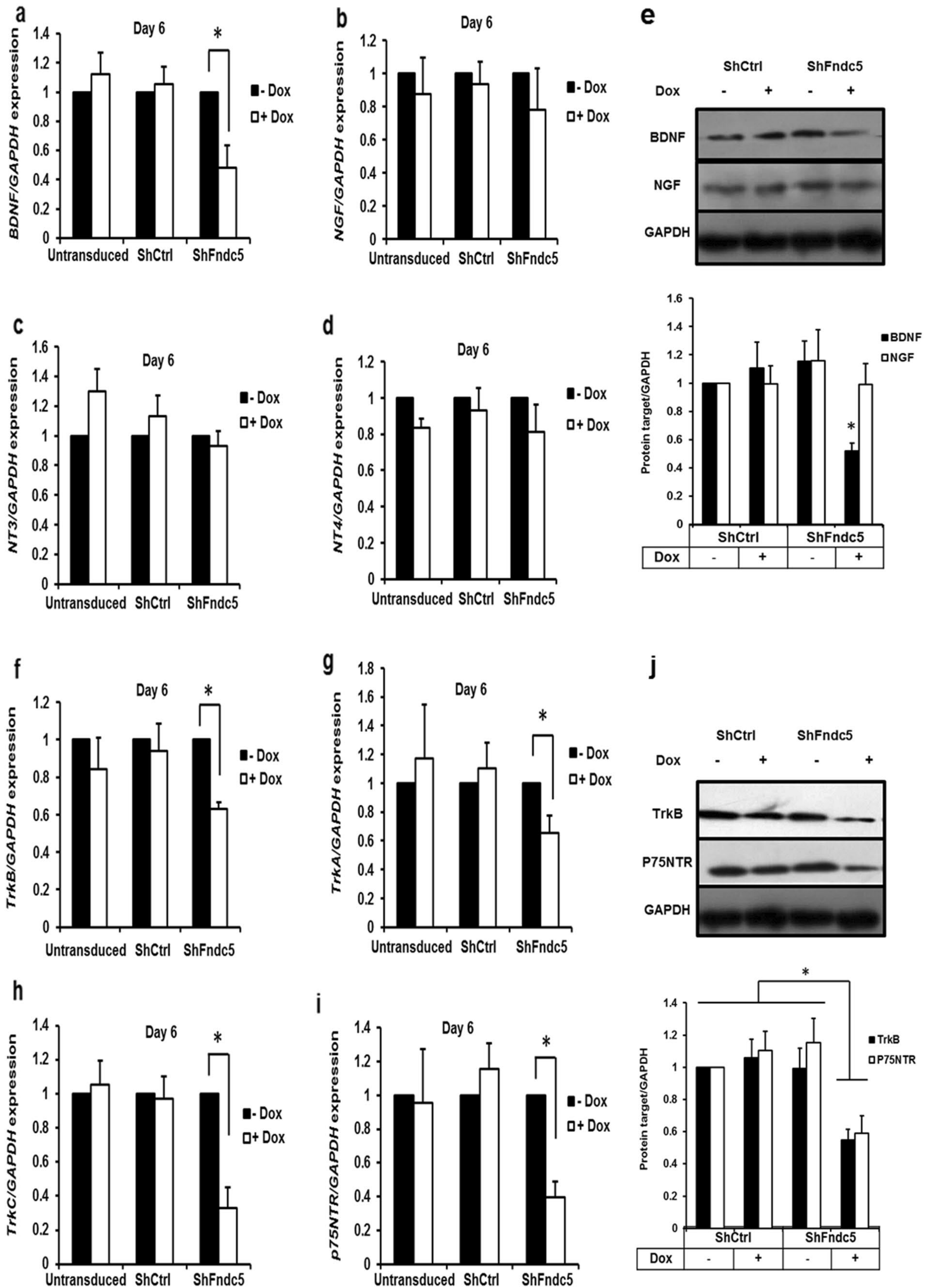
## Confirmation of mature neuronal markers expression reduction in sh*Fndc5* cell line

In previous study by Hashemi et al., it has shown that mature neuronal markers (*Map2* and*Tuj-1*) and consequently neural differentiation rate of mESCs decreased following *Fndc5* expression reduction in sh*Fndc5* cell line compared to control [6]. Therefore, in the present study, to confirm these data, we assessed the transcript levels of *Fndc5* and mature neuronal markers in mature neural cells (day 14) of transduced (shCtrl, sh*Fndc5*) and untransduced cell lines following Dox treatment compared to untreated groups. Figure 4a depicts the schematic procedure for neural differentiation of mESCs. As shown in Fig. 4, Dox induction reduces *Fndc5* RNA level and significantly decreases the transcript levels of *Map2* and *Tuj-1*as mature neuronal markers in sh*Fndc5* cell line compared to untreated control (Fig. 4b–d) which was further verified by western blot analysis (Fig. 4e), and morphological analysis of neural cells derived from mouse embryonic stem cells using phase contrast microscope and immunofluorescence staining against neuronal marker Map2 (Fig. 4f).

As mentioned in the case of progenitor cells, Dox-treated sh*Fndc5* cells in day 14 were also mature neural cells as mature neuronal markers (*Map2* and *Tuj-1*) expression increased significantly in these cells compared to NPs and mESCs like other groups (Supplementary Fig. 2a-b).

## Effect of *Fndc5* knockdown on expression of neurotrophins and their receptors expression in *Fndc5* knockdown neural progenitor cells

To investigate the effect of *Fndc5* knockdown on neurotrophins and their receptor expression in neural progenitors (day 6), transduced (shCtrl, sh*Fndc5*) and untransduced cell lines were differentiated according to aforementioned protocol and *Fndc5* knockdown was performed by Dox induction from day 2 until day 6. As shown in Fig. 5a–d, f–i, *Fndc5* knockdown in sh*Fndc5* cell line resulted in reduction of *BDNF* and all neurotrophin receptors (*TrkB*, *TrkA*, *TrkC*, and *p75NTR*) at mRNA levels in neural progenitors. Transcript level of other neurotrophins (*NGF*, *NT3*, and *NT4*) did not alter in neural progenitors following *Fndc5* knockdown in Dox-treated sh*Fndc5* cell line compared to untreated control. Western blot analysis further confirmed decreased protein levels of BDNF, TrkB, and p75NTR and unchanged protein level of NGF following *Fndc5* knockdown in neural progenitors (Fig. 5e, j).


a
Day 6
BDNF/GAPDH expression
- Dox
+ Dox
Untransduced
ShCtrl
ShFndc5
b
Day 6
NGF/GAPDH expression
c
Day 6
NT3/GAPDH expression
d
Day 6
NT4/GAPDH expression
e
ShCtrl
ShFndc5
Dox
BDNF
NGF
GAPDH
Protein target/GAPDH
f
Day 6
TrkB/GAPDH expression
g
Day 6
TrkA/GAPDH expression
h
Day 6
TrkC/GAPDH expression
i
Day 6
p75NTR/GAPDH expression
j
TrkB
P75NTR
GAPDH

◂**Fig. 5** Evaluation the effect of *Fndc5* knockdown on neurotrophins and their receptor expression in neural progenitors. RT-qPCR analysis of neurotrophins (**a**–**d**) and their receptors (**e**–**h**) mRNA expression in Dox-treated cell lines (untransduced, shCtrl and sh*Fndc5*) compared to untreated cells in neural progenitors (day 6). Western blot analysis for BDNF and NGF (**e**) and TrkB and p75NTR (**j**) in Dox-treated sh*Fndc5* cell line compared to untreated and shCtrl cell lines in neural progenitors (day 6). Data were reported as the mean ± SEM of three independent experiments. $*p < 0.05$

### Effect of *Fndc5* knockdown on expression of neurotrophins and their receptor expression in *Fndc5* knockdown mature neural cells

To verify the effect of *Fndc5* knockdown on neurotrophins and their receptor expression in mature neural cells (day 14), Dox induction was started from day 2 and continued until the end of the process. The expressions of neurotrophins and their receptors were evaluated following *Fndc5* knockdown by real-time PCR and western blot analysis. Contrary to our expectation, in mature neural cells, *BDNF* and *NGF* mRNA levels increased in Dox-treated sh*Fndc5* cell line compared to untreated control (Fig. 6a, b). Unlike *BDNF* and *NGF*, *NT3* and *NT4* transcript levels were not affected by *Fndc5* knockdown in mature neural cells (Fig. 6c, d). Interestingly, western blot analysis revealed a conflicting result with RNA expression analysis. As shown in Fig. 6e, inconsistent with mRNA levels of *BDNF* and *NGF*, protein levels of these two neurotrophins decreased following *Fndc5* knockdown in mature neural cells, as expected. Furthermore, all neurotrophin receptors (*TrkA*, *TrkB*, *TrkC*, and *p75NTR*) transcript levels decreased in Dox-treated sh*Fndc5* cell line compared to untreated control (Fig. 6f–i). Western blot analysis further confirmed the reduction of TrkB and p75NTR at protein levels following *Fndc5* knockdown in mature neural cells (Fig. 6j).

## Discussion

In recent years, transplantation of differentiated neural cells from embryonic stem cells into brain of animal models is an appropriate approach to treat neurodegenerative diseases such as Parkinson's and Alzheimer's diseases [33, 34]. However, functional recovery of nerve injury after transplant depends on appropriate function of neurotrophic factors by the grafted cells in host which induce survival and regeneration of neurons [34].

Neurotrophins, members of neurotrophic factor family, are the main mediators of survival, development, differentiation, repair, plasticity, and molecular synthesis pathways of neurons in both the central and peripheral nervous systems by signaling through specific receptors [35, 36]. It has been revealed that pathogenesis of significant number of psychiatric and neurodegenerative disorders is associated with alteration in neurotrophins and their receptors expression levels. Supply of neurotrophins as therapeutic agents through neurotrophin-producing cell transplantation or neurotrophin-encoding viral vector delivery is an effective strategy to restore neuronal function in neurodegenerative disorders [37, 38].

It has been reported that beneficial effects of exercise on brain performance were achieved by PGC1α/FNDC5/BDNF pathway [12]. Furthermore, in our laboratory, it has been shown so far that knockdown of *Fndc5* decreases neural differentiation rate of mESCs [6] and overexpression of it facilitates this process. In aforementioned study, it was reported that mRNA level of *BDNF* increased following *Fndc5* overexpression and this could be a cause of increased neurogenesis [7]. However, downstream molecular pathways and targets of FNDC5 which influence neuronal health, except BDNF, have not yet been determined.

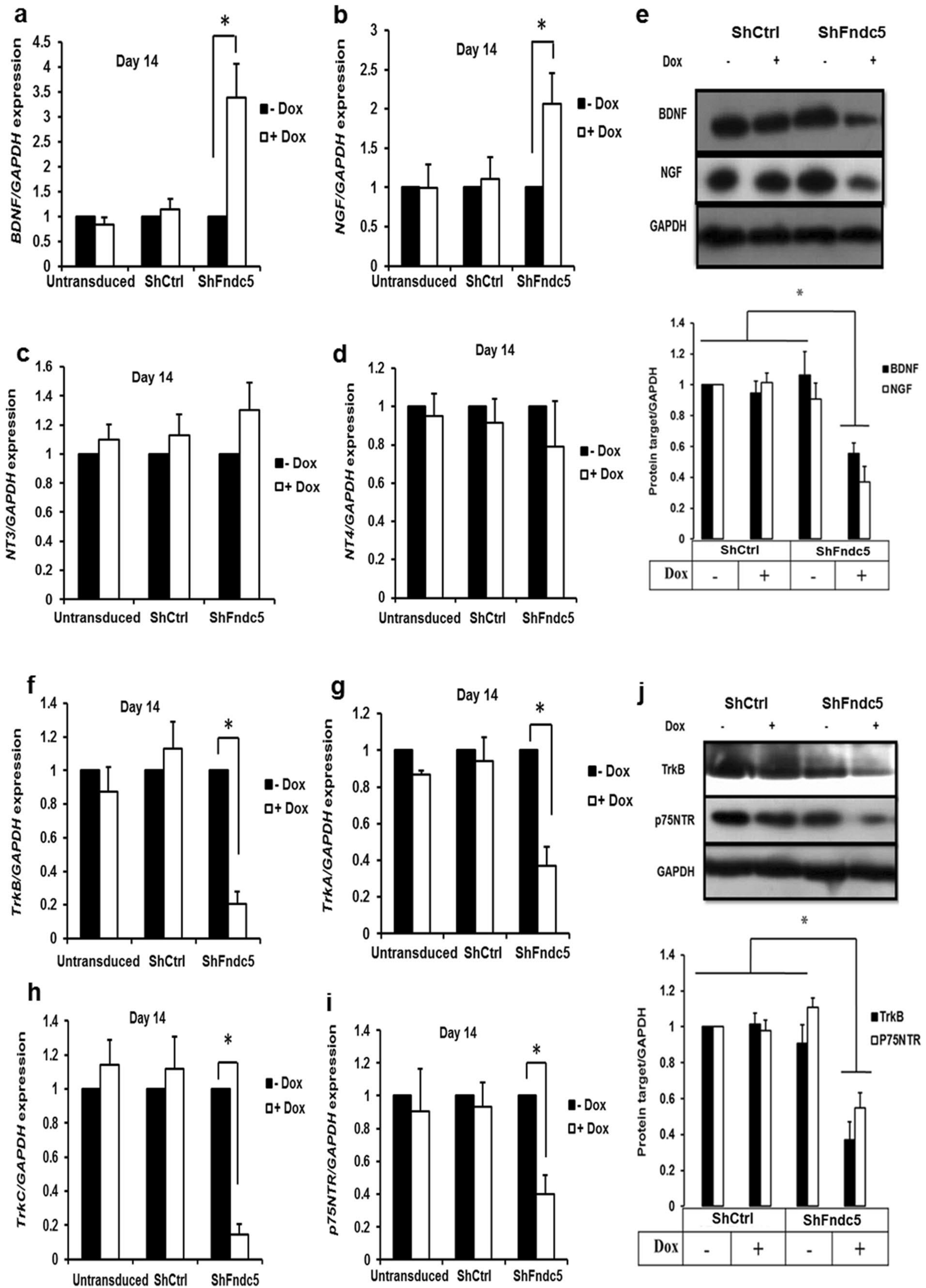
In this study, considering the crucial roles of neurotrophins and their receptors in neurogenesis and since gene silencing through RNA interference (RNAi) is a general method for analyzing gene function [39, 40], we used a stably transduced *Fndc5* knockdown cell line to assess whether decreased level of *Fndc5* reduced neural differentiation of mESCs by affecting the expression of neurotrophins and their receptors. Since neurotrophins have important role in neural tissue repair [13–15], it would be advantageous to modulate the neurotrophins through Fndc5 for further clinical approaches.

In this paper, we used a sh*Fndc5* expressing mESC line and short hairpin RNA interference (shRNAi) transcript products [9].

Our data were evident that mRNA expression level of neural progenitor markers, *Pax6*, *Nestin*, and *Sox1,* and mature neuronal markers *Map2* and *Tuj*-1 decreased significantly following *Fndc5* knockdown in Dox-treated sh*Fndc5* cell line compared to untreated control.

In the present study, among the neurotrophins which were studied, *BDNF* transcripts were sharply decreased upon downregulation of *Fndc5* in NPs. In this circumstance, also the expression of neurotrophin receptors decreased, but *NGF*, *NT3*, and *NT4* mRNA expression levels unchanged. Western blot analysis further confirmed these data in neural progenitors.

In mature neural cells, following *Fndc5* knockdown, the expression of *BDNF* and *NGF* at protein level and all neurotrophin receptors at transcript level decreased in Dox-treated sh*Fndc5* cell line compared to untreated control. Western blot analysis for TrkB and p75NTR protein confirmed these data. All neurotrophins mediate their effects via these receptors through activation of three important signaling cascades (ERK, PI3-kinase, and phospholipase C-γ1) and induction of the expression of bHLH transcription factors which are


a
Day 14
BDNF/GAPDH expression
- Dox
+ Dox
Untransduced ShCtrl ShFndc5
b
Day 14
NGF/GAPDH expression
- Dox
+ Dox
Untransduced ShCtrl ShFndc5
e
ShCtrl ShFndc5
Dox
BDNF
NGF
GAPDH
Protein target/GAPDH
BDNF
NGF
c
Day 14
NT3/GAPDH expression
d
Day 14
NT4/GAPDH expression
f
Day 14
TrkB/GAPDH expression
g
Day 14
TrkA/GAPDH expression
j
ShCtrl ShFndc5
Dox
TrkB
p75NTR
GAPDH
Protein target/GAPDH
TrkB
P75NTR
h
Day 14
TrkC/GAPDH expression
i
Day 14
p75NTR/GAPDH expression

◂**Fig. 6** Evaluation of the effect of *Fndc5* knockdown on neurotrophins and their receptor expression in mature neural cells. RT-qPCR analysis of neurotrophins (**a**–**d**) and their receptor (**f**–**i**) mRNA expression in Dox-treated cell lines (untransduced, shCtrl, and sh*Fndc5*) compared to untreated cells in mature neural cells (day 14). Western blot analysis for BDNF and NGF (**e**) and TrkB and p75NTR (**j**) in Dox-treated sh*Fndc5* cell line compared to untreated and shCtrl cell lines in mature neural cells (day 14). Data were reported as the mean ± SEM of three independent experiments. $*p < 0.05$

critical for neuronal growth, proliferation, maintenance, and differentiation [41, 42]. Hence, in the current study, the reduction of neurotrophins receptor expression following *Fndc5* knockdown results in unfunctional neurotrophins.

It has been revealed that activity-dependent mRNA translation and synthesis of endogenous BDNF is triggered by activation of Trk receptors and regulated by multiple signaling cascades, as inhibition of signaling mediated by Ras, Erk1/2, PI3K, mTOR, or PLC which all control translation through 5′ UTR- dependent mechanisms, inhibit BDNF translation [43].

On the other hand, various studies have already shown that Fndc5/irisin exerts its biological effects through several intracellular signaling pathways. MAPK signaling pathway including Erk1/2 is the major signaling pathway downstream of Fndc5 which is involved in neural and osteoblast differentiation and white adipocytes' browning [10, 11, 44]. Furthermore, a recent study revealed that recombinant irisin not only induced phosphorylation of ERK in cultured neuron but also stimulates the cAMP/PKA/CREB pathway in human cortical and mouse hippocampal slices [45]. CREB is one of the best-studied factor involved in regulation of BDNF expression. Downregulation of phosphorylated (p)-CREB reduces BDNF level [46]. Furthermore, it has been revealed that the expression of BDNF receptor, trkB, is regulated by the cAMP/CREB pathway in neurons [47].

In conclusion, decreased levels of BDNF and NGF and their receptors in Dox-treated sh*Fndc5* cell line compared to untreated control can be attributed to the decreased activity of signaling cascades such as Erk1/2 and cAMP/CREB following *Fndc5* knockdown in these cells.

In the current study, comparing western blot results *vs.* RT-PCR data, there was a RNA/protein discordant in BDNF and NGF expression when *Fndc5* decreased in mature neurons in Dox-treated sh*Fndc5* cell line. Like this discordance in NGF and BDNF RNA/protein levels, observed in our previous study which we used two different media for differentiation of NPs to mature neurons [48]. In the present study, discrepancy between mRNA and protein levels of BDNF and NGF in Dox-treated sh*Fndc5* cell line could presumably due to a compensatory mechanism or the possible miRNAs expression following *Fndc5* knockdown which suppress neurotrophins at protein levels [49, 50] or signaling pathways which contribute in neurotrophins translation. For example a study in 2017 revealed that irisin attenuates H2O2-induced apoptosis in cardiomyocyte through upregulation of miR-19b which activates AKT/mTOR signaling pathway [51]. Furthermore, the previous studies reported that miR-19 has functional implications in adult hippocampal neurogenesis as it is enriched in neural progenitor cells (NPCs) and is a key regulator of migration of newborn neurons in the adult brain. In addition, miR-19 affects the maturation of newborn neurons [52, 53]. As mentioned above, inhibition of multiple signaling cascades such as mTOR inhibits BDNF translation. Therefore, if Fndc5 impacts the expression of miR-19b in neural cells like cardiomyocyte, it is possible that *Fndc5* knockdown causes downregulation of miR-19b and thereby mTOR signaling activity and BDNF translation.

In the current study, the expressions of *NT3* and *NT4* did not affect by *Fndc5* knockdown. Previous studies revealed that neurotrophins have differences in temporal and spatial expression during the neurogenesis as NT3 is needed for early stages of this process and has important roles in immature neurons. The expression of NT3 is decreased with maturation of CNS neurons [54, 55]. Accordingly, we speculate that the same level of NT3 in Dox-treated sh*Fndc5* cell line compared to untreated control is likely due to the time of gene expression analysis (mature neural cells, day 14). NT3 gene expression analysis before day 14 is required to determine whether *Fndc5* knockdown can affect its expression or not.

Moreover, studies revealed that NT4 is widely expressed in non-neuronal tissues as highest level of NT4 has been detected in testis and skeletal muscle and much lower levels in the CNS. NT4 null mice show minimal neurological phenotypes compared with BDNF knockout mice. Surprisingly, it has been revealed that NT4 is not activity regulated in the brain [56]. Furthermore, data obtained from previous studies suggest that NT4 expression is important in the absence of BDNF [57]. As we showed in our previous study, mRNA expression level of *NT4* did not change during neural differentiation of mESCs by RA and it seemed that there was no need to increase NT4 expression, because BDNF upregulated during this process [48]. Accordingly, in the present study, the expression of NT4 has not been affected by *Fndc5* knockdown during neural differentiation of mESCs.

In conclusion, these data suggest that the decreased efficiency of neural differentiation following *Fndc5* knockdown can be attributed to decreased levels of NGF and BDNF proteins in addition to their cognate receptors. Although, these findings are not sufficient to

declare whether neurotrophins and their cognate receptor are direct downstream targets of Fndc5 or not, because molecular mechanisms involved in the control of neural differentiation by Fndc5 are still unclear. Further investigations including overexpression of *Fndc5* or treatment with candidate neurotrophins (such as recombinant proteins of NGF and BDNF) to see the recovery of neural differentiation rate in *Fndc5* knockdown mouse ES cells will be required to clarify the molecular event of this process.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s13577-021-00517-z.

**Acknowledgements** Authors would like to thank University of Sistan and Baluchestan and Royan Institute for the financial support of this project. The authors are thankful to other members of Royan Institute for their excellent technical assistance and advice.

**Author contributions** RE: experimental design, data collection, data analysis, data interpretation, and manuscript writing. FR: data analysis, data interpretation. DMKT: experimental design, financial support, data analysis, data interpretation, and final approval of the manuscript. MHNE: experimental design, financial support, data analysis, data interpretation, and final approval of manuscript. KG: experimental design, data analysis, data interpretation, manuscript writing, and final approval of the manuscript.

## Declarations

**Conflict of interest** The authors indicate no potential conflicts of interest.

**Ethical approval** Approval for this study was obtained from the Institutional Review Board of Royan Institute (Tehran, Iran).

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