Zinc finger protein 521 overexpression increased transcript levels of *Fndc5* in mouse embryonic stem cells

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Zinc finger protein 521 is highly expressed in brain, neural stem cells and early progenitors of the human hematopoietic cells. Zfp521 triggers the cascade of neurogenesis in mouse embryonic stem cells through inducing expression of the early neuroectodermal genes *Sox1*, *Sox3* and *Pax6*. Fndc5, a precursor of Irisin has inducing effects on the expression level of *brain derived neurotrophic factor* in hippocampus. Therefore, it is most likely that Fndc5 may play an important role in neural differentiation. To exhibit whether the expression of this protein is under regulation with Zfp521, we overexpressed *Zfp521* in a stable transformants of mESCs expressing *EGFP* under control of *Fndc5* promoter. Increased expression of *Zfp521* enhanced transcription levels of both *EGFP* and endogenous *Fndc5*. This result was confirmed by overexpression the aforementioned vectors in HEK cells and indicated that Zfp521 functions upstream of *Fndc5* expression. It is most likely that Zfp521 may act through the binding to its response element on *Fndc5* core promoter. Therefore it is concluding that an enhanced expression of *Fndc5* in neural progenitor cells is stimulated by *Zfp521* overexpression in these cells.

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1. Introduction

Zinc finger protein 521 (Zfp521; also called Evi3 in mice and EHZF/ZNF521 in human) is a 180 kDa transcriptional co-regulator protein which was detected to be highly expressed in brain, neural stem cells and early progenitors of the human hematopoietic cells (Bond *et al.* 2004). Zfp521 includes 30 domains of Krüppel-like C2H2 zinc fingers (Bond *et al.* 2004;

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Abbreviations used: Bdnf, brain-derived neurotrophic factor; BSA, bovine serum albumin; DAPI, 4, 6-diamidio-2-phenylindole; DMEM F12, Dulbecco's modified Eagle medium/Ham's-Nutrient Mixture F-12; EB, embryoid body; EBF1, early B-cell factor 1; FITC, fluorescein isothiocyanate; Fndc5, firbonectin type III domain containing 5; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; LIF, leukemia inhibitory factor; mESCs, mouse embryonic stem cells; MOI, multiplicity of infection; NPs, neural progenitors; PEP, peroxisomal protein; PBS, phosphate buffered saline; PVDF, polyvinylidenedifluoride; TBST, tris-buffered saline with Tween 20; Zfp521, zinc finger protein 521

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Lin *et al.* 2004) and a stretch of 12 amino acid residues at N-terminus responsible for binding to some transcriptional repressors. In hematopoietic stem cells, Zfp521 is highly expressed while its transcription levels decrease during differentiation to mature leukocytes (Bond *et al.* 2004, 2008). Further studies revealed that Zfp521 inhibits process of erythroid differentiation through the repression of GATA1 target genes (Matsubara *et al.* 2009) and inhibits the early B-cell factor 1 (EBF1) as the transcriptional factor. Meanwhile, Zfp521 stimulates osteogenesis via interaction with Zfp423, Ebf1 and Runx2 and numerous chromatin remodelling factors (Wu *et al*. 2009; Hesse *et al.* 2010; Correa *et al*. 2010; Kang *et al.* 2012; Kiviranta *et al.* 2013). Besides the aforementioned functions, Kamiya *et al.* have shown that Zfp521 has a critical role in neurogenesis through an induction on expression of the early neuroectodermal genes including *Sox1*, *Sox3* and *Pax6* which are responsible for neural differentiation of mouse embryonic stem cells (mESCs). The importance of Zfp521 in process of neural differentiation was confirmed by *Zfp521* knockdown which abolished neural differentiation (Kamiya *et al.* 2010).

Fndc5 [firbonectin type III domain containing 5; previously known as peroxisomal protein (PEP)], first identified by Ferrer-Martinez and colleagues, was shown to be expressed in brain of adult mice (Ferrer-Martinez *et al.* 2002). We previously showed that expression level of *Fndc5* was increased upon treating with retinoic acid (RA) during neural differentiation of mESCs, suggesting a possible role in neural differentiation (Ostadsharif *et al.* 2011). Furthermore, our group demonstrated that *Fndc5* knockdown significantly decreased neural differentiation of mESCs (Hashemi *et al.* 2013). Fndc5 could be cleaved and secreted from muscle cells as a type of myokine known as Irisin. Irisinis aglycosylated myokine with 112 amino acid residues (Bostrom *et al.* 2012). Pharmacological concentrations of Irisin have increasing effects of mouse H19-7 hippocampal cell proliferation (Moon *et al.* 2013). Furthermore, Fndc5 has inducing effects on the expression level of *brain-derived neurotropic factor* (*Bdnf*) in hippocampus (Wrann *et al*. 2013). Therefore it is most likely that Fndc5 may play an important role in neural differentiation. To exhibit whether this protein is under regulation of Zfp521, a key factor in neural differentiation, we overexpressed *Zfp521* CDS in a stable transformants of mESCs expressing *EGFP* under control of *Fndc5* promoter (Seifi *et al.* 2014). Increased expression of *Zfp521* enhanced transcription levels of both *EGFP* and endogenous *Fndc5*.

2. Materials and methods

2.1 *mESCs culture and neural differentiation stages*

Mouse embryonic derivative stem cell line, RoyanB20, was used in this study and retained in an undifferentiated state as described (Baharvand and Hassani 2013). Cells were cultured in serum free medium, a mixed cocktail of Dulbecco's modified Eagle medium/Ham's-Nutrient Mixture F-12 (DMEM F12) with Neurobasal media (Gibco, USA) supplemented with 0.5% BSA, 2 mM glutamine, 0.1 mM nonessential amino acids, 1% penicillin–streptomycin (all from Invitrogen, USA), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, USA) and 1000 U/mL leukemia inhibitory factor (LIF; Chemicon, USA), 1 μM PD0325901(Sigma, USA) and 10 μM SB431542 (Sigma, USA),1% N2 (Gibco, USA) , 2% B27 (Gibco, USA). The medium was changed everyday and cells were passaged every 2 days. Neural differentiation was carried out according to the previously described protocol through embryoid body (EB) formation in hanging drops for 2 days (Ostadsharif *et al.* 2011). Subsequently, EBs were plated on gelatin-coated 12-well plates (TPP, Switzerland) for an additional 6 days in a neurobasal medium (Invitrogen) that contained 5% ES-FCSand penicillin–streptomycin supplemented with 0.1 mM non-essential amino acids, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 2% B27 supplement (Invitrogen).

2.2 *Production of lentiviral particles and titration*

In this study, packaging vectors were psPAX2 and pMD2G (Addgene, USA) and recombinant vector pLenti6.3/TO/V5-DEST/Zfp521 was obtained from Department of Stem Cells and Developmental Biology (Royan Institute, Tehran, Iran). HEK293T cells were transfected with packaging and recombinant vectors using Lipofectamine LTX (Invitrogen) as described in manual protocol. Supernatant fraction of transfected cell culture was collected after 72 h and filtered with 0.2 μm filter (Millipore, USA). Produced lentiviral particles were concentrated at 25000 rpm for 2 h at 4°C. The concentrated lentiviral particles of pLenti6.3/TO/V5-DEST/Zfp521or the control vector were used to transduce mESCs.

2.3 *mESCs transduction to generate a stable cell line overexpressing Zfp521*

In the present study stable transformant of mESCs, Fndc5 Promoter-EGFP cells, were used which expressed *EGFP* under tight control of proximal part of *Fndc5* promoter (Seifi *et al.* 2014). Approximately 2×10^5 mESCs were seeded in 6-wells coated with 0.1% gelatin, and 24 h later, they were transduced [Multiplicity of infection (MOI) was 10] with the lentiviral particles that contained pLenti6.3/TO/V5-DEST/Zfp521. Two days post transduction, to select stable cell line, cells were treated with Blasticidin (4 μg/mL) (Sigma) for 2 weeks and the medium was changed every day until stable colonies were observed. One of the colonies, named ZFP521 cell line, with the ability to overexpress *Zfp521*, was

selected for further analyses. Meanwhile the stably transformed Fndc5 Promoter-EGFP cells with the empty pLenti6.3/TO/V5-DEST vector, as mock cells, was selected for experiments as negative control.

2.4 *Western blot analysis*

Protein isolation was carried out with Trizol (TRI) reagent (Sigma-Aldrich). SDS-PAGE was performed at 120 V for 2 h using a Mini-PROTEAN Tetra cell (Bio-Rad, China). Separated proteins were transferred to a polyvinylidenedifluoride (PVDF; Biorad, USA) membrane by wet blotting (Bio-Rad). Membranes were blocked for 1 h with 10% skim milk and incubated for 1.5 h at room temperature (RT) with the respective primary antibodies: anti-GFP (Santa Cruz, SC-14033, 1:100) and anti-glyceraldehyde-3-phosphate dehydrogenase (Gapdh; Sigma-Aldrich, A2228, 1:5000). At the end of the incubation period, membranes were rinsed 3 times for 15 min with a mixture of tris-buffered saline with Tween 20 (0.1%v/v; TBST) and incubated with the second antibody: HRP-conjugated goat anti-mouse antibody (Dako, 1:5000) or HRP-conjugated goat anti rabbit antibody (Santa Cruz, SC-2301,1:16000) for 1 h. Subsequently, membranes were washed with TBST and target protein bands were visualized by an Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare, USA).

2.5 *RNA isolation and real-time quantitative PCR(RT-qPCR)*

Total RNA was extracted from the cultured cells using the RNeasy MiniKit (Qiagen, Germany) and treated with *DNase*I (Thermo Scientific, USA). cDNA synthesis was performed with 1 μg of total RNA applying random hexamer primer and the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). RT-qPCR was carried out with SYBR green (TaKaRa, Japan) in a thermal Cycler Rotor-Gene 6000 (Corbett, Australia). For each reaction, PCR mixture contained 10 μL Rotor-Gene SYBR Green PCR Master Mix (TaKaRa), 25 ng cDNA and 3 pmol of each primer in a final volume of 20 μL. Analysis of gene expressions was carried out by the ΔΔCT method and the relative levels of expression were normalized to *Gapdh* gene expression level. The primer sequences for real-time analysis are as follows: Amplification of a part of *Fndc5* was achieved by using a forward primer, 5′-GACAGTAGAGAATG CGAGAGG-3′ and a reverse primer, 5′-5′-CCGATGATAG GAGAAGATGGAG-3′. Meanwhile RT-qPCR was carried out for *Zfp521* using sequences: 5′-GAACTCGTGGT GGATGACT-3′ (Sense) and 5′-GCAGCAGAAGC AGAACTAAC-3′ (Antisense). On the other hand the subsequent primer pair (Forward) 5′-CAAGCAGAAGA ACGGCATCAAG-3′ and (Reverse) 5′-GGTGCTCAGGTA GTGGTTGTC-3′ were used for EGFP amplification. For *MapII* expression analysis primer pair was as: 5′-AAGT CACTGATGGAATAAGC-3′ (Sense) and 5′-CTCTGCGA ATTGGTTCTG-3′ (antisense) and for Nestin the primers were: 5′-CACACCTCAAGATGTCCC-3′ (Sense) and 5′-GAAAGCCAAGAGAAGCCT-3′ (Antisense). For assessment of the *Gapdh* gene expression level following primers were used: 5′-TGCCGCCTGGAGAAACC-3′ (Sense) and 5′-TGAAGTCGCAGGAGACAACC-3′(Antisense).

2.6 *Co-transfection of Plasmids*

pLenti6.3/TO/V5-DEST/Zfp521 or the control vector (same vector without *Zfp521* CDS) was used along with pDB2 vector encompassing core promoter of *Fndc5* upstream of EGFP (Seifi *et al.* 2014), for co-transfection into the HEK293T cells using Lipofectamine LTX reagent. Cell numbers and the amount of plasmids for each transfection were determined according to the manufacturer's protocol. At 2 days post-transfection, we used the cells for flow cytometry analysis.

2.7 *Flow cytometry analysis*

To quantify the *EGFP* intensity, Fndc5 Promoter-EGFP cells expressing enhanced green fluorescence protein (EGFP) under the regulation of *Fndc5* Promoter were simultaneously cultured in the presence LIF 1000 IU/mL for 3 days. The fluorescence intensity of cells, which represented *EGFP* expression levels were analysed by a Becton DickinsonFACSCalibur flow cytometer (USA). For each sample, 10^4 events were recorded in the forward light scatter/side light scatter (FSC/SSC) dot plot. *EGFP* was detected in a fluorescence detector 1 (FL-1) with a 530/30 nm band pass filter. Data were analysed, using CellQuest Pro and WinMDI 2.9 softwares, respectively. The same approach was performed with the HEK293T cells which were co-transfected with pLenti6.3/TO/V5-DEST/Zfp521 or the control vector along with pDB2 vector encompassing core promoter of *Fndc5* upstream of *EGFP*.

2.8 *Immunocytochemistry*

Cells were grown on cover slips were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 30 min. Fixed cells were permeabilized utilizing 0.1% Triton X-100 in PBS for 40 min. Primary and secondary antibodies were diluted in blocking buffer [10% goat serum and 1 mg/mL bovine serum albumin (BSA; Sigma-Aldrich) in PBS] for 2 h and 1 h at room temperature, respectively. Finally, 4, 6-diamidio-2-phenylindole (DAPI; Sigma-Aldrich) was used for 3 min

for nuclear counterstaining. The stained cells on the cover slip were mounted on glass slides and analysed under a fluorescent microscope (Olympus, Japan). Images were captured with an Olympus DP70 camera (Olympus, Japan). Primary and secondary antibodies used for immunofluorescence staining were anti-mouse antibodies against MapII (1:200 v/v; Sigma-Aldrich) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:50 v/v; Chemicon) as already described (Hashemi *et al.* 2013).

2.9 *Statistical analysis*

Quantitative experiments including RT-qPCR were repeated inbiologically triplicate independent experiments. Significant differences between groups were examined by *t*-test analysis. Data were presented as mean±standard error of mean (SEM) and $p \leq 0.05$ was considered statistically significant.

3. Results

3.1 *Expression profile analysis of Zfp521 and Fndc5 during neural differentiation of mESCs cells*

As depicted in the figure 1A, neural differentiation of mESCs was carried out by an established protocol using RA induction as already described (Hashemi *et al.* 2013). Emerged neural progenitors (NPs) were verified by RT-qPCR for *Nestin* as a NP marker (supplementary figure 1B). Similar to s previous study (Hashemi *et al.* 2013), expression levels of *Nestin* was optimal on day 6 and mature neural cells were emerged on day 14 as verified by RT-qPCR and immunostaining of *MapII* as a neural marker (supplementary figure 1A and C). Transcript levels of *Fndc5* (figure 1B) and *Zfp521* (figure 1C) were assessed on day 0, 6 and 14 from the beginning of neural differentiation process. The expression levels of both genes, *Fndc5* and *Zfp521*, increased in NPs and then decreased in mature neural cells.

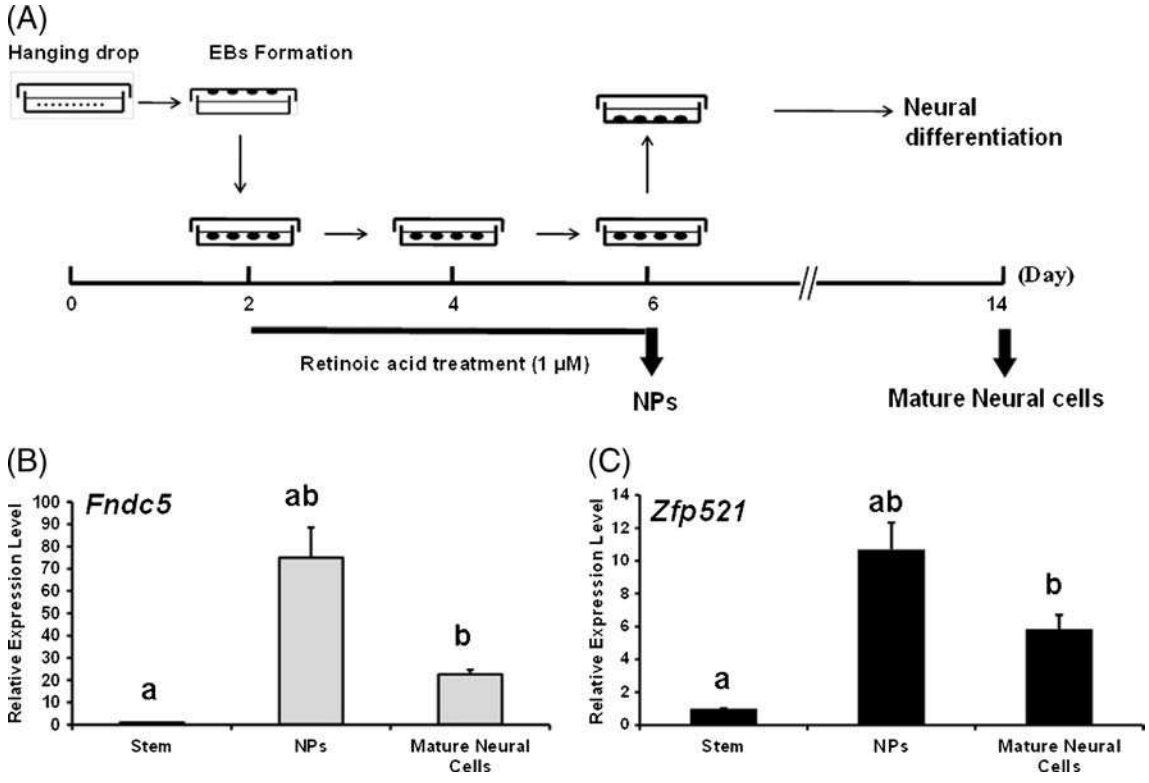

Figure 1. Modulation of *Zfp521* and *Fndc5* transcripts during neural differentiation of mESCs. (**A**) Illustrated protocol of neural differentiation of mESCs. As depicted neural differentiation was induced by adding of retinoic acid (1 µM) to the cell culture and cells were grown to produce NPs. Mature neural cells were immerged in differentiated state. (**B** and **C**) RT-qPCR analysis of *Fndc5* and *Zfp521* in NPs and mature neural cells compare to stem cells. Relative expression of target genes were quantified and normalized with *Gapdh*. The number of independent repeats were 3 for each experiment (n=3). Similar alphabets indicate significant difference between same samples at $p < 0.05$.

3.2 *Establishment of stable transduced mESCs-Fndc5 promoter–EGFP cell line expressing Zfp521*

Lentiviral-mediated gene transduction was carried out to transfer pLenti6.3/TO/V5-DEST/Zfp521or the empty pLenti6.3/TO/V5-DEST vectors to Fndc5 promoter–EGFP cells (figure 2A). As described in the materials and methods section, 2 days post-transduction, stable cells were selected by adding of Blasticidin (4 µg/mL) for 2 weeks when stable colonies appeared. The expression level of *Zfp521* in pLenti6.3/TO/V5-DEST/Zfp521 transduced cells (Zfp521), was significantly higher than mock cells, pLenti6.3/TO/V5-DEST transduced cells (figure 2B). Essentially, expression levels of the pluripotency markers, *Nanog* and *Oct4* in ZFP521 and mock cells (Mock), were comparable with those data observed for untransfected mESCs (Untransfect) (figure 2C).

3.3 *Zfp521 overexpression enhanced transcript levels of EGFP and Fndc5in ZFP521 cells*

The forced expression of *Zfp521* induced an increment in mRNA levels of *EGFP* (figure 2D) which was under tight control of *Fndc5* core promoter. This observation was also confirmed by the Western blot analysis of lysate cells (ZFP521 and Mock cell lines) (figure 2F and G). To estimate whether similar increment in transcription level was also apparent for endogenous *Fndc5*, quantitative real-time PCR analysis was performed for the cDNA derived from both

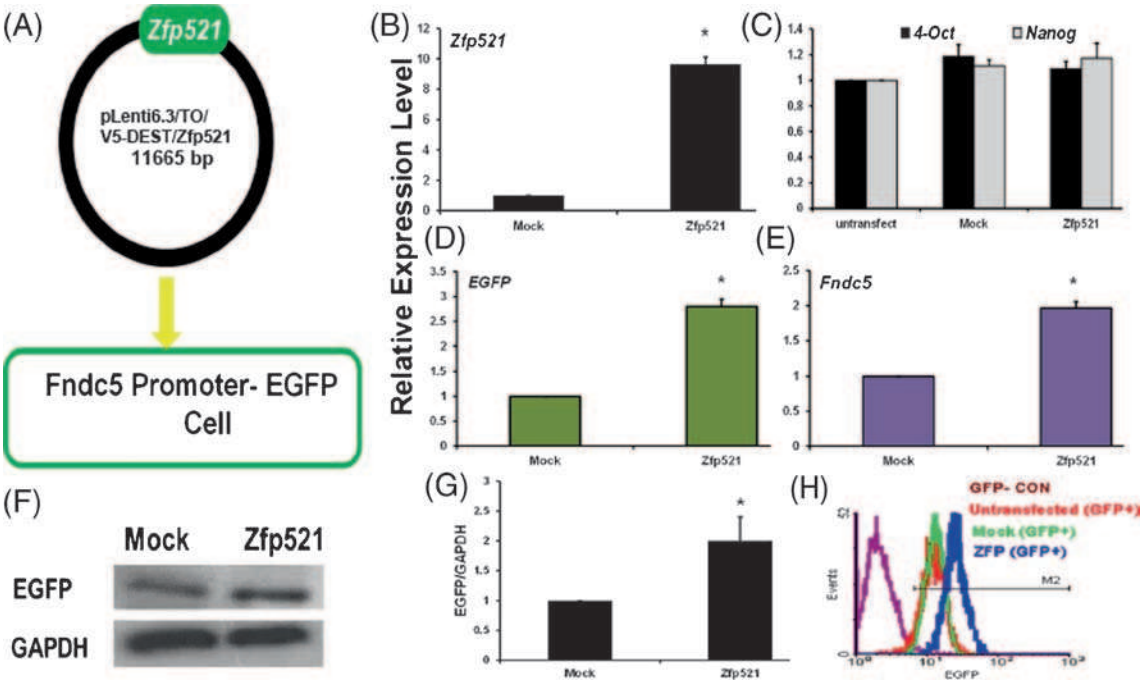

Figure 2. Overexpression of *Zfp521*enhanced expression level of *Fndc5*. (**A**) Schematic representation of a viral based vector which was used for transduction of *Zfp521* CDS into Fndc5 promoter-EGFP cells. RT-qPCR analysis of gene markers upon *Zfp521* overexpression was carried out to assess the transcript levels of *Zfp521* (**B**), *Oct4* and *Nanog* (**C**), *EGFP* (**D**) and *Fndc5* (**E**). Relative expression of target genes was quantified and normalized with *Gapdh*. Mock and Zfp521 cells are stably transformed Fndc5 Promoter-EGFP cells with the empty pLenti6.3/TO/V5-DEST vector, and pLenti6.3/TO/V5-DEST/Zfp521 vector respectively. (**F** and **G**) The protein content of EGFP was estimated and compared in Mock and Zfp521 cells. The number of independent repeats was 3 for each experiment (n=3). Star indicates significant difference between samples at $p<0.05$. (**H**) EGFP fluorescence intensity was assessed in intact mESCs [GFP-CON, purple colour], Fndc5 Promoter-EGFP mESCs [Untransfected (GFP+), red colour], Fndc5 Promoter-EGFP mESCs transduced with the empty pLenti6.3/TO/V5-DEST vector [Mock (GFP+), green colour] and Fndc5 Promoter-EGFP mESCs transduced with thepLenti6.3/TO/V5-DEST/Zfp521 [ZFP (GFP+), blue colour] The GMean of EGFP intensity for the aforementioned cell lines was estimated as: 0, 37, 36.49 and 75.17 respectively as notified in results.

ZFP521 and mock cells. Results indicated a similar increasing pattern of *Fndc5* mRNA levels in ZFP521 overexpressed cells (figure 2E). These data were confirmed by flow cytometry, as shown in figure 2H; EGFP intensity was examined in four different cell lines, intact mESCs [GFP-CON, purple colour], Fndc5 Promoter-EGFP mESCs [Untransfected (GFP+), red colour], Fndc5 Promoter-EGFP mESCs transduced with the empty pLenti6.3/TO/V5-DEST vector [Mock (GFP+), green colour] and Fndc5 Promoter-EGFP mESCs transduced with the pLenti6.3/TO/V5-DEST/Zfp521 [ZFP (GFP+), blue colour). The mean of EGFP intensity for the aforementioned cell lines was estimated as 0, 37, 36.49 and 75.17, respectively, indicating an enhancement in EGFP intensity upon *Zfp521* expression into the Fndc5 Promoter–EGFP mESCs.

3.4 *Zfp521 overexpression enhanced transcript levels of EGFP in HEK cell line.*

To examine whether induced EGFP expression driven by Fndc5 promoter under ectopic expression of *Zfp521* was not cell line dependent, we extend our experiments to another cell line, HEK293T cells, as described in the materials and methods section. The intensity of EGFP fluorescence in co-transfected cells with pLenti6.3/TO/V5-DEST/Zfp521 vehi-

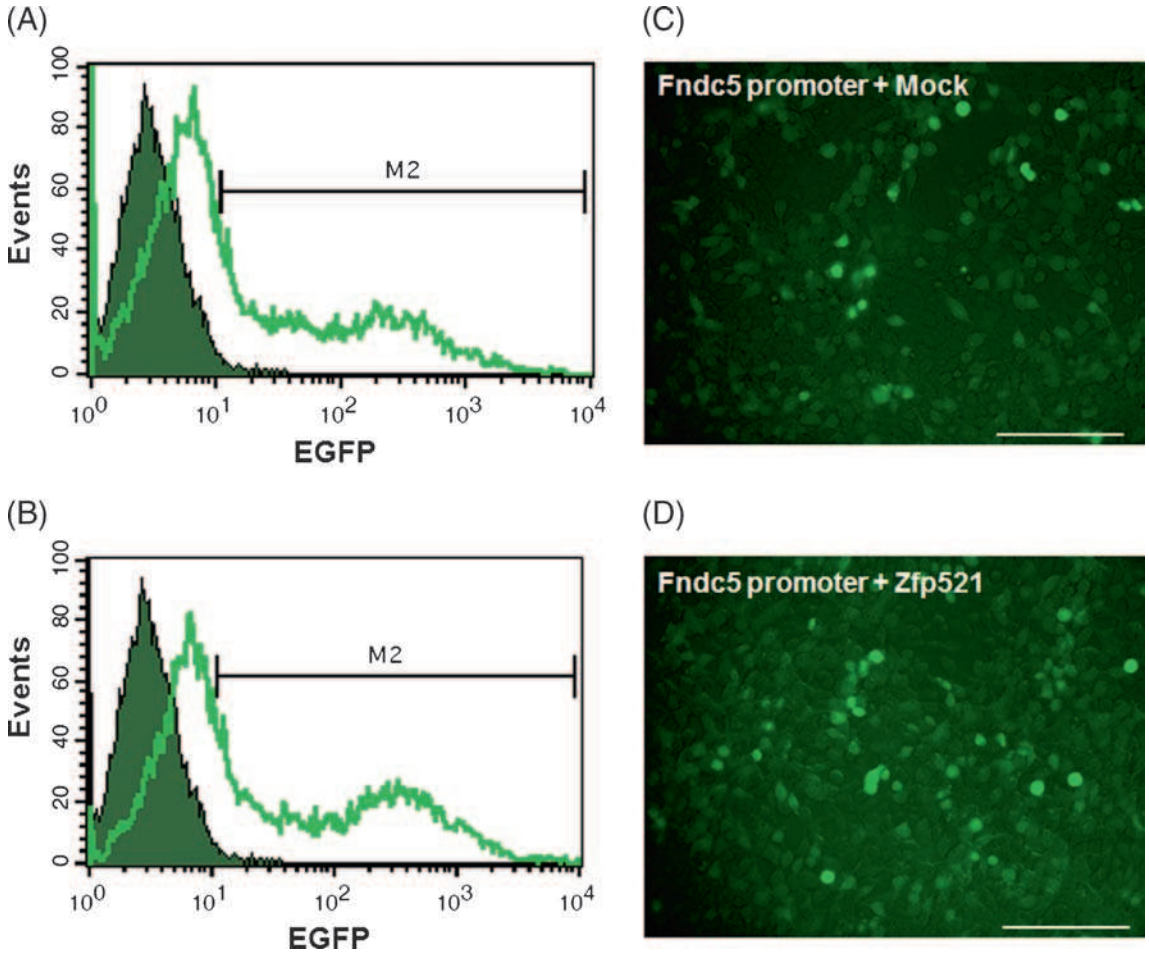

Figure 3. The forced expression of *Zfp521* induced an increment in levels of EGFP driven by Fndc5 promoter in HEK 293T cells. Flow cytometry analysis indicated an enhance in EGFP intensity of co-transfected HEKcells with pLenti6.3/TO/V5-DEST/Zfp521 vehicle and pDB2 vector encompassing core promoter of *Fndc5* upstream of EGFP [Fndc5 promoter+Zfp521, G Mean:112.21] (**B**) compared to the co-transfected HEK cells with pLenti6.3/TO/V5-DEST vehicle and pDB2 vector encompassing core promoter of *Fndc5* upstream of EGFP [Fndc5 promoter+Mock, G Mean:83.89] (**A**) as described in the results. Moreover the G Mean of untransfected HEK cells was estimated as 0. The fluorescence microscopy of the same cells is shown as (**D**) [Fndc5 promoter+Zfp521] and (**C**) [Fndc5 promoter+Mock] respectively. Bar is 100 μm.

cle and pDB2 vector encompassing core promoter of *Fndc5* upstream of EGFP (figure 3B and D; Fndc5 promoter+ Zfp521) was more than co-transfected cells with pLenti6.3/TO/V5-DEST vehicle and pDB2 vector encompassing core promoter of *Fndc5* upstream of EGFP (figure 3A and C; Fndc5 promoter+ Mock) (G Mean 112.21 versus 83.89).

4. Discussion

Zfp521 is a zinc finger protein with 180 kDa weight which includes 30 zinc finger motifs. Zfp521 involves in different process such as neuronal differentiation, hematopoietic cells and osteoblasts (Warming *et al.* 2003; Bond *et al.* 2004; Wu *et al.* 2009). Zfp521 is the critical co-activator for neural differentiation of ES cells which induces the ES-cell-derived definitive ectodermal progenitors. Zfp521 and p300 work together and induce neural differentiation by activating early neural marker genes *Sox1*, *Sox3*, *Pax6*. Fndc5 is a myokine hormone that could be cleaved to form Irisin. PGC1α is a key regulator in mitochondrial biogenesis which acts upstream of Fndc5. Hence, *PGC1α* overexpression induces *Fndc5* transcription level. Irisin, induces thermogenesis and energy expenditure (Bostrom *et al.* 2012). Our previous studies have shown that expression level of *Fndc5* was increased during neural differentiation of mESCs upon treatment with RA (Ostadsharif *et al.* 2011). Furthermore, *Fndc5* knockdown before NP formation reduced transcript levels of early neural marker genes *Sox1*, *Sox3*, *Pax6*, and *Nestin*. On the other hand, *Fndc5* knockdown post NPs formation reduced mRNA and protein levels of both neuronal and astrocytes markers, *Map2*, *Tuj1* and *Gfap* (Hashemi *et al.* 2013). Recently it has been demonstrated that *Fndc5* expression enhances expression level of hippocampal *Bdnf* during exercise (Wrann *et al.* 2013). The first results of this study indicated a similar pattern for expression of both *Zfp521* and *Fndc5* in different stages of neural differentiation. On the other hand our previous bioinformatics studies predicted a response element site for zinc finger proteins from position –193 to –217 with respect to translation start site inmouse *FNDC5* putative core promoter (with high matrix similarity of 0.825) (Seifi *et al.* 2014). Hence, to investigate whether Zfp521 activates *Fndc5* promoter during neural differentiation, we used mESCs that expressed *EGFP* under regulation of *Fndc5* core promoter. The influence of Zfp521 overexpression on *Fndc5* promoter was assessed in this study. Here we have shown that the overexpression *Zfp521* in mESCs not only increased EGFP expression under *Fndc5* core promoter but also enhanced RNA levels of endogenous *Fndc5*. As Zfp521 is the principal factor to trigger neural differentiation, we concluded its activity locates upstream of *Fndc5* expression. Therefore it is most likely that Zfp521 may act through the binding to its receptor on *Fndc5* putative core promoter. However, further studies are required to explore such hypothesis. Of note is that the overexpression *Zfp521* in undifferentiated state, including the presence of LIF and other components could not trigger the enhancement of ectodermal markers expression. This phenomenon is consistent with the data of Kamiya *et al.* (2010), who reported the promoting of neural differentiation of mESCs by overexpression of *Zfp521* in a differentiation medium in the absence of LIF. They have also notified that ZFP521 did not induce neural differentiation in the presence of LIF (Kamiya *et al.* 2010). Our data is in agreement with their observations, thus Zfp521 overexpression did not change the basic transcription levels of stem cells markers, *Oct4* and *Nanog.* Taken together, these data have implied that Zfp521 induces *Fndc5* transcript levels in neural differentiation cascade of mESCs. However, the physiologic significance of this phenomenon should be more investigated in future studies.

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