*Neuroscience* 231 (2013) 296–304

# *FNDC5* KNOCKDOWN SIGNIFICANTLY DECREASED NEURAL DIFFERENTIATION RATE OF MOUSE EMBRYONIC STEM CELLS

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**Abstract—Fibronectin type III domain-containing 5 protein (Fndc5) or peroxisomal protein, is a type I membrane protein that has 209 amino acid residues. Previous studies by our group have shown an increase in its expression after retinoic acid treatment of mouse embryonic stem cells (mESCs) during the process of neural differentiation, leading us to conclude that it might be involved in neurogenesis. In the present study, we have constructed an inducible short hairpin RNA (shRNA) vector that is expressed under induction by doxycycline. Next, we generated a stably transformed mESCs line that expressed shRNA against the *Fndc5* gene. The knockdown of *Fndc5* was performed in two stages of mESC neural differentiation during and post-neural progenitor (NP) formation. Our results indicated that in the process of NPs formation, decreased *Fndc5* expression significantly reduced expression of NPs and mature neuronal markers which modulated neuronal differentiation. Decreased *Fndc5* expression during the post-NPs formation stage also caused significant reduction in the levels of mature neuronal markers. *Fndc5* knockdown during both stages significantly affected both neuronal and astrocytes maturation. We have concluded that *Fndc5* expression is required for the appropriate neural differentiation of mESCs. These data confirm the importance of Fndc5 in the generation and development of the nervous system.** 

**Key words: *Fndc5*, neurogenesis, peroxisomal protein, RNAi.**

## INTRODUCTION

Fibronectin type III domain-containing 5 protein (Fndc5), also known as peroxisomal protein (PeP), is a type one membrane protein comprised of 209 amino acid residues, initially cloned by Ferrer-Martinez et al. (2002) and Teufel et al. (2002). There are three peptides, serine, lysine, isoleucine (SKI), that resemble the peroxisomal targeting signal type 1 at the C-terminus of its coding sequence. Previously, it has been suggested that SKI directs this protein into the matrix of peroxisomes (Ferrer-Martinez et al., 2002; Ostadsharif et al., 2009, 2010). According to other studies, Fndc5 could be a secreted protein that is further cleaved and glycosylated to produce irisin, a myokine with 112 amino acid residues (Boström et al., 2012). mRNA expression levels of *Fndc5* are elevated in the heart, skeletal muscle, and brain of adult mice (Ferrer-Martinez et al., 2002). Recently, we have reported a significant increase in *Fndc5* expression in the differentiation of mouse embryonic stem cells (mESCs) into neural cells following retinoic acid (RA) treatment (Ostadsharif et al., 2011).

In order to further understand *Fndc5* function during neurogenesis of mESCs we constructed a pSingle-tetracyline-controlled transcriptional suppressor (tTS)-short hairpin RNA (-shRNA) vector. This vector generated inducible shPeP in a stably transformed mESCs line that constitutively expressed tTS under the control of a cytomegalovirus (CMV) promoter. In this cell line the suppression of *Fndc5* expression during and post-neural progenitor NP resulted in reduction of neural markers, which implied a functional role for *Fndc5* in neurogenesis.

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*Abbreviations:* BSA, bovine serum albumin; CMV, cytomegalovirus; DAPI, 4,6-diamidio-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; Dox, doxycycline; EBs, embryoid bodies; ES-FCS, ES-qualified fetal calf serum; *Fndc5*, fibronectin type III domain-containing 5 protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; LIF, leukemia inhibitory factor; MAP2, microtubule-associated protein 2; MEF, mouse embryo fibroblast cells; mESCs, mouse embryonic stem cells; MTS/PMS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)/phenazine methosulfate; NPs, neural progenitors; NeuN, neuronal nuclei; PBS, phosphate-buffered saline; PeP, peroxisomal protein; RA, retinoic acid; SEM, standard error of mean; shRNA, short hairpin RNA; shCtrl, control shRNA; siRNA, small interfering RNA; SKI, serine, lysine, isoleucine; TRI, total RNA isolation; tTS, tetracyline-controlled transcriptional suppressor.

0306-4522/12 $36.00 
http://dx.doi.org/10.1016/j.neuroscience.2012.11.041

## EXPERIMENTAL PROCEDURES

### mESCs culture and neural differentiation stages

We obtained mESCs (Royan B1) from the C57BL/6 strain as reported (Baharvand and Matthaei, 2004). Cells were maintained in an undifferentiated state on mouse embryo fibroblast cells (MEF) as previously described (Ghoochani et al., 2012) and grown in knockout-Dulbecco's modified Eagle's medium (Ko-DMEM) that contained 15% ES-qualified fetal calf serum (ES-FCS), 2 mM glutamine, 0.1 mM non-essential 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). The medium was changed daily and cells passaged every 2 days. mESCs underwent neural differentiation according to a previously described protocol that used embryoid body (EB) formation in hanging drops for 2 days (Ostadsharif et al., 2011) in a medium that consisted of Ko-DMEM supplemented with 10% ES-FCS and 1 μM RA (Sigma–Aldrich) for 4 days. This medium was renewed every other day. Subsequently, we plated EBs on gelatin-coated 12-well plates (TPP, Switzerland) for an additional 6 days in a neurobasal medium (Invitrogen) that contained 5% ES-FCS and 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).

### pSingle-tTS-shRNA vector construction to induce sh*Fndc5* expression in mESCs

We used short hairpin RNA interference (shRNAi) transcript products because they provide more efficient long-term knockdown (Brummelkamp et al., 2002; McManus et al., 2002). Three shRNAs were designed to knockdown the mouse *Fndc5* gene (Gene Bank accession no. NM_027402) along with a control shRNA (shCtrl). We used the following online small interfering RNA (siRNA) design tools: Dharmacon, (www.dharmacon.com), Ambion (www.invitrogen.com/.../Ambion-Silencer-Select-siRNAs.html), and siDirect (sidirect2.rnai.jp) as seen in Table 1 and Supplementary Fig. 1. The shRNAs (shRNA1, shRNA2 and shRNA3) encoded nucleotides against various parts of *Fndc5* mRNA (Supplementary Fig. 1). According to the vector manufacturer's protocol (Clontech, USA), we considered a stretch of nona-nucleotides between the sense and antisense domains in addition to terminator sequences for the cessation of RNA polymerase III, and one *Mlu*I site for additional analysis in the backbone of the oligonucleotides. For annealing, we mixed equal volumes of upper and lower strands of oligonucleotides (100 μM) and heated them at 95 °C for 30 s, 72 °C for 2 min, 37 °C for 2 min, and 25 °C for 2 min. The annealed oligonucleotides have been previously designed to carry *Hind*III (Fermentas, Lithuania) and *Xho*I (Fermentas) and could generate compatible overhangs at both ends to allow for appropriate insertion into the pSingle-tTS-shRNA vector (Clontech; Supplementary Fig. 1). Recombinant vectors were transformed into *Escherichia coli* DH5α (Invitrogen). For plasmid extraction, we chose the resistant bacterial colonies that grew in LB/ampicillin (100 μg/ml, Sigma–Aldrich) on an agar plate and cultured them in the same medium at 37 °C for an additional 16 h in a shaker incubator. The Qiaprep Spin Miniprep Kit (Qiagen, Germany) was used. We confirmed recombinant vector construction by means of *Mlu*I (Fermentas, Lithuania) digestion due to the presence of a unique site in the designated shRNAs that produced oligonucleotides (Table 1).

### Generation of stably transformed mESCs that expressed sh*Fndc5* and shCtrl

Approximately $5 \times 10^4$ mESCs were seeded on neomycin-resistant mitotically inactivated MEF for a 3-h incubation period with 10 μg/ml mitomycin C (Sigma–Aldrich) in 12-well dishes. Four hours post-seeding we transfected the cells with a cocktail that included 20 μl of lipofectamine 2000 (Invitrogen) and 8 μg each of the recombinant vectors, according to the manufacturer's instructions (Invitrogen). In order to isolate stable transformants, the transfected cells that showed a significant reduction in *Fndc5* expression upon induction were chosen to grow in the presence of G418 (400 μg/ml). The medium was changed daily until stable colonies appeared (after 10–14 days). To isolate transformant cell lines that highly expressed efficient sh*Fndc5* and shCtrl, we performed an insert check experiment on total genomic DNA. This genomic DNA was isolated from resistant colonies with the DNeasy Blood and Tissue Kit (Qiagen). PCR analysis verified the presence of three different regions of recombinant vectors in isolated genomic samples (Supplementary Table 1). For each set of PCR, we used 0.15 μg of the DNA template in a 25-μl total reaction volume that contained appropriate primers and *Taq* DNA polymerase. The cycling program was 94 °C for 45 s, 70 °C for 45 s, and 72 °C for 1 min, repeated for 35–40 cycles. PCR began with an initial denaturation step at 94 °C for 4 min and lasted until a final extension at 72 °C for 10 min. We chose significant stable, expressed sh*Fndc5*- and shCtrl-transformed mESC colonies and cultivated them for additional neuronal differentiation. We called these cell lines, sh*Fndc5* and shCtrl, respectively.

**Table 1.** The three designated *Fndc5*-targeted shRNAs and shCtrl sequences.

| Name | | *Xho*I | Sense | Hairpin loop | Anti-sense | Terminator | *Mlu*I, *Hind*III |
|---|---|---|---|---|---|---|---|
| shRNA1 | U | 5'-TCGAGg | CATGTGGGCAGGTGTTATA | (TTCAAGAGA) | TATAACACCTGCCCACATG | cTTTTTT | ACGCGTa-3' |
| | L | 3'-cc | GTACACCCGTCCACAATAT | (AAGTTCTCT) | ATATTGTGGACGGGTGTAC | gAAAAAA | TGCGCA,TTCGA-5 |
| shRNA2 | U | 5'-TCGAGg | AGATATGATGACACATTTA | (TTCAAGAGA) | TAAATGTGTCATCATATCT | cTTTTTT | ACGCGTa-3' |
| | L | 3'-cc | TCTATACTACTGTGTAAAT | (AAGTTCTCT) | ATTTACACAGTAGTATAGA | gAAAAAA | TGCGCA,TTCGA-5' |
| shRNA3 | U | 5'-TCGAGg | CAATCCAGTTAGAGCAATA | (TTCAAGAGA) | TATTGCTCTAACTGGATTG | cTTTTTT | ACGCGTa-3' |
| | L | 3'-cc | GTTAGGTCAATCTCGTTAT | (AAGTTCTCT) | ATAACGAGATTGACCTAAC | gAAAAAA | TGCGCA,TTCGA-5 |
| shCtrl | U | 5'-TCGAGg | GACCATCAATATGACTAGA | (TTCAAGAGA) | TCTAGTCATATTGATGGTC | TTTTTT | ACGCGTa-3' |
| | L | 3'-cc | CTGGTAGTTATACTGATCT | (AAGTTCTCT) | AGATCAGTATAACTACCAG | AAAAAA | TGCGCA,TTCGA-5' |

U: Upper oligonucleotide strands. L: Lower oligonucleotide strands. Partial restriction site of *Xho*I and whole sequence of *Mlu*I enzymes are shown in grey box upstream and downstream of upper strand of oligonucleotides, respectively. Partial restriction sites of *Hind*III and *Mlu*I enzymes are shown in grey box downstream of lower strand of oligonucleotides, respectively. Terminator repeats are underlined.