Original Article

http://dx.doi.org/10.3349/ymj.2013.54.3.772
pISSN: 0513-5796, eISSN: 1976-2437

Yonsei Med J 54(3):772-777, 2013

YMJ

Human *SNF2L* Gene Is Regulated Constitutively and Inducibly in Neural Cells via a cAMP-Response Element

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Received: July 3, 2012
Revised: August 16, 2012
Accepted: August 16, 2012
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· The authors have no financial conflicts of interest.

Purpose: SNF2L belongs to Imitation Switch family and plays an essential role in neural tissues and gonads. In our previous studies, we have demonstrated that the basal transcription of human *SNF2L* gene is regulated by two cis-elements, cAMP response element (CRE)- and Sp1-binding sites. Recent studies suggested that cyclic adenosine monophosphate (cAMP) stimulation significantly up-regulated *SNF2L* expression in ovarian granulose cells. These data suggested that protein kinase-mediated signal pathways might also regulate *SNF2L* expression in neural cells. We therefore investigated the effects of agents that activate protein kinases A on *SNF2L* gene expression in neural cells. **Materials and Methods:** To increase intracellular cAMP levels, all neural cells were treated with forskolin and dbcAMP, two cAMP response activators. We exmined the effects of cAMP on the promoter activity of human SNF2L gene by luciferase reporter gene assays, and further examined the effects of cAMP on endogenous SNF2L mRNA levels by qPCR. **Results:** Transient expression of a luciferase fusion gene under the control of the *SNF2L* promoter was significantly increased by treatment of rat primary neurons with forskolin or dbcAMP, but not PC12, C6 and SH-SY5Y cells. Consistently, treatment with forskolin or dbcAMP could enhance endogenous *SNF2L* mRNA levels also only in rat primary neurons. **Conclusion:** These results suggest that the CRE consensus sequence in the *SNF2L* proximal promoter most likely confers constitutive activation and regulation by cAMP in neural cells.

Key Words: *SNF2L*, CRE, cAMP stimulation, neural cells

INTRODUCTION

Imitation Switch (ISWI), the biggest subfamily of the SWI2/SNF2 superfamily of ATPases, contains SANT and SLIDE (SANT-like ISWI) domains in addition to highly conserved ATPase domains.[1] The ISWI chromotin remodeling complexes play important roles in multiple nuclear functions, including transcriptional regulation, chromatin assembly and DNA replication.[2]

The two ISWI orthologs in mammals, SNF2H and SNF2L, perform different functions *in vivo*. The expression patterns of SNF2H and SNF2L are different in

mouse and human.[3,4] In mouse, Snf2l is expressed only in the brain and gonads, while human SNF2L is ubiquitously expressed. This different expression pattern probably suggests that they might have different developmental functions.[5] Furthermore, *Snf2h* expresses in proliferating cells within neural tissue and gonads, while *SNF2L* is prevalent in differentiated cell in these tissues.[6] Recently, different functions of SNF2L have been revealed in both neural tissues and gonads. The human nucleosome-remodeling factor complex containing SNF2L is enriched in the brain and regulates human *Engrailed* genes that are necessary for neuronal development in the midbrain.[7] Moreover, ectopic expression of *SNF2L* in proliferating neuroblastoma cells can promote differentiation.[8] SNF2L has different roles in the events of ovarian germ- and somatic-cell development and differentiation.[9] Other studies suggest that the inhibition of SNF2L expression selectively leads to DNA damage, growth inhibition, and cancer cell death.[10] Snf2l mutant mice exhibit forebrain hypercellularity arising from increased Foxg1 expression, increased progenitor-cell expansion, and delayed differentiation.[11] Recent studies indicate that SNF2L is broadly expressed in primary human tissues and suppresses cell proliferation and migration and attenuates Wnt signaling.[12]

In our previous studies, we demonstrated that the basal transcription of human *SNF2L* gene is regulated by two cis-elements, cAMP response element (CRE)- and Sp1-binding sites, located close to the transcription start site.[13] More recent studies suggested that cAMP stimulation significantly up-regulated *SNF2L* expression in ovarian granulose cells.[14] However, compared with TATA-less promoters of cAMP response element-binding protein (CREB) target genes, TATA-containing genes are more responsive to cAMP through genome-wide analysis.[15] Very little is known about the psysiological functions of SNF2L in nervous system, therefore, we investigated the effects of agents that activate protein kinase A (PKA) on *SNF2L* gene expression in neural cells.

MATERIALS AND METHODS

Reagents

Forskolin (SigmaF6886) stock solution was made in Dulbecco's modified Eagle's medium (DMEM) at 100 Mm, and N^6-2'-O Dibutyryl-cAMP (dbcAMP) (SIGMA, #D0627, Deisenhofen, Deutschland) stock solution was made in phosphate buffered saline at 100 mM. Working solution was 20 uM for forskolin and 1 mM for dbcAMP.

Cell culture

Rat pheochromocytoma (PC12) cells were cultured on collagen coated dishes in complete medium containing RPMI-1640, 15% horse serum, 2.5% fetal calf serum and 1% antibiotics. C6 cells were grown in DMEM containing 10% fetal bovine serum (FBS) and 1% antibiotics. Human SH-SY5Y neuroblastoma cells were cultured in DMEM/F-12 (F-12, GIBCO, Grand Island, NY, USA) supplemented with 15% FBS. Cortical primary neuronal cultures were prepared from E15 Wistar rats. After dissection of brains, cortical hemispheres were detached and meninges were removed. Fresh tissues were then digested with trypsin (0.25% trypsin in EDTA maintained at 37°C) for 10 min. The cortical neurons were suspended in neuron-defined culture medium supplemented with 1 mL B27, 0.5 mM L-glutamine and antibiotics. Cells were incubated in the humidified incubator equilibrated with 5% CO_2 at 37°C and passaged using standard cell culture techniques.

Plasmid and constructs

A fragment that contains CRE site was subcloned into the promoterless luciferase reporter plasmid pGL3-basic (Promega, Madison, WI, USA), and the mutant type was prepared by polymerase chain reaction (PCR) using the primer which carries point mutations as described previously.[13]

Transient transfections and luciferase assays

All of the cells were cultured in medium without FBS and antibiotics for 24 h before transfection, and 6×10^4 cells were seeded in each 24-well plate. 0.2 μg of the *SNF2L* reporter constructs and 0.02 μg of pRL-TK Renilla luciferase reporter were co-transfected into each well using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). 24 h after transfection, the cells were either left untreated or treated with forskolin (stimulator of adenylate cyclase) or dbcAMP (cAMP analogue) and incubated at 37°C for additional 6 h. Then, the lysates were analyzed for luciferase activity using the dual luciferase reporter assay system (Promega) in a Perkin-Elmer 1420 multilabel counter (Perkin-Elmer, Wellesley, MA, USA).

Western blot analysis

Total extracts from SH-SY5Y cells or rat primary neurons, prepared with or without cAMP activator forskolin (20 uM) or dbcAMP (1 mM) treatment for 24 h, to serve as negative

and positive controls. Phospho-CREB (Ser133) antibody and CREB antibody were used as primary antibodies. All the procedures were the same as described previously.[13]

RNA isolation and reverse transcription

PC12, C6, SH-SY5Y and rat primary neurons cultured in 6-well plates were stimulated with forskolin or dbcAMP for 24 h. Total RNA was isolated from cultured cells by using the RNeasy Midi Kit (Qiagen, Hilden, Germany). The integrity RNA was segregated and purified by the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip(R) reagent set (Agilent Technologie, Santa Clara, CA, USA). The RNA was determined spectrophotometrically and then stored at -80°C. Reverse transcription reactions were performed for 10 min at 70°C, using 2 μg RNA, 4 μL 5×first strand buffer (Invitrogen, Karlsruhe, Germany), 2 μL 0.1 mol/L dithiothreitol, 1 μL dN6 primer (10 mmol/L), 1 μL dNTPs (10 mmol/L), and diethypyrocarbonate water. The synthesized cDNA was used as a template for PCR amplification. RT-PCR was carried out using 1 μL cDNA for 1 h at 37°C, and inactivated at 70°C for 10 min. RNA was finally degraded by incubation with 1 μL RNase A (10 mg/mL) at 37°C for 30 min.

Realtime quantitative RT-PCR (TaqMan) analysis

After reverse transcription reaction, qPCR was performed using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The reaction solution was assembled in a volume of 20 μL, which comprised TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM primers, 200 nM TaqMan probe and 50 ng cDNA. 18S rRNA was used as endogenous control. SNF2L and 18S rRNA reactions were run in triplicates and in parallel. The thermal cycler conditions for qPCR were preheating at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles of shuttle heating at 95°C for 15 s and at 60°C for 1 min. All cDNA was measured in trilicate.

The primer and TaqMan probe for SNF2L were designed using the Primer Express program version 2.0 (Applied Biosystems). The primer and TaqMan probe for 18S rRNA were synthesized according to a previous report. TaqMan probe and primer oligonucleotide sequences were as follows, SNF2L: forward primer 5'-GCTTGGTACGGTG GAATGGAT-3', reverse primer 5'-ACATTTGGCTGT TTTGGAGGC-3'; probe 5'-FAM-CCTTTGGGACCTTT GGCTCGCTGACAC-TAMRA-3'; 18S rRNA: forward primer 5'-CGGCTACCACATCCAAGGAA-3', reverse primer 5'-GCTGGAATTACCGCGGCT-3'; Probe 5'-FAM-TGCTGGCACCAGACTTGCCCTC-TAMRA-3'.

Statistical analysis

The statistical significance of differences between experimental groups was calculated using Student's test. $p<0.05$ was considered statistically different.

RESULTS

Effects of cAMP on the promoter activity of human *SNF2L* gene in neural cells

Our previous study identified a functional CRE site in proximal promoter of human *SNF2L*, suggesting that protein kinase-mediated signal pathways might also regulate *SNF2L* expression. Therefore, we exmined the effects of cAMP on

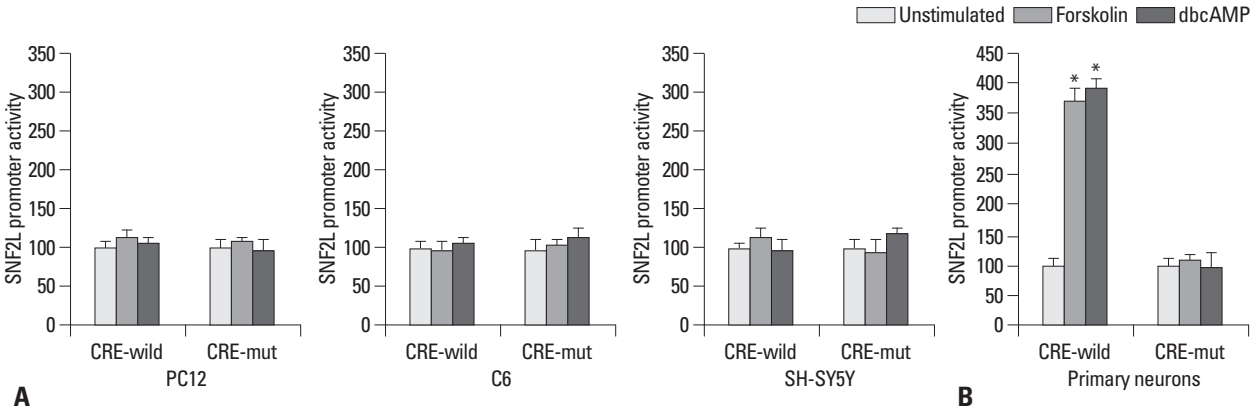

Fig. 1. Effects of forskolin or dbcAMP on the promoter activity of human SNF2L gene in PC12, C6, SH-SY5Y and rat primary neurons. We investigated the activity of the CRE-Wild and CRE-Mut promoters in response to cAMP activator forskolin or dbcAMP. (A) The addition of forskolin or dbcAMP to transiently transfected cells resulted in no activation of the CRE-Wild and CRE-Mut constructs in PC12 cells, and the same effects were deteted in C6 and SH-SY5Y cells. (B) Treatment with forskolin or dbcAMP increased luciferase activity nearly 4.0-fold in the CRE-Wild construct, In contrast, the response to cAMP activator was diminished in the CRE-Mut construct. *Values of statistical significance (Student t-test; $p<0.05$).

the promoter activity of human *SNF2L* gene in three neural cell lines, PC12, C6 and SH-SY5Y as well as rat primary neurons. Two luciferase reporters driven by the promoter containing CRE site (CRE-Wild) or mutant CRE site (CRE-Mut) were transiently transfected into these neural cells. A CRE-Wild plasmid was used as a positive control for cAMP responsiveness. Twenty-four hours after transfection, the cells were treated with forskolin or dbcAMP for 6 h, and luciferase activity was determined. As shown in Fig. 1A, treatment with forskolin or dbcAMP did not alter the promoter activities of both wild-type or mutant CRE constructs in PC12 cells. No changes of promoter activities were also observed in C6 and SH-SY5Y cells. However, in rat primary neurons, treatment with cAMP activator significantly increased the promoter activities of CRE-Wild promoter (nearly 4.0-fold), but not CRE-Mut construct (Fig. 1B). These results show that protein kinase-mediated signal pathways can regulate *SNF2L* expression in neural cells, but this regulation depends on cell types.

Effects of cAMP on endogenous *SNF2L* mRNA levels in neural cells

To confirm the stimulatory effect of cAMP on endogenous *SNF2L* expression, we further examined the *SNF2L* mRNA levels in PC12, C6, SH-SY5Y and rat primary neurons, by treatment with forskolin or dbcAMP. Thus, all cells were grown in 6-well plates, and treated with forskolin or dbcAMP for 24 h, and the level of *SNF2L* mRNA was then measured by qPCR. As shown in Fig. 2, treatment with forskolin or dbcAMP did not enhance endogenous *SNF2L* mRNA levels in PC12, C6 and SH-SY5Y cells. In contrast, however, the significant cAMP stimulatory effect was observed in rat primary neurons (more than 4.0-fold), appearing to be cAMP responsive. These results also suggest that the cAMP responsive activity of *SNF2L* depends on cell types, in agreement with cAMP effects on the promoter activity of human *SNF2L* gene.

Our previous study demonstrated that CREB is one of the proteins from CREB/Activating transcription factors (ATF) family that stimulates *SNF2L* promoter activity.[13] Therefore, we investigated the phosphorylation effects of forskolin or dbcAMP on CREB in the *SNF2L* promoter. Thus, nuclear extracts from SH-SY5Y cells or rat primary neurons were prepared with or without forskolin or dbcAMP treatment for 24 h, and phospho-CREB (Ser133) antibody and CREB antibody were used for western blot analysis. As shown in Fig. 3, the levels of phosphorylated CREB significantly increased with forskolin or dbcAMP treatment in both SH-SY5Y cells or rat primary neurons, but this effects were not detected in total CREB levels. These results demonstrate that CREB phosphorylation occurs in all cell lines, however, that *SNF2L* is stimulated only by protein kinase A (PKA) in rat primary neurons.

DISCUSSION

We have previously identified a CRE consensus sequence in the proximal promoter of human *SNF2L* gene, and demonstrated that it is highly conserved among different species and plays an important role in the control of *SNF2L* basal promoter.[13] A CRE site can act as a constitutive and/or inducible element to regulate the target genes transcription through binding the CREB/ATF family members.[16] Increasing number of studies suggest that cAMP stimulation significantly up-regulates *SNF2L* expression in ovarian granulose cells.[14]

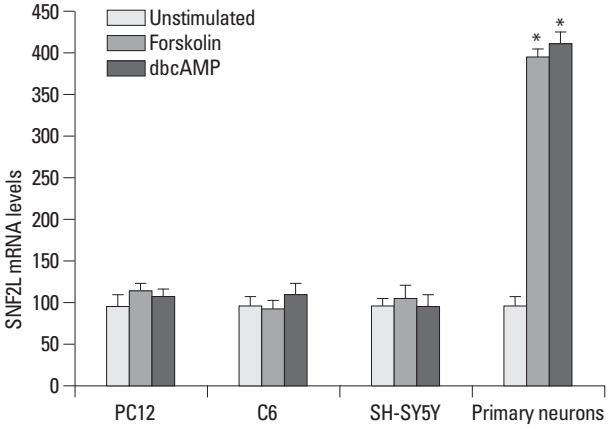

Fig. 2. Effect of cAMP on endogenous SNF2L gene expression in PC12, C6, SH-SY5Y and rat primary neurons. The SNF2L mRNA levels in neuron cells were detected by qPCR, with or without cAMP activator forskolin or dbcAMP treatment. Treatment with forskolin or dbcAMP did not enhance endogenous SNF2L mRNA levels in PC12, C6 and SH-SY5Y cells, but the SNF2L mRNA levels with cAMP induced showed more than 4.0-fold higher than the levels unstimulated. *Values of statistical significance (Student t-test; $p<0.05$).

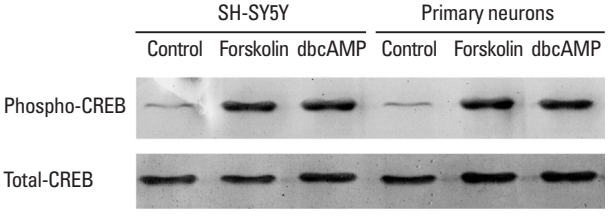

Fig. 3. Effects of the cAMP activator forskolin or dbcAMP. Western blot analyses of nuclear extracts from neuron cells treated with or without cAMP activator for 24 h. Whether in SH-SY5Y cells or rat primary neurons, levels of phosphorylated CREB significantly increased with forskolin or dbcAMP treatment, but this effects were not detected in total CREB levels.

Therefore, we reasoned that protein kinase-mediated signal pathways might also regulate *SNF2L* expression in neural cells, and investigated the effects of agents that activate protein kinases on *SNF2L* gene expression in neural cells. Results showed that forskolin or dbcAMP did not alter the promoter activities of wild type or mutant CRE constructs in PC12, C6 and SH-SY5Y cells, however, a significant induction was observed on CRE-Wild construct in rat primary neurons. To confirm the stimulatory effect of cAMP on *SNF2L* gene expression, we examined endogenous *SNF2L* mRNA level in these cells. Consistently, neither forskolin nor dbcAMP enhanced *SNF2L* expression in PC12, C6 and SH-SY5Y cells. However, the significant cAMP stimulatory effect was observed in rat primary neurons, thus appearing to be cAMP responsive.

Cyclic AMP is an ubiquitous secondary messenger. It regulates gene expression mostly through the activation of the CREB/ATF family of proteins, which belong to the leucine-zipper (bZIP) transcription factors.[17] The CREB/ATF family member binds as a dimer to the CRE. Upon phosphorylation, CREB recruits a co-activator, CREB-binding protein (CBP), and CBP can induce transcription.[18] To increase intracellular cAMP levels, the cells were treated with forskolin, an activator of adenylyl cyclase, and dbcAMP, cAMP analogue, both of which are widely used to stimulate the cAMP response genes.

Recent studies suggest that cAMP stimulation significantly up-regulates *SNF2L* expression in ovarian granulose cells. In primary cultures of rat granulosa cells, dbcAMP stimulation induced a significant increase in SNF2L protein levels. However, in immortalized granulosa cell line (SVOG-4o), only a slight increase of SNF2L protein was evident after dbcAMP treatment; but this increase did not reach significance.[14] In our present study, we did not observe any inducible effect of either forskolin or dbcAMP on *SNF2L* promoter activity in PC12, C6 and SH-SY5Y cells, and also did not detect any stimulatory effects on *SNF2L* mRNA level in these cells. On the other hand, significant effect of cAMP stimulation was observed in rat primary neurons, indicating that the response to cAMP of human *SNF2L* proximal promoter and the cAMP stimulatory effects on endogenous *SNF2L* mRNA levels depends on cell types. Our result in neural cells is in agreement with previous observations in ovarian cells. This result was most likely due to difference between neurons and differentiated neurons in the proliferative status, but not due to neuron specificity (PC12, C6 and SH-SY5Y are all neural sources cells). PC12 and SH-SY5Y cells can be differentiated into a functionally mature neuronal phenotype in the presence of various inducible agents,[19-21] so it is more meaningful to investigate the cAMP response effect in differentiated and undifferentiated states of neuron cell lines.

In the present study, we investigated the phosphorylation effects of forskolin or dbcAMP on CREB in the *SNF2L* promoter. Phosphorylated CREB levels were significantly increased in both SH-SY5Y cells and rat primary neurons, despite not being able to demonstrate the inducible effect of cAMP activitor in SH-SY5Y cells. A question arises why the levels of CREB phosphorylation in cell lines and primary neurons are different, but SNF2L performs inconsistent result. There may be other mechanisms; for example, inducible effect requires the participation of other molecules, and these molecules appear only in differentiated neurons. We, therefore, are in a process to investigate molecular mechanisms in more detail.

Primary neurons, which are mature differentiated neuronal phenotype, can better represent biochemical characteristics of neuron tissues, therefore, they more accurately reflect the reality. Many studies indicate that CRE can act as a constitutive and/or inducible element in controlling gene expression.[22-24] In conclusion, our present results indicate that the CRE consensus sequence in the proximal promoter of *SNF2L* gene likely confers constitutive activation and regulation by cAMP in neural cells.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (81001338) and the Promotive research fund for excellent young and middle-aged scientisits of Shandong Province (BS2009SW039).

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