Experimental Neurobiology
Vol. 19, pages 75~82, September 2010

A Winged-Helix Transcription Factor Foxg1 Induces Expression of Mss4 Gene in Rat Hippocampal Progenitor Cells

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ABSTRACT

Foxg1 (previously named BF1) is a winged-helix transcription factor with restricted expression pattern in the telencephalic neuroepithelium of the neural tube and in the anterior half of the developing optic vesicle. Previous studies have shown that the targeted disruption of the Foxg1 gene leads to hypoplasia of the cerebral hemispheres with severe defect in the structures of the ventral telencephalon. To further investigate the molecular mechanisms by which Foxg1 plays essential roles during brain development, we have adopted a strategy to isolate genes whose expression changes immediately after introduction of Foxg1 in cultured neural precursor cell line, HiB5. Here, we report that seventeen genes were isolated by ordered differential displays that are up-regulated by over-expression of Foxg1, in cultured neuronal precursor cells. By nucleotide sequence comparison to known genes in the GeneBank database, we find that nine of these clones represent novel genes whose DNA sequences have not been reported. The results suggest that these genes are closely related to developmental regulation of Foxg1.

Key words: Foxg1, telencephalon development, Mss4, ordered-differential display

INTRODUCTION

The telencephalon is the most complex and divergent structure of the vertebrate central nervous system. A crucial regulator of telencephalic development is Foxg1 (previously named BF1), for which mutations were very recently identified as the cause of a congenital form of the severe human neurodegenerative disease Rett syndrome (Ariani et al., 2008). This Forkhead transcriptional repressor, which encodes a winged helix transcription factor expressed in telencephalon, is thought to participate in diverse developmental processes such as proliferation, differentiation, patterning and neurogenesis (Seoane et al., 2004; Regad et al., 2007; Danesin et al., 2009). Foxg1 expression in the developing brain is restricted to the telencephalic neuroepithelium and the nasal half of the retina and optic stalk (Tao and Lai, 1992). It was previously reported that mice lacking Foxg1 die at birth with hypoplasia of the telencephalon, therefore, Foxg1 controls the morphogenesis of the telencephalon by regulating the rate of neuroepithelial cell prolifera-

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Received September 21, 2010
Accepted for publication September 25, 2010

tion and the timing of neuronal differentiation (Xuan et al., 1995). In Foxg1(−/−) mutant, defects in the patterning of the dorsal-ventral axis of the optic vesicle are correlated with a localized loss of shh expression in the ventral telencephalon and abnormal expression of Pax6 and Pax2 (Huh et al., 1999). In controlling the pattern of neurogenesis in *Xenopus* ectoderm, XFoxg1 regulates the expression of XSox3, X-ngnr-1, XMyt-1 and X-Delta-1 (Bourguignon et al., 1998). These findings raised the possibility that lots of genes controlling development are regulated by Foxg1.

The growth and specification of forebrain during development require the regulation of Foxg1, but the distinct molecular mechanism about developmental regulation of Foxg1 has not yet been elucidated, and few genes involved in the function of Foxg1 have been identified. To investigate the molecular mechanisms of Foxg1 *in vitro* system, a stable cell line that has developmental potency is necessary. We used neuronal precursor cell line of hippocampus, HiB5 (Renfranz et al., 1991). In this neuronal precursor cell line, Foxg1 may function as developmental control molecule like *in vivo*.

The aim of this study is to evaluate the alterations in gene expression in neuronal precursor cell line of hippocampus after over-expression of Foxg1. It is believed that the function of Foxg1 is to control downstream-genes as transcription factor, and many genes have been demonstrated to contribute to the development of the forebrain. However, little is known about the complete expression profile of genes that are regulated by Foxg1 during the forebrain development. mRNA differential display is an effective method for isolating genes that are expressed differentially among different conditions (Liang and Pardee, 1992). The differential display technique was applied to compare expression of mRNAs between GFP and GFP-Foxg1 transfected neuronal precursor cell line. Although some of the approaches related to differential display which have been proposed could be used for several systematic investigation, these methods are rather complicated or may not have enough sensitivity. So, we tried ordered differential display (ODD), which does not involve arbitrary priming for pattern generation and provides a possibility for a through step-by-step comparison of all mRNAs, was developed to search differentially expressed genes (Matz et al., 1997; Matz and Lukyanov, 1998).

We isolated seventeen differentially expressed genes, nine that were novel and eight that were already known. These data provide basic information necessary to understand the gene expression profiles regulated by Foxg1. Further characterization of these genes will help to clarify the molecular mechanisms of Foxg1 during the forebrain development.

MATERIALS AND METHODS

Cell culture

The HiB5 neuronal precursor cell line was cultured by previously described methods (Renfranz et al., 1991). Cells were maintained in Dulbecco's modified Eagle's medium (Gibco) with 10% fetal bovine serum (Gibco) supplemented with 0.11 g/l sodium pyruvate, 3.7 g/l $NaHCO_3$, 0.29 g/l glutamine, 0.6 g/l penicillin, and 0.1 g/l streptomycin. Medium was changed every three days and cells were incubated at 33°C in 5% CO_2. HiB5 cells proliferate at 33°C, the permissive temperature of the oncogenic tsA58 allele of the SV40 large T antigen.

Transfection

HiB5 cell line was cultured in 100 mm dishes for RNA isolation and plated onto glass coverslips in 24-well plates for immunocytochemistry at 75～80% confluence. The cells were transiently transfected using the calcium-phosphate co-precipitation method (Pear et al., 1993). Two μg/ml of the vector encoding 2.5 kb of the mouse Foxg1 cDNA, which was subcloned into the pEGFP C2 (clontech) expression vector, was used for transfection. After transfection, cells were incubated in serum-free medium including transfection solution for 12 hr. For transient gene expression assay, cells were incubated in complete medium after removing calcium-phosphate containing medium for 24 hr.

Immunocytochemistry

R18F1 and HiB5 cells were plated onto glass coverslips at a density of 75～80% confluence in 24-well plates. The transfected cells were fixed in 4% paraformaldehyde and permeabilized with 0.1%

TritonX-100. Coverslips were incubated with the primary antibody (anti-Foxg1, 1 : 1,500) for 3 hr, and then incubated for 1 hr with a goat anti-rabbit rhodamine-conjugated antibody (Leinco, 1 : 300). The stained coverslips were observed under fluorescence microscope (Olympus PROVIS AX-70, X200).

RT-PCR

Total RNA was isolated from cultured HiB5 cell line using TRIzol (GibcoBRL), and cDNA was made using 1 μg of RNA and AMV reverse-transcriptase (Promega). Primer sequences for Foxg1 were forward (5'-GGGCAACAACCACTCCTTCTCCAC-3') and reverse (5'-GACCCCTGATTTTGATGTGTGAAA-3'). The expected size of the Foxg1 product was 396 bp. PCR cycling conditions were 94°C for 30 s, 65°C for 30 s, and 72°C for 90 s, for a total of 30 cycles. The PCR products were electrophoresis on the 1.5% agarose gel and stained with ethidium bromide.

Ordered differential display

Foxg1-overexpressing HiB5 cell line was used as sources of RNA for ODD. The techniques used for ODD have been described (Matz et al., 1997). In brief, total RNA used for differential display was further purified to remove DNA contamination using the MessageClean Kit (GenHunter Corp) and a commercially available kit (Boehringer Mannheim) was used for the synthesis of double-stranded cDNA from 1 μg of total RNA, except the T-primer for first strand synthesis provided with the kit that should be substituted for non-extended ODD T-primer. cDNA species were discriminated by the length of fragment between polyA attachment site and the first occurrence of site for restrictase, RsaI, and then ligated to 5'-ends of full length cDNA digests with ODD adaptor. After the generation of amplified cDNA, the subsets of cDNA were produced by PCR with both a T-primer and an adaptor-specific primer extended by two arbitrary bases at their 3'-ends. The population of cDNA was subdivided into 192 subsets (as there exist 16 possible variants of adaptor-specific primer extension and 12 of T-primer), which were displayed on an ordinary 6% sequencing gel. The gel was transferred to 3 M filter paper and dried. By terminal labeling of extended adaptor-specific primer with γ-^{33}P[dATP], the displayed bands were detected on the exposed X-ray films.

Reverse northern blotting

The PCR products were excised from the dried sequencing gel and eluted by boiling in 20 μl of H_2O for 10 min. The eluted cDNA fragments were subjected to PCR reamplification using the appropriate extended T-primers and adaptor-specific primers. The reamplified fragments of cDNA were fractionated on 2% agarose gels and extracted. One μl of the reamplified cDNA fragments directly applied by diffusion as targets on a nitrocellulose membrane and cross-linked by UV-illuminator. Two copies of the membrane were prepared and hybridized overnight with [α-^{32}P]dCTP-labeled cDNA probes reverse-transcribed from the original RNA preparation of either the GFP or the GFP-Foxg1 transfected cells. After hybridization, the membranes were exposed to X-ray films at −80°C. If differential expression was confirmed by reverse northern blot analysis, the band was cloned and sequenced as described below.

Cloning and sequencing

The differentially expressed cDNA fragments confirmed by reverse northern blotting were cloned into plasmid vector pCRII using the TOPO TA cloning system (Invitrogen). The resulting plasmids were confirmed by restriction enzyme digestion and performed automatic sequencing (KAIST BioMedical Research Center). Partial length sequences were compared to all previously reported gene sequences in GeneBank.

In situ hybridization

Mouse brains (P0) were fixed at 4°C in 4% paraformaldehyde for 4 hr, immersed in 20% sucrose overnight and embedded in OCT (Tissue-Tek). Sections were cut at 10 μm on a cryostat. Non-radioactive *in situ* hybridization was performed as described by Gradwohl (Gradwohl et al., 1996). Antisense RNA probe from partial fragment of Mss4 were prepared by *in vitro* transcription of the linearized DNA templete in the presence of digoxigenin-11-UTP (Boehringer Mannheim). The substrate for the chromogenic reaction of alkaline

phosphatase was BM Purple (Boehringer Mannheim). Stained sections were mounted and photographed with Olympus PROVIS AX-70.

RESULTS

Over-expression of Foxg1 in neuronal precursor cell line

It is known that the Foxg1 is highly expressed in the developing mammalian forebrain. Homozygous null Foxg1 mutants die at birth, and have a dramatic reduction in the size of the cerebral hemispheres and multiple developmental anomalies of the eyes (Tao and Lai, 1992; Xuan et al., 1995; Huh et al., 1999). To study the developmental mechanism of Foxg1 in the hippocampus through the regulation of the downstream genes, we used neuronal precursor cell line which was originated in the hippocampus. As the first step to examine the molecular mechanisms of Foxg1, HiB5 cell line was transfected with Foxg1 subcloned into the pEGFP C2. Total RNAs from the transfected cells were reverse-transcribed into cDNAs and the cDNAs were used in PCR reaction. Foxg1 was weakly expressed in the normal state but increased in the transfected cells (Fig. 1A). According to the results of immunocytochemistry using specific antibody, Foxg1-expressing cells were identical with the GFP-expressing cells under the fluorescence microscopy. Especially, Foxg1 expressed in the transfected cells was localized in the nucleus (Fig. 1B). These results indicate that mRNA and protein level of Foxg1 were simultaneously increased in the Foxg1-transfected cells.

Differential display of mRNAs isolated from the Foxg1-overexpressing cells

We compared the profiles of gene expression between the GFP- and GFP-Foxg1-transfected cell line by ordered differential display, using 192 sets of primer pairs as described previously (Matz et al., 1997). ODD often shows false positive bands because of differences in the efficiency of reverse transcription or PCR among each sample. To overcome this problem, we duplicated each sample and remove contaminating DNA in total RNA using DNase I. Double-stranded cDNAs synthesized from isolated RNAs were used to prepare representative pools of 3' cDNA fragment from polyA-trac to the first occurrence of RsaI recognition site, and 3' cDNA fragments were amplified (Fig. 2A). Adapter-ligated 3'-end cDNA fragments were subjected to ODD. For each sample, 192 different combinations of primer sets made of 12 extended T-primers and 16 adaptor-specific primers were used for PCR amplification to generate differential displays. PCR products were separated by PAGE and visualized on the exposed X-ray films. The vast majority of the PCR products were common in all of the samples (Fig. 2B). A total of 98 differentially displayed bands designated as 1 to 100 were excised and recovered from the dried gels, and re-amplified using the corresponding primer sets. All

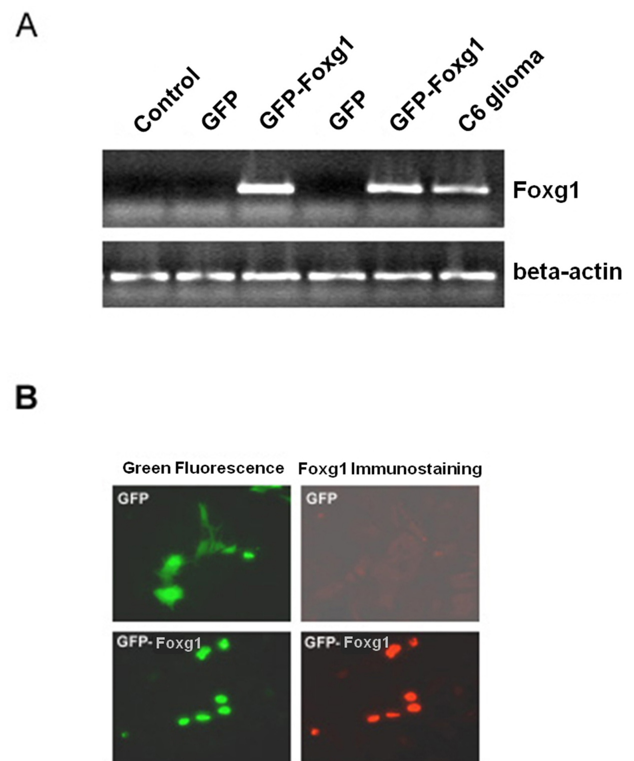

Fig. 1. Detection of Foxg1 over-expressed in level of both mRNA and protein. The expression level of Foxg1 was increased in HiB5 cell line transfected with constructs encoding GFP or Foxg1 tagged with GFP by calcium-phosphate method. Twenty-four hours after transfection, cells were fixed or harvested and their total RNA was isolated. RT-PCR result indicated that mRNA level of Foxg1 was increased in HiB5 and R18F1 cells transfected with Foxg1 (A). mRNA from C6 cell line was used for positive control of Foxg1 and β-actin was used for internal control. Increased protein and cellular localization of Foxg1 were detected in HiB5 cell line transfected with Foxg1 by immunocytochemistry with anti-Foxg1 antibody (B). GFP was visualized as green fluorescence and Foxg1 was visualized as red fluorescence.

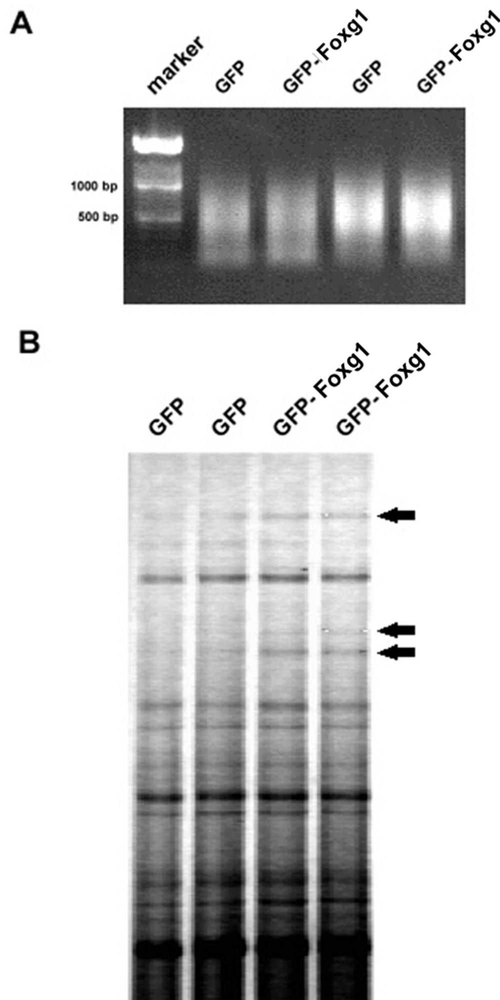

Fig. 2. Example of the ordered differential display. Double-stranded cDNA was synthesized with non-extended ODD T-primer (5'-GCGAGTCGACCG(T)$_{13}$) using double-stranded cDNA synthesis kit (Boehringer Mannheim) and the cDNA fragment pools, ligated with pseudo-double-stranded adaptor: long oligo (5'-GCGTGA-AGACGACAGAAAGGGCGTGGTGCGGAGGGC GGT) and short one (5'-AC-CGCCCTCCGC), were amplified with adaptor-specific primer (5'-TGTAGCGTGAA GACGACAGAA) and non-extended ODD T-primer (A). Several primer combinations were used to amplify the cDNA fragments as described in materials and methods, and ^{33}P-labeled PCR products were separated by PAGE (B). Each sample was prepared as duplication. Arrowheads indicate differential expressed bands.

of cDNAs were re-amplified with sizes between 100 ~500 bp.

Reverse northern blot analysis of the differential expressed cDNA fragments

Those cDNA fragments were recovered from the gel and reamplified by PCR with the same primer sets. To confirm the expression patterns observed in the sequencing gels, RNAs from the GFP- and

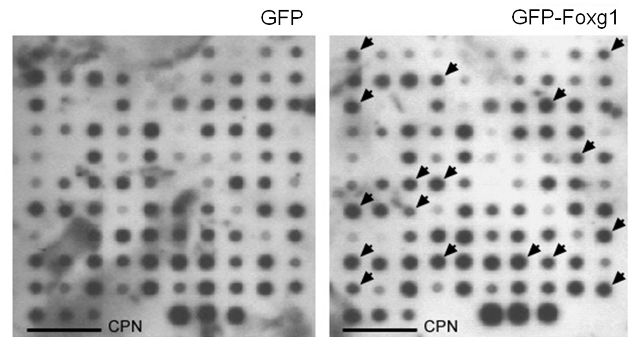

Fig. 3. Reverse-northern blot analysis of 17 confirmed cDNA fragments differentially expressed in Foxg1-transfected cells. Out of 100 Fragments, 17 were confirmed for their differential expression patterns. Note that the RNA for the cDNA probe was derived from another cell culture preparation than the one used for the original ODD. Arrows indicate the differentially expressed dots. Below the blots are the controls DNA, cyclophiline (CPN), demonstrating that equivalent amounts of total RNA were hybridized on each blots.

GFP-Foxg1-transfected cell line was radioactively labeled and used as probes for reverse-northern blot analysis of all 100 reamplified cDNA fragments. Seventeen percent (17 out of 100) of these reamplified cDNA fragments were certified to the original observed displayed patterns (Fig. 3). For internal control, cyclophilin was used. Reverse-northern blot analysis confirmed that these clones corresponded to mRNA that was differentially expressed in response to Foxg1.

Identification of the cloned cDNA fragments

Differentially expressed cDNA fragments were subcloned into pCRII cloning vector and sequenced automatically using M13 primer. The unique sequence of the differentially expressed cDNA fragments were compared to GeneBank entries using BLASTn (Altschul et al., 1997). All 17 confirmed cDNA fragments were successfully cloned into TA cloning vectors for further analysis. The above cloned cDNA fragments were subjected to DNA sequencing. All sequences of 17 cDNA fragments were flanked by sequences derived from the extended T-primers and adaptor-specific primers used in PCR amplification. The features of each clone are summarized in Table 1. Homology searches against GeneBank database revealed that nine fragments are not homologous to any known genes and the other eight fragments contain partial cDNA sequences for known genes (Table 1).

Table 1. Identification of Foxg1-responsive genes (up-regulated) by ordered differential display

Clone	Homology with known genes or EST clones	Function (putative)
1	Rat AI502605 388 bp mRNA EST	Unknown
11	Rat AI710681 362 bp mRNA EST	Unknown
16	Rat AW253495 334 bp mRNA EST	Unknown
18	Human gadd45	DNA damage-repair
22-L	Rat AA900991 516 bp mRNA EST	Unknown
22-S	Rat mss4	Guanine nucleotide-releasing protein, regulation of synaptic secreation
24	Rat CEP52	Ubiquitin/60S ribosomal subunit protein
26	Rat Galectin 8 (RL-30)	S-type lectin, sugar binding and hemaggulutination activity
27	Rat (XL alpha s)	Guanine nucleotide-binding protein, secretory vesicle formation
37	Mouse M-beta-2	Beta-tubulin gene, cytoskeleton formation
50	Not found in any other database	Unknown
51	Mouse W44112 335 bp mRNA EST	Unknown
84	Rat NADH:ubiquinone oxidoreductase subunit	Electron transfer
86	Mouse MITF-2B	bHLH family, inhibition of MyoD activity
90	Rat AI711590 397 bp mRNA EST	Unknown
92	Rat AI556121 358 bp mRNA EST	Unknown
98	Rat AI145929 414 bp mRNA EST	Unknown

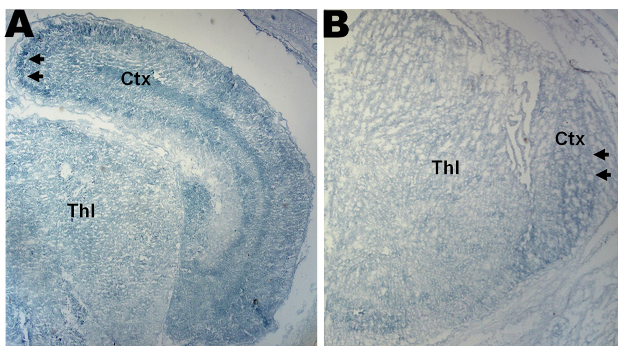

Fig. 4. Expression of Mss4 in the heterozygote and homozygote Foxg1 mutant mouse brain. Sections from Foxg1(+/−) heterozygote (A) and Foxg1(−/−) mutant (B) at P0 were probed by *in situ* hybridization for Mss4 expression. cDNA fragment of Mss4 cloned in pCRII was used for dig-labeled probe by *in vitro* transcription. (A) A coronal section of the telencephalon from the Foxg1(+/−) heterozygote mice. (B) A coronal section of the telencephalon from the Foxg1(−/−) mutant mice. Thl: thalamus, Ctx: cortex.

Down-regulation of Mss4 mRNA expression in the hippocampus of the Foxg1(−/−) mutant mice

Using non-radioactive *in-situ* hybridization, Mss4 mRNA level was examined in the heterozygote and homozygote Foxg1 mutant mouse brain. In the Foxg1(+/−) mouse hippocampus, the specific Mss4 signal was detected (arrows in Fig. 4A). However, in the Foxg1(−/−) mutant hippocampus, the specific Mss4 massage was not detected in the presumptive hippocampal region (arrows in Fig. 4), suggesting that Mss4 expression might be dependent on the normal expression of Foxg1 during development of hippocampus.

DISCUSSION

Identification of genes whose expression is altered in Foxg1-overexpressing cells would be instrumental in elucidating the molecular mechanism of developmental regulation by Foxg1. Here, using an ordered-differential differential display screening, we isolated seventeen distinct genes that are differentially expressed after screening of 100 differential bands from the differential sequencing gels. Of these, eight genes represent known genes or homologues of genes characterized previously, and the other nine genes represent genes that are not related to any sequences in the databases. All seventeen genes have not been found to be associated with the Foxg1 gene prior to our study. In our experimental condition, over-expression of Foxg1 in hippocampal progenitor cells did not affect marked changes in cell morphology. However, the gain-of-Foxg1 functions in these cells were found to be modifying the level of expression of genes that are involved in cell proliferation, differentiation or survival. Foxg1 increased the expression of Mss4,

XL alpha-s, NADH:ubiquinone oxidoreductase subunit, Gadd45, CEP52, M-beta-2, Galectin 8, and several unknown genes. Our observations indicate that Foxg1 modifies the expression of several genes in neuronal precursor cell line. The information on such genes may facilitate our understanding of the spectrum of the functional genetic changes in central nervous system development.

Mss4 binds to, and stimulates GDP-GTP exchange on, a subset of the Rab GTPase that belongs to the Ras superfamily (Burton et al., 1994). The involvement of Rab proteins in the regulation of intracellular membrane traffic and secretion of hormone and neurotransmitters has been previously demonstrated using both genetic and biochemical approaches. 'Extra large' G protein (XL alpha s), which consists of a new 51K XL-portion linked to the alpha s subunit of heterotrimeric G proteins truncated at the amino terminus, is specifically associated with the trans-Golgi network and occurs selectively in cells containing both the regulated and constitutive pathway of protein secretion (Kehlenbach et al., 1994). Hence, Mss4 and XL alpha s may mediate the effects of Foxg1 on the function related to synaptic secretion and secretory vesicle formation.

NADH:ubiquinone oxidoreductase (complex I), the first and largest enzyme of the mitochondrial respiratory chain, transfers electrons from NADH to ubiquinone-10 (UQ-10) (Kitahara et al., 1996). This finding suggest that up-regulation of the NADH: ubiquinone oxidoreductase subunit after Foxg1-overexpression plays an important role in the effective electron transfer from NADH to UQ-10 in neuronal precursor cell line.

Gadd45, which encodes a protein associated with PCNA to stimulate DNA repair machinery (Kastan et al., 1992), is a DNA damage-inducible gene involved in DNA excision repair that is induced by a variety of DNA-damaging stimuli in mammalian cells (Fornace et al., 1988). Only gadd45, of the five gadd genes, is induced in the central nervous system by DNA-damages (Zhu et al., 1997). At the permissive temperature, TUNEL-stained cells were detected in the small number of HiB5 cells (data not shown). Thus, the induction of gadd45 may suggests that the Foxg1-overexpressing cells better respond to repair DNA damage and that Foxg1 has survival effect through the involvement of DNA repair systems.

The functions of other up-regulated genes in response to Foxg1 are not elucidated in detail and thus the biological relevance of the expression of these genes in relation to the expression of Foxg1 has not been assessed yet. However, because these genes might play important roles as a downstream mediator of the effects of Foxg1, a detailed cellular signaling cascades exerted by Foxg1 are worth to investigate in future studies. In addition, since Foxg1 is a transcription factor with cognate DNA binding sites, examination of the promoter region of these Foxg1-regulated genes would enlighten novel downstream target genes of Foxg1 during brain development. Furthermore, functional analysis of the gene products would provide clues in understanding the molecular mechanisms during telencephalic morphogenesis exerted by Foxg1. Finally, examination of the spatio-temporal expression pattern of these Foxg1-regulated genes by in situ hybridization would further enlighten the complicated gene-gene interactions during development of telencephalon.

ACKNOWLEDGEMENTS

We thank Professor Yunhee Kim-Kwon for providing us with HiB5 cell line. This work was supported by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0094073).

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