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Transcriptional Regulation of Human *FE65*, a Ligand of Alzheimer's Disease Amyloid Precursor Protein, by Sp1

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ABSTRACT

FE65 is a neuronal-enriched adaptor protein that binds to the Alzheimer's disease amyloid precursor protein (APP). FE65 forms a transcriptionally active complex with the APP intracellular domain (AICD). The precise gene targets for this complex are unclear but several Alzheimer's disease-linked genes have been proposed. Additionally, evidence suggests that FE65 influences APP metabolism. The mechanism by which FE65 expression is regulated is as yet unknown. To gain insight into the regulatory mechanism, we cloned a 1.6 kb fragment upstream of the human *FE65* gene and found that it possesses particularly strong promoter activity in neurones. To delineate essential regions in the human *FE65* promoter, a series of deletion mutants were generated. The minimal *FE65* promoter was located between −100 and +5, which contains a functional Sp1 site. Overexpression of the transcription factor Sp1 potentiates the *FE65* promoter activity. Conversely, suppression of the *FE65* promoter was observed in cells either treated with an Sp1 inhibitor or in which Sp1 was knocked down. Furthermore, reduced levels of Sp1 resulted in downregulation of endogenous FE65 mRNA and protein. These findings reveal that Sp1 plays a crucial role in transcriptional control of the human *FE65* gene. J. Cell. Biochem. 109: 782–793, 2010.

KEY WORDS: FE65; AMYLOID PRECURSOR PROTEIN; Sp1; PROMOTER

Alzheimer's disease is the most common cause of dementia in the elderly. Aggregation of amyloid β (Aβ) derived from the amyloid-β precursor protein (APP), is believed to be central to the pathogenesis of the disease (see review [Selkoe, 2000]). APP is a ubiquitously expressed type I integral transmembrane protein with a large ectodomain and a short intracellular domain [Kang et al., 1987; Tanzi et al., 1987]. APP is processed first by either α- or β-secretase and then by γ-secretase. Cleavage of APP by α- and γ-secretases produces non-amyloidgenic p3 peptide and APP intracellular domain (AICD) while β- and γ-secretase processing of APP results in the generation of Aβ and AICD.

FE65 is a brain-enriched adaptor protein with multiple protein–protein interaction domains including a WW domain and two C-terminal phosphotyrosine binding (PTB) domains. FE65 has been shown to interact with the intracellular domain of APP (see review [McLoughlin and Miller, 2008]). Human FE65 is encoded by the gene

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Hoi-Tin Yu, William Wai-Lun Chan, and Ka-Ho Chai contributed equally to this work.

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amyloid precursor protein-binding, family B member 1 (*APBB1*) which contains 14 exons [Hu et al., 1998] and is located on chromosome 11 [Bressler et al., 1996]. Although the mechanism is not known, FE65 has been shown to alter APP processing and β-amyloid (Aβ) production [Sabo et al., 1999; Wang et al., 2004; Santiard-Baron et al., 2005; Guenette et al., 2006; Wiley et al., 2007]. In addition to altering APP processing, FE65 has been found to complex with APP intracellular domain (AICD), and to stimulate transcription of a GAL4-dependent reporter system [Cao and Sudhof, 2001; Kimberly et al., 2001; Baek et al., 2002; Kinoshita et al., 2002; Scheinfeld et al., 2003]. Although the role of the FE65-AICD complex in transcription remains controversial, several Alzheimer's disease linked genes have been reported to be regulated by FE65-APP signaling including *GSK3*β, *BACE1*, *acetylcholinesterase*, *neprilysin* and *APP* [Kim et al., 2003; Bimonte et al., 2004; von Rotz et al., 2004; Pardossi-Piquard et al., 2005; Telese et al., 2005; Chang et al., 2006].

FE65 is highly expressed in the brain regions affected by Alzheimer's disease [Esposito et al., 1990; Simeone et al., 1994; Bressler et al., 1996; Kesavapany et al., 2002]. Furthermore, it has been reported that FE65 levels are increased in the hippocampal area CA4 of Alzheimer's disease brain [Delatour et al., 2001]. Therefore aberrant expression of FE65 may influence APP processing and the transcription of FE65-APP regulated genes. To date, the mechanism(s) by which human FE65 expression is regulated is still unclear. However, there are several lines of evidence to suggest that FE65 can be regulated at the transcriptional level; firstly, FE65 mRNA is differentially expressed in different tissues [Duilio et al., 1991], secondly, the expression of FE65 mRNA has been shown to be developmentally regulated [Simeone et al., 1994] and finally the mRNA level of FE65 has been found to increase dramatically in rats with traumatic brain injury [Iino et al., 2003]. As FE65 appears to play an important role in the pathogenesis of Alzheimer's disease, it is therefore of great interest to understand the transcriptional control of human *FE65*. To this end, we have attempted to clone and characterize the human *FE65* gene promoter.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

CHO cells, HEK293 cells, and primary rat cortical neurones were cultured as described previously [Lau et al., 2008]. CHO and HEK293 cells were transfected using Fugene 6 (Roche), and rat cortical neurones were transfected using Lipofectamine 2000 (Invitrogen). Mithramycin A (Sigma) was added to 7-day-old cultured neurones 48 h before harvesting. siRNA knockdown in HEK293 was performed as described previously [Lau et al., 2008] using human Sp1 (Dharmacon), Sp3 (Santa Cruz) and, control non-targeting siRNAs (Dharmacon). Sp1 knockdown in rat cortical neurones was performed using rat Sp1 and non-targeting Accell siRNA according to manufacturers' instruction (Dharmacon).

MAPPING OF THE TRANSCRIPTION START SITE OF *FE65*

The transcription start site of *FE65* was identified by RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) using a human brain FirstChoice RACE-Ready cDNA kit (Ambion). The cDNA was used as a template for 5′RACE using FE65 RACE primer 1 (5′-GCATCTGGGTCAGCTTATC-3′) and RACE outer primer (5′-GCTGATGGCGATGAATGAACACTG-3′). For nested PCR, FE65 RACE primer 2 (5′-GCCTGCAGCTTGGCGTTGAGCAGCTG-3′) and 5′ RACE inner primer (5′-CGCGGATCCGAACACTGCGTTTGC-TGGCTTTGATG-3′) were employed. Confirmatory nested PCR was performed using FE65 RACE primer 3 (5′-CCGACTGGCTCAGT-GATGATGGAACAG-3′) and 5′ RACE inner primer. Both RACE outer and inner primers were supplied with the kit. PCR conditions for 30 cycles were denaturation at 94°C for 30 s, annealing at 62°C for 30 s and polymerization at 72°C for 2 min. The PCR product was cloned and sequenced.

ISOLATION OF THE HUMAN *FE65* PROMOTER

The putative human *FE65* promoter was amplified from human genomic DNA by PCR using FE65 specific primer 1 (5′-TCAC-CCCAGTGAAACTCCTTAAAAGACT) and FE65 specific primer 2 (5′-CCGGACGAGGGGCAGGAGGTAAAG-3′) which were designed according to the sequence of clone NT_009237 in Genbank. FE65 specific primer 1 and primer 2 are located 1316 nucleotides upstream and 328 nucleotides downstream of the identified transcription start site, respectively. PCR conditions for 30 cycles were denaturation at 94°C for 1 min, annealing at 62°C for 1 min and polymerization at 72°C for 1.5 min. The PCR product was cloned and sequenced.

ASSAY OF PROMOTER ACTIVITY

The putative *FE65* promoter DNA fragment was cloned, in both positive and reverse orientations, into the vector pGL4.17 [*luc2*/Neo] (Promega) which contains a promoterless firefly luciferase gene. CHO, HEK293, and primary rat cortical neurones were transfected with *FE65* promoter/luciferase and pRL-TK (Promega) constructs. pRL-TK which expresses *Renilla* luciferase was used as a control to quantify transfection efficiency. The firefly luciferase activities produced by the *FE65* promoter/luciferase constructs were measured with a luminometer (Wallace). Then, the *Renilla* luciferase activities produced by the pRL-TK were assayed by adding equal volumes of Dual-Glo Stop & Glo substrate (comprising the stop solution for firefly luciferase and substrate for *Renilla* luciferase) and analyzed with the luminometer. The firefly luciferase activity was normalized to the corresponding *Renilla* luciferase activity.

GENERATION OF *FE65* PROMOTER MUTANT CONSTRUCTS

The 5′ deletion mutants of the human *FE65* promoter were generated by either restriction digestions or PCR. *FE65* promoter deletion constructs p−369Luc, p−100Luc and pΔ−369 to +331Luc were generated by cutting the 1.6 kb FE65 genomic DNA with appropriate restriction endonucleases, blunt-ended, and then subcloning into pGL4.17 [*luc2*/Neo] (Promega). p−796Luc was generated by PCR using FE65 specific primer 3 (5′-TTTACACTTTT-CATTCACAATATCCCCTTC-3′) and FE65 specific primer 2. p+5Luc was generated by PCR using FE65 specific primer 4 (5-ATGTTGT-GATGGAGAAGCCG-3′) and FE65 specific primer 2. p−1316 Δ−100/+5Luc was created by subcloning a PCR fragment containing −1316/−101 into p+5Luc. p−100Luc Sp1 mutant was generated by mutagenesis using a QuikChange mutagenesis kit

(Stratagene). Mutagenic oligonucleotides were 5′-CCGCGCGCT-CCTGAATTCGGGATGTTGTGATGGAGAAG-3′ and 5′-CTTCTCCAT-CACAACATCCCGAATTCAGGAGCGCGCGG-3′. The sequences of the deletion mutants were confirmed by DNA sequencing.

SEMI-QUANTITATIVE POLYMERASE CHAIN REACTION (PCR) ANALYZES

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) and was reverse transcribed into first strand cDNA in the presence of oligo-dT primer using the 1st Strand cDNA synthesis kit for RT-PCR (Roche). Amplification of FE65 was performed by using the following two primers: 5′-CGGAACGCCAATCCAGGGATCAAG-3′; 5′-TTTACCAAGCAGCGAGCATTGCG-3′. PCR was performed as follows: 28 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 68°C. PCR of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was performed as previously described [Lau et al., 2008].

IMMUNOBLOT ANALYZES

Total cell lysates were prepared by harvesting cells using SDS sample buffer. The lysates were separated by SDS–PAGE and transferred electrophoretically onto nitrocellulose membranes. The membranes were probed with appropriate primary antibodies and then followed by secondary antibodies conjugated with horseradish peroxidise (GE Healthcare). Antibodies were detected using enhanced chemiluminescence (GE Healthcare). Primary antibodies were as follows: FE65 (Cell Signaling Technology); α-tubulin DM1A (Sigma); Sp1, Sp3, and Sp4 (Santa Cruz).

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

EMSA was performed using a gel shift assay system (Promega). To prepare rat cortical neurone nuclear extract, the nuclei were isolated from the neurones as described [Thomas et al., 1996]. The isolated nuclei were resuspended in nuclear extraction buffer (20 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 400 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 25% glycerol) and sonicated for 5 s. The extract was cleared by centrifugation at 13,000 rpm for 10 min at 4°C. Recombinant Sp1 protein and Sp1 consensus probe (5′-ATTCGATCGGGGCGGGGC-GAGC-3′) were purchased from Promega. Double-stranded FE65-Sp1, FE65-Sp1 mutant, and HSE probes were prepared by incubating oligonucelotides FE65-Sp1-f 5′-CGCGCTCCTGGGCGGGGGATG-TTGT-3′ + FE65-Sp1-r 5′-ACAACATCCCCCGCCCAGGAGCGCG-3′, FE65-Sp1-f mutant 5′-CGCGCTCCTGAATTCGGGATGTTGT-3′ + FE65-Sp1-r mutant 5′-ACAACATCCCGAATTCAGGAGCGCG-3′ and HSE-f 5′-GCAGAATCTTCTAGAATCAGC-3′ + HSE-r 5′-GCTGATTCTAGAGATTCTGC-3′ in annealing buffer (20 mM Tris pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl) at 80°C for 10 min and then cooled gradually to room temperature. The consensus binding sequences for Sp1 on the Sp1 consensus and FE65-Sp1 probes were underlined. The Sp1 consensus and annealed probes were then labeled with [γ-32P] ATP by T4 polynucleotide kinase at 37°C for 1 h. The labeled probes were incubated with corresponding components in 1× gel shift binding buffer at room temperature for 10 min. Competition assays were performed by adding 250× molar excess of either unlabeled probes (competitor) or unlabeled mutant probe (non-specific competitor). Supershift assays were performed by adding anti-Sp1 polyclonal antibody (Santa Cruz). The samples were then loaded into a 4% non-denaturing polyacrylamide gel. The signals on the gel were detected by autoradiography.

STATISTICAL ANALYSIS

All data were analyzed by Instat software (GraphPad) using either Student's *t*-test or one-way analysis of variance (ANOVA). Significance is indicated between different treatments as $^{*}P < 0.001$, $^{**}P < 0.0001$. Error bars shown are standard deviations (SD).

RESULTS

MAPPING OF THE TRANSCRIPTION START SITE OF HUMAN *FE65*

In order to identify the *FE65* promoter, we first attempted to locate the transcription start site. As premature termination may occur in traditional transcription site mapping methods including primer extension and classic RACE, RLM-RACE was employed in this study. A major advantage of RLM-RACE over other approaches is that the 5′ RLM-RACE adaptor can only ligate to full-length capped mRNA, and therefore selective amplification of full-length mRNA is guaranteed. In primary PCR, no specific DNA fragment was amplified (Fig. 1A). A DNA fragment with a molecular weight of about 0.25 kb was amplified from nested PCR using FE65 RACE primer 2 and 5′ RACE inner primer (Fig. 1B, lane 1). Sequencing results revealed that the DNA fragment contained the 5′ untranslated region of human FE65 mRNA. The transcription start site is located 105 nucleotides upstream of the translation start codon of FE65 mRNA (Fig. 1C). To confirm the transcription start site, further RACE was performed using an upstream primer (FE65 RACE primer 3) which is located closer to the identified start site (Fig. 1, lane 2). The second RACE product ended at the same position as the first RACE product. The FE65 RLM-RACE product contains exon 1 and the 5′ region of exon 2 of the human *FE65* gene [Hu et al., 1998].

MOLECULAR CLONING AND FUNCTIONAL ANALYSIS OF THE HUMAN *FE65* PROMOTER

Most gene promoters are located upstream of their transcription start sites. Therefore we performed a Genbank sequence search and found that contig sequence NT_009237 contains the sequence that is upstream of the *FE65* transcription start site. According to the contig sequence, the size of intron 1 of the *FE65* gene is about 7.6 kb (The location of intron 1 in the RLM-RACE product is indicated in Fig. 1C). In silico analyzes using various online tools including PROSCAN (http://www-bimas.cit.nih.gov/molbio/proscan/) and Neural Network Promoter Prediction (http://promotor.biosino.org/) revealed that a putative promoter region is located upstream of the transcription start site (PROSCAN promoter score = 78.97; cutoff = 53). To obtain experimental evidence, we amplified a 1.6 kb fragment from human genomic DNA containing the 5′ end of intron 1, exon 1 and the putative *FE65* promoter region (Fig. 2A,B). Then the fragment was cloned into the promoterless pGL4.17[*luc2*/Neo] vector to form p−1316Luc. HEK293 cells were transfected with the putative *FE65* promoter luciferase construct. phRL-TK plasmid was cotransfected to monitor and normalize the transfection efficiency.

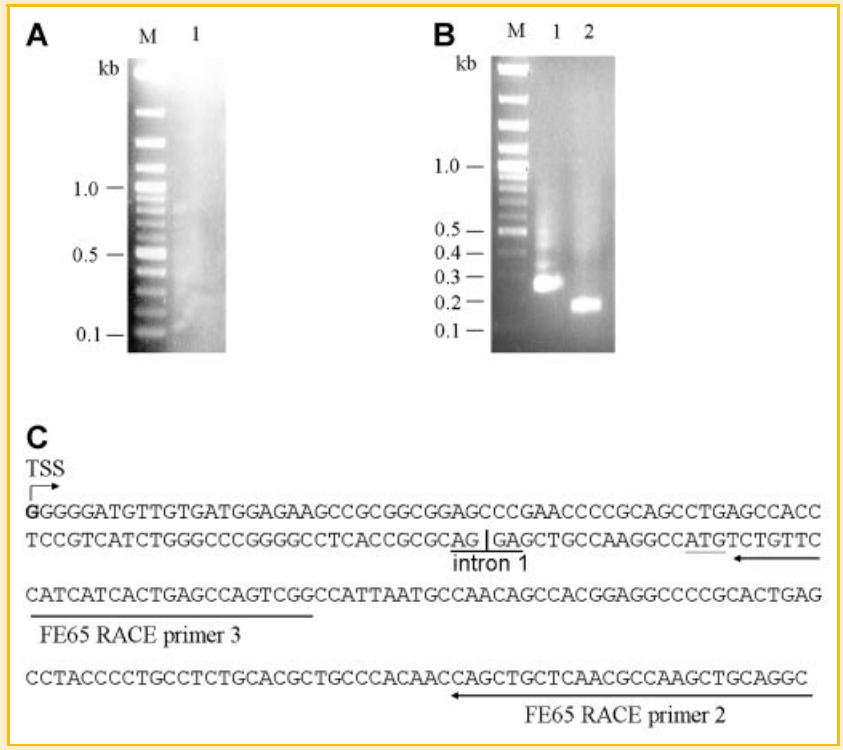

Fig. 1. Identification of the transcription start site of human *FE65* using RLM-RACE. A: 5′ RACE using FE65 RACE primer 1 which is complementary to sequences located at the coding region of human FE65. PCR was performed using RACE primer 1 and RACE outer primer (lane 1). M; 2-Log DNA Ladder (NEB). B: Nested-PCR using RACE inner primer with either FE65 RACE primer 2 (lane 1) or FE65 RACE primer 3 (lane 2). M; 2-log DNA Ladder. C: Nucleotide sequence of the 5′ RACE product from (B, lane 1). Bent arrow indicates the putative transcription start site (TSS). The locations of primers used in (B) are indicated. The start codon (ATG) of FE65 is underlined. The position of intron 1 defined by Hu et al. [1998] is indicated.

There was a significant fivefold increase in luciferase activity in HEK293 cells transfected with p−1316Luc compared with the pGL4.17[*luc2*/Neo] transfected control cells (Fig. 2C). The putative *FE65* promoter was also cloned into pGL4.17[*luc2*/Neo] in reverse orientation (p−1316Luc-R) and analyzed in the promoter assay. The reverse-oriented construct did not show any significant promoter activity (Fig. 2C). A similar result was observed in CHO cells (data not shown). As FE65 is a brain enriched protein, the *FE65* promoter constructs were also tested in rat cortical neurones. A 50-fold increase in promoter activity was detected in neurones transfected with p−1316Luc. Again, no promoter activity was observed in p−1316Luc-R (Fig. 2C). These findings suggest that the 1.6 kb fragment contains a functional promoter, and *FE65* promoter is more active in neurones than other tested cell types. We also confirmed by RT-PCR that the endogenous FE65 mRNA expression level is higher in neurones than in HEK293 cells (Fig. 2D).

DELETION ANALYSIS OF THE HUMAN *FE65* PROMOTER

To determine the essential region(s) in the human *FE65* promoter, deletion constructs were generated and used to transfect cortical neurones (Fig. 3). There was no significant difference in promoter activities of deletion constructs p−796Luc, p−369Luc, and p−100Luc compared to p−1316Luc (Fig. 3A). However, promoter activity was markedly reduced when the region from −100 to +5 (i.e., p+5Luc) was deleted suggesting that an essential promoter region is located in the deleted region. No promoter activity was also seen in p−1316Δ−369/+331. To further confirm the importance of −100 to +5, a deletion mutant which lacks this region was generated (i.e., p−1316 Δ−100/+5Luc). Again, the mutant did not show any significant promoter activity. Similar results were observed in other cell types including CHO and HEK293 (data not shown).

AN Sp1 BINDING SITE RESIDES IN THE MINIMAL PROMOTER REGION OF *FE65*

Our deletion analysis indicates that the region −100 to +5 contains the minimal promoter and is required for *FE65* promoter activity. In silico analysis using PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) indicates that a putative Sp1 binding element (PROMO dissimilarity rate = 0%) resides within the minimal promoter (Fig. 2B). The putative Sp1 site is conserved among several species of animals including chimpanzee, monkey, and mouse (Fig. 3B). Sp1 is a member of a small transcription factor family containing Sp1–Sp8 (see reviews [Suske, 1999; Safe and Abdelrahim, 2005; Suske et al., 2005]). Immunoblot analyzes revealed that the level of Sp3 was slightly lower in neurones than that in CHO and HEK293. Sp4 levels were similar in the three cell types. However, higher level of Sp1 was detected in neurones than in the CHO and HEK293 (Fig. 4). This may suggest that Sp1 is the major Sp protein involves in the regulation of the human

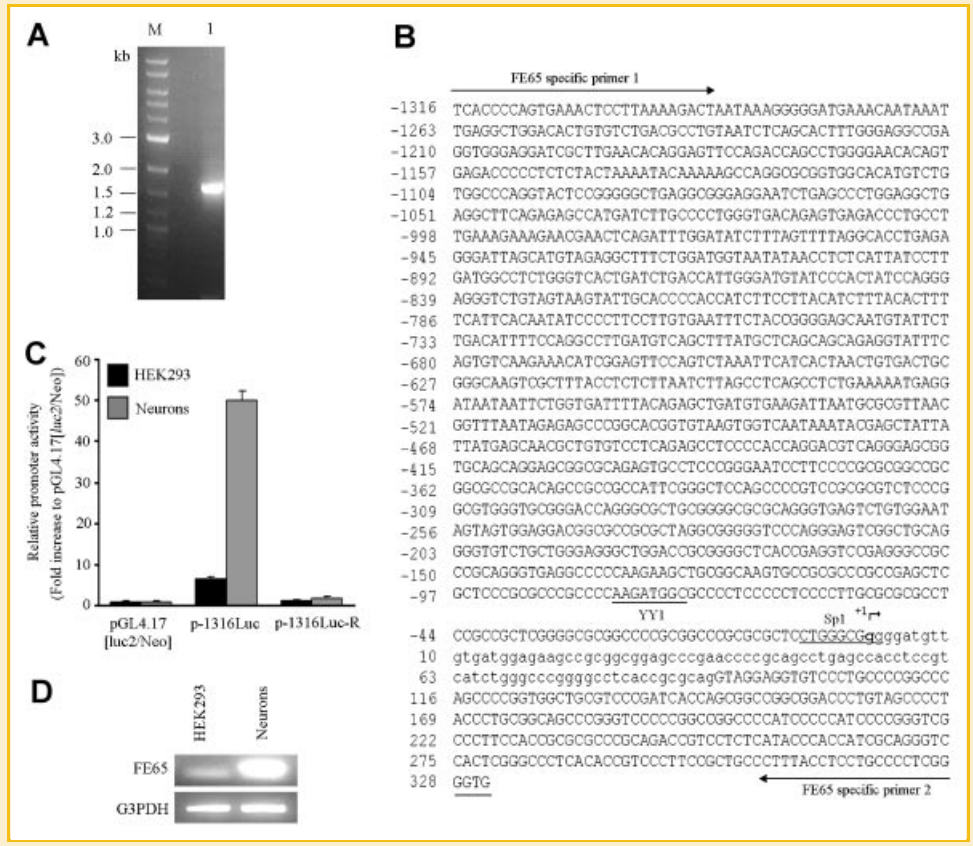

Fig. 2. Amplification and functional analysis of the human *FE65* promoter. A: Sequence upstream of the *FE65* transcription start site was amplified by PCR using FE65 specific primer 1 and primer 2 from human genomic DNA. B: Nucleotide sequence of the amplified DNA from 2A. Transcription start site is indicated by the bent arrow and is denoted as +1. Exon 1 sequence is shown in lowercase letters. The putative Sp1 and YY1 site are underlined. The locations of primers used for PCR in 2A are indicated. C: The 1.6 kb fragment in 2A was cloned into pGL4.17[*luc2*/Neo] in both sense and antisense orientations, that is, p−1316Luc and p−1316Luc-R, respectively, and these were used to transfect HEK293 and rat cortical neurones. Cells were co-transfected with pRL-TK to normalize the transfection efficiency, n = 12. D: RT-PCR to determine the expressions of endogenous FE65 in HEK293 and rat cortical neurones. G3PDH was used as an internal control.

FE65 transcription in neurones. To test if the Sp1 binding element is essential to the basal promoter activity, we generated a p−100Luc Sp1 mutant, in which the Sp1 site was disrupted, and its promoter activity was examined in cortical neurones. We found that the promoter activity of the p−100Luc Sp1 mutant was markedly decreased by 67% (Fig. 5A).

To investigate whether the element in the human *FE65* promoter is an Sp1 binding site, EMSA was performed. Sp1 consensus and FE65-Sp1 probes were end-labeled and incubated with cortical neurone nuclear extract. Shifted DNA-Sp1 complexes were detected with Sp1 consensus and FE65-Sp1 probes in the reactions with nuclear extract (Fig. 5B, lanes 2 and 7). To test if the shifted complexes contain Sp1, a polyclonal Sp1 antibody was added. Although addition of Sp1 antibody did not induce a supershift band, the signals of DNA-Sp1 complexes were reduced significantly in both Sp1 consensus and FE65-Sp1 probes (Fig. 5B, lanes 3 and 8). A similar phenomenon has been reported for Sp1 supershift assays [Chung et al., 1995; Imai et al., 2006; Li et al., 2008; Liu et al., 2002; Schwarzenbach et al., 2004; Wu et al., 2007]. Addition of 250-fold molar excess of unlabeled probes (competitor) inhibited the shifts in the probes. (Fig. 5B, lanes 4 and 9). However, addition of unlabeled mutant probes (mutant competitor) did not affect the formation of the complexes (Fig. 5B, lanes 5 and 10). These findings indicate that the binding between Sp1 and FE65-Sp1 probe is specific.

To further confirm that the putative FE65-Sp1 element binds to Sp1, recombinant Sp1 was used in EMSA. Again, shifted DNA-Sp1 complexes were observed in Sp1 consensus and FE65-Sp1 probes (Fig. 5C, lanes 2 and 6) but not with the negative control HSE consensus probes (Fig. 5C, lane 10) when incubated with recombinant Sp1. The signals of DNA-Sp1 were abolished with the incubation of excess unlabeled competitors (Fig. 5C, lanes 3 and 7). Addition of unlabeled mutant competitors did not show any competitive effect on the DNA-Sp1 complexes (Fig. 5C, lanes 4 and 8).

Sp1 INFLUENCES *FE65* PROMOTER ACTIVITY

To further investigate the effect of Sp1 on the activity of the *FE65* promoter, HEK293 cells and cortical neurones were transfected with p−100Luc *FE65* promoter reporter either with or without Sp1. There was a significant increase in *FE65* promoter activity in both Sp1 transfected HEK293 cells and cortical neurones (Fig. 6A). A similar

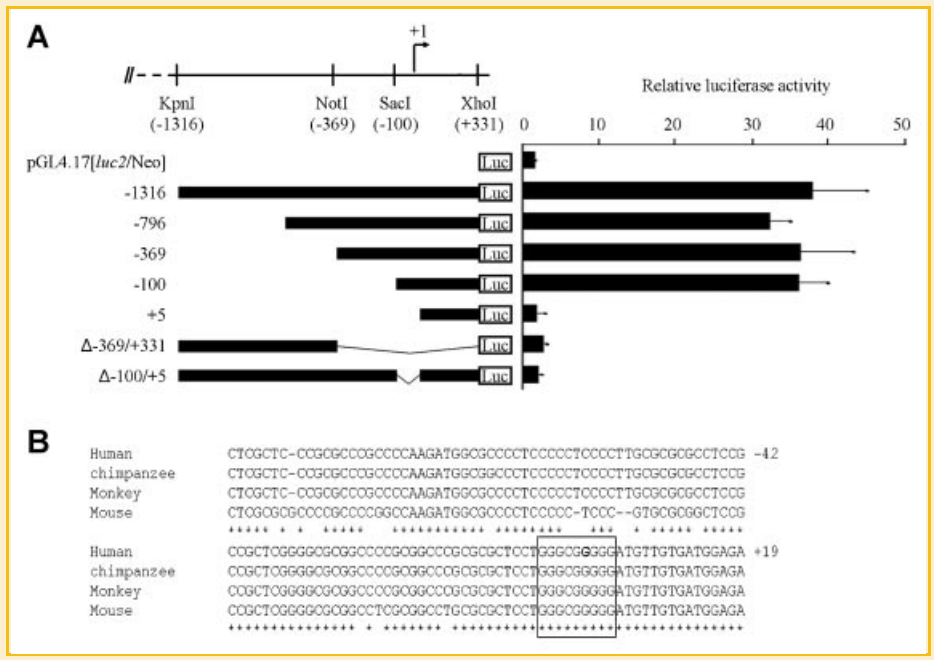

Fig. 3. Deletion analysis of the human *FE65* promoter. A: Schematic diagram of the *FE65* promoter deletion constructs (left panel). The transcription start site is indicated (+1). The restriction sites used for the generation of various deletion mutants are indicated. The bar chart shows the promoter activities of different FE65 deletion constructs in transfected cortical neurones. pRL-TK was co-transfected to normalize the transfection efficiency, n = 12. B: Sequence comparison of the minimal *FE65* promoter from human, chimpanzee, monkey and, mouse. Asterisks indicate identical nucleotide. The conserved putative Sp1 sites were boxed.

effect was observed in cells transfected with p−1316Luc reporter constructs (data not shown).

In a complementary experiment, HEK293 cells and cortical neurones were transfected with p−100Luc and then treated with mithramycin A, a suppressor of Sp1, which can inhibit Sp1 binding to DNA [Ray et al., 1989; Blume et al., 1991] and the expression of Sp1 [Jia et al., 2007; Yuan et al., 2007; Wang et al., 2008; Zimmermann et al., 2008]. *FE65* promoter activity dropped significantly in mithramycin A treated HEK293 cells and cortical neurones (Fig. 6B). A reduced level of Sp1 in the mithramycin A treated cells was confirmed by Western blot analysis. We further tested the effect of Sp1 on the *FE65* promoter using a knock-down approach. The promoter activitiy of p−100Luc was significantly suppressed in the HEK293 cells co-transfected with Sp1 siRNA but not with the non-targeting control siRNA (Fig. 6C). Reduced levels of Sp1 in Sp1 siRNA transfected cells were shown by Western blot analysis. Furthermore, in consistent with the reporter assay, the endogenous levels of FE65 mRNA in the Sp1 knock-down HEK293 cells and neurones were markedly decreased (Fig. 6D,F).

Since Sp3 has been reported to influence the Sp1-mediated transcription (see reviews [Black et al., 2001; Li et al., 2004; Safe and Abdelrahim, 2005]), we therefore tested the effect of Sp3 on the human *FE65* promoter. In HEK293 cells, knock-down of Sp3 enhanced the transcription of p−100Luc *FE65* promoter reporter significantly (Fig. 7). This observation suggests that Sp3 might function as a FE65 transcription repressor, and work cooperatively with Sp1 to control the expression of FE65.

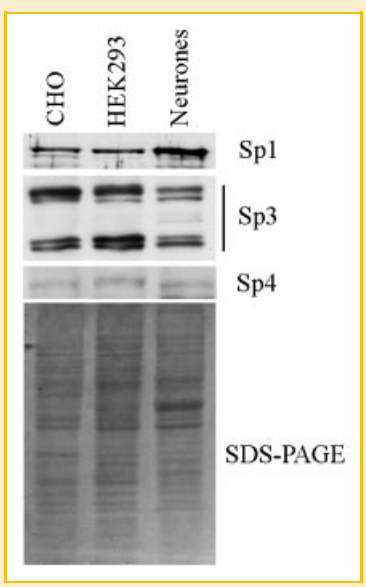

Fig. 4. Immunoblot analyzes of Sp1, Sp3, and Sp4 from CHO, HEK293, and rat cortical neurones. Coomassie blue stained SDS–PAGE gel (bottom panel) shows the amounts of protein loaded.

INHIBITION OF Sp1 BY MITHRAMYCIN A REDUCES THE EXPRESSION OF FE65

As mithramycin A inhibits *FE65* promoter activity, we therefore investigated whether mithramycin A influences the expression of

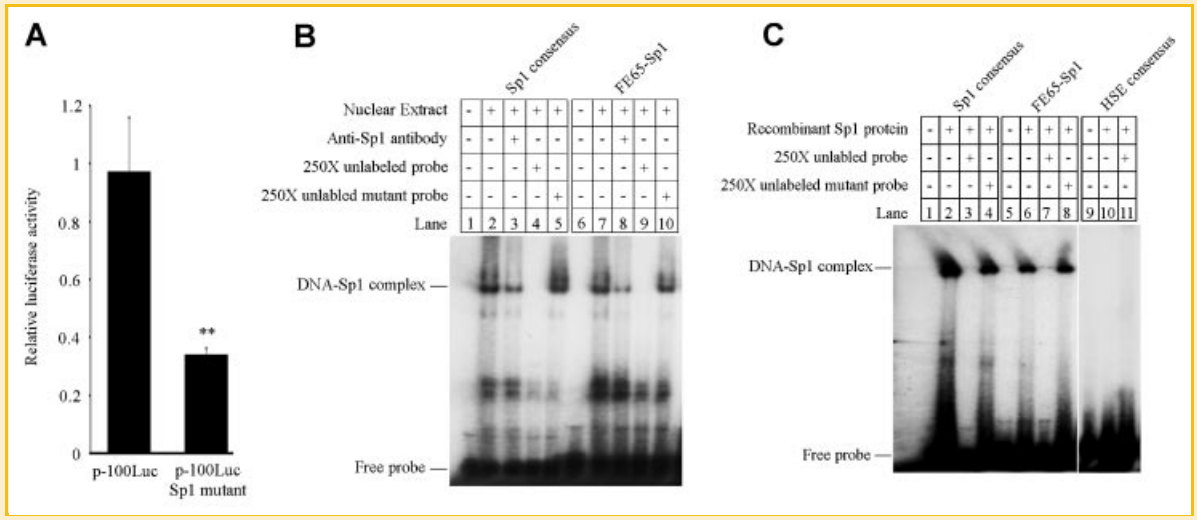

Fig. 5. Sp1 binding site is located within the minimal FE65 promoter. A: Cortical neurones were tranfected with p−100Luc and p−100Luc Sp1 mutant, n = 12. EMSA was performed by incubating ^{32}P-labeled probes with (B) cortical neurone nuclear extract and (C) recombinant Sp1. The components of the EMSA reactions are indicated. The DNA-Sp1 complexes were separated by 4% non-denaturing PAGE and visualized by autoradiography. Competition assays were performed by adding 250× molar excess of either unlabeled probes (competitor) or unlabeled mutant probe (non-specific competitor). Supershift assays were performed by adding anti-Sp1 polyclonal antibody.

endogenous FE65. RT-PCR and immunoblot analyzes revealed that treatment of neurones with mithramycin A led to a significant decrease in endogenous FE65 mRNA and protein levels (Fig. 8). Again, the amount of Sp1 in the mithramycin A treated neurones was confirmed to be reduced (Fig. 8). These findings suggest that Sp1 plays a role, at least partially, in the activation of the transcription of *FE65*.

DISCUSSION

Aberrant processing of APP is believed to be central to the pathogenesis of Alzheimer's disease. FE65 is an adaptor protein that binds to the cytoplasmic domain of APP and alters APP processing. Moreover, FE65-AICD complex has been reported to influence the expression of several Alzheimer's disease associated genes (see review [McLoughlin and Miller, 2008]). Therefore, proper expression of FE65 appears to be crucial for the physiological functions of FE65. In order to understand the molecular mechanism by which FE65 expression is controlled, we attempted to clone the human *FE65* promoter. A 1.6 kb 5′ UTR of the human *FE65* gene, which contains a putative promoter region, was amplified from human genomic DNA. The functionality of the putative human *FE65* promoter was determined in several cell types. In our promoter reporter assays, the *FE65* promoter was more active in cortical neurones than in non-neuronal cells including HEK293 and CHO. This observation explains the fact, at least in part, that FE65 mRNA is expressed abundantly in brain but not in other tissues [Duilio et al., 1991].

To identify the essential regulatory region(s) within the cloned human *FE65* promoter, progressive deletion mutants were generated. The promoter activity was retained until the region −100 to +5 was removed. This indicates that the minimal promoter region required for transcriptional activity of *FE65* is located within −100 to +5. In silico sequence analysis reveals that the minimal *FE65* promoter has a high CG content (84.7%) and does not possess any canonical TATA box or initiator sequence. The absence of both a TATA box and an initiator suggests that the *FE65* promoter is a null promoter. The mechanism by which the transcription of a null promoter is initiated is still not fully understood [Novina and Roy, 1996], however, transcription factor Sp1 has been shown to mediate the transcription of many neuronal TATA-less genes (see review [Myers et al., 1999]). It has been found that Sp1 functions in recruiting TATA-binding protein in the absence of a TATA box (see review [Butler and Kadonaga, 2002]). As FE65 is abundantly expressed in brain and a highly conserved Sp1 binding site (PROMO dissimilarity rate = 0%) was identified within the minimal promoter region, we therefore investigated whether Sp1 influences the transcription of *FE65*. In fact, overexpression of Sp1 enhances the *FE65* promoter activity significantly. Conversely, *FE65* promoter activity was markedly reduced after treatment with mithramycin A, an inhibitor of Sp1 [Ray et al., 1989; Blume et al., 1991; Jia et al., 2007; Zimmermann et al., 2008]. A similar effect was observed in Sp1 knock-down cells. The binding of the FE65-Sp1 element was confirmed by EMSA using both nuclear extract and recombinant Sp1. In addition to promoter reporter assays, both FE65 mRNA and protein levels were found to be reduced in the mithramycin A treated neurones. Thus, our findings clearly indicate that Sp1 plays an essential role in the regulation of *FE65* gene expression. Sp1 is a widely expressed transcription factor which binds to CG boxes and functions in controlling the expression of many genes (see review [Wierstra, 2008]). Aberrant expression of Sp1 has been implicated in several human diseases including neurodegenerative disorders [Qiu et al., 2006; Santpere et al., 2006; Citron et al., 2008]. Recently, it has been found that the expression of Sp1 is up-regulated in several Alzheimer's disease mouse models and Alzheimer's disease brains

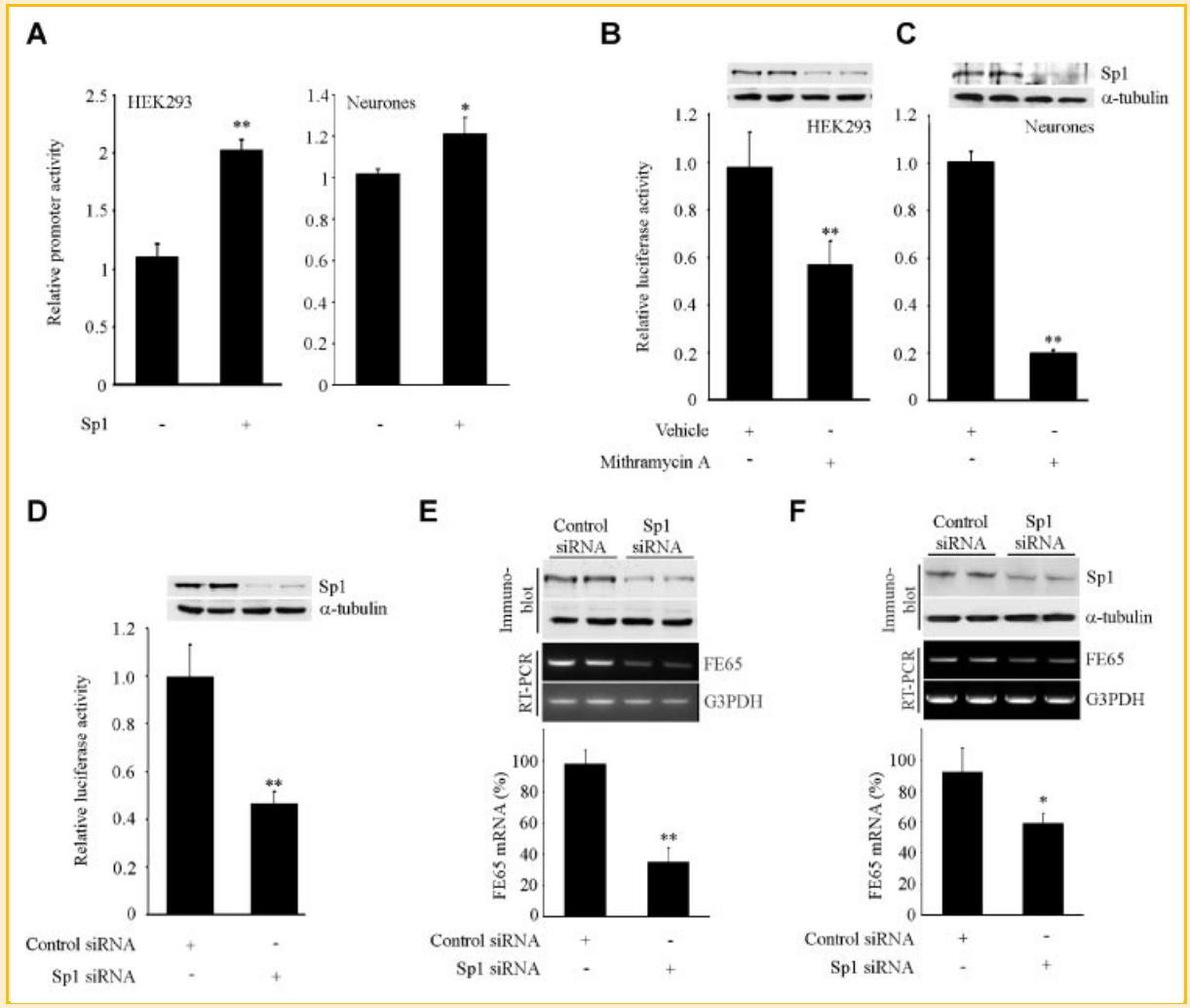

Fig. 6. Sp1 stimulates the human *FE65* expression. A: HEK293 and rat cortical neurones were co-transfected with p−100Luc *FE65* promoter reporter and either empty vector (pN3) or mammalian expression construct of Sp1 (pN3-Sp1), n = 8. B: HEK293 cells or (C) rat cortical neurones were tranfected with p−100Luc and then treated with either vehicle (methanol) or 100 nM mithramycin A for 48 h, n = 8. Western blots above show Sp1 and α-tubulin levels in vehicle and mithramycin A treated cells. α-tubulin was used as an internal control. D: HEK293 cells were transfected with p−100Luc along with either with non-targeting control or Sp1 siRNA. The amounts of Sp1 and α-tubulin in the cells were determined by Western blot analyzes. RT-PCR to determine FE65 mRNA levels in (E) HEK293 cells and (F) rat cortical neurones transfected either with non-targeting control or Sp1 siRNA. G3PDH was used as an internal control, n = 5.

[Citron et al., 2008]. As our findings show that the expression of FE65 can be regulated, at least in part, by Sp1, it is possible that dysregulation of Sp1 in Alzheimer's disease influences the expression of *FE65* (and maybe other Alzheimer's disease linked genes) which in turn alters Aβ generation.

In addition to Sp1, Sp protein family (a subfamily of Sp/KLF transcription factors) also includes Sp2–Sp8. Sp1–Sp4 have similar structural features (see reviews [Suske, 1999; Safe and Abdelrahim, 2005; Suske et al., 2005]). Sp1, Sp3, and Sp4 have been shown to recognize GC-rich boxes and to regulate the expression of GC-rich gene promoters [Hagen et al., 1992, 1994; Phillips et al., 2005; Abdelrahim et al., 2007; Chintharlapalli et al., 2007; Mertens-Talcott et al., 2007; Shatnawi et al., 2007; Chadalapaka et al., 2008; Khalil et al., 2008; Valin et al., 2009]. Although Sp3 and Sp4 show similar DNA-binding specificities to Sp1, they are not just functional redundants of Sp1. Many studies have shown that Sp3 can enhance or inhibit Sp1-mediated transcription, and the ratio of Sp1:Sp3 is essential in gene regulation (see reviews [Black et al., 2001; Li et al., 2004; Safe and Abdelrahim, 2005]). Similarly, Sp4 has been shown to influence Sp1 mediated transcription [Hagen et al., 1995; Kwon et al., 1999; Lerner et al., 2005]. In fact, our pilot study suggests that Sp3 downregulates the transcription of FE65 (Fig. 6G). Further investigation is therefore worthwhile to explore the roles of other Sp family proteins in the transcription control of human *FE65*.

Currently there is no cure for Alzheimer's disease, which is going to become an even greater social problem in the near future as the aging population increases. One possible therapeutic strategy for Alzheimer's disease is to lower Aβ production by inhibiting the APP processing secretases. However, this approach may have adverse effects on cells. For example γ-secretase has multiple cellular targets (see review [Selkoe and Wolfe, 2007]), and inhibition of γ-secretase is likely to affect the proteolysis and normal functions of its target

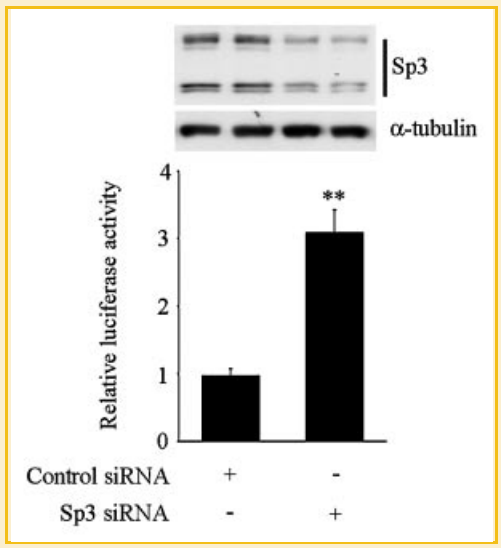

Fig. 7. Sp3 suppresses the human *FE65* promoter. HEK293 cells were transfected with p−100Luc along with either with non-targeting control or Sp3 siRNA. The amounts of Sp3 and α-tubulin in the cells were determined by Western blot analyzes, n = 6.

proteins such as Notch1. In fact, severe side-effects of a γ-secretase inhibitor due to inhibition of Notch processing and signaling have been observed in mice [Searfoss et al., 2003; Wong et al., 2004]. Therefore, therapeutic approaches for Alzheimer's disease other than inhibition of the APP proteases are desirable. As FE65 has been shown to influence the production of Aβ, it may provide a novel avenue for treatment of Alzheimer's disease. However, the effect of FE65 on APP processing remains controversial [Sabo et al., 1999; Wang et al., 2004; Santiard-Baron et al., 2005; Guenette et al., 2006; Wiley et al., 2007]. The reason for such conflicting findings is largely unknown but may be due to differences between cell types or models employed. Nevertheless, modulation of FE65 levels may be a useful pharmaceutical approach for treating Alzheimer's disease. In the future, it is essential to further dissect the molecular mechanism of *FE65* transcriptional control, including identification of transcription factor(s) responsible for the high level expression of FE65 in neurones. These studies will provide more insights into the feasibility of targeting *FE65* transcription in Alzheimer's disease treatment.

In this study, the 5′ UTR of the FE65 mRNA was also determined. Interestingly, in addition to the main open reading frame (ORF), two other upstream ORFs (uORFs) exist in the 5′ UTR (Fig. 1). uORFs have been shown to regulate gene expression (see review [Morris and Geballe, 2000]). For example, the existence of multiple ATGs in BACE-1 impairs the translation of the main ORF [De Pietri Tonelli et al., 2004]. It has been reported that an uORF inhibits the translation of huntingtin [Lee et al., 2002]. Further investigation is required to determine whether the FE65 uORFs involve in the human *FE65* transcription control.

We note that a 0.3 kb rat *FE65* promoter sequence has been reported [Faraonio et al., 1994]. Interestingly, the human *FE65* promoter herein is located upstream of the exon 1 whereas part of the rat promoter sequence (from −41 to +26) corresponds to the 5′ untranslated region in exon 2 of the human *FE65* gene. Such discrepancies between rat and human *FE65* promoters may be due to differences in genomic organization of the two genes as they are located on different chromosomes (human, chromosome 11; rat, chromosome 1). It is also possible that the rat *FE65* gene contains more than one promoter as multiple transcription start sites have been identified for rat *FE65* [Faraonio et al., 1994]. Alternatively, it has been suggested that the rat *FE65* promoter was mis-defined due to pre-mature primer extension in the mapping of the transcription start site [Hu et al., 1999]. However, the rat *FE65* promoter sequence

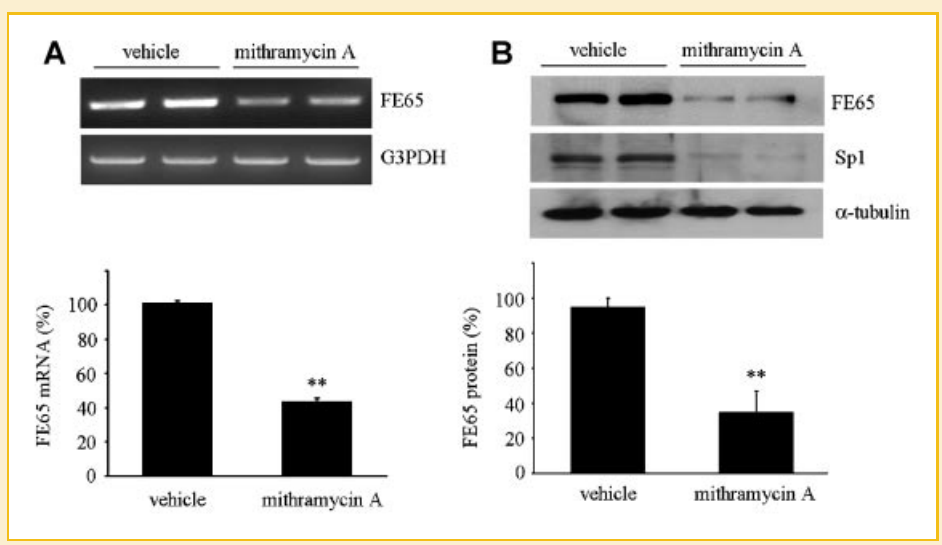

Fig. 8. Suppression of Sp1 by mithramycin A reduces endogenous FE65 expression. Rat cortical neurones were treated with either vehicle or 100 nM of mithramycin A. A: RT-PCR to determine FE65 mRNA levels. The bar chart shows the relative amounts of FE65 mRNA which were normalized against the expression of G3PDH, n = 8. B: Western blot analyzes of FE65 protein levels. The bar chart shows the relative amounts of FE65 protein which were normalized against the amount of α-tubulin, n = 3.

has been shown to form several DNA-protein complexes including YY1 [Zambrano et al., 1997], and therefore the region may represent an essential exonic/intronic cis-regulatory region for the rat *FE65* gene. It is worthy of note that in silico analysis reveals the presence of a putative YY1 binding site in the human *FE65* promoter (Fig. 2B). YY1 is a bifunctional transcription factor that can induce or suppress gene expression (see review [He and Casaccia-Bonnefil, 2008]). A potential role for YY1 in the Alzheimer's disease pathogenesis has been suggested as YY1 stimulates the BACE-1 promoter [Nowak et al., 2006] and the rat FE65 promoter [Zambrano et al., 1997]. It is worthwhile to investigate the role of YY1 in the human FE65 transcription control. Nevertheless, our study has improved our understanding of the transcription regulation of the human *FE65* gene. In summary, we have cloned and functionally tested the human *FE65* promoter, and shown that the transcription of *FE65* is regulated by Sp1.

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