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Ciliogenic RFX Transcription Factors Regulate *FGF1* Gene Promoter

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ABSTRACT

Fibroblast growth factor 1 (FGF1) has been shown to regulate cell proliferation, cell division, and neurogenesis. Human *FGF1* gene 1B promoter (−540 to +31)-driven green fluorescence (F1BGFP) was shown to recapitulate endogenous *FGF1* gene expression. It can also be used to isolate neural stem/progenitor cells (NSPCs) and glioblastoma stem cells (GBM-SCs) from developing mouse brains and human glioblastoma tissues, respectively. However, the regulatory mechanisms of *FGF-1B* promoter and F1BGFP(+) cells are not clear. In this study, we present several lines of evidence to show the roles of ciliogenic RFX transcription factors in the regulation of *FGF-1B* gene promoter and F1BGFP(+) cells: (i) RFX1, RFX2, and RFX3 transcription factors could directly bind the 18-bp *cis*-element (−484 to −467), and contribute to the regulation of *FGF1* promoter and neurosphere formation. (ii) We demonstrated RFX2/RFX3 complex could only be detected in the nuclear extract of *FGF-1B* positive cells, but not in *FGF-1B* negative cells. (iii) Protein kinase C inhibitors, staurosporine and rottlerin, could decrease the percentage of F1BGFP(+) cells and their neurosphere formation efficiency through reducing the RFX2/3 complex. (iv) RNA interference knockdown of *RFX2* could significantly reduce the percentage of F1BGFP(+) cells and their neurosphere formation efficiency whereas overexpression of RFX2 resulted in the opposite effects. Taken together, this study suggests ciliogenic RFX transcription factors regulate *FGF-1B* promoter activity and the maintenance of F1BGFP(+) NSPCs and GBM-SCs. J. Cell. Biochem. 113: 2511–2522, 2012.

KEY WORDS: *FGF1*; RFX; F1BGFP; NEURAL STEM CELLS; NEUROGENESIS

The human *FGF1* gene is over 120 kb long and contains three protein-coding exons as well as a long 3′-untranslated region. It also contains at least four upstream untranslated exons, designated 1A, 1B, 1C, and 1D, which are alternatively spliced to the first protein-coding exon [Myers et al., 1993; Payson et al., 1993; Chiu et al., 2001]. This tissue-specific expression of the four mRNA generated by alternative splicing relies on the use of different promoters [Myers et al., 1993, 1995ab]. Thus, *FGF-1A* transcript predominates in the human kidney [Myers et al., 1993], and *FGF-1C* and *-1D* transcripts predominate in vascular smooth muscle cells and fibroblasts [Chotani et al., 1995]. *FGF-1B* is the major transcript within the human brain [Myers et al., 1993] and retina [Myers et al., 1995a]. Previous studies also showed that most malignant gliomas express *FGF1*, utilizing the 1B promoter [Chiu et al., 2001]. Moreover, the expression of *FGF-1B* mRNA is restricted to sensory and motor nuclei in the brain stem, subventricular zone, spinal cord, and other areas that are known to be abundant for neural stem/progenitor cells (NSPCs) [Alam et al., 1996; Chiu et al., 2000]. Interestingly, it has been shown that *FGF-1B* mRNA is upregulated for the maintenance of NSPCs in hippocampus dentate gyrus in response to activity-induced neurogenesis [Ma et al., 2009]. The 540-bp (−540 to +31) sequence upstream of the 1B transcription start site has been demonstrated to drive the expression of luciferase [Myers et al., 1995b], green fluorescence protein (GFP) reporter genes in cultured cells [Hsu et al., 2009b, 2010; Lee et al., 2009] and the SV40 large T-antigen in transgenic mice [Chiu et al., 2000].

The regulatory factor of X-box (*RFX*) gene family is characterized by a highly conserved DNA-binding domain (DBD) and consists of seven members in mammals (*RFX1* to *7*) [Aftab et al., 2008]. The *RFX* family is conserved throughout the evolution in eukaryotic

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species and contains one member each from yeast [Emery et al., 1996ab], *C. elegans* (*daf-19*) [Senti and Swoboda, 2008], two members from *Drosophila* (*dRfx*, *dRfx2*) [Dubruille et al., 2002; Laurencon et al., 2007], and seven members each from mouse and human [Aftab et al., 2008]. These RFX proteins feature a characteristic 76 amino acid DBD with a wing-helix structure [Gajiwala et al., 2000]. RFX1, RFX2, and RFX3 are similar to daf-19 at the amino acid sequence [Emery et al., 1996a; Aftab et al., 2008]. Phylogenetic analysis on the DNA-binding domain of RFX family also reveals that *RFX1*, *RFX2*, and *RFX3* in mammals are highly similar to the *dRFX* and *daf-19* [Aftab et al., 2008]. *dRFX* and *daf-19* have been shown to regulate genes involved in the assembly, maintenance and function of primary cilia [Laurencon et al., 2007; Aftab et al., 2008; Senti and Swoboda, 2008]. RFX3 directs nodal cilium development and left-right asymmetry specification [Bonnafe et al., 2004]. RFX3 deficient mice have defective cilia [Baas et al., 2006], indicating conserved ciliogenic functions for this protein in mammals [Baas et al., 2006; Ait-Lounis et al., 2007]. It is notable that RFX1 and RFX2 have similar functional domains and DNA-binding specificities to RFX3 [Aftab et al., 2008], suggesting that RFX1 and RFX2 may also activate genes of ciliogenic pathway and mediate transcriptional rewiring of ciliary genes [Piasecki et al., 2010]. Notably, RFX1, RFX2, and RFX3 are shown to regulate *ALMS1* gene [Purvis et al., 2010], which encodes a centrosomal protein [Andersen et al., 2003; Hearn et al., 2005] and is required for the proper function of primary cilia [Graser et al., 2007; Li et al., 2007]. Mutations in *ALMS1* cause Alström syndrome [Collin et al., 2002, 2005; Hearn et al., 2002], a disorder characterized by neurosensory degeneration, metabolic defects, and cardiomyopathy [Alstrom et al., 1959; Marshall et al., 2007]. Of note, primary cilia are crucial for cell division and cell cycle progression [Pan and Snell, 2007].

We have previously demonstrated that *FGF-1B* promoter (−540 to +31)-driven GFP reporter (F1BGFP) could be used to isolate NSPCs and GBM-SCs with self-renewal and multipotent capacities from human glioblastoma tissues [Hsu et al., 2009b], developing (E11.5, E14.5, E17.5), neonatal, or adult mouse brains [Hsu et al., 2009b; Lee et al., 2009]. We also showed that F1BGFP-selected NSPCs from mouse brains were able to repair the damaged sciatic nerve of paraplegic rats [Lin et al., 2008; Hsu et al., 2009a]. Here, our results show that RFX2/3 complex is crucial for the activation of *FGF-1B* promoter, and RFX2 and RFX3 have different roles from RFX1 in the regulation of *FGF-1B* gene promoter and neurosphere formation of F1BGFP(+) cells. This study suggests ciliogenic RFX transcription factors regulate *FGF-1B* promoter activity and the maintenance of F1BGFP(+) NSPCs and GBM-SCs.

MATERIALS AND METHODS

F1BGFP REPORTER

Nucleotides −540 to +31 of the 1B promoter of human *FGF1* gene [Myers et al., 1995b] and nucleotides 5,171–2,533 of the SV40 immediate early gene were cloned into the *Sma*I-*Bam*HI sites of pGL2-Basic (Promega) and designated pF1BTag [Chiu et al., 2000]. We also cloned the nucleotides −540 to +31 of the human *FGF-1B* promoter into the pEGFP1 (Clontech) vectors to construct the pF1BGFP reporter [Lee et al., 2009; Hsu et al., 2009b, 2010]. All constructs were verified by DNA sequencing. pF1BGFP was prepared using the EndoFree Plasmid Maxi Kit (Qiagen Inc., Chatsworth, CA). For generation of U-1240 MG/F1BGFP(+) cells [Hsu et al., 2010], human glioblastoma U-1240 MG cells were plated in 60-mm tissue culture dishes (BD Labware) to achieve 60–80% confluence by day 2. On day 2, cells were transfected with 10 μg of pF1BGFP using GeneJuice transfection reagent (Merck). Percentage of F1BGFP-positive cells was analyzed by using flow cytometry according to the procedures described previously [Ducrest et al., 2002; Hsu et al., 2010]. A total of 1×10^4 cells were gated on a dot plot forward side scatter on the X-axis and side scatter on the Y-axis. The gated cells were evaluated on a histogram displaying GFP intensity on the X-axis and side scatter on the Y-axis.

CELL CULTURE AND TRANSFECTION

Human glioblastoma cell lines, U-1240 MG and U-1242 MG, were cultured in minimal essential media (MEM; Invitrogen) supplemented with 10% calf serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37°C as described [Myers et al., 1995b]. U-1240 MG/F1BGFP cells were further cultured in culture medium containing 100 μg/ml G418. The pSG5-RFX1, pcDNA3-RFX2, and pSG5-RFX3 constructs were provided by Dr. Shaul [Lubelsky et al., 2005] and Dr. Iwama [Iwama et al., 1999]. Cells were transfected with these plasmids using the GeneJuice transfection reagents according the manufacturer's instructions. Cells were harvested 3 days post-transfection for the analysis of flow cytometry, total RNA, and protein isolation. Protein kinase C inhibitors, staurosporine, and rottlerin, were purchased from Sigma and Tocris, respectively.

CHROMATIN IMMUNOPRECIPITATION ASSAY

The ChIP assay was performed using the EZ ChIP chromatin immunoprecipitation kit (Upstate Biotechnology, Inc., Lake Placid, NY) according to the manufacturer's description. Briefly, U-1240 MG cells were cross-linked with 1% formaldehyde in the medium for 5 min at room temperature, and this reaction was stopped by adding glycine to a final concentration of 125 mM. Subsequently, cells were rinsed with PBS twice, scraped in PBS, pelleted at 700*g* at 4°C for 5 min, and lysed in SDS-lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1). DNA was fragmented into around 200-bp pieces using the sonicator (Sonicor, Deer Park, NY). Sheared chromatin was diluted 10 times and pre-cleared with protein G agarose at 4°C for 1 h with rotation. After pelleting the protein G agarose at 4,000 × *g* for 1 min, 10 μl of the supernatant was removed as 1% input group and saved until the reverse-crosslinking step. Each reaction mixture was reacted with 5 μg of polyclonal anti-RFX1 (D-19X), anti-RFX2 (C-15X), anti-RFX3 (T-17X) (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies; anti-acetyl-histone H3 (Upstate, 06-599B) antibody was used for positive control, and non-specific rabbit IgG (PP64B) (Upstate) served as a negative control. The immunoprecipitated products were washed sequentially with low-salt immune complex wash buffer, high-salt immune complex wash buffer, LiCl immune complex wash buffer, and twice with TE buffer. The chromatin was eluted from the agarose by incubating with elution buffer (1% SDS, 100 mM $NaHCO_3$); and the DNA–protein complexes were reversely

cross-linked by high-salt solution containing 200 mM NaCl at 65°C for at least 5 h. To eliminate contaminations of proteins and RNAs, the mixture was treated with 10 μg RNase A at 37°C for 30 min and then treated with protease K for 2 h at 45°C. Finally, the precipitated DNA was recovered using the spin column provided in the ChIP kit, and eluted with 50 μl elution buffer. PCR reaction was conducted using Taq DNA polymerase (Roche Applied Science). Two microliters of the precipitated DNA was used as template. The sequence of the primers used in the ChIP assay were as follows: (5′-GCAGGGATGCCAGATGACA-3′) and (5′-TGTGTGAGCCGAATG GACTTC-3′) with the amplicon size of 166 bp.

PREPARATION OF NUCLEAR EXTRACTS

Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagent according to the manufacturer's instructions (Pierce). Briefly, 1×10^6 cells were trypsinized, followed by lysing in 100 μl CERI buffer. After the lysates were vortexed for 15 s and incubated on ice for 10 min, 5.5 μl of CERII was added. The lysates were vortexed for 5 s, incubated on ice for 1 min, and vortexed again for 5 s. The nuclei were pelleted at 16,000*g* at 4°C for 5 min, and the cytoplasmic extracts were removed. Nuclei were resuspended in 25 μl nuclei extraction buffer and vortexed 15 s. The nuclei were extracted on ice and vortexed for 15 s every 10 min, for a total of 40 min. The extracts were centrifuged at 16,000*g* at 4°C for 5 min and the supernatant were collected as nuclear extracts. Protein concentration was determined by the Bradford method using BSA as a standard (Bio-Rad).

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Binding reaction containing 20 μl binding buffer (10 mM Tris, 50 mM KCl, 1 mM DTT, and 5 mM $MgCl_2$, 1 μg poly(dI·dC), 10 μg nuclear extracts, 200-fold excess of cold competitors, and 20 femtomoles of 5′ biotin-labeled oligonucleotide probes) was added sequentially and incubated at room temperature for 20 min. The reaction mixture was separated on 4% native polyacrylamide gel at 50 V for 8–10 h. The resolved probes were transferred onto nylon membranes (Hybond-N^+ nylon transfer membrane, RPN303B; Amersham Biosciences) at 600 mA for 2.5 h. Biotin-labeled probe on the membranes was detected using the streptavidin-horseradish peroxidase conjugate and the chemiluminescent substrate provided in the chemiluminescent nucleic acid detection module (Pierce) according to the manufacturer's instructions; subsequently, the membranes were exposed to X-ray film. For EMSA supershift assay, 2 μg of anti-RFX1 (D-19X), anti-RFX2 (C-15X), anti-RFX3 (T-17X), or anti-RFX5 (A-19X) antibodies (Santa Cruz Biotechnology) was added, respectively. Finally, the probe was added and incubated for another 15 min. The sequences of probes and cold competitors used in the EMSA experiment were as follows: 26-bp, (5′-ACGACCTGCTGTTTCCCTGGCAACTC-3′); *AP-1*, (5′-CGCTTGAT-GAGTC AGCCGGAA-3′); 18-bp, (5′-CTGTTTCCCTGGCAACTC-3′); 18-bp-mut, (5′-CT TTTTCCCTTTCAACTC-3′); *MAP1A*, (5′-CGGCGTTGCCATGGAGACAACTC CG-3′); *PyEP*, (5′-GGCCAGTT GCCTAGCAACTAATAC-3′); m26-bp, (5′-AC AACCAGTTGTTTCC-CTGGTGACAG-3′); and m18-bp, (5′-TTGTTTCCCTGGT GACAG-3′) (Protech Technology, Taipei, Taiwan). The above oligonucleotides were incubated with respective complementary oligonucleotides in 10 mM Tris, 1 mM EDTA, 50 mM NaCl (pH 8.0) reaction buffer at 95°C for 5 min, and then the temperature was decreased by 1° per second to 4°C to anneal the complementary oligonucleotides.

QUANTITATIVE REVERSE TRANSCRIPTION AND POLYMERASE CHAIN REACTION (RT-RCR)

For analysis of human *FGF-1B* transcripts, RNA extracted from U-1240 MG and U-1242 MG cells was primed with oligo (dT) and reverse transcribed using SuperScript II RT (Invitrogen). Each cDNA transcribed from 500 ng RNA was amplified using specific primer pairs with Taq DNA polymerase (Roche Applied Science) under the conditions of initial denaturing at 95°C for 10 min, followed by 30 cycles denaturing at 95°C for 15 s and extension at 60°C for 30 s, and finally extension at 60°C for 1 min for completing the polymerization. Quantitative PCR analysis was performed using an ABI prism 7500 HT Sequence Detection System (Applied Biosystems). We used SyBR Green method to analyze the expression levels of human *FGF-1B*, *RFX1*, *RFX2* and *RFX3*. *FGF-1B* primers: (5′-TGAGCGAGTGTG-GAGAGAGGTA-3′) and (5′-GCTGTGAAGGTGGTGATTTCC-3′) with amplicon size of 114 bp; *RFX1* primers: (5′-AGACCGGCGTTCC-TACTCA-3′) and (5′-GGGGCACTTGGATGTT GGT-3′) with amplicon size of 129 bp; *RFX2*, (5′-GCGATTGAA AACCTCCAAAA-3′) and (5′-GGCTTCAGACGAATCCCATA-3′) with amplicon size of 290 bp; *RFX3*, (5′-AAACTGGACCCAGTCAATGC-3′) and (5′-TGTT GCA-TGGGTTGTTGTCT-3′) with amplicon size of 197 bp.

RNAi EXPERIMENTS

Double-stranded small interfering RNA oligos were designed using BLOCK-iT™ RNAi Designer software (Invitrogen). Stealth RNAi duplex oligonucleotides against *RFX1* (GenBank™ accession number NM_002918.3 and *FGF1* (GenBank™ accession number NM_000800.2, NM_033136.1, and NM_033137.1) were synthesized by Invitrogen. Representative Stealth RNAi oligos selected from three different RNAi against targeted gene are listed as follows: RFX1-RNAi (HSS109206), RFX2-RNAi (HSS109207), RFX3-RNAi (HSS184279), *FGF1*-RNAi (HSS142002), and stealth RNAi GFP reporter control (GFP-RNAi). U-1240 MG/F1BGFP(+) cells were used in RNAi knockdown experiments. Cells were transfected with siRNA against *RFX1*, *RFX2*, or *RFX3* using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. Three different RNAi (I, II, and III) against *RFX1*, *RFX2*, and *RFX3* were tested, and representative results for RNAi knockdown using RFX1-RNAi (HSS109206), RFX2-RNAi (HSS109207), RFX3-RNAi (HSS184279) were shown in the experiments of U-1240 MG/F1BGFP(+) cells.

NEUROSPHERE ASSAY

U-1240 MG/F1BGFP(+) cells were washed with basal medium and seeded at a maximal density of 1×10^4 cells in 60-mm Petri dish (Falcon Inductries, Oxnard, CA) with 5 ml neurosphere medium (NS medium): DMEMHG/F12 supplemented with B27 (Invitrogen), 50 ng/ml epidermal growth factor, 20 ng/ml FGF2, 10 ng/ml leukemia inhibitory factor, and 5 μg/ml heparin [Hsu et al., 2009b, 2010]. Subsequently, cells were cultured in 5% CO_2 at 37°C incubator. The spheres (diameter larger than 50 μm) were counted directly under microscope after 7 days in vitro.

STATISTICAL ANALYSES

Data are expressed as means ± SEM. One-way analysis of variance was used for comparison of multi-groups. The data were considered statistically significant at $P < 0.05$.

RESULTS

RFX2 AND RFX3 COULD BIND TO 18-BP *cis*-ELEMENT OF *FGF-1B* PROMOTER

Two RFX1-binding sites, namely X-box, locate within 18-bp sequence (−484 to −467). The 18-bp sequence is similar to the imperfect palindromic RFX consensus sequence, which contains a 6-bp half site. Each sequence in the two complementary strands is separated by a spacer region from 0 to 3 bp (Fig. 1A). The 140-kDa RFX1 is the prototype member of RFX family, and the structure of RFX2 and RFX3 are closely related to RFX1 [Aftab et al., 2008]. In addition to the DNA-binding domain, several functional regions of RFX family are shown here (Fig. 1B). RFX1, RFX2, and RFX3 proteins contain an N-terminal glutamine-rich transcription activation domain and B-C-D region, which mediate the dimerization.

To investigate the roles of RFX transcription factors in the regulation of *FGF-1B* gene promoter, we performed EMSA experiment with nuclear extracts isolated from *FGF-1B*-positive human glioblastoma U-1240 MG cells (Fig. 2A). Several complexes (A, B, C, and X) were observed when incubating U-1240 MG nuclear extracts with the 26-bp probe (−492 to −467) (Fig. 2A, lane 2). The specificity of complex formation was analyzed by EMSA competition assay. Four DNA–protein complexes were competed away by the 200-fold excess of cold 18-bp probe (Fig. 2A, lane 2, complexes A, B, C, and X). Interestingly, the complex X was not observed in the

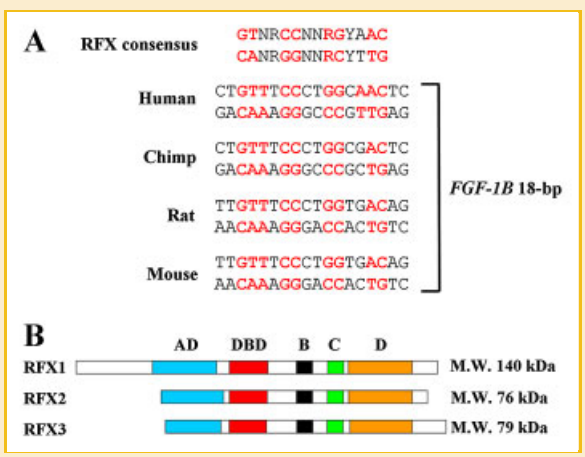

Fig. 1. A: Alignment of RFX consensus sequence with 18-bp sequence of the *FGF-1B* promoter in different species. Palindrome sequences are in red. B: Functional domains of human RFX1, RFX2, and RFX3 proteins. RFX1 is the prototype of RFX family and comprises several functional regions. RFXs include a highly conserved DNA-binding domain (DBD), an activation domain (AD) and B-C-D domain that participates in dimerization and transcriptional repression. The organization and function of each domain in RFX2 and RFX3 is closely related to RFX1.

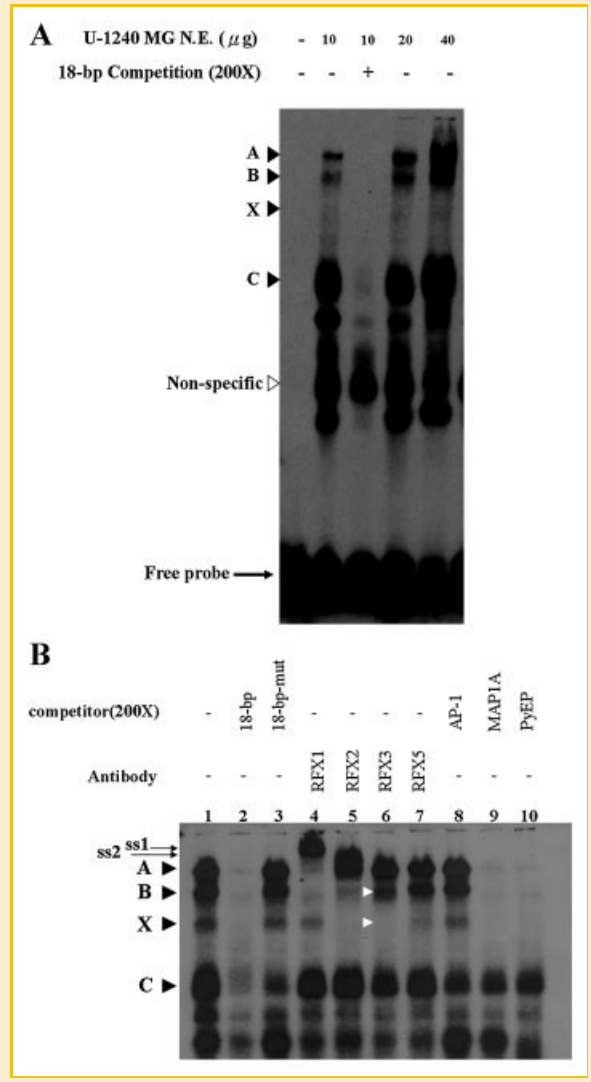

Fig. 2. RFX1, RFX2, and RFX3 could bind the 18-bp *cis*-element of *FGF-1B* promoter. The biotin-labeled 26-bp (−492 to −467) probe was incubated with nuclear extract from U-1240 MG cells and assayed in EMSA. A: EMSA assays were performed with different amounts of nuclear extracts (10, 20, 40 μg; lanes 1, 2, 4). An EMSA competition experiment (lane 2) was performed using 10 μg nuclear extract and 18-bp cold probe (200× molar excess). The arrowheads indicate the specific DNA–protein complex, including A, B, C, and X. B: The specificity of complex formation was confirmed by competition and three specific complexes competed by the 18-bp but not by the non-relevant competitor, AP-1 (lane 8). To examine whether RFX family members involve in the complex formation on the 18-bp, we incubated the EMSA-binding reaction mixtures with specific antibody, respectively. The ss1 and ss2 asterisks mark the band supershifted by anti-RFX1 and anti-RFX2, respectively. The white arrowheads indicate the complexes B and X which are influenced by anti-RFX3 antibody. EMSA supershift assay revealed that complex A was RFX1 homodimers; complex B included RFX1/2 and RFX1/3 heterodimers, and complex X was RFX2/3 heterodimer. Oligonucleotides, *MAP1A* or PyEP, containing the known RFX1-binding site could diminish the specific RFX complexes A (RFX1/1), B (RFX1/2 and RFX1/3), and X (RFX2/3) (lanes 9 and 10) but not the oligonucleotides, 18-bp-mut (lane 3), with mutated RFX-binding sequence.

previous study due to its low amount (Fig. 2A, indicated by arrowhead and named X). However, this complex could be detected when increasing the amount of U-1240 MG nuclear extract used in EMSA (Fig. 2A).

Oligonucleotides, *MAP1A* and *PyEP*, containing RFX1-binding site could diminish complex A, B, and X (Fig. 2B, lanes 9, 10), but non-relevant cold probe (Fig. 2B, lane 8), *AP-1* could not diminish these complexes. In addition, the 18-bp-mut (Fig. 2B, lane 3), with mutated RFX1-binding site could not compete with the wildtype 26-bp probe for the binding. These results indicated that specific complex formation depends on the core RFX-binding sequence. Complexes A and B shown in Figure 2 have been identified previously as RFX1-specific complexes. DNA-binding domain of the RFX2 and RFX3 are closely related to RFX1 in sequence. RFX1 has been shown to form dimeric complex either with itself, RFX2 or RFX3. At least four RFX family genes, RFX1, RFX2, RFX3, and RFX5 were detected in U-1240 MG cells by RT-PCR (data not shown). Therefore, we further examine whether these RFX proteins could also bind to the 18-bp. EMSA supershift assays were performed by adding anti-RFX2, RFX3, or RFX5 antibody to the binding reaction (Fig. 2B). We found that complexes A and B were supershifted with anti-RFX1 antibody (Fig. 2B, lane 4), indicating RFX1 was involved in the formation of these two complexes. It was noted that complex B could be supershifted by anti-RFX1, anti-RFX2 or anti-RFX3 antibody, respectively (Fig. 2B, lanes 4–6). Due to the fact that the molecular weight of RFX2 and RFX3 are very similar (Fig. 1B), our result here suggested that complex B may contain both RFX1/2 and RFX1/3 complex. In addition, complex X cloud be supershifted with either anti-RFX2 or anti-RFX3 antibody, indicating that it was RFX2/3 complex (Fig. 2B, lanes 5–6). RFX5 might not bind the 18-bp since no supershift band was observed when using anti-RFX5 antibody (Fig. 2B, lane 7). To further compare the formation of dimeric RFX1, RFX2, and RFX3 complexes binding to 18-bp and X-box sequence, the EMSA assays were carried out with the 18-bp sequence or *MAP1A* probe containing X-box sequence, respectively. EMSA supershift experiments suggested that dimeric RFX could form similar complexes when binding to 18-bp (Fig. 3A, lane 2) and *MAP1A* probe (Fig. 3A, lanes 6–8). Together, our results above suggested that dimeric RFX1/1, RFX1/2, RFX1/3, and RFX2/3 were specific DNA–protein complexes binding the 18-bp in U-1240 MG cells.

To verify whether RFX2 and RFX3 binds to the 18-bp in cultured cells, we performed the ChIP assay using anti-RFX2 and RFX3 specific antibody to precipitate the chromatin in U-1240 MG/F1BGFP(+) cells. Specific primer pairs were designed to examine the precipitated DNA. The sequence containing the 18-bp was precipitated by anti-RFX2 or RFX3 antibody and the positive control antibody, anti-RFX1 and anti-acetyl-H3, but not by the negative control antibody, IgG (Fig. 3B). These results further demonstrated that RFX2 and RFX3 could also bind the 18-bp of F1B promoter in U-1240 MG/F1BGFP(+) cells.

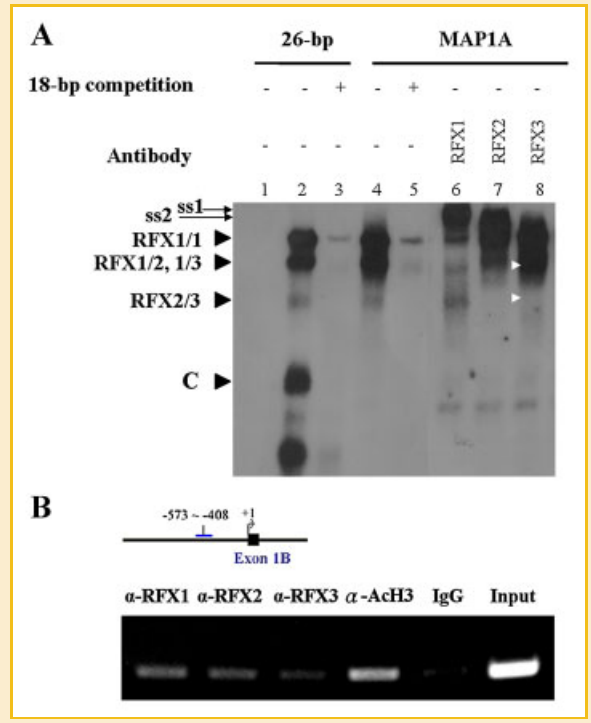

Fig. 3. A: EMSA supershift assay revealed the in vitro interaction between RFX complex and the 18-bp in nuclear extracts isolated from human U-1240 MG cells. EMSA experiment was carried out using *MAP1A*, which contains the RFX1-binding site, and human 26-bp probes. RFX1/1 and RFX1/2; RFX1/3 complexes were formed with these two probes, diminished by human 18-bp competition (lane 5), and supershifted by anti-RFX1, RFX2, or RFX3 antibody (lanes 6–8). The result of supershift assay demonstrated the evidence of in vitro interaction between 18-bp and RFX1, RFX2 and RFX3 proteins. The bands supershifted by anti-RFX1 antibody and anti-RFX2 antibody are designated as ss1 and ss2, respectively. The complex diminished by anti-RFX3 antibody is indicated as white arrowheads. Lane 1 was without nuclear protein and served as negative control. B: Chromatin immunoprecipitation assay confirms that RFX1, RFX2 and RFX3 could bind the 18-bp sequence in human glioblastoma U-1240 MG cells. Schematic representation of the localization of the primer-amplified region on the *FGF1* gene genomic sequences. The exons and introns are shown as black boxes and lines, respectively. The sequences containing the 18-bp sequence are precipitated by the anti-RFX1, RFX2, or RFX3 antibody and positive control antibody, as well as by the anti-acetyl H3 (α-AcH3) antibody, but not by the negative control antibody, IgG.

COMPARISON OF RFX COMPLEXES BETWEEN *FGF-1B* POSITIVE AND NEGATIVE CELLS

Comparison of RFX complexes between *FGF-1B* positive and negative cells may help identify the important regulatory complex for *FGF-1B* promoter activation. Thus, we compared the RFX complex formation between *FGF-1B* positive U-1240 MG and negative U-1242 MG cells. Using quantitative PCR, we showed that *FGF-1B* mRNA expression level was 36-fold higher in U-1240 MG cells than in U-1242 MG cells (Fig. 4A). We further compared RFX complex formation between U-1240 MG and U-1242 MG cells (Fig. 4B), and these RFX complexes were confirmed by supershift assay with anti-RFX1, RFX2 or RFX3 antibody (Fig. 4B, lanes 6, 8, and 10). Notably, the RFX2/3 complex was only detected in the U-1240 MG cells but not in U-1242 MG cells (Fig. 4B, lanes 2 and 4). Our results suggested that the formation of RFX2/3 complex on 18-bp was directly correlated with the expression of *FGF-1B* in U-1240 MG cells. Interestingly, when comparing the nuclear and cytosol extracts by EMSA, we demonstrated that RFX2/3 complex was not

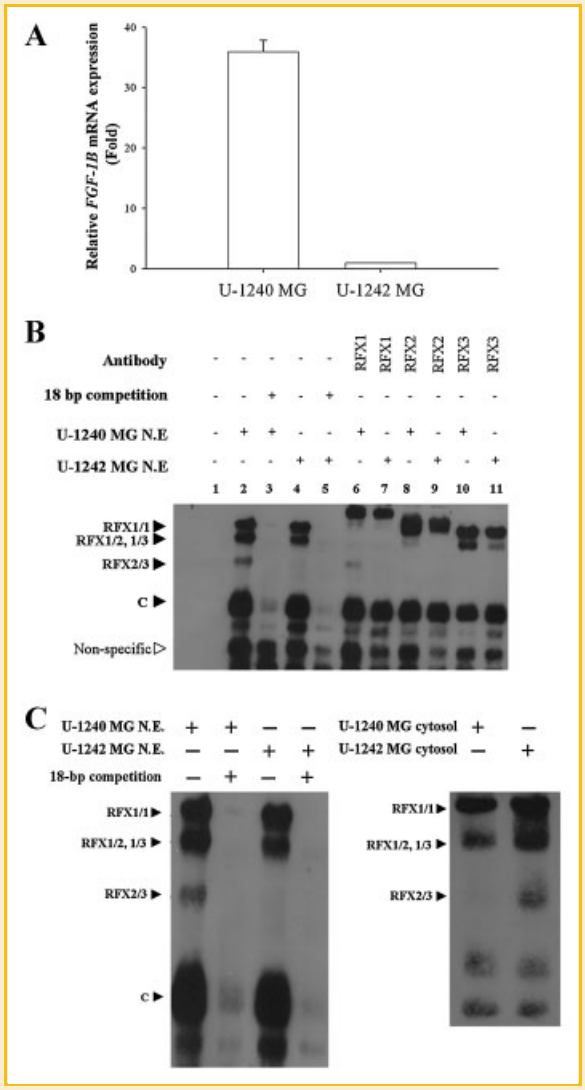

Fig. 4. A: Comparison of *FGF-1B* mRNA expression level between U-1240 MG and U-1242 MG cells. *FGF-1B* transcript levels were 36-fold higher in U-1240 MG cells than in U-1242 MG cells. *FGF-1B* mRNA expression was analyzed by quantitative PCR and normalized to *β-actin* gene expression. Representative result with triplicates was shown here. B: Comparison of dimeric RFX complexes between U-1240 MG and U-1242 MG cells. Nuclear extracts from *FGF-1B* positive (U-1240 MG) and negative (U-1242 MG) cells were assayed by EMSA using the 26-bp probe and 18-bp as specific cold competitor. Several distinct RFX complexes (RFX1/1, RFX1/2, RFX1/3, and RFX2/3) were formed with U-1240 MG nuclear extract (lane 2). These RFX complexes were competed by 18-bp (lanes 3 and 5) and supershifted by specific RFX antibodies (lanes 6–11). Notably, the fastest mobility RFX2/3 complex was only detected in the *FGF-1B* positive U-1240 MG cells but not in the *FGF-1B* negative U-1242 MG cells. C: Comparison of dimeric RFX complexes in the nuclear and cytosol extracts between U-1240 MG and U-1242 MG cells. RFX2/3 complex was only detected in the nuclear extract of U-1240 MG cells but also in the cytosol extract of U-1242 MG cells.

only detected in the nuclear extract of U-1240 MG cells, but also in the cytosol extract of U-1242 MG cells (Fig. 4C). Using immunofluorescence staining, we also found that RFX2 and RFX3 were strongly stained in the nucleus of U-1240 MG cells, but mostly in the cytosol of U-1242 MG cells (data not shown), indicating the difference of RFX2/3 complex between U-1240 MG and U-1242 MG cells may be due to different location of RFX2 and RFX3 in these two cell lines.

To further confirm the importance of the RFX2/3 complex, we transfected U-1240 MG cells with F1BGFP reporter and sorted U-1240 MG/F1BGFP(+) and (−) cells by fluorescence-activated cell sorting (FACS; Fig. 5A), and analyzed by EMSA. As shown in Figure 5A, the U-1240 MG/F1BGFP(+) cells have significantly higher GFP fluorescence intensity than U-1240 MG/F1BGFP(−) cells (87.3% vs. 7.6%). We further compared RFX complex formation between U-1240 MG/F1BGFP(+) and (−) cells by EMSA. RFX2/3 complex formation in U-1240 MG/F1BGFP(+) cells was higher than in U-1240 MG/F1BGFP(−) (Fig. 5B). In addition, we also observed the same result that RFX2/3 complex formation was in F1BGFP(+) cells, but not in F1BGFP(−) cells when using primary cells from human glioblastoma tissues (data not shown). Our results consistently showed RFX2/3 complex binding to the 18-bp in *FGF-1B*(+) cells.

PROTEIN KINASE C INHIBITORS, STAUROSPORINE AND ROTTLERIN, COULD DECREASE THE PERCENTAGE OF F1BGFP(+) CELLS, RFX2/3 COMPLEX FORMATION AND NEUROSPHERE FORMATION OF U-1240 MG/F1BGFP(+) CELLS

We further screened different kinase inhibitors to investigate which signal pathway may regulate RFX transcription factors binding the *FGF-1B* promoter, Intriguingly, we found that protein kinase C inhibitors, staurosporine, and rottlerin, could significantly decrease the percentage of F1BGFP(+) cells (Fig. 6A), RFX2/3 complex formation (Fig. 6B), and neurosphere formation (Fig. 6C). Notably, exogenous FGF1 treatment could also significantly rescue neurosphere formation that was reduced by staurosporine treatment (Fig. 6D).

REGULATORY EFFECTS OF RFX2 AND RFX3 ON F1BGFP IN U-1240 MG/F1BGFP(+) CELLS

Since the RFX2/3 complex could bind the 18-bp sequence and was directly correlated with the activation of *FGF-1B* promoter, we further studied the regulatory effects of RFX2 and RFX3 on U-1240 MG/F1BGFP(+) cells by gain and loss of function assays in 10% serum and serum-free culture conditions. We transfected U-1240 MG/F1BGFP(+) cells with double-stranded siRNA (RFX1-RNAi, RFX2-RNAi, and RFX3-RNAi) to knockdown target gene expression. Three different RNAi against *RFX1*, *RFX2*, or *RFX3* were tested, and representative results for RNAi knockdown using RFX1-RNAi, RFX2-RNAi, RFX3-RNAi were shown here. GFP-RNAi was transfected as positive control for flow cytometry experiment and negative control for quantitative PCR experiment. As shown in Figure 7A, quantitative PCR analysis of cells transfected with RFX2-RNAi or RFX3-RNAi showed a significant decrease in the expression of *RFX2* or *RFX3*, respectively (Fig. 7A). In addition, *RFX2* and *RFX3* mRNA expression levels are not altered by GFP-RNAi. F1B promoter activity was analyzed by flow cytometry for the expression of green fluorescent protein driven from the *FGF-1B* promoter. Consistent with our previous report [Hsu et al., 2010], when RFX1 expression level was effectively reduced by siRNA transfection, the percentage of F1BGFP(+) cells was significantly up-regulated (Fig. 7B). Of note,

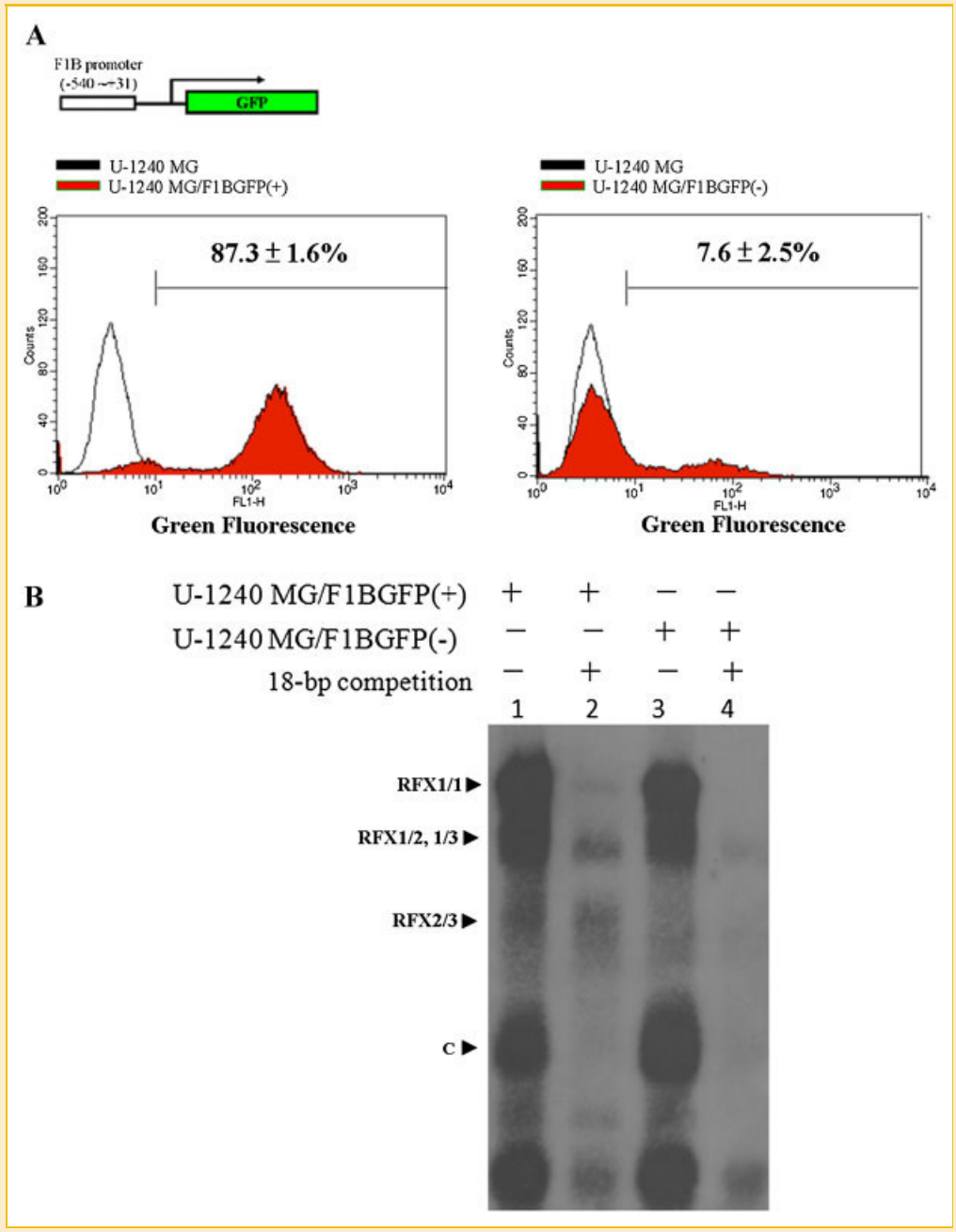

Fig. 5. Comparison of dimeric RFX complexes between U-1240 MG/F1BGFP(+) and U-1240 MG/F1BGFP(−) cells. A: F1BGFP reporter was transfected into U-1240 MG cells, and then maintained in G418 selection medium. The GFP-positive and -negative cells were sorted by FACS. U-1240 MG/F1BGFP(+) cells have significantly higher GFP fluorescence intensity than U-1240 MG/F1BGFP(−) cells. B: U-1240 MG/F1BGFP(+) and U-1240 MG/F1BGFP(−) cells were analyzed by EMSA using the 26-bp probe and competed with 18-bp. Three specific RFX complexes (RFX1/1, RFX1/2, RFX1/3, and RFX2/3), as shown in U-1240 MG cells, were also detected in U-1240 MG/F1BGFP(+) and (−) cells (lanes 1 and 3). Of note, level of RFX2/3 complex formation in U-1240 MG/F1BGFP(+) was higher than in U-1240 MG/F1BGFP(−) cells.

the percentage of F1BGFP(+) cells was significantly decreased when the *RFX2* expression was effectively reduced by *RFX2*-RNAi (Fig. 7B). The regulatory effects of siRNA on F1BGFP were similar in both 10% serum and serum-free culture conditions. Overexpression of RFX2 or RFX3 protein in 10% serum culture condition showed only marginal effects on the percentage of F1BGFP(+) cells. However, in serum-free culture condition, the percentage of F1BGFP(+) cells were significantly increased when RFX2 were overexpressed (Fig. 7C). In addition, we further studied RFX complex formation of U-1240 MG/F1BGFP(+) cells in 10% serum and serum-free culture conditions by EMSA. Here, we provide the evidence that the levels of RFX2/3, 1/2, 1/3, and 1/1 complexes were gradually and significantly decreased when cells were cultured in serum-free condition (Fig. 7D).

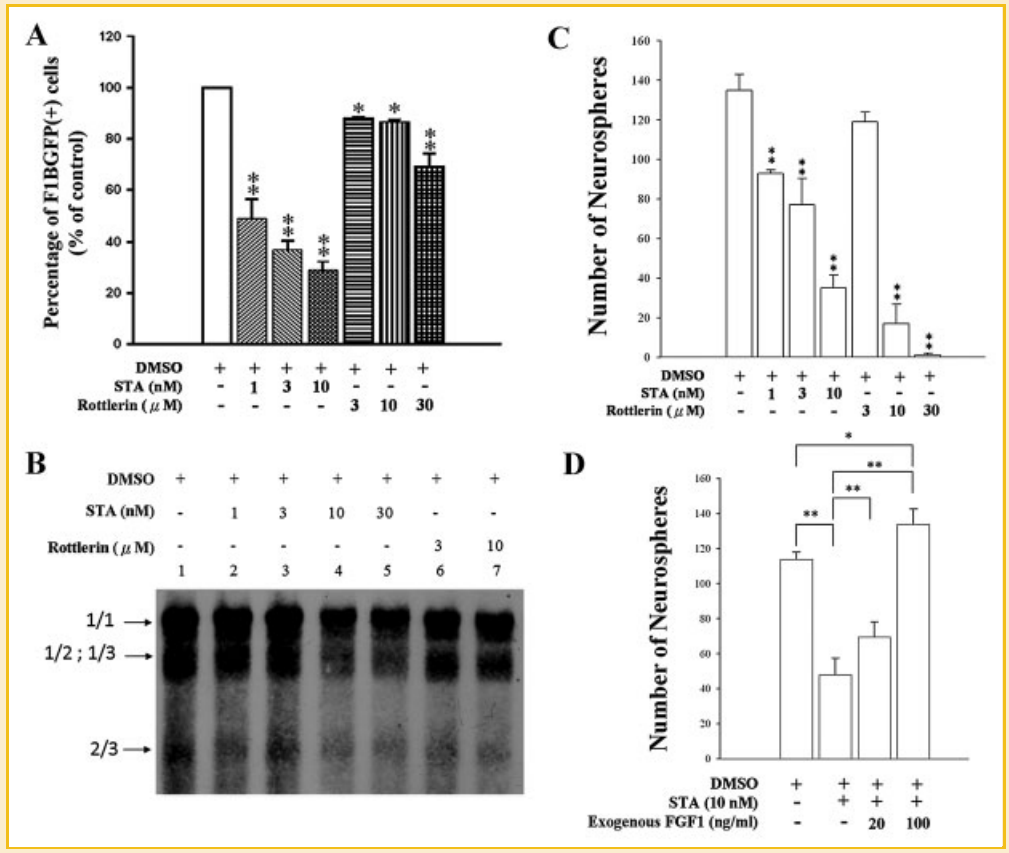

Fig. 6. Protein kinase C inhibitors, staurosporine and rottlerin, could decrease the percentage of F1BGFP(+) cells, *FGF1* gene expression and neurosphere formation of U-1240 MG/F1BGFP(+) cells. U-1240 MG/F1BGFP(+) cells were treated with staurosporine and rottlerin. Cells were harvested at 24 h after treatment. A: Using U-1240 MG/F1BGFP(+) cells, we analyzed the percentage of F1BGFP(+) cells in U-1240 MG/F1BGFP(+) cells by flow cytometry. Staurosporine and rottlerin could significantly decrease the percentage of F1BGFP(+) cells in both 10% serum culture condition. Data are shown as mean ± SEM, n = 3, $^{*}P < 0.05$ versus vehicle. B: Staurosporine and rottlerin could downregulate RFX2/3 complex formation. C: Quantification of neurospheres generated by U-1240 MG/F1BGFP(+) cells treated with staurosporine and rottlerin. Data are shown as mean ± SEM, n = 3, $^{*}P < 0.05$ versus vehicle. D: Exogenous FGF1 treatment could significantly rescue the neurosphere formation that was reduced by staurosporine treatment. Data are shown as mean ± SEM, n = 3, $^{*}P < 0.05$ versus vehicle.

REGULATORY EFFECTS OF RFX TRANSCRIPTION FACTORS ON NEUROSPHERE FORMATION OF U-1240 MG/F1BGFP(+) CELLS

We further used the cells that were transfected with different RNAi in Figure 7B for neurosphere assay. Knockdown of *FGF1* significantly decreased the number of neurospheres, suggesting that endogenous *FGF1* is important for neurosphere formation (Fig. 8B). Concomitantly, knockdown of *RFX2* significantly decreased the number of neurospheres (Fig. 8A and B). GFP-RNAi was used as a negative control. We also observed that exogenous FGF1 could significantly rescue the inhibitory effect of RFX2-RNAi on neurosphere formation. When comparing the neurosphere formation efficiency of the cells that are pre-cultured in 10% serum and serum-free medium, we observed that serum-deprivation could not only significantly reduce the percentage of F1BGFP(+) cells (Fig. 7B), but also decreased the neurosphere formation of U-1240 MG/F1BGFP(+) cells (Fig. 8B).

DISCUSSION

FGF1 belongs to the FGF1 subfamily [Itoh and Ornitz, 2004], comprising FGF1 and FGF2, of fibroblast growth factor family. FGF1 functions as a mitogen for various cell types [Giacobini et al., 1991; Hisajima et al., 1991; Bryckaert et al., 2000] and plays an important role in neurogenesis [Giacobini et al., 1991; Hisajima et al., 1991; Lin et al., 2009; Hsu et al., 2009b]. An 18-bp *cis*-acting element (−484 to −467) in *FGF1* promoter is essential for promoter activity and transcription factor binding [Ray et al., 1997]. DNA sequence alignment showed that the 18-bp *cis*-element of the *FGF-1B* promoter matches the consensus RFX-binding site (Fig. 1A). RFX1 transcription factor is the prototype member of RFX family [Aftab et al., 2008]. RFX1, RFX2, and RFX3 are highly conserved in DNA-binding domain, therefore, it is worthwhile to investigate the roles of RFX transcription factors in the regulation of *FGF-1B* promoter. In this study, we refine the interaction of RFX isoforms with the 18-bp *cis*-element of *FGF-1B* promoter: (i) RFX1, RFX2, and RFX3 transcription factors could directly bind the 18-bp *cis*-element (−484 to −467), and contribute to the regulation of *FGF1* promoter and neurosphere formation (Figs. 2 and 3). (ii) We demonstrated RFX2/RFX3 complex could only be detected in the nuclear extract of *FGF-1B* positive cells, but not in *FGF-1B* negative cells (Figs. 4 and 5). (iii) Protein kinase C inhibitors, staurosporine, and rottlerin, could decrease the percentage of F1BGFP(+) cells and their

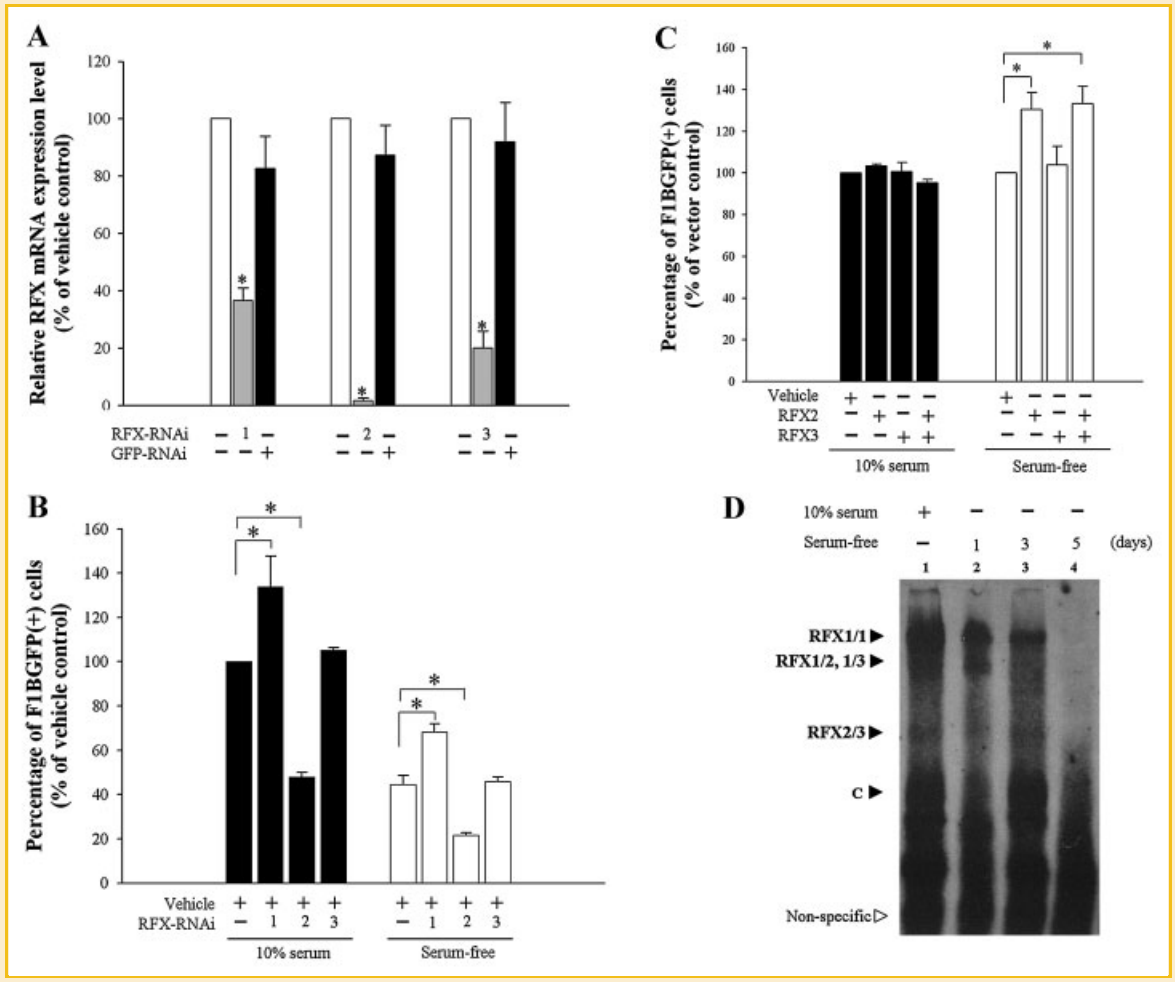

Fig. 7. Regulatory effects of RFX2 and RFX3 on the percentage of F1BGFP(+) cells in U-1240 MG/F1BGFP(+) cells in 10% serum and serum-free condition. U-1240 MG/F1BGFP(+) cells were transiently transfected with RFX1-RNAi, RFX2-RNAi, RFX3-RNAi, and GFP RNAi (30 nM). Cells were harvested at 72 h after transfection. A: mRNA expression levels after RNAi knockdown were analyzed by quantitative real-time PCR, and data were normalized to *β-actin* gene expression. RFX1, RFX2 and RFX3-RNAi could efficiently knockdown the endogenous *RFX1*, *RFX2*, and *RFX3* expression, respectively. Data are represented as mean ± SEM and $^{*}P < 0.05$ versus vehicle (lipofectamine RNAiMAX). B: Using U-1240 MG/F1BGFP(+) cells, we analyzed the percentage of F1BGFP(+) cells in U-1240 MG/F1BGFP(+) cells by flow cytometry. RFX1-RNAi could significantly increase the percentage of F1BGFP(+) cells, whereas RFX2-RNAi treatment decreased the percentage of F1BGFP(+) cells in both 10% serum and serum-free condition. Data are shown as mean ± SEM, n = 5, $^{*}P < 0.05$ versus lipofectamine control. C: Overexpression of wildtype RFX2, RFX3, or RFX2 + RFX3 proteins in U-1240 MG/F1BGFP(+) cells in both 10% serum and serum-free culture conditions. Data are shown as mean ± SEM, n = 3, $^{*}P < 0.05$ versus vector control. D: Comparison of dimeric RFX complexes in the nuclear extracts of U-1240 MG/F1BGFP(+) cells that were cultured in different culture conditions. Lane 1: cells were cultured in 10% serum culture condition; lanes 2–4: cells were cultured in serum-free culture condition for 3, 5, 7 days, respectively.

neurosphere formation efficiency through reducing the RFX2/3 complex (Fig. 6). (iv) RNA interference knockdown of *RFX2* could significantly reduce the percentage of F1BGFP(+) cells and their neurosphere formation efficiency, whereas overexpression of RFX2 resulted in the opposite effects (Figs. 7 and 8).

To further address the roles of RFX proteins in the regulation of *FGF-1B* promoter, we performed EMSA to compare the RFX complex formation between U-1240 MG and U-1242 MG cells (Fig. 4B). Our results showed that the RFX2/3 complex was only detected in the nuclear extract of *FGF-1B*(+) U-1240 MG cells. Similar results were observed when comparing the dimeric RFX complexes between U-1240 MG/F1BGFP(+) and (−) cells (Fig. 5B) and another primary human glioblastoma cells (data not shown). It has been shown that RFX1 alone or dimerized with RFX2 or RFX3 could regulate several cellular genes, such as MHC class II [Fontes et al., 1997], *c-Myc* [Chen et al., 2000], *RPL30* [Safrany and Perry, 1993], *IL5Rα* [Iwama et al., 1999], *MAP1A* [Nakayama et al., 2003]. Our results suggested that RFX2/3 complex binding to the 18-bp *cis*-element is a critical step in the activation of *FGF-1B* promoter. RFX2 played a different role from RFX1 in the regulation of *FGF1* promoter; RFX3 may function as a bystander and cooperate with RFX1 and RFX2 to form dimeric complex binding the 18-bp *cis*-element of *FGF-1B* promoter. Ciliogenic RFX transcription factor family members may differentially regulate cell proliferation and F1BGFP-dependent cellular processes through modulating *FGF1* expression. It has been reported that nuclear translocation of RFX1 protein is regulated by PKC [Chen et al., 2000]. Interestingly, we found that PKC inhibitor, staurosporine (STA) and rottlerin, could

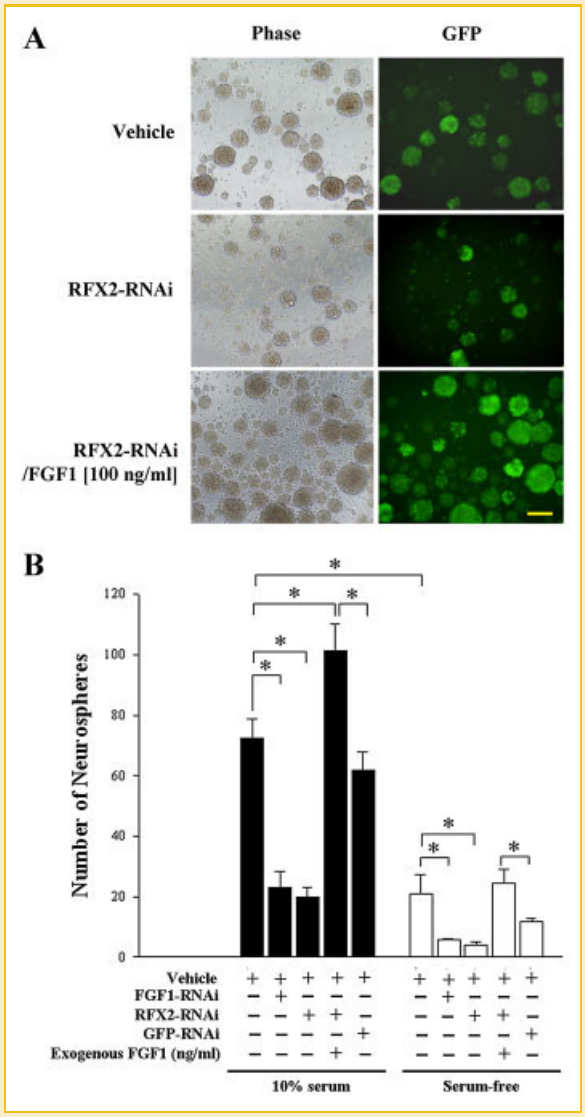

Fig. 8. Knockdown of RFX2 activator could significantly decrease the neurosphere formation of U-1240 MG/F1BGFP(+) cells. A: Representative images of neurospheres with different treatments are shown. *Scale bar*, 100 μm. B: Quantification of neurospheres generated by U-1240 MG/F1BGFP(+) cells treated with *FGF1*-RNAi, *RFX2*-RNAi, and GFP-RNAi. Data are shown as mean ± SEM, n = 3, $^{*}P < 0.05$ versus vehicle.

decrease the percentage of F1BGFP(+) cells, *FGF1* gene expression and neurosphere formation of U-1240 MG/F1BGFP(+) cells (Fig. 6A–C). As a proof of principle, we also demonstrated that staurosporine and rottlerin reduce the RFX2/3 complex formation in U-1240 MG/F1BGFP(+) cells, indicating the importance of RFX2/3 complex in the expression of F1BGFP (Fig. 6D).

In this study, we observed that overexpression of RFX2 only showed marginal effects on the percentage of F1BGFP(+) cells in 10% serum culture condition, however, overexpression of RFX2 could significantly increase F1BGFP expression in serum-free culture condition (Fig. 8C), suggesting the fact that the concentration of dimeric RFX complexes in 10% serum culture condition is abundant, so overexpression only showed marginal effects (Figs. 2–5). We further showed the difference of RFX complexes between 10% serum and serum-free culture conditions by EMSA experiment. RFX2/3, RFX1/2, RFX1/3, and RFX1/1 complexes were gradually and significantly decreased, with RFX2/3 first, and is consistent with our speculation (Fig. 7D).

It has been shown that intracrine/autocrine signals in cell division require *FGF1*. Endogenous *FGF1* is expressed during cytokinesis and plays a fundamental role in cell division [Chotani et al., 2000]. FGF1 also plays an important role in neurogenesis. In adult neurogenesis, the ventricular contacts of SVZ B cells can be observed as small apical surfaces containing a short, single primary cilium extending into the ventricle [Mirzadeh et al., 2008]. This feature is very different from ependymal cells that line most of the ventricular surface and extend a large number of motile cilia. The short primary cilia may facilitate the self-renewal of neural stem cells [Mirzadeh et al., 2008] and are important sites for signal receptions, particular sonic hedgehog and Fgf signaling. We have an ongoing study to characterize the F1BGFP(+) cells in the brain of F1BGFP transgenic mice. Interestingly, we observed that the F1BGFP(+) cells in adult brain exhibit characteristics of NSPCs. In addition, we also observed that Rfx1, Rfx2, and Rfx3 transcription factors differentially colocalized with F1BGFP(+) cells in adult mouse brain (data not shown). Future work on characterization of F1BGFP(+) cells in vivo will help clarify the relationship among NSPCs, F1BGFP(+) cells and Rfx transcription factors in adult neurogenesis. In this study, we mainly showed the regulatory effects of RFX transcription factors on *FGF-1B* promoter and the maintenance of F1BGFP(+) cells. The identification of ciliogenic RFX transcription factors in binding and regulating *FGF1* promoter further suggested the interplay of primary cilia, ciliogenic RFX proteins and *FGF1* in cell division and cell cycle progression. Given the significance of FGF1 in cell proliferation, cell division, and neurogenesis, understanding of *FGF1* gene regulation bring us closer to understanding FGF1-dependent cellular processes.

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