Basic Fibroblast Growth Factor-induced Neuronal Differentiation of Mouse Bone Marrow Stromal Cells Requires FGFR-1, MAPK/ERK, and Transcription Factor AP-1*

Haijie Yang, Yinyan Xia, Song Qing Lu, Tuck Wah Soong, and Zhi Wei Feng

From the Research Laboratories, National Neuroscience Institute, Singapore 308433

It has been reported recently that bone marrow stromal cells (BMSCs) are able to differentiate into various neural cells both in vivo and in vitro (Egusa, H., Schweizer, F. E., Wang, C. C., Matsuka, Y., and Nishimura, I. (2005) J. Biol. Chem. 280, 23691–23697). However, the underlying mechanisms remain largely unknown. In this report, we have demonstrated that basic fibroblast growth factor (bFGF) alone effectively induces mouse BMSC neuronal differentiation. These differentiated neuronal cells exhibit characteristic electrophysiological properties and elevated levels of the neuronal differentiation marker, growth-associated protein-43 (GAP-43). To explore possible signaling pathways, we first analyzed the expression of various FGF receptors in mouse BMSCs. FGF receptor-1, -2, and -3 were detected, but only FGFR-1 was shown to be activated by bFGF. Small interfering RNA knock down of FGFR-1 in BMSCs significantly inhibited neuronal differentiation. Moreover, we have shown that the mitogen-activated protein kinase (ERK1/2) is persistently activated and blockade of ERK activity with the ERK-specific inhibitor U0126 prevents neuronal differentiation. It appears that activation of ERK cascade and neuronal differentiation of BMSCs induced by bFGF are independent of Ras activity but require functions of phospholipase C-γ pathway. Lastly, we examined the role of the immediate-early transcription factors AP-1 and NF-κB and have found that phospholipase C-γ-dependent c-Jun and ERK-dependent c-fos, but not the NF-κB, are strongly activated by bFGF, which in turn regulates the neuronal differentiation of BMSCs.

Bone marrow stromal cells (BMSCs) of mesodermal origin provide a conductive environment for the development of hematopoietic cells in the bone marrow (1). Recent studies have demonstrated that BMSCs can be induced to generate not only the progenies of mesodermal lineages, such as adipocytes, chondrocytes, and myogenic cells, but also cells from different germ layers, such as neuronal cells (2–4). These neurogenic capacities of BMSCs have attracted tremendous attention because of their great potential as donor cells for cell supplementary therapy of neuronal disorders, with the advantages of fewer ethical concerns and less immune rejection (5, 6). However, the molecular mechanisms underlying the neuronal differentiation of BMSCs are not clear and the “transdifferentiation” hypothesis (7) between different germ layers is yet to be proven with more solid data. A recent challenge indicated that such neuronal differentiation was attributed to the stress response of BMSCs to the chemicals used in neuronal differentiation protocols (8). It will be therefore worthwhile to study neuronal differentiation of BMSCs under physiological conditions and elucidate the relevant signaling pathways and nuclear events.

The basic fibroblast growth factor (bFGF) was the first to be used in studies elucidating the neuronal differentiation of BMSCs (9). bFGF has a wide range of biological effects on cell growth, differentiation, and survival. Recent studies revealed the neurogenic function of bFGF in animals (10) and the induction of neuronal tube formation from embryonic stem cells by bFGF in vitro (11). More interestingly, cells from non-neuronal origin can also be induced by bFGF to adopt neuronal phenotypes, such as neuronal transdifferentiation of retina epithelium (12) and BMSCs (9), indicating its potent neuronal-inductive effects.

The function of bFGF is achieved by binding to the fibroblast growth factor receptors (FGFRs). There are at least four structurally related high affinity FGFRs that are expressed differentially in various tissues and cell types (13). The affinity of bFGF with various FGFRs is different, and the downstream signaling pathways of different FGFRs are also varied (14), although the signaling domains of FGFRs are highly conserved. Several signaling pathways can be activated by FGFRs, such as the cascades of phospholipase C-γ (PLC-γ), Src, Crk, and SNT-1/FRS2 (15), some of which are implicated in bFGF-induced neurogenesis and differentiation (16). A recent study demonstrated that the MAPK/ERK cascade, an important target for the SNT-1/FRS2 signaling pathway, plays a crucial role in mediating the neurotrophic effect of bFGF (17). Activated ERK kinase was also found to induce the neuronal transdifferentiation of retinal pigment cells (18). These findings suggest a central role of ERK in the neuronal induction by bFGF. However, whether ERK is involved in bFGF-induced neuronal differentiation of BMSCs needs to be clarified.

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1 To whom correspondence should be addressed: NNI Research, 11 Jalan Tan Tock Seng, Singapore. Tel.: 65-6357-7528; Fax: 65-62569178; E-mail: Zhi_Wei_Feng@nni.com.sg.

2 The abbreviations used are: BMSC, bone marrow stromal cell; bFGF, basic fibroblast growth factor; ERK, extracellular signal-regulated kinase; FGF, FGF receptor; FRS2, FGF receptor substrate 2; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; PLC-γ, phospholipase C-γ; siRNA, small interfering RNA.
Neuronal differentiation is a complex process involving many molecules, some of which need to be newly transcribed and translated (19). It has been well established that the activated ERK induced by bFGF can be translocated into the nucleus and activate transcription factors (20). Both bFGF and ERK can activate the immediate-early transcription factors AP-1 and NF-κB (21, 22), which in turn regulate neuronal differentiation (23, 24). For instance, in BMSCs the DNA binding activity of AP-1 was greatly induced by cytokines (25) and NF-κB was shown to be activated during neuronal differentiation (26). However, the exact functions of these factors in bFGF-induced neuronal differentiation of BMSCs have not been documented.

In this study, we demonstrated that bFGF effectively induces neuronal differentiation of mouse BMSCs and that FGFR-1 is the main FGF receptor recognized and activated by bFGF. The signal transduction evoked by bFGF/FGFR-1 in BMSCs is mainly mediated by MAPK/ERK, which is downstream of signal transduction evoked by bFGF/FGFR-1 in BMSCs is documented.

bFGF-induced Differentiation Requires FGFR-1, MAPK, and AP-1

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human bFGF was purchased from i-DNA Biotechnology. Insulin-like growth factor I, nerve growth factor, and platelet-derived growth factor were from iDNA Biotechnology. Insulin-like growth factor I, nerve growth factor, and platelet-derived growth factor were from Sigma. FGFR inhibitor SU5402, Src inhibitor PP2, Ras inhibitor FTI-277, PLC-γ inhibitor U0126 and phosphoinositide 3-kinase inhibitor LY294002 (succinimidyl propionate) were from Calbiochem. MEK inhibitor U0126 and phosphoinositide 3-kinase inhibitor LY294002 were from Cell Signaling Technology. Superscript™ III RT, Lipofectamine 2000, G418, and Hygromycin B were from Invitrogen. Mouse monoclonal antibodies against phospho-MAP kinase (Thr-180/Tyr-182), phospho-PKC (Thr-505), and phospho-PLC-γ1 (Tyr-196), phospho-c-Raf (Ser-338), phospho-p90RSK (Ser-380), phospho-p38 MAP kinase (Thr-180/Tyr-182), phosphor-PKCθ (Thr-538), phospho-PKCδ (Thr-505), and phospho-PLC-γ1 (Tyr-783) were from Cell Signaling Technology. Mouse monoclonal anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology. Rabbit polyclonal antibodies against FGFR-1, FGFR-2, and FGFR-3 were from Santa Cruz Biotechnology. Goat polyclonal antibody against FGFR-4 and mouse monoclonal antibody against bFGF were from R&D Systems (Abingdon, UK). Goat polyclonal antibody against GAP-43 and mouse monoclonal antibody against β-actin were from Chemicon. Other chemicals were obtained from Sigma.

**Cell Culture**—Mouse BMSCs were obtained from 8-week-old male mice. Briefly, cells were harvested from mouse bone marrow and cultured in Dulbecco modified Eagle’s medium-low glucose supplemented with 10% fetal bovine serum, 10% fetal calf serum, and 1% penicillin-streptomycin. The adhered cells were eventually purified by passing, and a homogenous cell population with flat and fibroblast-like morphology at passages 10–20 was used in this study.

**Plasmids and DNA Constructs**—The short interfering RNAs (siRNAs) for mouse FGFRs were designed to target 19 nucleotides of the mouse FGFR transcripts (FGFR-1 siRNA, nucleotides 1542–1560, 5′-GATGTTGAAGTCCGACGCA-3′; FGFR-2 siRNA, nucleotides 1555–1573, 5′-GCAGTGGAGATTGAGATAGC-3′; FGFR-3 siRNA, nucleotides 1453–1461, 5′-GCTATTGGCATCGACAAGG-3′, which were cloned into a siRNA expression vector pSilencer 1.0-U6 (Ambion). The same vector containing a hairpin siRNA whose sequence is not found in the mouse data base supplied in the vector kit was used as a control. A dominant negative mutant of c-Jun, TAM67 (27), was cloned into Xhol/KpnI sites of pREP10 (Invitrogen) and tagged with V5 epitope at the C-terminal.

**Reverse Transcription PCR Analysis of FGFR Expression in BMSCs**—Total RNA was extracted from mouse BMSCs using TRIzol reagent following the manufacturer’s instructions (Invitrogen), and 2 μg of RNA was reverse-transcribed and amplified by PCR. The primers for FGFRs were as follows: FGFR-1: sense, 5′-CGCTCTACCTGGAAGATCATT-3′, anti-sense, 5′-ATAAAGGAGACCATCTGTT-3′; FGFR-2: sense, 5′-AGAACGCTAGCTGTTGCC-3′, antisense, 5′-GCTCGTCCTCAAACTCCCTC-3′; FGFR-3: sense, 5′-AGCTAGAGGTTCTGTCCTTG-3′, antisense, 5′-TCTTTGTGAGTGGCC-3′; FGFR-4: sense, 5′-GGTGGAATCTGACCTGCCCTC-3′, antisense, 5′-CACTTCCGAGACTCCAGAT-3′. β-actin was included in the PCR as an internal control. The PCR reaction consisted of 35 cycles of denaturing at 94 °C for 45 s, annealing at 54 °C for 45 s, and extension at 72 °C for 90 s. The PCR products for FGFR-1, 2, 3, and 4 were 560, 464, 532, and 476 bp, respectively. The PCR products of expected sizes were gel-purified and cloned into pGEM T-easy and were confirmed to be FRGR-related by sequencing.

**Immunoprecipitation and Western Blotting**—Cells were washed twice with cold phosphate-buffered saline and then extracted with lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM potassium phosphate, 1 mM β-glycerophosphate, 1 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride, and Roche Applied Science’s complete protease inhibitors) and centrifuged at 14,000 × g for 20 min at 4 °C. The proteins in the supernatant were measured using a Protein Assay Kit II (Bio-Rad). For immunoprecipitation analysis, 500 μg of total cell lysates was precleared with protein A plus G-Sepharose before incubation with specific antibodies, followed by addition of protein A plus G-Sepharose. The precipitated proteins were resolved in 2× SDS-PAGE sample buffer before electrophoresis.

For Western blotting analysis, samples were separated by electrophoresis on 10–14% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore Corp.). After blocking with PBST (3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 140 mM NaCl, 0.1% Tween-20, pH 7.4) containing 5% skim milk, membranes were incubated with primary antibodies. The membranes were further incubated with hors eradish peroxidase-conjugated secondary antibodies and developed using Pierce’s West Pico Chemiluminescence substrate. To determine the equivalence of protein amounts loaded among different samples, the developed membranes were stripped with a buffer consisting of 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol at 50 °C for 45 min,
followed by incubation with control antibodies such as anti-β-actin for further blotting. In some cases, immunoblots were quantified by measuring the immunoreactive protein band density with the software ImageJ 1.34s.

Cross-linking Experiment—After incubation with 10 ng/ml bFGF for 2 min, BMSCs were washed twice with cold phosphate-buffered saline and cross-linked with 1 mM thiol-cleavable cross-linking reagent dithiobis (succinimidyl propionate) at 4 °C for 2 h. Dithiobis (succinimidyl propionate) stock solution was freshly prepared in dimethyl sulfoxide. The reactions were stopped by the addition of glycine at a final concentration of 100 mM for 15 min at room temperature. The cells were extracted with lysis buffer, and the lysates were immunoprecipitated with antibodies against FGFR-1, 2, and 3. Finally, the precipitated proteins were treated or untreated with DDT (200 mM), followed by immunoblotting.

Ras Activation Assay—Ras activity was detected using the Ras activation assay kit (Upstate Biotechnology) according to the manufacturer’s specifications. Briefly, cell lysates were incubated with glutathione S-transferase-Raf-1 Ras binding domain-agarose beads and the GTP-bound Ras was precipitated, followed by immunoblotting with anti-pan Ras antibody.

Electrophysiology—For whole-cell current recording, cells were clamped as previously described (28). The bath solution contained (in mM): NaCl 150, KCl 5, MgCl2 1, CaCl2 2.2, Hapes 10, pH 7.3, with NaOH, and osmolarity was adjusted to 310 mosM with glucose. The pipette solution contained (in mM): aspartic acid potassium salt 120, MgCl2 5, EGTA 0.5, Hepes 10, ATP 2, GTP 0.3, pH 7.3, with KOH, and osmolarity was adjusted to 295 mosM. Whole-cell current was obtained under voltage clamp with Axopatch 200B amplifier (Axon Instruments), and the results were analyzed using the software pClamp8.1 (Axon Instruments). The current was sampled at 10 kHz and filtered at 1 kHz. Leak was subtracted on-line by using P/4 protocol. The pipette used was 1–2 mΩ, and the series resistance was typically <5 mΩ and compensated by 75%. The holding potential was set at −70 mV and depolarized with different voltages to evoke channel opening.

Transfection and Gene Silencing with siRNA—5 × 105 cells were seeded on 60-mm tissue culture dishes and cultured overnight. 8 μg of pSilencer 1.0-U6-based recombinant plasmids (each containing a unique 19-bp insert targeting FGFR-1–3) and 0.8 μg of pEGFP-C2 vector were co-transfected into BMSCs using Lipofectamine 2000 following the manufacturer’s instructions. Transfected cells were selected with G418 at 500 μg/ml, and the expression of FGFR-1, 2, and 3 in selected cell clones was determined by Western blotting with specific antibodies. Cell clones in which the expression of FGFR was significantly silenced were selected for further study. Similarly, cell clones with TAM67 were obtained by selection with 500 μg/ml hygromycin, and the expression of TAM67 was determined by Western blotting using antibodies against V5 epitope and C-terminal of c-Jun.

Transcription Factor Report Assay—The DNA binding activity of c-Fos, c-Jun, and NF-κB were determined with the TransAM™ Assay kits (Active Motif) according to instructions provided by the manufacturer. Briefly, cells were stimulated by bFGF and 2–10 μg of nuclear extracts was incubated in oligonucleotide-coated 96-well plate for 1 h. After washing, the bound complexes were incubated with antibodies against c-Fos, phospho-c-Jun, or NF-κB p65 for 1 h. The plates were then incubated with horseradish peroxidase-conjugated secondary antibodies for another 1 h. Finally, the developing solution was added for 2–10 min, followed by the measurement of absorbance at 450 nm.

Statistical Analysis—Data were expressed as means ± S.E. values. The group means were compared by analysis of variance, and significance of differences was determined by post-hoc testing using Bonferroni’s method. A p value of <0.05 was considered significant.

RESULTS

bFGF Induces Mouse BMSC Neuronal Differentiation—In vitro studies, BMSCs can be induced and differentiated into neuronal phenotype by several protocols, using various growth factors and even chemicals such as β-mercaptoethanol and dimethyl sulfoxide (5, 6). Thus, the neuronal differentiation of BMSCs has been recently challenged as a stress response of BMSCs to the toxicity of chemicals (7). However, the pilot observation of neuronal differentiation of BMSCs after transplantation in the brain (29) strongly argues that BMSCs can generate neuronal cells under physiological conditions. To seek more solid in vitro evidence, we examined several growth factors for their ability to induce neuronal differentiation of mouse BMSCs. Under normal culture conditions, mouse BMSCs at passages 10–20 were treated with various molecules for 1–9 days. Cell morphological changes were monitored under the microscope, and images were taken every 24 h. Among tested molecules, only bFGF was found to be able to potentiate neuronal differentiation of mouse BMSCs (Fig. 1A). The bFGF-treated cells exhibited a gradual shrinkage of cell body and long cell processes connecting to the neighboring cells. These morphological changes started from day 2 and could be maintained for at least 9 days (data not shown). In contrast, cells treated with all other growth factors, such as insulin-like growth factor 1, platelet-derived growth factor, and nerve growth factor, did not show any obvious morphological change within 3 days (Fig. 1A), and a longer treatment (up to 9 days) did not change our conclusion (data not shown).

To confirm that the neuronal differentiation is induced specifically by bFGF, we applied the FGFR inhibitor SU5402 (30) in the neuronal differentiation experiment. As shown in Fig. 1A, the neuronal morphological change induced by bFGF was completely inhibited by SU5402 and cells remained flattened with fibroblast-like morphology similar to the untreated cells. We also analyzed the expression of the neuronal differentiation marker growth-associated protein-43 (GAP-43), which has been shown to be involved in the axon growth (31). As expected, the expression level of GAP-43 was substantially up-regulated from day 1 of treatment and increased steadily afterward (Fig. 1B). In contrast, the bFGF-induced expression of GAP-43 was completely blocked by FGFR inhibitor SU5402.

The differentiated BMSCs exhibited not only neuronal morphology and marker expression but also the relevant electrophysiological properties. The membrane current of differentiated BMSCs was measured with the whole-cell patch clamp.
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A

Control
IGF-1
NGF

PDGF
bFGF
bFGF+SU5402

B

GAP-43
Actin

SU5402
0
1d
2d

C

90mV
-90mV
-70mV
-30mV
50pA
20ms

bFGF
Control

FIGURE 1. bFGF induces neuronal differentiation of mouse BMSCs. A, mouse BMSCs were treated with medium (Control), 10 ng/ml insulin-like growth factor I (IGF-I), nerve growth factor (NGF), platelet-derived growth factor (PDGF), bFGF, or 10 ng/ml bFGF + 25 μM SU5402 (FGFR inhibitor). Cells were photographed live at day 3 of treatment, and pictures are shown at ×100 magnification. B, cells were pretreated with 25 μM SU5402 for 1 h prior to incubation with 10 ng/ml bFGF for 2 days, and protein levels of neuronal differentiation marker GAP-43 were determined by immuno blotting. The actin level was shown as an indicator for equal loading of proteins. C, the electrophysiological properties of bFGF-treated BMSCs at day 3 were determined by the patch clamp method. The whole-cell current was recorded by a 100-ms stimulus from −30 to 90 mV in 30-mV increments, and the holding potential was −70 mV. The upper left picture shows the voltage clamp recording protocol used, and the upper right one is the standard bar of the current.

technique. As shown in Fig. 1C, after 3 days of treatment with bFGF, the current of BMSCs was evoked by giving a train of stimulated pulses ranging from 30 to 90 mV, and the outward current from the differentiated cells was likely to be the K⁺ current. In contrast, no detectable outward current was observed in untreated BMSCs under the same conditions. These results indicate that the neuronal cells differentiated from BMSCs have characteristic electrophysiological properties.

FGFR-1 is the Major Receptor Mediating Neuronal Differentiation—To determine whether FGFRs in mouse BMSCs contribute to the specific neurogenic effects of bFGF, we measured the expression levels of various FGFRs. Semi-quantitative reverse transcription PCR experiments showed that the mRNA expression levels of four FGFRs were markedly different (Fig. 2A). The level of receptor-1 was high and levels of receptor-2 and -3 were relatively weak. The mRNA level of FGFR receptor-4 was nearly undetectable. Western blotting analysis showed similar FGFR protein pattern (Fig. 2B). Because the binding affinities between various FGFRs and bFGF were different (14), we then analyzed the binding properties of bFGF with each FGFR in BMSCs. Results from the chemical cross-linking and immunoprecipitation experiments showed that bFGF mainly bound to the FGFR-1 under our experimental conditions (Fig. 2C).

Upon the binding of bFGF, the cytoplasmic domain of FGFR is phosphorylated (15), which in turn activates its downstream molecules. We examined the tyrosine phosphorylation of total cellular proteins in bFGF-treated BMSCs. As expected, protein phosphorylation was detected in BMSCs within 2 min after bFGF treatment (Fig. 3A). Similarly, phosphorylation of FRS2, a key initiator in FGFR signaling (15), was also observed (Fig. 3B). To determine which FGFR initiated the activation of FRS2, we carried out co-immunoprecipitation experiments. As shown in Fig. 3C, the phosphorylated FRS2 only formed a complex with FGFR-1, whereas neither FGFR-2 nor FGFR-3 interacted with FRS2. These data further indicate that FGFR-1 is the major FGFR receptor to transduce signals from bFGF engagement to cytoplasmic cascades in neuronal differentiation of BMSCs.

To determine precisely the role of FGFR-1 in neuronal induction, we generated stable cell lines transfected with siRNA constructs specific to FGFR1–3. The control cell line was transfected with the same vector containing a random sequence that was not found in the mouse genome data base. Several cell clones from each siRNA transfection were obtained, and the expression levels of FGFR-1, 2, and 3 were evaluated (Fig. 4A). The clones with the most knockdown were chosen for the following experiments. In the presence of bFGF, both the wild-type BMSCs and control siRNA-transfected cells exhibited the neuronal morphology within 3 days (Fig. 4B). However, bFGF failed to induce neuronal morphology in the FGFR-1 siRNA cells (clone 6) (Fig. 4B). In contrast, under the same conditions, both FGFR-2 and FGFR-3 siRNA cells exhibited neuronal morphology induced by bFGF (data not shown). Besides neuronal morphology, the expression of the neuronal differentiation marker GAP-43 was undetectable in FGFR-1 siRNA cells in day 1 cells and dramatically decreased in day 2 cells as compared with that of control cells (Fig. 4C). These results indicate that both neuronal morphological changes and expression of GAP-43 are FGFR-1-dependent.

ERK1/2 Pathway Plays a Central Role in Neuronal Differentiation of BMSCs—The activated FGFRs will initiate various signaling pathways to fulfill their biological functions (15).
Because ERK has been shown to be required for neuronal transdifferentiation of pigment cells (18), we first determined the activity of the ERK pathway. As shown in Fig. 5A, the phosphorylation of ERK1/2 was first detected within 2 min after bFGF treatment and sustained for more than 4 h. A similar activation was also observed for ERK1/2, immediately upstream molecule MEK, and downstream target p90RSK. To clarify signaling pathways mediating the activation of ERK1/2, we sequentially analyzed the activation of Ras, Raf, p38 MAP kinase, JNK, AKT, and protein kinase C in this experiment. Interestingly, we detected the activation of Ras (Fig. 5B), Raf, and PLC-γ/H9253 but not AKT, protein kinase C, p38, and JNK (Fig. 5A). These results suggest that both ERK activation and neuronal differentiation of BMSCs rely on neither phosphoinositide 3-kinase nor protein kinase C pathways and are also not involved in the stress-signaling pathways.

To determine how bFGF activates the ERK pathway, we applied selected inhibitors in the ERK activation experiment. As shown in Fig. 6A, the phosphorylation of ERK was completely blocked by the FGFR inhibitor SU5402 but was not affected by the Ras inhibitors FTI-277 (32), which drastically inhibited Ras activation (Fig. 6B). Similar inhibitory experiments were also carried out with LY294002 (for phosphoinositide 3-kinase) and PP2 (for Src family). The unfavorable results excluded their involvement in the activation of ERK (Fig. 6B).

Interestingly, in the presence of PLC-γ inhibitor U-73122, the phosphorylation of ERK and p90RSK induced by bFGF was significantly inhibited. Conclusively, the activation of ERK cascade in bFGF-induced neuronal differentiation is largely dependent on the PLC-γ, but not the Ras, pathway.
To elucidate the effect of ERK1/2 on neuronal induction by bFGF, we applied the MEK inhibitor U0126 in differentiation experiments. As compared with bFGF-induced neuronal morphology of BMSCs, the morphology of cells treated with bFGF plus U0126 remained largely as flattened as those of untreated ones (Fig. 6C). The neuronal marker GAP-43 was also drastically blocked in both day 1 and day 2 cells (Fig. 6D). In contrast, the presence of Ras inhibitor FTI-227 did not prevent neuronal morphology changes in the experiment. These results strongly suggest that ERK plays a crucial role in mediating bFGF-induced neuronal differentiation of BMSCs.

AP-1 Is Required for bFGF-induced Neuronal Differentiation of BMSCs—It is known that ERKs can stimulate the immediate-early transcription factors such as AP-1 and NF-κB (21, 22). NF-κB was activated during the neuronal differentiation of BMSCs (26), but its significance remains unclear. To determine the effects of these two molecules on neuronal differentiation of BMSCs, we examined the activation of c-Jun, c-Fos, and p65 with report assay. The DNA binding activities of c-Jun and c-Fos increased greatly within 30 min after bFGF treatment and reached maximum levels around 1 h (Fig. 7A). Under the same conditions, the DNA binding activity of NF-κB p65 remained unchanged in the presence of bFGF (Fig. 7A). To determine the association of the PLC-γ-ERK pathway with the activation of c-Jun and c-Fos, we applied inhibitors in the report assay. Interestingly, the activation of c-Fos was coupled with the activation of both PLC-γ and ERK, whereas the activity of c-Jun was only dependent on PLC-γ (Fig. 7B). Furthermore, the activation of both c-Jun and c-Fos was not affected by Ras.

To determine the role of AP-1 in neuronal differentiation of BMSCs, the stable BMSC cell lines expressing the dominant negative c-Jun (TAM67) were generated (27). In these cells, bFGF-stimulated c-Jun and c-Fos activity was dramatically decreased (Fig. 7C). When these cells were subjected to neuronal differentiation, the high levels of GAP-43 observed in bFGF-induced controls were significantly reduced in TAM67 cells (Fig. 7C). In conclusion, these data indicate that activation of AP-1 is required for bFGF-induced neuronal differentiation.

**DISCUSSION**

The work presented here demonstrates that bFGF alone can effectively induce neuronal differentiation of mouse BMSCs...
and provides solid evidence for the physiological properties of the neurogenic effect of BMSCs (8). Moreover, we found that FGFR-1 plays a crucial role in neuronal differentiation via activating PLC-γ, ERK1/2, and AP-1 axis. These findings may represent a signal transduction mechanism responsible for the neuronal differentiation of mouse BMSCs.

It has been known that different cells respond distinctly to bFGF stimulation, partly due to the expression profile of the FGFR and different affinity between FGFs and FGFRs (33). However, the contribution of individual FGFRs in the neural induction by bFGF is still unclear. It has been demonstrated that the differentiation of neuronal stem cells is regulated by a specific FGFR (34) and FGFR-1 is required for the development of the auditory sensory epithelium (35). In this study, we found that bFGF-evoked signal transduction is mainly dependent on FGFR-1. Down-regulating the expression of FGFR-1 could significantly block the neuronal differentiation, suggesting that the neurogenic function of bFGF on BMSCs is mainly mediated by FGFR-1. These data demonstrate for the first time the drastic variation of neurogenic capacity among different FGFRs in mouse BMSCs. Given the fact that bFGF has a wide range of biological effects, it would be of significance to determine whether a specific FGF receptor is involved in neural cell specification. This work also argues that neuronal differentiation of BMSCs is a stringently regulated physiological phenomenon rather than a stress response to toxic chemicals used in some neuronal differentiation protocols (8).

Even though FGFRs belong to the same receptor family, the downstream effects of these receptors are not equivalent (13, 14). FGFRs are shown to be expressed differentially in various cell types, which may also influence the variations in intensity and in the nature of cell response to bFGF (36). Human BMSCs have been shown to express all four FGFRs (37) and be able to differentiate into neuronal cells. However, we found that mouse BMSCs express a high level of FGFR-1 and relatively low levels of FGFR-2 and FGFR-3. In contrast, FGFR-4 remained nearly undetectable under the same experimental conditions. The difference between the expression patterns of FGFRs in mouse and human BMSCs may be due to the different organism strains or culture conditions. One recent study showed that mouse osteoclasts, differentiated from BMSCs, only express FGFR-1 (38). This suggests that the expression levels or pattern of FGFRs in BMSCs may vary at different development stages of stromal lineage. As a result, these cells may respond distinctly to the same stimulation of FGF. On the other hand, different FGFRs may play the same role in a given biological phenomenon, via different pathways. For example, FGFR-3 induces gene expression and neuronal phenotype via a Ras-independent signaling pathway, whereas FGFR-1 does that via a Ras-dependent mechanism (39). Nevertheless, the specific function of individual FGFR in a given biological phenomenon or development stage needs to be further investigated.

The MAPK/ERK cascade plays a central role in mediating signals from membrane receptors to the nucleus and is involved in cell growth, differentiation, and apoptosis (40, 41). Previous studies have suggested an important role of the MAPK/ERK pathway in bFGF-induced neurogenesis (17) and neurite outgrowth (42). More recent work revealed that the activation of ERK is required for the neuronal transdifferentiation of pigment epithelium (18), but the related mechanisms remain largely unknown. In this work, we observed a fast and sustained activation of the ERK and its downstream molecule, which was also demonstrated to be required for neuronal differentiation of BMSCs. Interestingly, it has been recently suggested that a sus-
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tained ERK activity leads to neuronal differentiation, whereas its transient activation only results in cell proliferation (43). These findings indicate that the sustained ERK activation may contribute to the cell differentiation.

How does bFGF activate the ERK cascade? Once bound by bFGF, FGFRs will activate a wide spectrum of signaling molecules, including Src (44), PLC-γ (45), phosphoinositide 3-kinase (46), and Ras (47). In our experiment, we only observed the activation of Ras and PLC-γ, which also indicates the characteristic of the signal transduction downstream of FGFR-1. Ras has been shown to activate ERK and contribute to bFGF-induced endothelium differentiation (48). Interestingly, in our study, inhibition of Ras does not affect the ERK activation and neuronal differentiation induced by bFGF. In fact, the activity of ERK is found to be dependent on PLC-γ in mouse BMSCs, demonstrating a complicated cross-talk downstream of FGFRs.

Both AP-1 and NF-κB have been shown to play important roles in neuronal differentiation (23, 24), but the functions of these two factors in the neuronal differentiation of mouse BMSCs are not clear. Yang et al. (26) observed the activation of NF-κB in the neuronal differentiation of rat BMSCs. However, no activation of NF-κB was detected in bFGF-treated mouse BMSCs in our observations. The discrepancy between these observations might be because the former used the baicalin to differentiate rat BMSCs whereas we employed bFGF to induce mouse cells.

AP-1 functions in many biological processes, including neuronal differentiation (49). Recent studies have demonstrated that AP-1 was involved in the inflammatory response in BMSCs and associated with bone formation (25, 50). AP-1 is composed of c-Jun and c-Fos family members. Jun family members can form homodimers, whereas Fos can only form heterodimers with Jun family members to bind DNA (51). In BMSCs, both c-Jun and c-Fos activation was significantly induced by bFGF (Fig. 7A). However, the activation of c-Fos appears to be ERK-dependent whereas the induction of c-Jun involves PLC-γ (Fig. 7B), indicating that AP-1 components are regulated by different signaling pathways. Furthermore, the application of TAM67, which can inhibit both Jun/Jun and Jun/Fos dimers, was found to suppress the neuronal differentiation of BMSCs, suggesting that different AP-1 dimers may act together in the neuronal differentiation of BMSCs.

In summary, we have provided evidence that bFGF could induce neuronal differentiation of BMSCs in vitro, which is mainly mediated by the activation of FGFR-1, MAPK/ERK, and transcription factor AP-1. Our results provide an insight into the understanding of neuronal differentiation of adult stem cells induced by bFGF.

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