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Basic Fibroblast Growth Factor Inhibits Mineralization but Induces Neuronal Differentiation by Human Dental Pulp Stem Cells Through a FGFR and PLCγ Signaling Pathway

Thanaphum Osathanon,[1,2,3] Nunthawan Nowwarote,[2] and Prasit Pavasant[2,3]*

[1] *Developing Research Unit of Genetic and Anatomical Analyses of Craniofacial Structures, Chulalongkorn University, Bangkok 10330, Thailand*

[2] *Mineralized Tissue Research Unit, Chulalongkorn University, Bangkok 10330, Thailand*

[3] *Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Bangkok 10330, Thailand*

ABSTRACT

Basic fibroblast growth factor (basic FGF) has pivotal roles in the function of various cell types. Here, we report the effects of basic FGF in the regulation of dental pulp stem cell (DPSC) behaviors including maintaining stemness and directing differentiation. Cells isolated from human dental pulp tissues exhibited stem cell properties including the expression of mRNA markers for embryonic and mesenchymal stem cells, the expression of Stro-1, and the multipotential differentiation. Basic FGF stimulated colony-forming units of DPSCs and up-regulated the expression of the embryonic stem cell markers; Oct4, Rex-1, and Nanog. Moreover, osteogenic medium containing basic FGF inhibited alkaline phosphatase enzymatic activity and mineralization of DPSCs. On the contrary, basic FGF appeared to be an influential growth factor in the neurogenic differentiation of DPSCs. In the presence of basic FGF, increased DPSCs neurosphere size and the up-regulation of neurogenic markers were noted. Inhibitors of FGFR or PLCγ were able to ablate the basic FGF-induced neuronal differentiation of DPSCs. Taken together, these results suggest basic FGF may be involved in the mechanisms controlling DPSCs cell fate decisions. J. Cell. Biochem. 112: 1807–1816, 2011.

KEY WORDS: DENTAL PULP STEM CELLS; BASIC FIBROBLAST GROWTH FACTOR; SELF-RENEWAL; DIFFERENTIATION

Dental pulp stem cells (DPSCs) were firstly identified in 2000 [Gronthos et al., 2000]. To date, many investigations of their characteristics and applications have been substantially published. DPSCs were shown to differentiate into osteo/dentinogenic, adipogenic, chondrogenic, myogenic, and neurogenic lineages in vitro [Gronthos et al., 2002; Arthur et al., 2008; Zhang et al., 2008; Huang et al., 2009; Spath et al., 2010]. In addition, the formation of dentin-pulp-like structures and bone-like tissues after the in vivo implantation of DPSCs has been reported [Gronthos et al., 2002; Huang et al., 2009]. Various potential therapeutic applications of dental pulp stem cells have been documented. For example, Gandia et al. [2008] showed that intramyocardial transplantation of DPSCs improved overall ventricular function and angiogenesis in the infarction area. A pilot clinical trail using DPSCs for mandibular bone repair was reported [d'Aquino et al., 2009]. This study demonstrated better bone formation in extraction sockets using transplanted collagen sponges containing DPSCs compared with those treated with vehicle sponge alone [d'Aquino et al., 2009]. Intriguingly, it has also been shown that the untreated DPSCs were able to differentiate into functional neurons upon injection in the developing avian nervous system [Arthur et al., 2008]. These implanted cells exhibited neuronal morphology with the strong

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*Correspondence to: Prasit Pavasant, DDS, PhD, Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Henri-Dunant Rd. Pathumwan, Bangkok 10330, Thailand. E-mail: prasit215@gmail.com

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expression of neuronal markers [Arthur et al., 2008], indicating DPSCs can differentiate along the neurogenic lineage in vivo in the appropriate environment and may be used for the treatment of neuronal diseases. Together, these data imply the prospective utilization of DPSC in number of clinical circumstances.

It has been reported that DPSCs contained a neural crest cell-derived subpopulation [Waddington et al., 2009]. Low-affinity nerve growth factor receptor (LANGFR) positive DPSCs exhibited Stro-1 expression and were able to differentiate along multiple lineages, including osteoblasts, adipocytes, and chondrocytes [Waddington et al., 2009], suggesting a neural crest cell origin of DPSCs. Neural crest cells are known to be able to differentiate into various cell types especially neuronal precursor cells [Le Douarin et al., 2004], thus, DPSCs may have the potential for neuronal induction of DPSCs. Recently, Govindasamy et al. [2010] reported that DPSCs exhibited an inherent tendency toward the neuronal lineage as DPSCs had higher expression of neuroectodermal markers in the undifferentiated state compared with stem cells derived from human deciduous teeth (SHEDs). Upon neuronal differentiation, the number of neurospheres and the mRNA expression of neuronal markers were greater for DPSCs compared with those of SHEDs [Govindasamy et al., 2010]. In addition, several publications have been illustrated the differentiation ability of DPSCs along the neurogenic pathway [Arthur et al., 2008; Sasaki et al., 2008; Kadar et al., 2009; Kiraly et al., 2009; Ryu et al., 2009]. These data suggest that DPSCs have high-neurogenic potential and should be investigated as a candidate cell source for neuronal repair.

Several methods have been employed to induce the neuronal differentiation of mesenchymal stem cells, that is, chemical and cytokine induction. As some investigators noted, the chemical-induced neuronal differentiation of mesenchymal stem cells led to a change of cytoskeleton and gene expression pattern in response to the chemical stimuli but not true differentiation into mature functional neuron [Lu et al., 2004; Neuhuber et al., 2004; Barnabe et al., 2009]. Therefore, the cytokine-induced neuronal differentiation is more extensively employed.

Basic fibroblast growth factor (basic FGF), also known as fibroblast growth factor-2 (FGF-2), is one of the supplemented cytokine used in neuroinduction medium along with others such as epidermal growth factor (EGF). Basic FGF is a member of the fibroblast growth factor family. It has substantial roles in various cell and tissue functions including proliferation, migration [Yang et al., 2010], and angiogenesis [Zhao et al., 2011]. Interestingly, it has been reported that basic FGF knockout mice exhibited a lower volume and lower proliferative rate of dorsal pseudostratified ventricular epithelium compared with the wild type mice [Raballo et al., 2000]. In addition, decreased neural and glia cell number and density were noted in basic FGF null mice [Vaccarino et al., 1999], implying that basic FGF has a significant role in neurogenesis.

Although, the function and influence of basic FGF on various cell types have been extensively reported. Investigation into the effects of basic FGF on DPSCs is still lacking. In the present study, we aimed to evaluate the effects of basic FGF on DPSCs' behaviors, especially its ability to induce neuronal differentiation in this cell type.

MATERIALS AND METHODS

DPSCs ISOLATION AND CULTURE

The protocol for the isolation of DPSCs was approved by the Ethical Committee, Faculty of Dentistry, Chulalongkorn University. Healthy adult subjects undergoing surgical treatment for tooth removal due to third molar impaction were recruited for the isolation of DPSCs. Dental pulp tissues were washed with sterile PBS, cut into small pieces and digested with type I collagenase (Gibco, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml amphotericin B (Gibco) in 100% humidity, 37°C and 5% carbon dioxide. Medium was changed every 48 h. After reaching confluence, the cells were sub-cultured at a 1:3 ratio. The expressions of stem cells markers were evaluated using reverse transcriptase polymerase chain reaction (RT-PCR). In addition, Stro-1 immunocytochemistry staining was performed using mouse anti-human Stro-1 monoclonal antibody (Chemicon, USA).

COLONY-FORMING UNIT ASSAY

The cells were seeded into 35-mm-diameter culture dishes at a density of 500 cells per dish and maintained in the medium described above but supplemented with 5% FBS. Recombinant human basic FGF (Invitrogen, USA) was added in the cultured medium at concentrations of 0, 1, and 20 ng/ml. After 14 days, the cells were fixed with 10% buffered formalin (MERCK, Germany) for 10 min, washed twice with PBS and stained with methylene blue (Sigma, USA). Aggregates of approximately $\geq$50 cells were scored as a colony and counted under a microscope (Axiovert 40CFL, Carl Zeiss, Göttingen, Germany).

OSTEOGENIC DIFFERENTIATION

To examine the osteogenic differentiation, the cells were seeded at a density of 25,000 cells/wells in a 24-well-plate and maintained in an osteogenic medium [growth medium supplemented with ascorbic acid (50 μg/ml), dexamethasone (100 nM), and β-glyceraphosphate (10 mM)]. In the test wells, basic FGF (20 ng/ml) was added in the culture medium, untreated wells served as controls. The medium was changed every 48 h. Alkaline phosphatase activity, osteoblast marker gene expression, and calcium deposition were investigated using the methods described below.

ALKALINE PHOSPHATASE ACTIVITY ASSAY

Alkaline phosphatase activity was determined using *p*-nitrophenol phosphate as the assay substrate. The cells were lysed in alkaline lysis buffer. Aliquots were incubated at 37°C in a solution containing 2 mg/ml *p*-nitrophenol phosphate (Invitrogen), 0.1 M 2-amino-2-methyl-1-propanol (Sigma) and 2 mM $MgCl_2$. After 30 min, 50 mM NaOH was added to stop the reaction. The presence of *p*-nitrophenol was measured at an absorbance of 410 nm. Total cellular protein was determined using a BCA assay (Thermo Scientific, USA). The enzyme activity was normalized to total cellular protein.

MINERALIZATION ASSAY

The cells were fixed with cold methanol for 10 min, washed with deionized water, and stained with 1% Alizarin Red S solution (Sigma) for 3 min at room temperature on a shaker. The amount of calcium deposition was quantified by destaining with 10% cetylpyridinium chloride monohydrate (Sigma) in 10 mM sodium phosphate at room temperature for 15 min. The absorbance was measured at 570 nm.

NEUROSPHERE FORMATION ASSAY

The cells were seeded in 60-mm-petri dishes (5×10^5 cells/plate) and maintained in neurobasal medium containing B27 (2%), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (5 μg/ml) (neuroinductive medium) as well as supplemented with basic FGF (20 ng/ml), EGF (20 ng/ml) for 7 days. To evaluate the influence of growth factors on neuronal differentiation, cells were divided into four groups; group 1: the control neuroinductive medium, group 2: the neuroinductive medium supplemented with EGF (20 ng/ml), group 3: the neuroinductive medium supplemented with basic FGF (20 ng/ml) and group 4: the neuroinductive medium supplemented with EGF (20 ng/ml) and basic FGF (20 ng/ml). For determination of intracellular transduction pathways, the FGFR inhibitor (SU5402; Calbiochem, USA) or PLCγ inhibitor (U73122; Calbiochem) was added in the culture condition. The images of the neurosphere cultures were randomly captured using a phase contrast microscope. The diameter of each sphere was determined using ImageJ software and then the percentage of neurosphere diameter was calculated and categorized into three groups; group 1: the spheres with a diameter <50 μm, group 2: the spheres with a diameter 50–100 μm, and group 3: the spheres with a diameter >100 μm.

For the cell migration study, similar sizes of neurospheres were placed on Col IV coated dishes for 48 h under various culture conditions as described above. The descriptive evaluation was performed by image analysis.

In some experiments, the neurospheres were dissociated into a single cell suspension by pipetting. The cells were then plated on Col IV coated 24 well plates at a density of 2×10^4 cells/well and maintained in the same culture conditions described previously for 7 days. Subsequently, the mRNA expression of neurogenic markers was evaluated using RT-PCR. The expression human β3-tubulin was also evaluated by immunocytochemical analysis.

REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Total cellular RNA was extracted with Trizol reagent (Roche Diagnostics, USA). RNA samples (1 μg) were converted to cDNA by avian myeloblastosis virus (AMV) reverse transcriptase (Promega, USA). A semi-quantitative polymerase-chain reaction (PCR) was performed using Tag polymerase (Invitrogen). The oligonucleotide sequences of the primers, annealing temperature, and number of cycles were shown in Supplementary Table S1. The amplified DNA was then electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide (Sigma)fluorostaining.

IMMUNOCYTOCHEMISTRY STAINING

The cells were fixed in 4% paraformaldehyde (MERCK) at room temperature for 30 min, permeabilized with 0.15% Triton®-X100 (USB Corp., Cleveland) in PBS and 10% horse serum for 1 h at room temperature. The cells were then incubated with primary antibody, mouse anti-β3-Tubulin (cat. No. G7121, Promega, USA) at a 1:200 dilution for 18 h at room temperature. After washing with PBS, the cells were incubated with biotinylated rabbit anti-mouse antibody (Zymed, San Francisco) for 30 min. Subsequently, the antibody was detected using Strep-FITC (Sigma) and the nuclei were counter-stained with DAPI (0.1 μg/ml, cat. No. D8417, Sigma). The cells were analyzed with a fluorescent microscope (Axiovert 40CFL, Carl Zeiss). In each experiment, the acquiring parameters were consistently set. The images were acquired using Axiocam MRc5 and the AxioVs40v4.7.2.0 software.

STATISTICAL ANALYSES

All experiments were performed in triplicate. Data were reported as mean ± standard deviation. Statistical analyses were performed using two-independent Student *t*-test for two-group comparison. A one-way analysis of variance (ANOVA) followed by Tukey HSD test was employed in some experiments (containing ≥three-group comparison). Differences at $P < 0.05$ were considered to be statistically significant.

RESULTS

CHARACTERIZATION OF DPSCs

The isolated cells exhibited a spindle shaped, fibroblast-like morphology (Fig. 1Aa) and were positively stained with Stro-1 antibody (Fig. 1Ab). These cells expressed mRNA markers for both embryonic (Oct4 and Nanog) and mesenchymal stem cells (CD44 and CD73) (Fig. 1Ac). The Oct4 protein expression was also observed by immunohistochemical staining (data not shown). These results imply that the isolated cells have stem cell-like characteristics as shown by stem cell marker gene expression.

The multipotency of the DPSCs was evaluated by osteogenic and neurogenic induction, representing mesodermal and ectodermal differentiation potency, respectively. Upon cultured in osteogenic medium for 14 days, a significant increase in mineral deposition was noted (Fig. 1Ba). Further, increased expression of odontoblast/osteoblast marker genes; DMP-1, Col I, and OCN was observed (Fig. 1Bb). Under neurogenic differentiation conditions, neurosphere formation was seen (Fig. 1Ca,b). Small spheres were observed by day 1 with the size and cellular density of the spheres increasing over time. We also investigated the capacity of the neurospheres to continue clonal expansion by dissociating the spheres into single cells and further culturing in neurosphere inductive conditions. The results showed that the dissociated cells from the spheres were able to form secondary and tertiary neurospheres in culture with relatively similar morphology to those of primary spheres (Supplementary Fig. S1). After plating neurosphere-derived cells on Col IV coated dishes, the extension of long thin cytoplasmic processes was observed. By immunocytochemical analysis, the neurosphere-derived cells were also positively stained with an antibody to β3-tubulin, neuronal

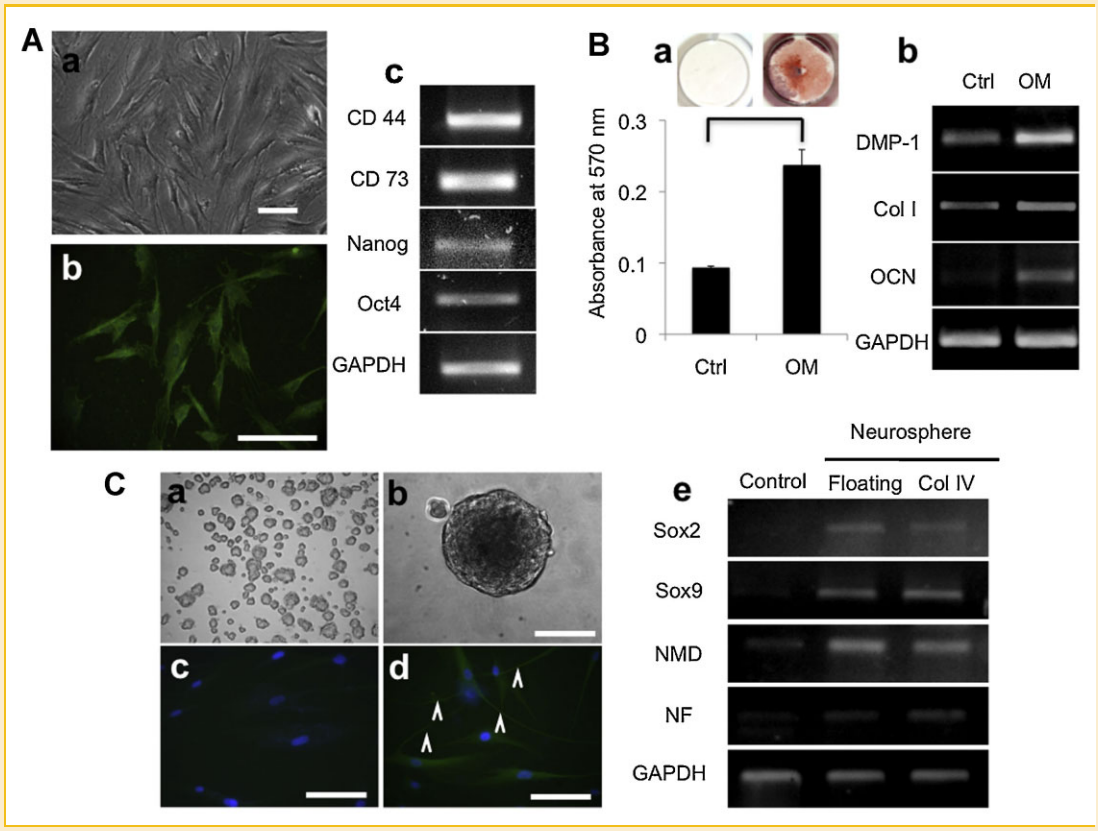

Fig. 1. Human dental pulp stem cells (DPSCs) characterization. A: DPSCs exhibited fibroblast-like morphology (a) and positive Stro-1 staining (b). Expression of CD44, CD73, Oct4, and Nanog was noted (c). B: Osteogenic differentiation potential was illustrated using Alizarin red staining (a) and the mRNA expression of DMP-1, Col I, and OCN (b). Bar indicated the statistical significant difference compared with control ($P < 0.05$). C: Neurosphere formation of DPSCs was observed at day 1 (a) and 7 (b). β3-tubulin immunocytochemical staining of control cells (c) and differentiated cells (d) were shown. The mRNA expression of Sox2, Sox9, NMD, and NF was observed in neurospheres (floating) and neurosphere-dissociated cells dishes on Col IV coated dish compared with control (e). The white arrowheads marked β3-tubulin positive, long, thin cytoplasmic processes of differentiated cells (Scale bars = 100 μm).

specific protein (Fig. 1Cd). In addition, floating neurospheres and adherent neurosphere-derived cells on Col IV expressed higher mRNA levels of neurogenic markers, including Sox2, Sox9, NMD, and NF compared with control (Fig. 1Ce). These data demonstrate the multipotential property of the isolated cells. As shown in these studies, the isolated unsorted heterogeneous cell population from human dental pulp tissues has several stem cell properties, suggesting that the isolated cells contain a stem cell population.

BASIC FGF-ENHANCED COLONY-FORMING UNIT AND THE EXPRESSION OF EMBRYONIC STEM CELL MARKERS

Using low-density culture to evaluate the colony-forming unit ability of DPSCs, the addition of basic FGF (20 ng/ml) in culture medium containing 5% fetal bovine serum significantly increased the DPSCs colony number (Fig. 2B). Descriptively, the colony formation in the presence of basic FGF had higher cell number and density compared with the control (Fig. 2A). In addition, the mRNA expression of stem cell markers (Oct4, Nanog, and Rex1) was increased in the cells exposed to basic FGF (Fig. 2C). No difference in mRNA expression of mesenchymal stem cell marker was observed (data not shown). However, after differentiation, the increase in these embryonic stem cell markers by basic FGF was attenuated (data not shown). Together, these results imply a role of basic FGF in self renewal and maintaining of stemness of DPSCs.

BASIC FGF INHIBITED MINERALIZATION BY DPSCs

As described above, DPSCs were able to differentiate into osteogenic cells. To evaluate the influence of basic FGF in osteogenic differentiation, DPSCs were cultured in osteogenic medium with or without basic FGF (20 ng/ml). The results illustrated that basic FGF significantly decreased ALP enzymatic activity at both day 7 and 14 (Fig. 3A). In addition, the dramatic reduction of mineralization of DPSCs was noted (Fig. 3B). Therefore, basic FGF might alter osteogenic differentiation of DPSCs, at least by attenuating the mineralization process.

BASIC FGF PARTICIPATED A SIGNIFICANT ROLE IN THE NEURONAL DIFFERENTIATION OF NEUROSPHERE-CULTURED DPSCs

Basic FGF and EGF are regularly used as cytokine supplementation in the neurobasal medium to induce neuronal differentiation. Therefore, we further investigated the influence of basic FGF and EGF on neurosphere formation by DPSCs. Culturing DPSCs with either basic FGF, both alone and in combination with EGF, resulted

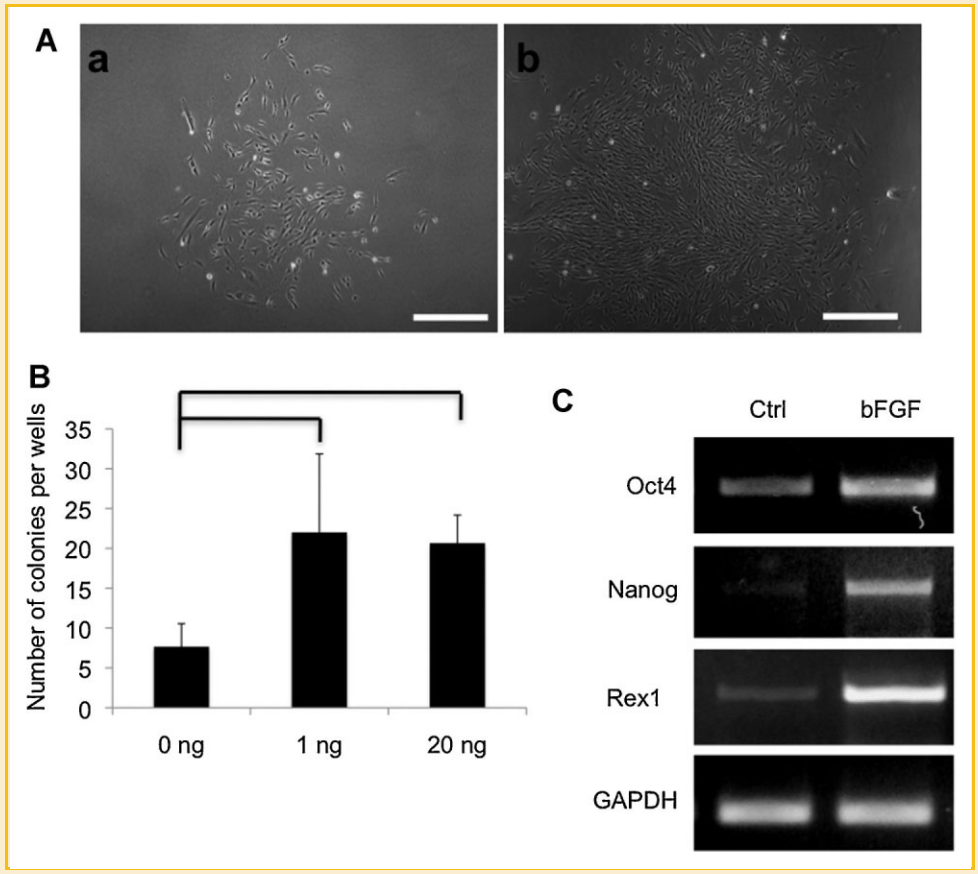

Fig. 2. Effect of basic fibroblast growth factor (basic FGF) on self-renewal ability of dental pulp stem cells (DPSCs). A: Colony-forming unit of DPSCs upon treated with 1 ng/ml (a) and 20 ng/ml (b) basic FGF. B: Graph illustrated the average number of colonies per well. Bars indicated the statistical significant difference compared to control ($P < 0.05$). C: The increase mRNA expression of Oct4, Nanog, and Rex1 in DPSCs treated with basic FGF (20 ng/ml) was noted (Scale bars = 500 μm).

in larger sized neurospheres than the control or EGF supplemented cultured conditions (Fig. 4A). A higher percentage of large neurospheres was observed with the supplementation of basic FGF alone and in combination with EGF compared with control (Fig. 4B). However, the percentage of neurosphere's size was not different between the control culture and the culture treated with EGF alone. To confirm the neurogenic induction property of basic FGF, the mRNA expression of neurogenic markers was examined in DPSC-derived neurospheres. In the presence of basic FGF, higher mRNA expression of Sox2 and β3-tubulin was noted (Fig. 5A). After 7 days of the sphere culture, neurospheres were dissociated into single cells and plated on Col IV coated dish for another 7 days. Cells in culture conditions containing basic FGF (both alone and in combination with EGF) demonstrated a neurite-like morphology with cellular processes (Fig. 5D,E). Moreover, these cells expressed β3-tubulin as determined by immunocytochemical staining. We also investigated the influence of basic FGF on cell migration out from the spheres (Fig. 4C). The neurospheres in all condition were able to attach on Col IV coated dishes. However, less cell migration was observed in the control and EGF culture conditions compared with the cell treated with basic FGF, either alone or in combination with EGF.

Monolayer-cultured DPSCs treated with basic FGF for 48 h exhibited long fine cellular processes with the reflecting cell body, while typical mesenchymal-like morphology was observed in the control culture. This phenomenon could be inhibited by SU5402, a FGFR inhibitor. Further, basic FGF treated monolayer-cultured DPSCs had increase Sox2, Sox9, β3-tubulin, and NMD mRNA levels than those of the control (Supplementary Fig. S2). The accumulated data indicates that basic FGF might be a prime factor influencing neuronal differentiation of DPSCs.

BASIC FGF-INDUCED NEURONAL DIFFERENTIATION OF DPSCs THROUGH THE FGFR/PLCγ PATHWAY

To further determine the participation of FGFR in the basic FGF-induced neuronal differentiation, SU5402 was added in culture to inhibit FGFR. The addition of SU5402 decreased the percentage of large-size neurospheres (Fig. 6A), inhibited cell migration from spheres when plated on Col IV coated dish (Fig. 6B) and reduced the mRNA expression levels of β3-tubulin and NMD (Fig. 6C). We further examined the intracellular signaling pathway as FGFR can activate various signaling molecules. U73122 was employed to inhibit PLCγ. The results showed that U73122 could decrease the percentage of large neurospheres and impeded the migration of cells

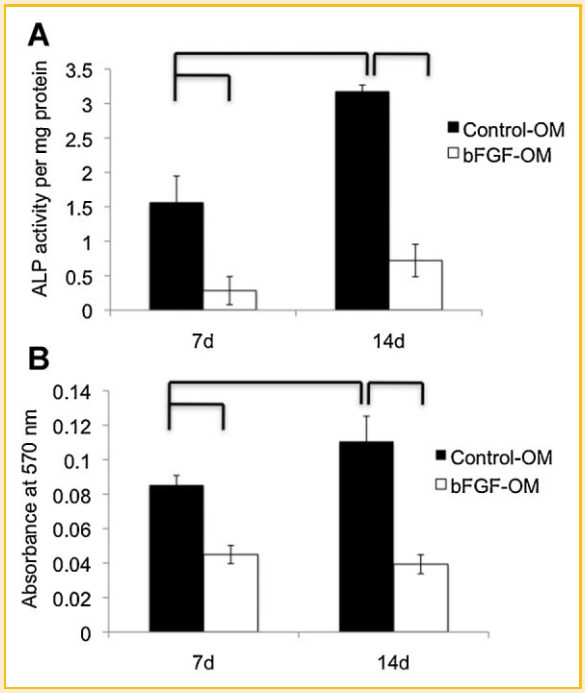

Fig. 3. Basic fibroblast growth factor (basic FGF) inhibited alkaline phosphatase (ALP) enzymatic activity, and mineralization of dental pulp stem cells (DPSCs). DPSCs were cultured in osteogenic medium (OM) with or without the supplementation of basic FGF (20 ng/ml) for 14 days. A: The ALP activity normalized to total cellular protein. B: The absorbance of solubilized alizarin red staining. Bars indicated the statistically significant difference at $P < 0.05$.

out from the spheres in the presence of bFGF. In addition, U73122 was able to decrease the neurogenic mRNA expression (Fig. 6C) and inhibit bFGF-induced neurite-like out growth after plating the dissociated cells on Col IV coated dish similar to those of cells treated with SU5402 (Fig. 7).

DISCUSSION

This study highlights an influence of basic FGF on human DPSCs, particularly on maintaining of pluripotency. In addition, the particular intracellular signal transduction, which was involved in the basic FGF controlling neuronal differentiation of human DPSCs, is illustrated.

In this study, we illustrated that basic FGF enhanced the expression of pluripotentcy marker; Oct4 and Nanog. In addition, Rex1 mRNA expression was increased after DPSCs was treated with basic FGF. As Rex1 has been shown as a target of Oct4 and Nanog [Ben-Shushan et al., 1998; Shi et al., 2006], these evidences may imply the function of Oct4. Thus, it suggests that basic FGF may influence in maintaining stem cells of human DPSCs. Although, Morito et al. [2009] reported that human dental pulp cells treated with basic FGF increased the expression of STRO-1, a marker for mesenchymal stem cells. We did not find the difference in mRNA expression of mesenchymal stem cell markers. Basic FGF may regulate stem cell self-renewal through several mechanisms, that is, the regulation of Nanog promoter activity [Xu et al., 2008], the preferentially activation of specific types of FGFR [Maric et al.,

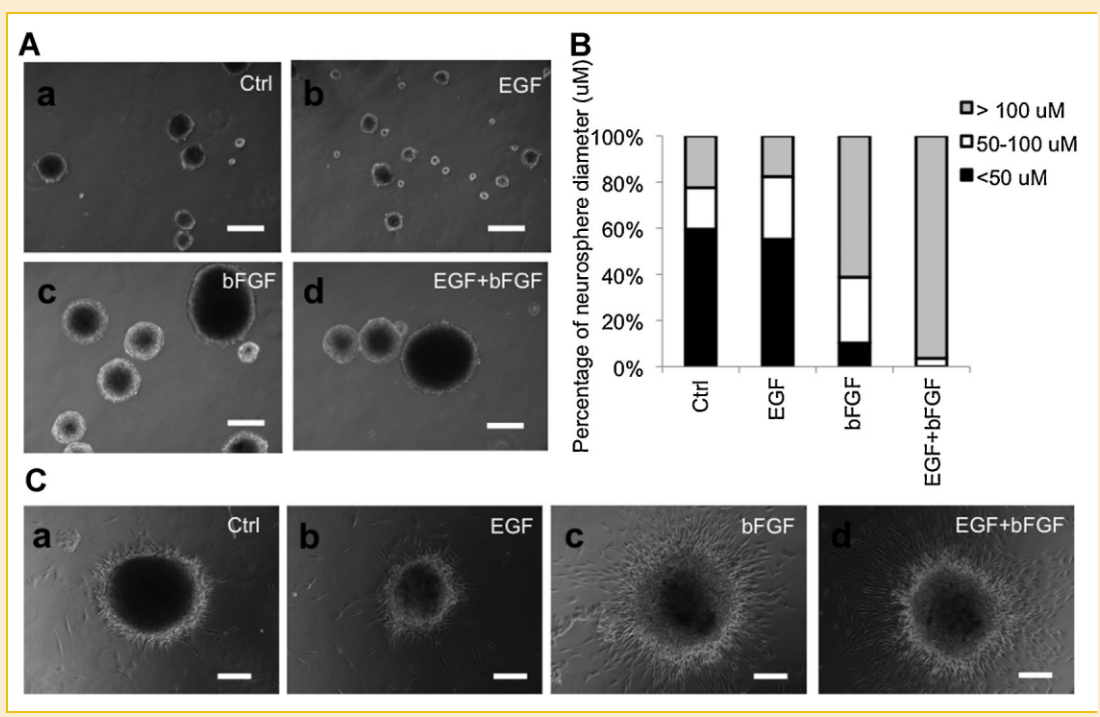

Fig. 4. Basic fibroblast growth factor (basic FGF) influenced neurosphere formation and cell migration. A: Representative neurospheres formed in neuroinductive medium (a), neuroinductive medium + 20 ng/ml EGF (b), neuroinductive medium + 20 ng/ml basic FGF (c), and neuroinductive medium + 20 ng/ml EGF, and basic FGF (d). B: The percentage of neurosphere diameter in each culture condition was illustrated. C: Cell migration on Col IV coated dishes under various culture conditions was also shown (Scale bars = 200 μm).

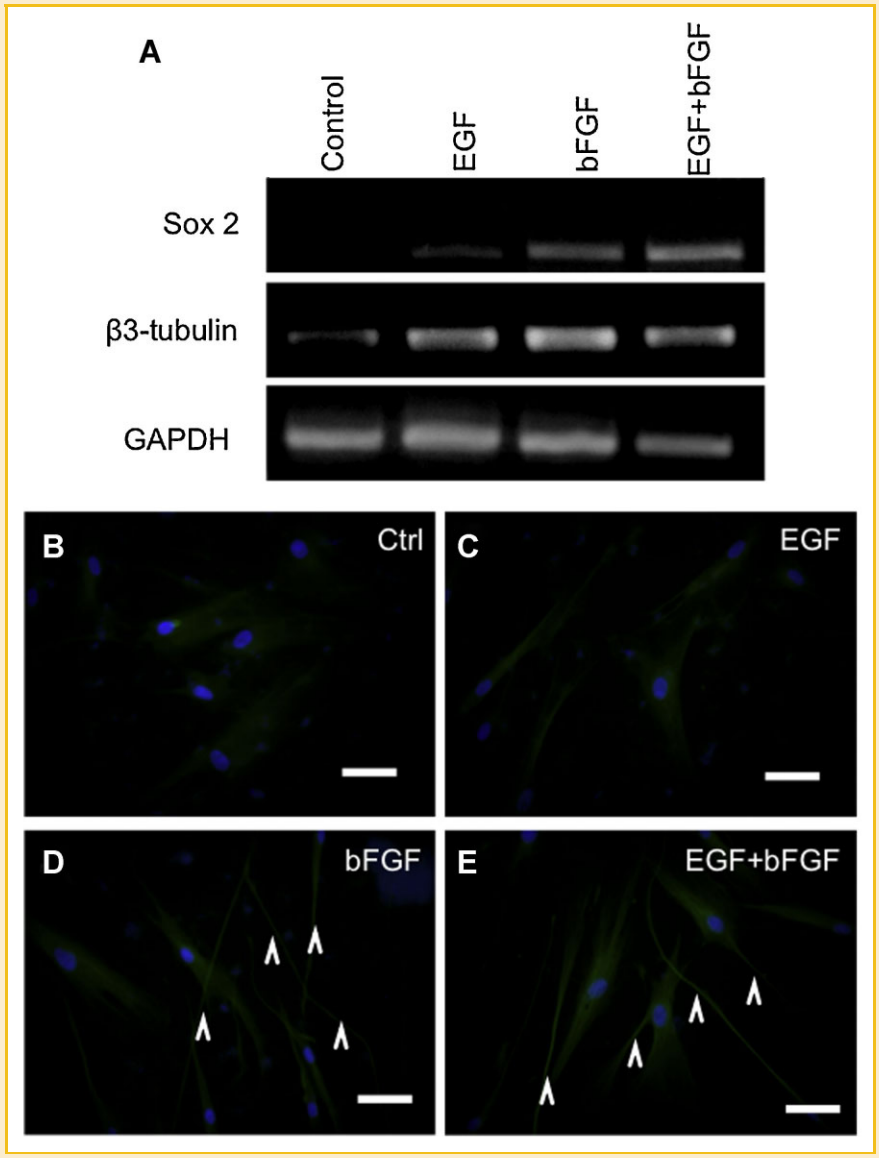

Fig. 5. Basic fibroblast growth factor (basic FGF) enhanced the expression of neuronal markers. A: The mRNA expression of DPSCs derived neurospheres cultured in various cultured conditions is shown. The dissociated cells from the neurospheres plated on Col IV coated dish for 7 days in various cultured conditions and further stained for β3-tubulin; neuroinductive medium (B), neuroinductive medium + 20 ng/ml EGF (C), neuroinductive medium + 20 ng/ml basic FGF (D), and neuroinductive medium + 20 ng/ml EGF and basic FGF (E). White arrowheads marked β3-tubulin positive, long, thin cytoplasmic processes of differentiated cells (Scale bars = 50 μm).

2007], and the regulation of other growth factors influencing self-renewal property of the cells [Park et al., 2009].

Basic FGF treated human DPSCs resulted in the decreased ALP enzymatic activity and mineralization after cultured in osteogenic medium, corresponding to previous reports [Shiba et al., 1995, 1998; Morito et al., 2009; Shimabukuro et al., 2009]. These suppressive effects of basic FGF in DPSCs may occur partly through the mechanism, which basic FGF regulated BMPR expression as similar to those observed in dental pulp stem cell from primary teeth (Supplementary Fig. S3). We hypothesize that basic FGF promotes self-renewing and inhibits abnormal mineralization in dental pulp cavity. Tran-Hung et al. [2008] reported that injured dental pulp cells increased the basic FGF release at early phase and then decreased in later phase. Together with our present data, the increase of basic FGF may play a role in stem cell proliferation to promote healing in early episode and further decrease in later phase to allow mineralized dentin bridge formation.

As shown in the present study, basic FGF promoted neuron-like morphology and expression of neurogenic markers in DPSCs, similar to those reported previously in other cell types [Sasaki et al., 2008; Yang et al., 2008; Govindasamy et al., 2010]. To further elucidate the intracellular transduction pathway, it has been demonstrated that basic FGF-induced neuronal differentiation of murine bone marrow stromal cells was mediated by FGFR1, MAPK/

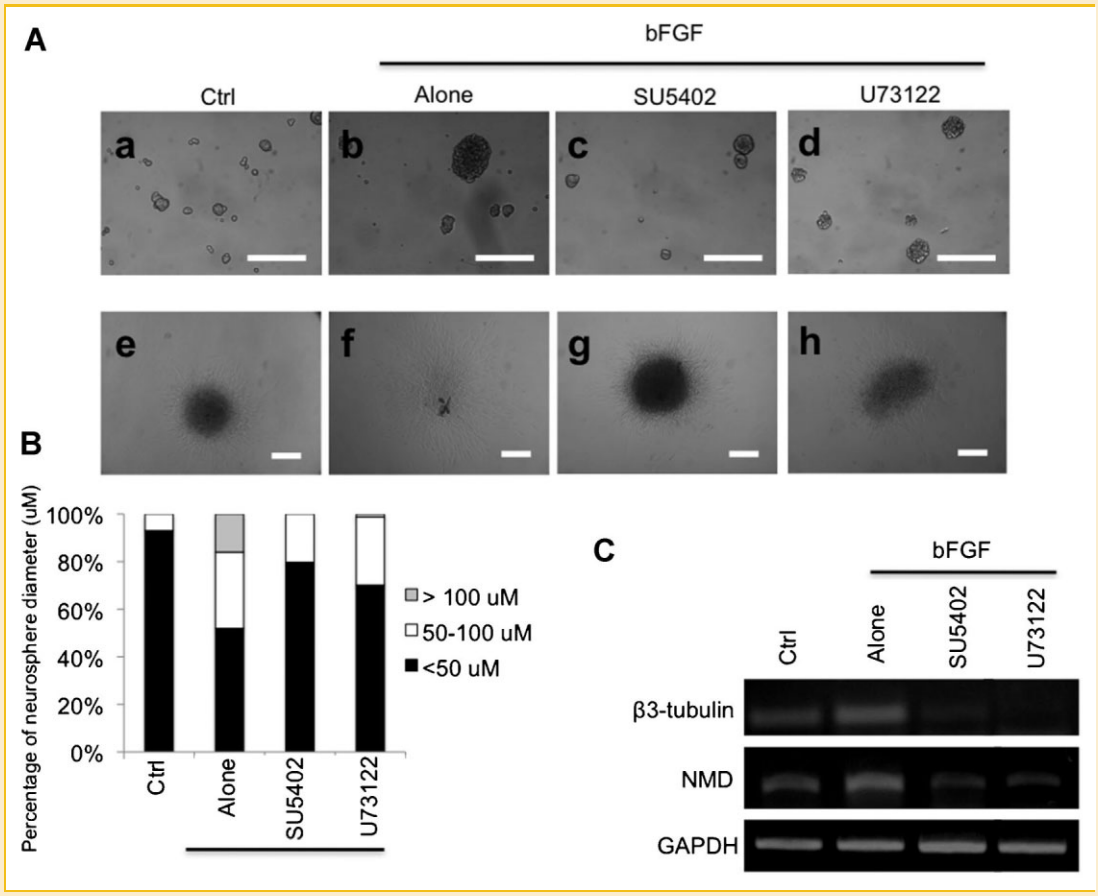

Fig. 6. Basic fibroblast growth factor (basic FGF) enhanced the neurosphere formation expression of neuronal markers through FGFR and PLCγ. A: Neurospheres formed in neuroinductive medium (a), neuroinductive medium + 20 ng/ml basic FGF (b), neuroinductive medium + 20 ng/ml basic FGF + 20 μM SU5402 (c), and neuroinductive medium + 20 ng/ml basic FGF + 2 μM U73122 (d). Cell migration out of the spheres when plated on Col IV coated dishes under previous conditions (e–h). B: Percentage of neurosphere diameter when treated with inhibitors is illustrated. C: The mRNA expression of β3-tubulin and neuromodulin (NMD) was decreased upon inhibited with SU5402 and U73122 (Scale bars = 200 μm).

ERK, and AP-1 with the ERK-activation dependent on PLCγ [Yang et al., 2008]. Similar to those results, our study reveals that basic FGF-induced neuronal differentiation of DPSCs using neurosphere culture was mediated by the FGFR and PLCγ pathway. We also investigated the involvement of MEK signaling using U0126, a MEK inhibitor. U0126 failed to inhibit the expression of β3-tubulin and NMD mRNA (data not shown). The different observations between studies may be due to several parameters including cell types, culture methods, and the concentration of inhibitor used.

Our results indicated basic FGF regulates self-renewal, maintains pluripotency, inhibits mineralization, and promotes neuronal differentiation of DPSCs. There are several plausible explanations of the multiple regulatory functions of basic FGF on DPSCs. First, the specific function of basic FGF occurred upon interaction with specific FGFR in particular cell types. We observed alteration of FGFRs expression in DPSCs when exposed to basic FGF. Increased expression of FGFR2 and FGFR3 was noted in basic FGF treated cells (Supplementary Fig. S4). This evidence suggests that the preferential interaction of basic FGF and FGFRs may occur in order to control of DPSCs functions. Second, a distinct subpopulation of stem cells may respond differently to basic FGF. The regulation of cell fate depended on the concentration of basic FGF, type of stem cells, and the preferential activation of FGFRs [Maric et al., 2007]. Last, the regulation of basic FGF in particular function may require the involvement of other stimulatory mechanisms. The increase in basic FGF released upon dental pulp cell injury might promote stem cell proliferation to facilitate healing rather than expediting the neuronal differentiation of DPSCs. These regulatory function definitely needs other molecules to co-regulate or switch off/on the particular functions. The mechanisms of how basic FGF preferentially regulates self-renewal or neuronal differentiation in DPSCs remains to be further elucidated.

In summary, basic FGF promoted DPSCs self-renewal and neuronal differentiation but inhibited mineralization under osteogenic conditions. In addition, basic FGF regulation of neuronal differentiation of DPSCs occurred through FGFR and PLCγ intracellular transduction pathways. The mechanisms involving the complex regulation of basic FGF in DPSCs requires further study.

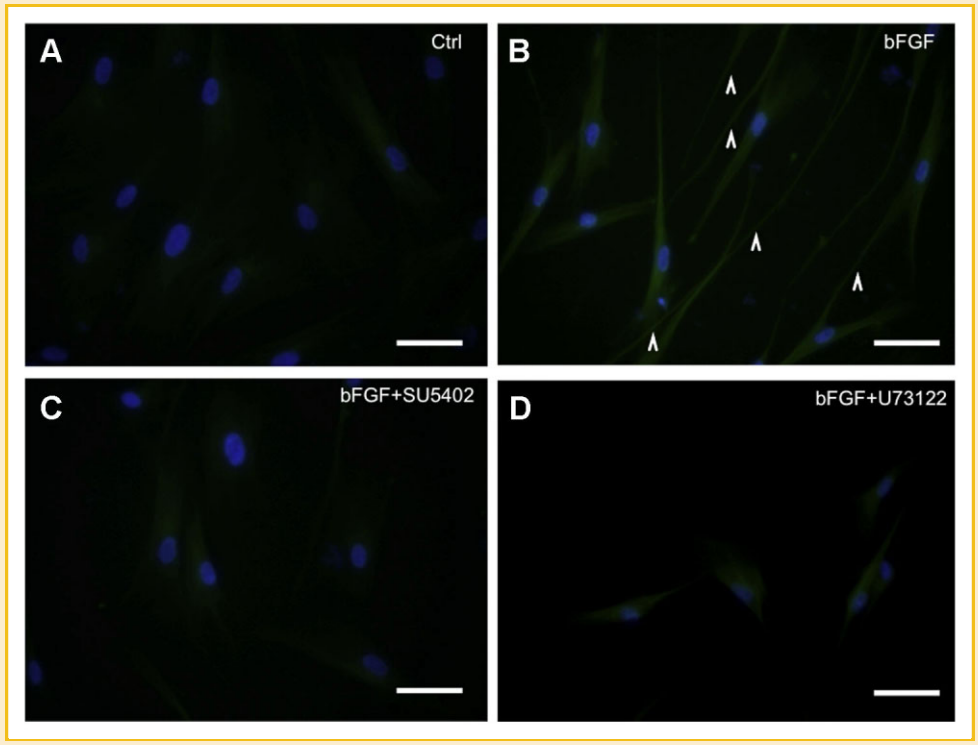

Fig. 7. Basic fibroblast growth factor (basic FGF) mediated the expression of β3-tubulin through FGFR and PLCγ. β3-tubulin expression in dissociated neurosphere cells after 1 week culture on COL IV coated dishes; (A) in neuroinductive medium, (B) neuroinductive medium + 20 ng/ml basic FGF, (C) neuroinductive medium + 20 ng/ml basic FGF + 20 μM SU5402, and (D) neuroinductive medium + 20 ng/ml basic FGF + 2 μM U73122. White arrowheads marked β3-tubulin positive, long, thin cytoplasmic processes of differentiated cells (Scale bars = 50 μm).

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