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Original Article

bFGF enhances activation of osteoblast differentiation and osteogenesis on titanium surfaces via PI3K/Akt signaling pathway

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Abstract: Objectives: The aim of this study was to investigate the role of bFGF during the differentiation of osteoblasts and in the course of osteogenesis on titanium surfaces. Materials and methods: Basic fibroblast growth factor (bFGF, FGF-2), a member of the fibroblast growth factor family, has many biological effects on cell growth, differentiation, and survival. It was added into this study and MC3T3-E1 Subclone14 was cultivated. The MTT method was used to detect the proliferation of osteoblasts. Alkaline phosphatase (ALP) and osteocalcin (OCN) were examined for indicators of osteoblast differentiation and osteogenesis. Immunofluorescence staining was used to observe protein fluorescence intensity and the Western-blot method was used to analyze the change of the proteins and study the PI3K/Akt signaling pathway. Results: The experimental results showed that the OD values of the MTT, ALP, and OCN rose and the expression levels of OPG, Runx2, and BMP-2 proteins increased when bFGF was added. Moreover, the concentration of 40 ng/ml bFGF was the optimum concentration. Furthermore, the results of signaling pathway analysis indicated that bFGF could promote expression of OPG, Runx2, p-Akt, and BMP-2 proteins via an activated PI3K/Akt signaling pathway. Conclusion: A certain concentration of bFGF could promote the proliferation, differentiation, and osteogenesis of osteoblasts on Ti surfaces via an activated PI3K/Akt signaling pathway.

Keywords: Titanium (Ti), osteoblast, basic fibroblast growth factor (bFGF), PI3K/Akt signaling pathway

Introduction

Endosseous titanium (Ti) implants have become an evidence-based treatment modality to replace missing teeth for patients who are either completely or partially edentulous. This treatment concept is based on the biological phenomenon of osseointegration, indicating a direct structural and functional connection between ordered, living bone, and the surface of a load-bearing implant [1, 2]. However, dental implants have low success rates for patients with low bone quality and quantity and for patients whose healing and regeneration capabilities are poor. To solve this problem, in recent years, studies on the integration of implants and bones have investigated the surface morphology and features of implants and bones and have attempted to promote their osseointegration via more microscopic physical and chemical surface treatment and even to biomimetically treat the surface [3, 4]. More recently, studies have focused on the surface upon which various growth factors are assembled. The growth factors delivery systems have been applied to facilitate bone regeneration by using various polysaccharides, such as hyaluronic acid, dextran, chitosan, heparin, etc [5-8]. Among them, studies on applying growth factors, such as the fibroblast growth factors (FGFs), to dental implants are underway.

FGFs can stimulate cell growth, migration, and differentiation. They are involved in development of skeleton, limbs, and the circulatory and central nervous systems [9-13]. FGFs can be classified into 23 structurally-related polypeptides, including the two prototypes, FGF-1 and

FGF-2. As a mitogen inducer, bFGF has a wide range of biological effect on the growth, differentiation, and survival of cells. Recently, many studies have reported that bFGF plays an important role in the proliferation and differentiation of osteoblasts and great efforts have been exerted on elucidating the underlying mechanisms.

However, the underlying mechanisms remain largely unknown on Ti surfaces and the osteoblast differentiation and osteogenesis are complex processes involving many molecules and signaling pathways, some of which need to be newly transcribed and translated. But the exact signaling taking place on Ti surfaces in bFGF-induced pathways have not been documented.

According to some reports, the fibroblast growth factor signaling pathway is one of the most ubiquitous elements in biology [14, 15]. In addition, bFGF exerts its functions by binding to and activating fibroblast growth factor receptors (FGFRs), which have at least four structurally-related intracellular isoforms: FGFR-1, FGFR-2, FGFR-3, and FGFR-4. Although the signaling domains of all four FGFR isoforms are highly conserved, the binding affinity varies significantly, depending on which isoform is bound to bFGF [16, 17]. Several signaling pathways, especially the phosphoinositide 3-kinase (PI3K)/Akt signal transduction pathway, play an important role in mitogenic signaling and cell survival, growth and motility, which can be activated by growth factors and other extracellular signals. At the plasma membrane, PI3Ks catalyze the phosphorylation of phosphoinositides on the 3'-OH (D3) position of the inositol ring to generate 3' phosphorylated phosphoinositides (3-PIs) as second messengers [18-20]. The 3-PIs bind to the pleckstrin homology domain-containing proteins, such as Akt, to activate them [21]. In osteoblasts, the PI3K/Akt signaling pathway is involved in growth and survival [22].

Regarding this study, bFGF was chosen for this experiment based on its features and function. In this study, we evaluated osteoblast functions, such as alkalinephosphatase (ALP) activity, osteocalcin (OCN) and protein expression, and analyzed the mechanism of bFGF activation of osteoblast proliferation, differentiation, and osteogenesis on Ti surfaces. And we seek to prove their relationship to the PI3K/Akt signal pathway taking place on Ti surfaces.

Materials and methods

Biomaterials

Pure titanium disks (22 mm and 34 mm in diameter, 1 mm in cylinder height) were prepared to fit snugly into 12-well plates and six-well plates. Ti disks were manufactured to a rough surface. Processing steps were as follows: (a) Polishing. Titanium plates were polished with #400, #600, #800, and #1200 crystal sand paper, step by step; (b) Sand blasting. White corundum sand blasted at 0.8 pressure (using 60-80 mesh), until the formation of an smooth surface; (c) Cleaning. Plates were washed in turn with acetone, absolute ethyl alcohol, 75% ethanol and ultrasonic cleaning with purified water twice, each sequence lasting 10 min. (d) Passivation. 40% nitric acid passivation for 30 min then double steaming water ultrasonic cleaning, 2 times-each time 10 min and, finally, drying at room temperature; (e) Disinfection. High temperature and high pressure sterilization were applied before irradiation with ultraviolet for 30 min.

Osteoblast culture and osteoblast seeding

MC3T3-E1 Subclone14 was obtained from Shanghai Bioleaf Bitech Co. Osteoblasts were incubated in α-MEM supplemented with 10% fetal bovine serum (Thermo Scientific HyClone, MA, USA) and 1% penicillin/streptomycin (Sigma-Aldrich Corp, MO, USA) under a humidified atmosphere of 5% CO_2 at 37°C. When osteoblasts were cultured to the third or fourth generation, they were seeded at a density of 20,000 cells per ml at 12-well plates and a density of 50,000 cells per ml at six-well plates. All Ti disks were placed in the bottom of plates whose surfaces had been already treated and disinfected. Then the osteoblasts were cultured on the sterilized slide glasses at a density of 20,000 cells per ml for the immunofluorescence staining assay.

Osteoblast viability

Osteoblasts were seeded on the Ti disks for analysis of osteoblast proliferation. After 24 h the 12-well plates were divided into five groups and the first group was marked as 10 ng/ml, 20

ng/ml, 40 ng/ml, and 80 ng/ml. Cell viability was investigated after seeding the osteoblasts for 1, 2, 3, 4 and 5 days using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay. At the selected incubation periods, 200 μl of MTT were added to a 12-well plate. After 4 h of incubation in 5% CO_2, the culture medium was removed carefully, 2 ml of dimethyl sulfoxide (DMSO) was added to each well, and the polystyrene plate was shaken for 20 min. The solutions were transferred (200 μl) to a 96-well plate and the absorbance of each solution was measured at the wavelength of 429 nm by spectrophotometer. The optical density (OD value) was recorded. Each group's (3 holes) sample readings average was recorded as the experimental results. Each step was repeated three times, and then we drew a bar graph of the OD values to observe the results.

Alkaline phosphatase activity assay

Alkaline phosphatase (ALP) activity was assayed by measuring the OD value. On the 4th, 8th and 12th day, the osteoblasts were lysed with a cell lysis reagent (80 μl) (Sigma-Aldrich, St Louis, MO, USA) for 20 min on the table concentrator at 4°C. Then, the 80 μl cell lysate samples were transferred to the centrifuge tubes. The samples were centrifuged for 10 minutes at 4°C. After completion of above steps, we used an alkaline phosphatase activity assay kit (Wako Pure Chemical Industries, Ltd. 1-2, Doshomachi 3-Chome, Chuo-Ku, Osaka, Japan) to detect the OD value. This kit consists of substrate tablets, standard solution, buffer solution and stop solution. The procedures were as follows: First, a piece of a substrate tablet was dissolved in 5 ml buffer solution to obtain a working fluid. Second, the standard solution was diluted with distilled water to the desired concentration (0.5 mol/ml, 0.25 mol/ml, 0.125 mol/ml and 0.0625 mol/ml). Third, taking a 96-well plate which to add 100 μl working fluid, 20 μl samples and dilution of standard solution to each hole was shaken for 1 min after 30 incubation min in the CO_2 incubator. Fourth, the 96-well plate received the 80 μl stop solution and shaken 1 min. to determine the OD values. Then we took the recorded OD values and the absorbance of different concentrations of standard solution to make a standard curve and calculate the ALP activity. Each step was repeated three times and graphs were drawn to observe the results.

Immunofluorescence staining

When osteoblasts were seeded, the plates were divided into two parts. One part received bFGF (40 ng/ml) and the other remained as a control group. The osteoblasts were twice rinsed in PBS 24 h later, and they were fixed for 30 min with 4% paraformaldehyde. The cytomembrane were ruptured by PBS/Triton X-100 (0.5%) and then incubated for 30 min with blocking PBS solution containing 3% BSA. The osteoblasts were subsequently incubated for 2 h with a mixture of 1:100 primary antibodies, including anti-BMP2, anti-Runx2, and anti-OPG in a blocking solution at 37°C temperature. After washing in PBS, the specimens were incubated for 1 h at room temperature in the dark with the respective secondary antibodies diluted 1:200 in PBS. The samples were viewed on Nikon ECLIPSE Ti microscope at the appropriate excitation wavelength, depending on the fluorochrome.

Western blotting

On the sixth day, the cultured osteoblasts were divided into five groups, four experimental groups and the other was the control group. The experimental groups received respective bFGF concentrations of 10 ng/ml, 20 ng/ml, 40 ng/ml, and 80 ng/ml. The next day MC-3T3-E1 cells were harvested at selected time points (Day 7) and were then lysed in a lysis buffer. Protein samples (80 μg) were mixed with 1/4 volume of 5×SDS loading buffer and purified water boiled for 10 min, and were then subject to a 10% gel. Following a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to polyvinylidene difluoride membranes. After blocking with 5% fat-free milk, the membranes were incubated with the following antibodies: anti-osteoprotegerin antibody (ab9986; Abcam, No.8 Science Park West Ave HKSP, New Territories, HK), anti-BMP2 antibody (ab14933; Abcam, No.8 Science Park West Ave HKSP, New Territories, HK), Runx2 (Cell Signaling, Danvers, MA) and GAPDH overnight at 4°C. The secondary antibodies used were goat anti-rabbit and rabbit anti-mouse for 1 h at room temperature. The signals were viewed with the ChemiDicTM XRS+ Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) and the band densities were quantified with Multi Gauge Software of Science Lab 2006 (FUJIFILM

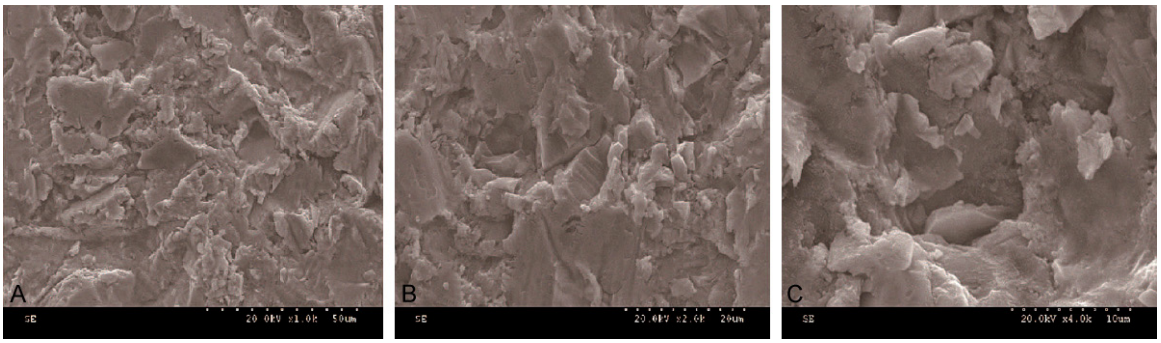


Figure 1. Surface morphology of titanium disks under different magnifications of scanning electron microscopy (SEM). A. 1000 times. B. 2000 times. C. 4000 times.

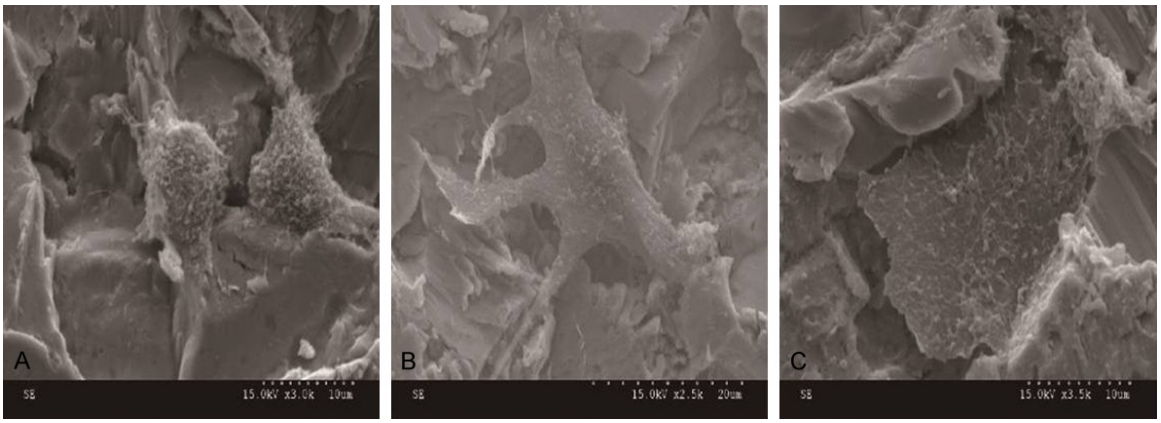


Figure 2. Three forms of osteoblasts at different magnifications of the scanning electron microscope. A. On the first day, under the SEM magnified 3000 times, osteoblasts were barely attached to the titanium surface; this type of osteoblast was small and either round or oval. B. Displayed under the SEM magnified 2500 times: on the second day, osteoblast was differentiating, but not yet fully spread. With time, it extended into the surrounding tissue. C. On the third day under the SEM magnified 3500 times, we can see osteoblasts had been deformed and fully spread; the volume of Type III osteoblast was bigger than the previous two types of osteoblasts and formed a typical triangular, polygonal shape.

Corporation, Tokyo, Japan). The experiment was repeated three times, at least.

Statistical analysis

Differences between conditions were examined for statistical significance using Student's t-test and one-way analysis of variance. *P* values of < 0.05 were considered statistically significant according to SPSS 19.0.

Results

Ti surface characteristics and osteoblasts growth

The SEM images showed that the processed surfaces had an uneven and rough morphology surface at different magnifications (**Figure 1**). At the 1, 2, 3, 4 and 5-day time points, there were significant differences in numbers of attached osteoblasts on Ti surfaces. The osteoblasts grew well on the Ti surface, and we observed their morphological growth using a scanning electron microscope on the second, third and fourth day. As seen from the pictures in **Figure 2**, three types of cells appeared. Type I: Osteoblasts were egg-like class. Cells had just attached to the surface of titanium, yet were not stretching. Type II: Osteoblasts appeared as deformed cells that gradually extended to the surrounding synapses, not stretching fully. Type III: Osteoblasts were completely spread, forming polygonal membranes and had the morphological characteristics of osteoblasts (**Figure 2**). In this study, there were more Type I and II osteoblasts than Type III on the

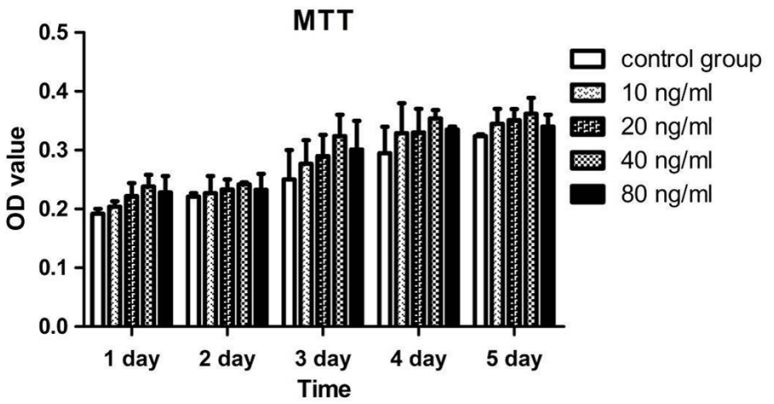


Figure 3. Effects of bFGF on osteoblast proliferation. On these five days the OD value of every group was continuously increasing over time. The OD value of all experimental groups were higher than the control group every day on the Ti surface and the OD values of 10 ng/ml, 20 ng/ml and 40 ng/ml showed an increased trend over time; the daily OD values of the 40 ng/ml bFGF group were the highest of all the groups.

first to the second day and more of Type II and III than of Type 1 on the third to the fourth day. On the fifth day, almost all cells found were the third type.

bFGF promoted proliferation, differentiation and osteogenesis of osteoblasts on Ti surface

The results of MTT showed that the OD values of the four groups that received bFGF were higher than those of the control group and the OD values of the 10 ng/ml, 20 ng/ml, and 40 ng/ml groups tended to rise over time. However, these values tended to decrease between the 40 ng/ml to 80 ng/ml group over time. The OD value of the 80 ng/ml bFGF group was still higher than the control group (**Figure 3**).

The line charts and the histograms had been drawn to detect the ALP activity tendency. For all groups with or without bFGF, ALP activities were starting to ascend at Day 4 and by Day 8 were significantly higher than at Days 4 and 12. The peak of ALP activation on the Ti surfaces appeared in the 40 ng/ml bFGF group compared with the other groups at Day 8. With 40 ng/ml bFGF, osteoblast differentiation on Ti surfaces demonstrated statistically higher ALP activities than those without bFGF ($P < 0.05$) (**Figure 4**).

The experimental results showed an increasing tendency for the expression of OCN from the 6th to 18th day. But on the 18th to 24th day, OD values showed a downward trend. However, the OD values of the experimental groups were higher than those of the control group, and in the experimental groups of 10 ng/ml, 20 ng/ml and 40 ng/ml bFGF, the OD values showed an increasing tendency over time a decreasing trend between 40 ng/ml and 80 ng/ml bFGF. But the OD values of the 80 ng/ml bFGF groups were still higher than those of the control group. The top value of OCN was reached at Day 18 with the concentration of 40 ng/ml bFGF on Ti surfaces (**Figure 5**).

bFGF accelerates osteoblasts secretion OPG, RUNX2 and BMP-2 proteins on Ti surface

The Western-blot results were consistent with immunofluorescence results. According to the immunofluorescence results, when the concentration of bFGF was 40 ng/ml, the fluorescence was significantly more intense than that of the control group and, when seen with the scanning electron microscope, the number of osteoblasts in the experimental groups was greater than those in control groups. Moreover, throughout the experiment, bFGF could alter osteoblast morphology, compared with the control group. With the concentration of 40 ng/ml bFGF, the shape of osteoblasts became more fusiform and slender, compared with the control group.

As shown in **Figure 6**, expression levels of bFGF experimental groups were markedly higher than the control group. The 40 ng/ml bFGF groups had significantly increased expression of Runx2, OPG and BMP-2, compared with the other bFGF groups. The relative expression tendency of the 10 ng/ml bFGF groups on the Ti surface was nearly the same as the 80 ng/ml bFGF group. The 40 ng/ml bFGF groups induced higher levels of expression of Runx2, OPG and BMP-2 proteins compared with the control groups; this difference were statistically significant ($P < 0.05$) on the Ti surface.

bFGF activated the PI3K/Akt signaling pathway to promote osteoblast differentiation and osteoblastogenesis on the Ti surface

To identify whether bFGF activates the PI3K/Akt signaling pathway to promote osteoblast

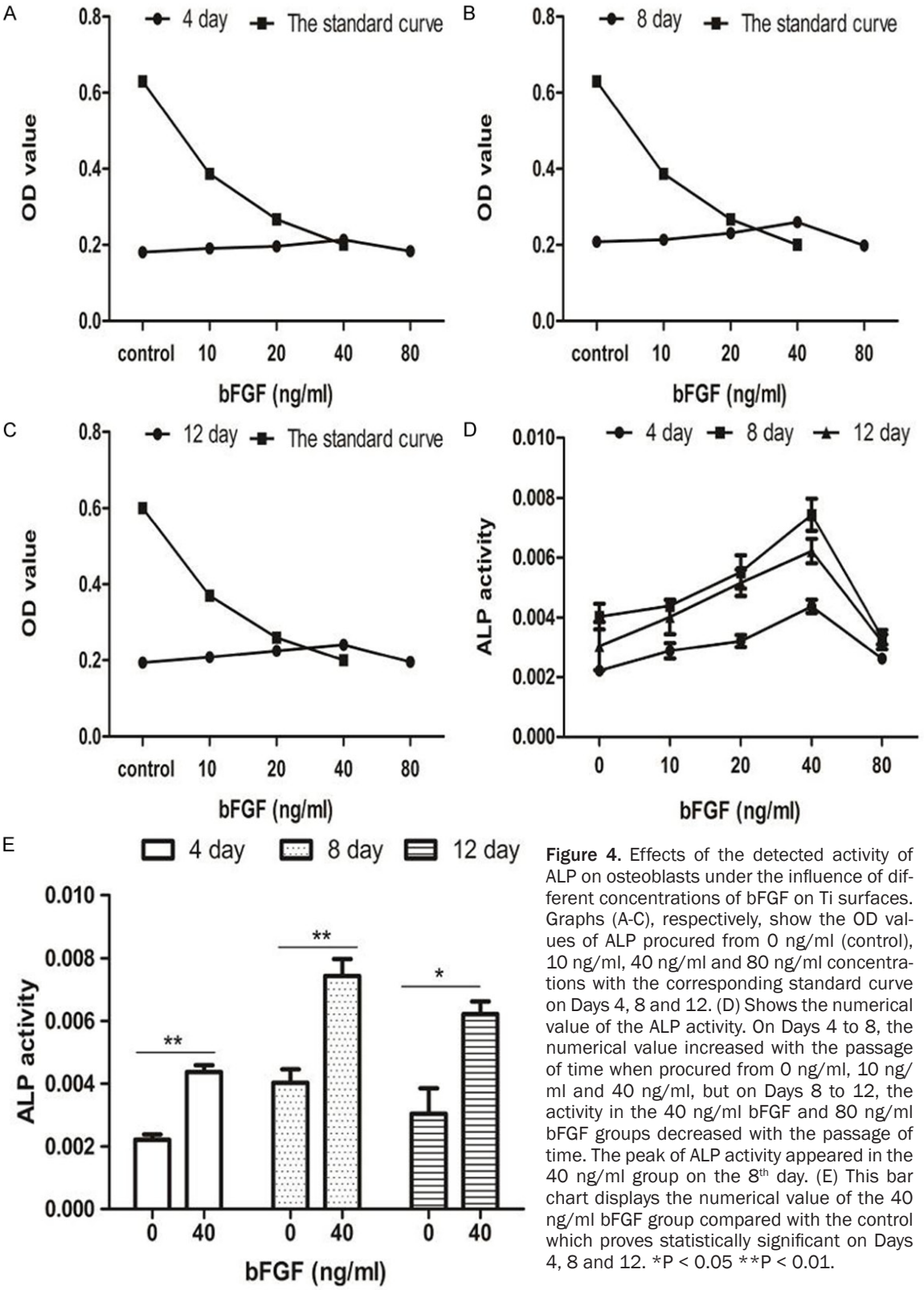


Figure 4. Effects of the detected activity of ALP on osteoblasts under the influence of different concentrations of bFGF on Ti surfaces. Graphs (A-C), respectively, show the OD values of ALP procured from 0 ng/ml (control), 10 ng/ml, 40 ng/ml and 80 ng/ml concentrations with the corresponding standard curve on Days 4, 8 and 12. (D) Shows the numerical value of the ALP activity. On Days 4 to 8, the numerical value increased with the passage of time when procured from 0 ng/ml, 10 ng/ml and 40 ng/ml, but on Days 8 to 12, the activity in the 40 ng/ml bFGF and 80 ng/ml bFGF groups decreased with the passage of time. The peak of ALP activity appeared in the 40 ng/ml group on the 8th day. (E) This bar chart displays the numerical value of the 40 ng/ml bFGF group compared with the control which proves statistically significant on Days 4, 8 and 12. *P < 0.05 **P < 0.01.

differentiation and osteoblastogenesis on the Ti surface, the osteoblasts were divided into two groups after completion of the culture. Only one group was treated with 10 μM LY 294002,

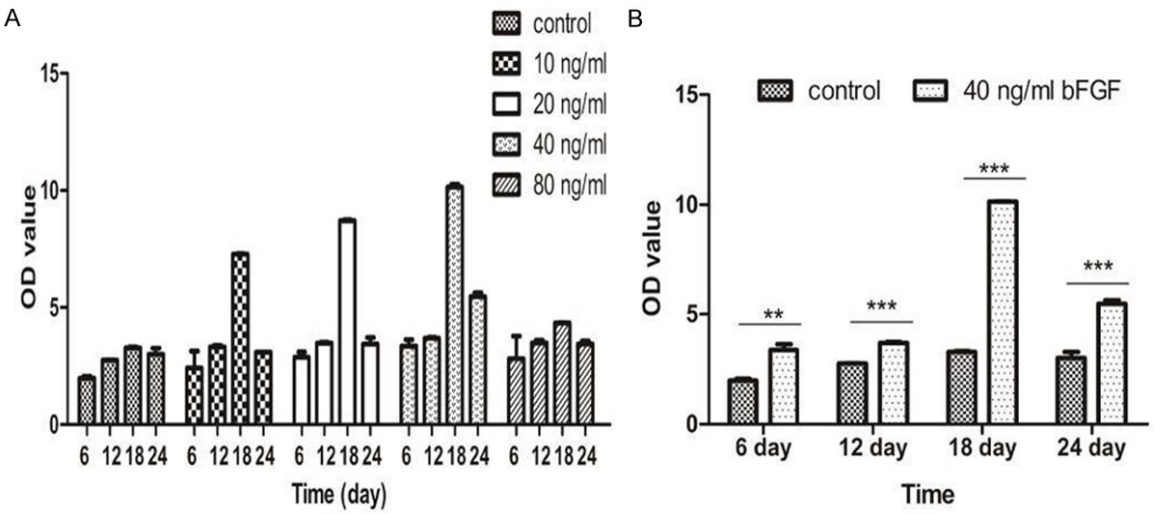


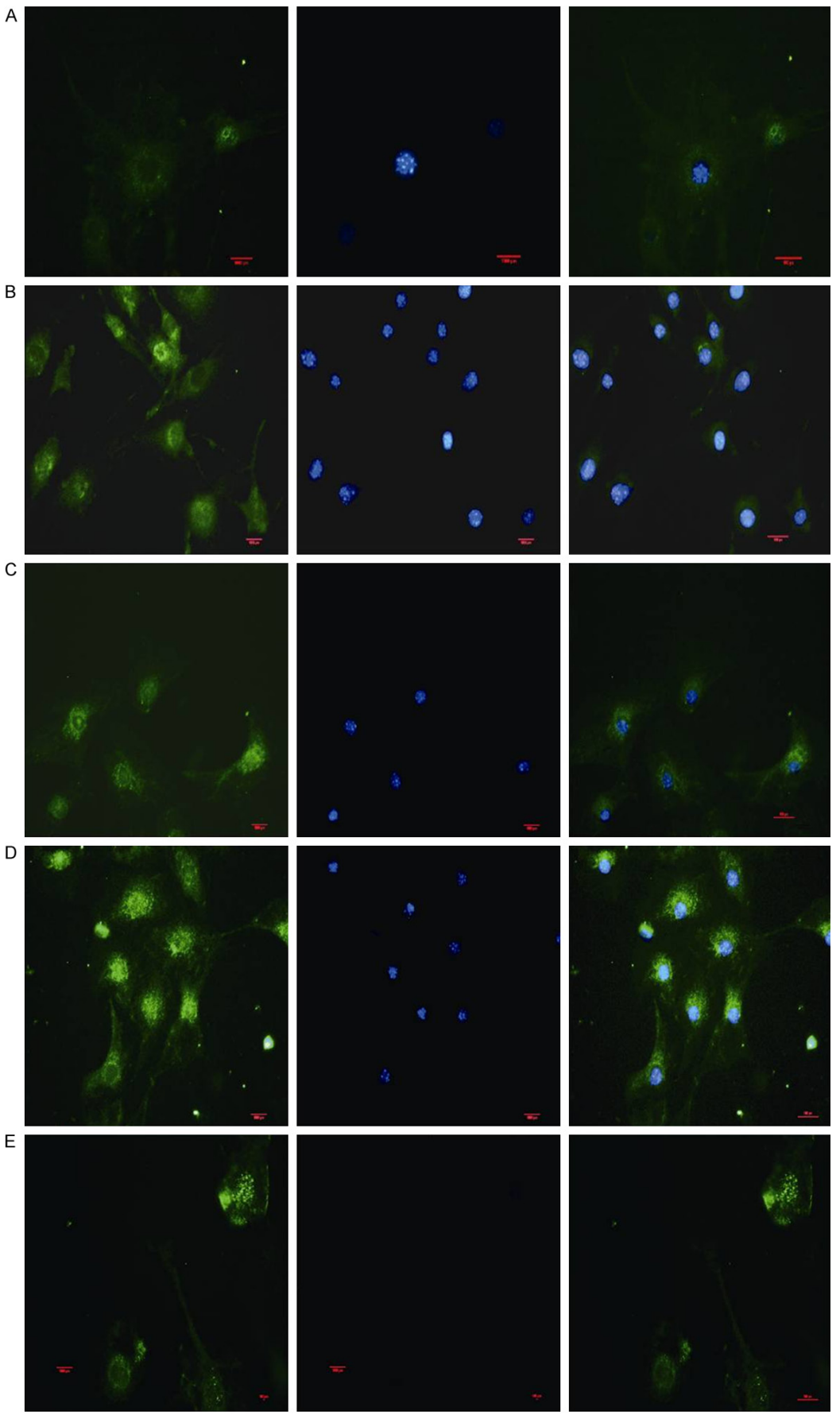
Figure 5. Effects of the osteocalcin quantitative detection on osteoblast under the influence of different concentrations of bFGF on Ti surface. A. The bar chart shows the OD values of OCN for the experimental groups and control group increased as the time passed from Days 6 to 18. From Days 18 to 24, OCN content decreased over time. The OD values of the experimental groups of 10 ng/ml, 20 ng/ml and 40 ng/ml bFGF concentrations all tended to increase, and they were all higher than the control. On Days 18 to 24, the 80 ng/ml group values were all lower than those of the 40 ng/ml group, but higher than the control. B. This chart showed the statistical comparison of the control group with 40 ng/ml bFGF group. On Days 6, 12, 18 and 24, the OD values for the 40 ng/ml group were higher than for the control and every two groups of contrast had statistical significance **P < 0.01; ***P < 0.001.

but both were used to examine the level of OPG, BMP-2, Runx2, p-Akt and total Akt. No marked alterations in total Akt on Ti surfaces were observed whether with or without LY 294002. When considering the change in levels, the total Akt and GAPDH were used as a control. According to the comparison of bFGF groups with the control groups, the levels of the OPG, BMP-2, Runx2, and p-Akt proteins had risen. Furthermore, we detected less expression of these proteins in the bFGF groups compared with the controls. Protein levels in the 40 ng/ml bFGF groups were obviously the lowest when compared with the other groups and the difference seemed statistically significant between them ($P < 0.05$) on the Ti surface (**Figure 7**).

Discussion

At present, the clinical success of dental implants is highly dependent on cell adhesion, matrix production, and anti-inflammatory and mineralization properties of the implant materials. This study mainly observed aspects of cell adhesion and cell proliferation. According to some reports, numerous surface modifications have been tried to optimize cell and tissue interactions and thereby obtain rapid bone response in endosseous implant therapy. Several studies have suggested that the combined use of submicron-scaled porous Ti oxide structure and a micro-roughened surface may have advantages in improving osseointegration of endosseous Ti implants. The SLActive surface, which is indicated by smaller contact angles and higher surface energy, has been shown to have superior osteogenic properties compared with the conventional SLA surface both in vitro and in vivo [23-25]. In this study, Ti disks were used to create a unified effective matrix for osteoblasts to culture upon. The SEM images showed that the processed surfaces had uneven and rough morphology when seen under different magnifications. As seen from the pictures 2, the osteoblasts grew well on the titanium surface. Therefore, through proper processing, titanium surface can effectively promote cell adhesion and growth.

However, in addition to improving the surface modifications of implants like the SLActive implant, adding the growth factors also can promote the cell adhesion and growth. In vivo, FGFs are responsible for a wide spectrum of biological effects induced in different types of cells. According to some reports, the basic fibroblast growth factor (bFGF), one member of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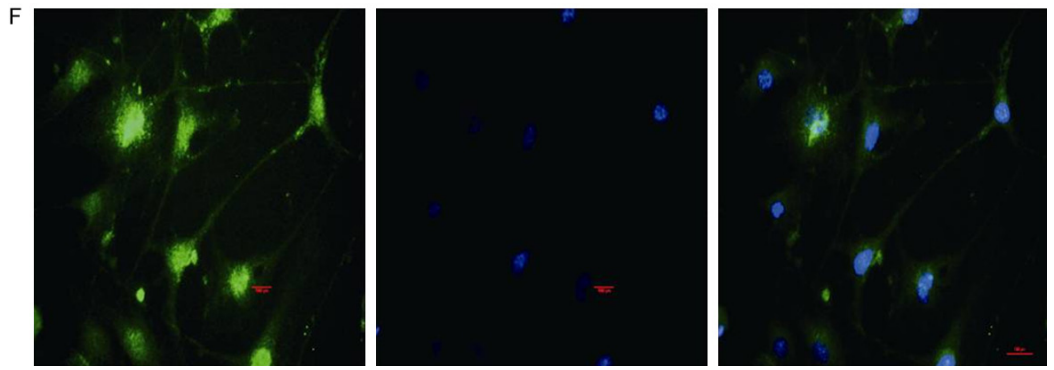

Figure 6. The fluorescence intensity effects and osteoblast density at a concentration of 40 ng/ml bFGF and at control group concentration through immunofluorescence staining assay. A. BMP-2 without bFGF. B. BMP-2 with 40 ng/ml bFGF. C. OPG without bFGF. D. OPG with 40 ng/ml bFGF. E. RUNX2 without bFGF. F. RUNX2 with 40 ng/ml bFGF.

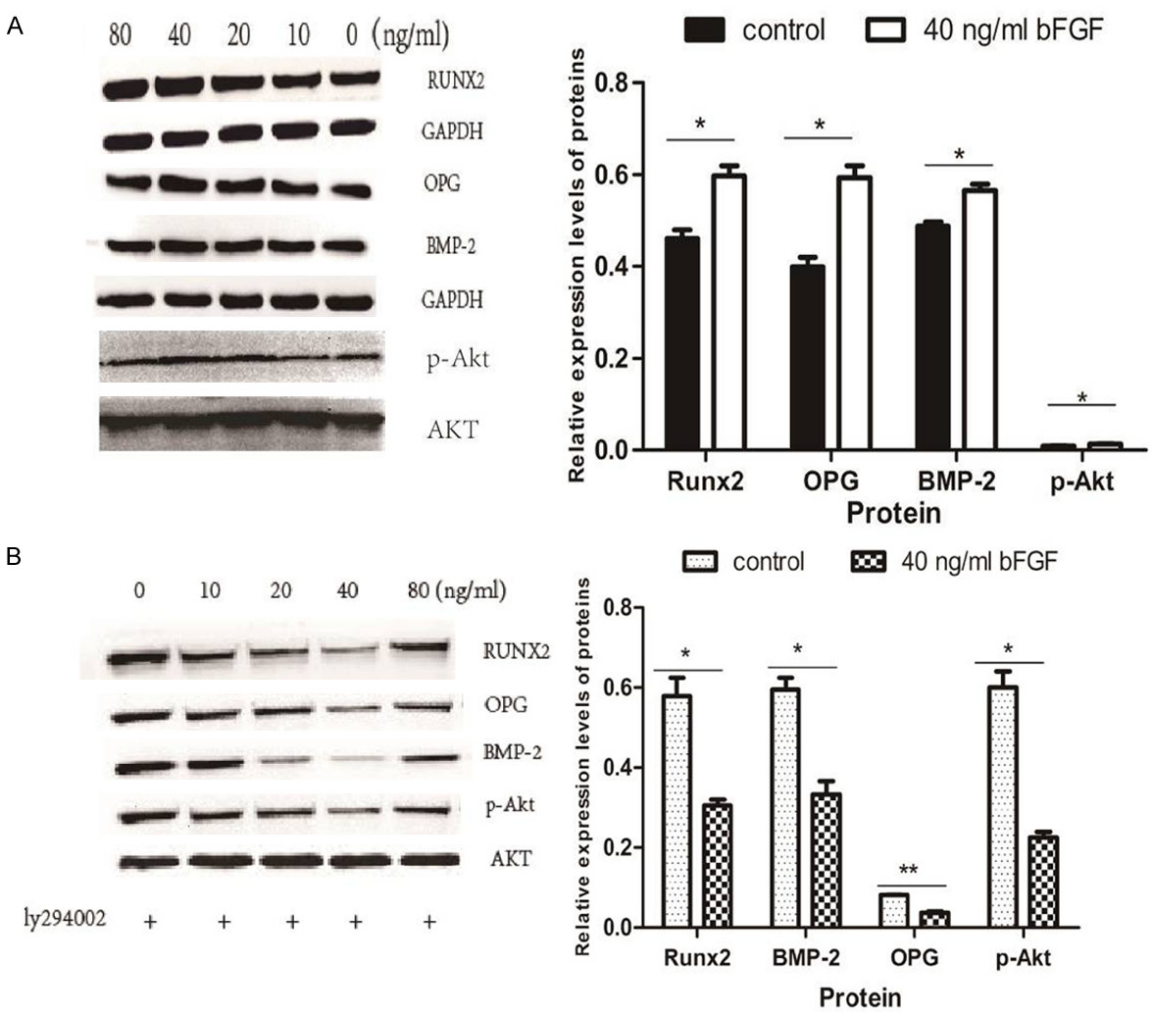

Figure 7. bFGF promotes osteoblast secreted proteins and suppresses OPG, BMP-2, and Runx2 expression through PI3K/Akt signaling pathway on Ti surfaces. A. Western blot analysis of expression of OPG, BMP-2, Runx2 and p-Akt proteins in osteoblasts on Ti surfaces procured from different concentrations of bFGF. B. Western blot assay was performed to detect expression of OPG, BMP-2, Runx2 and p-Akt proteins procured from different concentrations of bFGF with 10 μM Ly294002 added on Ti surfaces. *P < 0.05; **P < 0.01.

the FGFs, has a wide range of biological effects on cell growth, differentiation, and survival. Further, bFGF is associated with wound healing, tissue repair, angiogenesis and is involved in homeostatic regulation in an adult organism. It can promote new blood vessels formation and participate in cell proliferation [9, 12, 26, 27]. 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 [28]. The affinity of bFGF with various FGFRs is different, and the downstream signaling pathways of different FGFRs also vary, [16] although the signaling domains of FGFRs are highly conserved. Several signaling pathways can be activated by FGFRs, such as the cascades of phospholipase Src, Crk, and SNT-1/FRS2, [29] some of which are implicated in bFGF-induced neurogenesis and differentiation [30]. Based on the features and function of bFGF, it was chosen for this experiment from among all FGFs. The osteoblasts' compounded cultivation with bFGF has been reported, but it was very rare when added onto the titanium disks.

In this study, ALP activity and expression of OCN were evaluated. The ALP activity and OCN are widely used, respectively, as markers for early and late differentiation of osteoblasts, which indicates that bFGF stimulates early and late osteoblast differentiation. ALP is a marker for osteoblasts differentiation. As the osteoblasts' main osteogenic symbol of early differentiation, ALP reached peak value at the beginning of differentiation and rapidly downregulated in the process [31-33]. The ALP and OCN results demonstrated significantly higher expression levels of differentiation with bFGF and were compared with controls. The highest level of ALP activity and expression of OCN was observed in the 40 ng/ml bFGF groups on the Ti surface, suggesting that bFGF is superior in eliciting osteoblast proliferation and differentiation that favors osteogenesis. Furthermore, the peak of ALP activity appeared at Day 8. Increased ALP activity is an early marker of osteoblast differentiation and the expression of peak concentration in the 40 ng/ml solution on the Ti surface indicated the osteoblasts at this concentration switched quickly from proliferation to differentiation compared to that of the other concentrations. Therefore, the proliferation and differentiation of osteoblasts were observed to increase when osteoblasts were cultured with the bFGF on Ti surface.

Osteoprotegerin (OPG) is a soluble member of the tumor necrosis factor receptor family (TNFR family). It inhibits the differentiation and fusion of the osteoclastic precursor cells, locks RANKL and maintains control of the bone remodeling process. Osteoblasts differentiation is a process dependent on the presence of major transcription factor Cbfα-1/Runx-2 (core-binding factor-1/runt-related transcription factor-2) [34-36]. According to some reports, OPG can promote the growth of osteoblasts and the apoptosis of osteoclasts. From this study, we saw when bFGF was added, the expression of OPG increased, which was evidence for bFGF promoting osteoblast differentiation and protecting the formation of bone tissue. Further, bone morphogenetic proteins (BMPs) regulate osteoblast differentiation and bone formation. One of the most potent osteogenic signaling molecules is BMP-2, which has been shown to induce several transcription factors, such as Runx2, that promote osteoblast differentiation. Therefore, BMP-2 is potent secreted signaling molecule that plays a central role in bone formation [37]. BMP-2 and Runx2 have synergy with osteoblasts and, thus, are closely related with their differentiation, osteogenesis and signaling pathway. In this study, the expression of BMP-2 and Runx2 were all increased with bFGF, which provides more solid evidence for the bFGF promoted differentiation, osteogenesis of the osteoblasts on Ti surface. Moreover, we found that bFGF led to changing osteoblast forms. The underlying reasons remain unknown. According to some reports, the osteoblasts morphological changes may be associated with enhancement of the osteoblasts proliferation and differentiation.

Further, the fate of a cell depends on mutually exclusive extracellular signaling and the activation or repression of specific transcription factors that affect common intracellular signaling cascades. Osteoblasts function can be regulated by multiple signaling pathways such as MAPK and MEK/ERK signaling pathways, which may also interact with each other and serve as signal integrators [38, 39]. Neto et al. [40] reported a significant reduced phosphorylation of Akt in ascorbic acid/b-glycerophosphate-

induced MC3T3-E1 pre-osteoblast differentiation by using peptide microarray technique, which was further confirmed by Western blotting. PI3K/Akt activity is required during multiple phases of the cell cycle. PI3K had to be downregulated for proper G2/M progression. Thus, inhibition of PI3K/Akt signaling has been proposed as required for proper entry into and exit from mitosis. In addition, the PI3K/Akt pathways do not necessarily remain separate from other signaling pathways.

Suppressions of PI3K/Akt signaling pathway and downstream targets using specific inhibitors has been shown to decrease the expression of OPG, BMP-2, Runx2, and p-Akt. The results of this study indicate that Ly294002 could significantly downregulate the expression of OPG, BMP-2, Runx2 and p-Akt. The decreased expression of proteins treated with Ly294002 can be explained-bFGF activates the PI3K/Akt signaling pathway to promote osteoblast differentiation and osteoblastogenesis on the Ti surface. In this study, levels of Akt, a surrogate for PI3K activation, were analyzed with MC3T3-E1 cells cultured on Ti surfaces, which were found attenuated either by time or by treatment with Ly294002. The proteins with 40 ng/ml bFGF showed statistical significance ($P < 0.05$) compared with the controls with or without inhibitor, which indicated that bFGF via PI3K/Akt signaling pathway affected osteoblast function during the early stage of differentiation and bone formation. If the timing and degeneration rate of the bFGF used were controlled, a more favorable outcome would be expected. To determine how long such growth factors should be released need further studies, but the potential of growth factors effect on cells is believed to be great.

In summary, we have provided evidence that bFGF could promote osteoblast differentiation and osteogenesis in vitro, which occurs via an activated PI3K/Akt signaling pathway on Ti surfaces. Our results provide an insight into the understanding of osteoblasts differentiation, osteogenesis and the mechanism induced by bFGF.

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Disclosure of conflict of interest

None.

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