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# High-Power Nd:YAG Laser Triggers Osteogenesis Through BMP-2 and IGF-1 Signaling Pathways In Osteoblasts

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## Abstract

In the present study, we demonstrated that high-power laser irradiation significantly increased the proliferation of MC3T3-E1 cells. The alkaline phosphatase (ALP) activity of MC3T3-E1 cells was distinctly enhanced after high-power laser irradiation, with an optimal effect observed following treatment for 5 seconds. After the exposure of laser irradiation, MC3T3-E1 cells got a higher level of calcium precipitation. Referring to molecular mechanisms, we found the endogenous gene expression of bone morphogenetic protein-2 (BMP-2) and insulin-like growth factor-1 (IGF-1) was significantly increased by laser irradiation. Furthermore, the protein level of core-binding factor alpha 1 (CBFA1) rose obviously after high-power laser irradiation. As a whole, this study indicated that high-power neodymium-doped yttrium aluminium garnet (Nd:YAG) laser could significantly increase osteoblast activity and efficiently accelerate calcium precipitation. In addition, the activation of BMP-2 and IGF-1 associated signaling pathways might be a molecular mechanism of osteogenesis triggered by Nd:YAG laser in osteoblasts.

**Keywords:** Nd:YAG laser; Proliferation; Differentiation; BMP-2; IGF-1

## Abbreviations

Nd:YAG: Neodymium-Doped Yttrium Aluminium Garnet;

ALP: Alkaline Phosphatase;

BMP-2: Bone Morphogenetic Protein-2;

IGF-1: Insulin-Like Growth Factor-1;

CBFA1: Core-Binding Factor Alpha 1;

DMEM: Dulbecco's Modified Eagle's Medium;

FBS: Fetal Bovine Serum;

q-PCR: Quantitative Polymerase Chain Reaction;

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase;

SDS: Sodium Dodecyl Sulfate;

PBS: Phosphate-Buffered Saline;

ELISA: Enzyme-Linked Immunosorbent Assay;

PDGF-α: Platelet-Derived Growth Factor-A;

TGF-β: Transforming Growth Factor-B

## Introduction

Bone injury represents a variety of skeletal-related disorders, including defects that lead to major mobility hindrance and mortality in humans. It requires a long period of healing, which prolongs the patient's hospitalization and suffering, and increases the costs for their family and the society [1]. Certain risk factors, including poor blood circulation, loss of bone and soft tissue, metabolic disease, smoking, drinking, osteoporosis and high intraosseous pressure, may delay or impede bone healing in a clinical setting. Thus, in clinical, seeking ways to shorten healing periods and improve healing outcomes of bone injury is of great importance.

Nd:YAG laser therapy was first introduced into clinical settings by Berlien and Waldschmidt [2,3]. Using 17.7-mJ diode pump energy, 4.6-mJ long pulse energy was obtained from a 2-mm diameter and 20-mm long Nd:YAG crystal. The laser got an electrical-optical efficiency of 12.3% and was acousto-optically Q-switched. Furthermore, the Nd:YAG laser could generate 1.19 mJ and 108 ns pulses up to 100 Hz, when transversely pumped with a quasi-CW diode bar [4]. Currently, Nd:YAG laser is commonly used in clinical practice, with expanding roles including in pupilloplasty to widen a papillary phimosis and in dissection to lyse fibrotic vitreoretinal adhesions. The high-power pulse of Nd:YAG laser is partially attributed to the evolution of sharper focusing elements and shorter burst delivery times, enabling lower energy thresholds with which to perform procedures [5]. The advantage may be beneficial to bone formation in a restricted region such as bone loss in periodontal treatment and dental implant.

High-power lasers have also been adopted for bone regeneration [6]. However, how the high-power laser irradiation could affect osteoblast differentiation still remains unknown. Therefore, the present study aimed to explore the responses of osteoblasts to Nd:YAG laser irradiation and the underlying mechanisms for providing theory ground in bone formation with Nd:YAG laser.

## Materials and Methods

### Experimental design

Laser irradiation was conducted with a high peak power, Q-switched, pulsed Nd:YAG laser apparatus (Periolase Millennium Dental Technologies Inc., CA, USA). A 0.3-mm diameter optical fiber delivered the laser beam enabling uniform irradiation 3.5 cm over the cell layer. The parameters were as follows: Wavelength, 1,064 nm; output power, 0.8 W; and irradiation time, for 1, 2, 3, 4, 5 and 6 seconds. Laser irradiation was performed once per day for 7 days for the ALP activity assay and mineral deposition, or for the duration of the study in the other experiments. The control plate was placed on the non-irradiated bench during the laser exposure period of the experimental group.

### Cell culture

MC3T3-E1 cells were cultured in 24-well with growth medium supplemented with Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA), 10% fetal bovine serum (FBS; Gibco-BRL, Carlsbad, CV, USA) and 1% Penicillin-streptomycin (Gibco-BPL, Carlsbad, CV, USA) under cell culture conditions of $37^0$c in a humidified $CO_2$ air environment. Osteogenic studies were performed in differentiation medium supplemented with DMEM, ascorbic acid (50 μg/mL), β-glycerophosphate (10 mM) and dexamethasone (1:1000).

### Cell proliferation

Cell proliferation was assessed at day 1 and day 2 after laser irradiation by counting cells. The cell suspension was mixed with trypan blue solution. The first and second chambers of the hemocytometer (Yile Technologies Inc., Shanghai, China) were filled with the mixed suspension and the hemocytometer was placed onto the platform of an inverted microscope (Olympus Corporation, Tokyo, Japan). The cells were counted in grids with four angles. The cell concentration was calculated according to the following formula: Total number of four checks/$4\times10^4$.

### ALP activity

Cells cultured in differentiation medium (20,000 cells/$cm^2$) were irradiated with laser for 7 days and ALP activity was assessed at 3, 7 and 14 days. By adding RIPA buffer, the cell lysates were extracted and treated with ultrasonic radiation (Koncrete Engineering Testing Technology Co., Ltd., Beijing, China) at 4°C, then centrifuged at 10,000 g for 10 min at 4°C. The supernatant was transferred into a 96-well plate, followed by the addition of ALP substrates (6.7 mmol/L p-nitrophenyl phosphate, 25 mmol/L diethanol-amine and 1 mmol/L $MgCl_2$), then, the plate was agitated in the dark for 30 min. Finally, the reaction was terminated by 0.05 N NaOH, and the absorbance was read at 405 nm.

### Alizarin Red S Staining for Calcium Precipitation

Cells were fixed in 2.5% glutaraldehyde (freshly prepared in 1× phosphate-buffered saline (PBS) for 10-15 min at room temperature, then washed with 1× PBS 1-2 times. Filtered

2% Alizarin Red solution (Sigma-Aldrich; pH adjusted to 4.2) was added to the fixed cells and placed in a 37˚C incubator for 10-20 min. The dye was removed and cells were rinsed with PBS 1-2 times. The staining results (positive stained nodules were orange red) were recorded with microphotography (UV/Vis spectrophotometer; Beckman Coulter, Brea, CA, USA). For staining quantification, 800 μL of 10% acetic acid was added to each well and the plate was incubated at room temperature for 30 min with agitation. The monolayer was scraped from the plate and transferred with 10% (v/v) acetic acid into a microcentrifuge tube. Following vortexing for 30 sec, the slurry was overlaid with 500 μL mineral oil, heated to exactly 85˚C for 10 min and transferred onto ice for 5 min. The slurry was centrifuged at 20,000 g for 10 min and 500 μL supernatant was removed to a new microcentrifuge tube (Nalgene Labware, Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, 200 μL of 10% (v/v) ammonium hydroxide was added to neutralize the acid. Aliquots (150 μL) of the supernatant were read in triplicate at 405 nm in a 96-well format using opaque-walled, transparent-bottom plates.

### Quantitative polymerase chain reaction (q-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions, then diluted with diethylpyrocarbonate-treated water (1:40 dilution). Using a 10 μL microcuvette, the optical density was obtained at 260 and 280 nm to determine the sample concentration and purity; the required $A_{260}/A_{280}$ was >1.6. DNA was synthesized using a Transcriptor First Strand cDNA synthesis kit (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The expression levels of genes and the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by SYBR-Green I q-PCR (Applied Biosystems, Foster City, CA, USA). GAPDH was used as a housekeeping gene for internal control and gene expression in each reaction was normalized by GAPDH expression. The PCR procedures were performed as previously described by Chen *et al* [6]. Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA), and the following primer sequences were used: for mouse ALP, forward GTTGCCAAGCTGGGAAGAACAC, reverse CCCACCCCCGCTATTCCAAAC; for mouse BMP-2, forward GGTCACAGATAAGGCCATTGC, reverse GCTTCCGCTGTTTGTGTTTG; for mouse IGF-1, forward TGGATGCTCTTCAGTTCGTG, reverse CTTCAGTGGGGCACAGTACA; for mouse GAPDH, forward ACTCCACTCACGGCAAATTC, reverse TCTCCATGGTGGTGAAGACA.

### Western blot

Cells cultured in growth medium for 2 days after laser irradiation were solubilized in lysis buffer for 30 min. The lysates were centrifuged (15,000 g) at 4˚C for 15 min. Equal amounts of the soluble proteins were denatured in sodium dodecyl sulfate (SDS), electrophoresed on a 12% SDS-polyacrylamide gel, transferred onto nitrocellulose membranes and probed with antibodies, including rabbit anti-mouse antibodies and β-actin antibody (Abcam, Cambridge, MA, USA). The β-actin served as an internal control to monitor equal protein loading. Immunoblotting bands were observed using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). All the results were representatives of triplication.

### Statistic analysis

All the data were analyzed using SPSS version 20.0 (IBM, Armonk, NY, USA) and presented as the mean ± standard deviation. Statistical analysis was performed using Student's *t-test*. Values of $P<0.05$ were considered to be statistically significant.

## Results and Discussion

### Proliferation and differentiation of MC3T3-E1 cells were enhanced after Nd:YAG laser irradiation

Trypan blue analysis indicated that the proliferation of MC3T3-E1 cells was significantly enhanced in the experimental group compared with the non-laser control group 1 and 2 days after laser irradiation. Furthermore, the proliferation of MC3T3-E1 cells was enhanced most when cells were exposed to laser for 4 seconds compared with other durations (Figure 1A).

As for ALP activity assay, MC3T3-E1 cells were exposed to high-power Nd:YAG laser ranged from 1 to 6 seconds. Then, 3, 7 and 14 days after laser treatment ALP activity was measured. For all these above-mentioned detection time points, when the laser exposure lasted for 5 seconds, the ALP activity reached its peak level, which was significantly higher than that for the other exposure periods (1, 2, 3, 4 or 6 seconds). Furthermore, the ALP activity of MC3T3-E1 cells after laser irradiation was significantly increased compared with the non-laser control group (Figure 1B, C and D).

A, cells were exposed to laser irradiation for periods from 1 to 6 seconds. Then, cell proliferation was measured using a trypan blue assay 1 and 2 days after laser treatment. Compared with the non-laser control group, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$; compared with the irradiation lasting for 4 seconds, $^{\#}P<0.05$. B, C and D, cells were exposed to laser irradiation for periods from 1 to 6 seconds. After that, ALP activity was measured using enzyme-linked immunosorbent assay (ELISA) 3, 7 and 14 days after laser irradiation. Compared with the non-laser control group, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$; compared with the irradiation lasting for 5 seconds, $^{\#}P<0.05$.

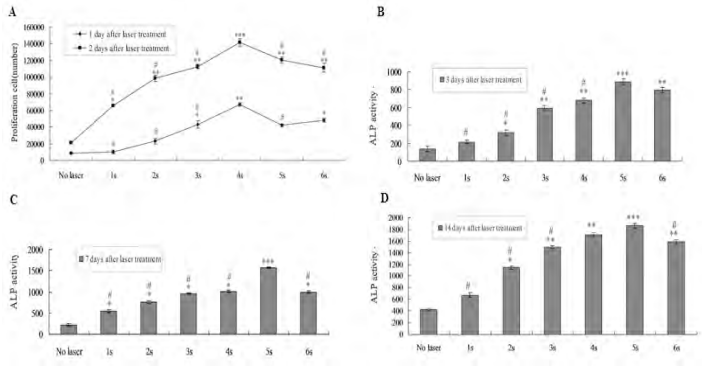

**Figure 1.** Proliferation and differentiation of MC3T3-E1 cells after high-power Nd:YAG laser irradiation

## Nd:YAG laser irradiation triggered the Calcium Precipitation

Alizarin Red S staining assay demonstrated that the calcium precipitation in the experimental group treated with laser was significantly higher than that in the non-laser control group 14 and 21 days after treatment. In addition, the maximum levels of calcium precipitation were observed at both time points mentioned above when the irradiation period was 5 seconds (Figure 2).

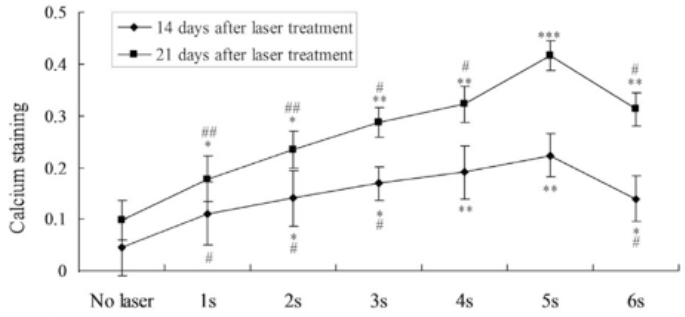


**Figure 2.** Calcium precipitation triggered by high-power Nd:YAG laser i*n virto.*

Cells were exposed to laser irradiation for periods from 1 to 6 seconds. Then, calcium precipitation was examined via Alizarin Red S staining method 14 and 21 days after laser irradiation. Compared with the non-laser control group, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$; compared with the irradiation lasting for 5 seconds, $^{\#}P<0.05$, and $^{\#\#}P<0.01$.

## Nd:YAG laser irradiation increased the mRNA expression levels of ALP, BMP-2 and IGF-1

The q-PCR results illustrated that ALP mRNA transcription in the experimental group was significantly increased compared with the non-laser control group 3 and 7 days after laser treatment. In addition, the highest ALP mRNA level was seen when cells were exposed to laser for 5 seconds (Figure 3A). Similarly, the mRNA expression levels of BMP-2 and IGF-1 were also significantly enhanced compared with the non-laser control group, with the highest levels observed when the irradiation lasting for 5 seconds (Figure 3B and C).

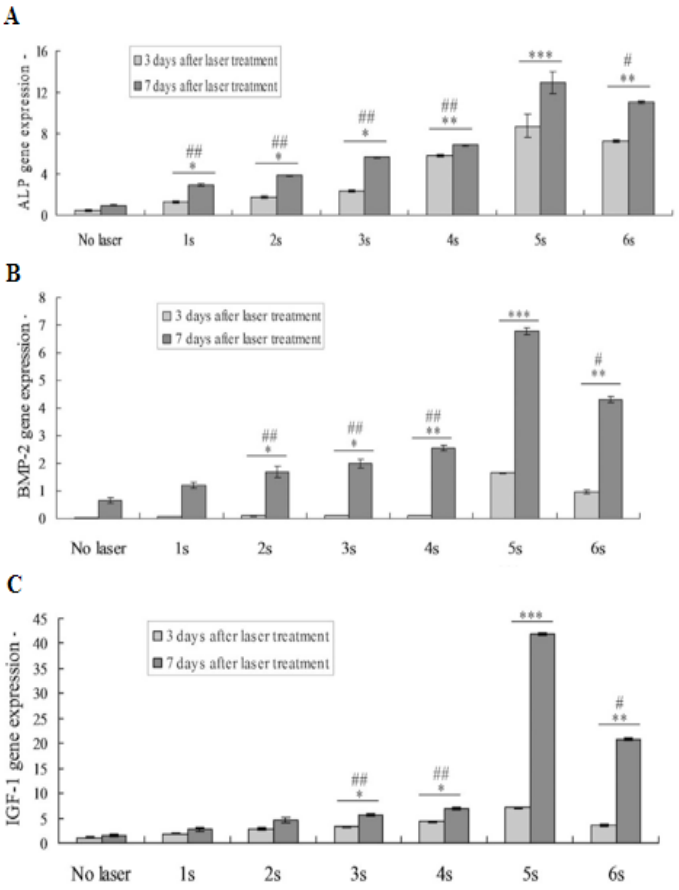


**Figure 3.** Gene expression of ALP, BMP-2 and IGF-1 induced by high-power Nd:YAG laser.

Cells were exposed to laser irradiation for periods from 1 to 6 seconds. Then, gene expression of ALP (A), BMP-2 (B) and IGF-1 (C) was detected using RT-PCR 3 and 7 days after laser irradiation. Compared with the non-laser control group, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$; compared with the irradiation lasting for 5 seconds, $^{\#}P<0.05$, and $^{\#\#}P<0.01$.

## Cellular CBFA-1 was upregulated by Nd:YAG laser irradiation

CBFA1 is considered to be associated with the osteoblastic gene expression and the formation of bone. Thus, the level of CBFA1 was detected in MC3T3-E1 cells after laser irradiation. The results demonstrated that both 3 and 7 days after laser treatment the protein level of CBFA1 was significantly enhanced compared with the non-laser control group. Besides, an optimal expression of CBFA1 was noticed when MC3T3-E1

cells experienced an irradiation lasting for 5 seconds (Figure 4).

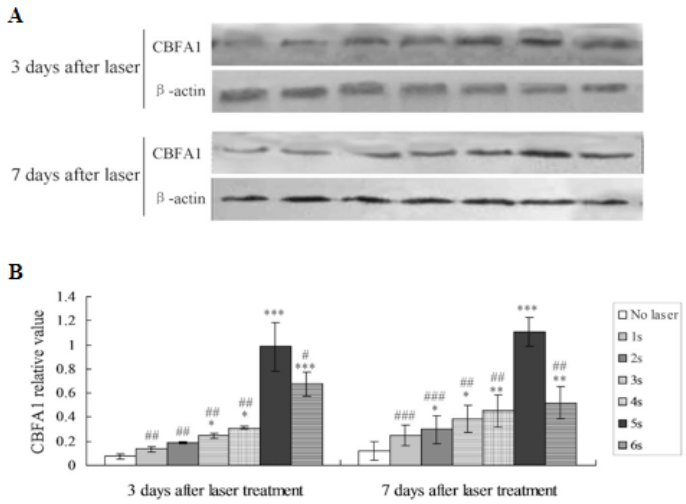


**Figure 4.** Protein expression of CBFA1 induced by high-power Nd:YAG laser

Cells were exposed to laser irradiation for periods from 1 to 6 seconds. CBFA1 protein expression was detected via western blot 3 and 7 days after laser irradiation (A) and statistically analyzed (B). Compared with the non-laser control group, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$; compared with the irradiation lasting for 5 seconds, $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$.

## Application of laser therapy and its role in cell proliferation

High-power laser therapy has been utilized in the clinical settings to optimize the wound environment, including stimulating angiogenesis and local blood flow, interstitial fluid flow and edema reduction [7,8]. The laser is also able to improve wound perfusion, stimulate growth factor induction and upregulate cytokines expression and cellular activity [9]. Thus, enhancing the formation of granulation tissue and improving wound-healing parameters are critical for increasing early blood supply and restarting bone tissue healing [10]. However, the specific function of the high-power laser in bone injury repair is elusive and requires investigation. Therefore, in the present study, the effects of the high-power laser on osteogenesis in MC3T3-E1 cells were examined.

The present study demonstrated that cell proliferation was increased significantly when cells were treated with the high-power laser. This result is consistent with previous studies demonstrating that the laser promoted the proliferation and migration of primary cultured human gingival epithelial cells [11]. However, there are also certain tissues demonstrating that only the low-power laser, and not the high-power laser, promotes the proliferation of human periodontal ligament cells [12]. Wu *et al* [13] also revealed that low-power laser irradiation was able to promote the proliferation of D1 cells. Thus, the underlying mechanisms of the induction of cell proliferation by the high- or low-power laser remain unknown.

The laser treatment period for stimulating cell proliferation is currently debated. The present study revealed a dose-dependent increase in MC3T3-E1 cell proliferation, with an optimal period of 4 seconds with high-power laser treatment. To verify whether a longer laser irradiation period promoted greater cell proliferation, the period was prolonged to 5 and 6 seconds. However, cell proliferation was reduced at these two longer periods. These results reinforce the observation that the high-power laser promotes the proliferation of MC3T3-E1 cells. However, the optimal high-power laser treatment conditions for cell proliferation require further investigation with distinct cell types and laser parameters.

## Role of laser in cell differentiation

In contrast to the unsure suppression of cell proliferation, the laser facilitated osteoblast differentiation. It demonstrated enhanced early and late stage osteogenic activities, as determined by ALP activity and in vitro matrix mineralization, respectively. ALP is recognized as an important early biomarker that is used to evaluate bone metabolism [14,15]. Khadra *et al* [16] demonstrated that ALP activity increased following exposure to 3 J/cm$^2$ of laser irradiation. Furthermore, Fukuhara *et al* [17] revealed that a GaAlAs laser at an energy density of 3.75 J/cm$^2$ significantly increased bone nodule area, as measured by von Kossa staining and subsequent determination of the number of ALP-positive colonies of calvarial cells. Similar results were also observed in the present study when the activity of ALP was assessed.

## Mechanisms underling high-power laser induced cell proliferation and differentiation

The specific mechanisms underlying the promotion of proliferation and the differentiation of cells by high-power laser irradiation remain to be elucidated. Numerous growth factors, including BMP-2 [18,19], IGF-1 [20], platelet-derived growth factor-α (PDGF-α) [21] and transforming growth factor-β (TGF-β) [22], have been reported to regulate stem cell proliferation and differentiation. As one of the strongest factor, BMP-2 plays a critical role in inducing bone formation. IGF-1 demonstrated an ability to form bone nudoles. The present data documented the gene expressions of ALP, BMP-2 and IGF-1 were gradually increased with the exposure time extension (from 1 to 5 second), especially later two factors peaked at 5 seconds, which indicated high-power laser irradiation regulates osteogenic differentiation via the BMP-2, IGF-1 and ALP. However, further studies are required to investigate the precise signaling pathways that are involved.

**Role of high-power laser in calcium precipitation**

In this study, calcium precipitation was detected via Alizarin Red S staining assay. The calcium precipitation induced by laser irradiation was obviously increased, with an optimal period of 5 seconds with laser treatment. The calcium precipitation in the high-power laser treated cells may result from the activation of ALP and increased IGF-1gene express, which is similar to results found in previous studies [23,24].

**Further influence of high-power laser on CBFA1**

CBFA1, an important transcription factor, is essential for osteoblast differentiation and thus for skeletal morphogenesis [25]. CBFA1 is stable during cell division, remains associated with chromosomes during mitosis and is important in the integration, organization and combinatorial assembly of DNA and its regulatory factors within the three dimensional context of the nuclear architecture. CBFA1, as an osteoblast-specific transcription factor, initiates the differentiation of osteoblasts and regulates the expression of osteoblast-specific genes during differentiation [26]. In the present study, the expression of CBFA1 increased as a result of treatment with high-power laser. By contrast, the present study and previous studies have demonstrated that high-power laser treatment increases the responsiveness of osteoblasts to cytokines that promote osteoblast proliferation, differentiation and bone formation, including BMP-2 and IGF-1. These cytokines regulate the expression and activity of CBFA1 during osteogenesis [27, 28].

## Conclusion

In this study, we demonstrated that high-power Nd:YAG laser irradiation promotes osteogenic activities, including ALP activity, gene expression of ALP, BMP-2 and IGF-1, as well as protein expression of CBFA1. The increased expression of BMP-2 and the upstream regulator CBFA1 may be important in the high-power Nd:YAG laser stimulated osteoblast differentiation. Taken together, the present findings indicate that high-power Nd:YAG laser is able to reinforce osteoblast differentiation and bone formation *in vitro*, and also its application for osteogenic therapy in the future.

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## Conflict of Interest Statement

The authors have stated explicitly that there is no conflicts interest.

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