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Abstract. Several studies have shown that low-level laser irradiation (LLLI) has beneficial effects on bone regeneration. The objective of this study was to examine the *in vitro* effects of LLLI on proliferation and differentiation of a human osteoblast-like cell line (Saos-2 cell line). Cultured cells were exposed to different doses of LLLI with a semiconductor diode laser (659 nm; 10 mW power output). The effects of laser on proliferation were assessed daily up to seven days of culture in cells irradiated once or for three consecutive days with laser doses of 1 or 3 J/cm^2. The obtained results showed that laser stimulation enhances the proliferation potential of Saos-2 cells without changing their telomerase pattern or morphological characteristics. The effects on cell differentiation were assessed after three consecutive laser irradiation treatments in the presence or absence of osteoinductive factors on day 14. Enhanced secretion of proteins specific for differentiation toward bone as well as calcium deposition and alkaline phosphatase activity were observed in irradiated cells cultured in a medium not supplemented with osteogenic factors. Taken together these findings indicate that laser treatment enhances the *in vitro* proliferation of Saos-2 cells, and also influences their osteogenic maturation, which suggest it is a helpful application for bone tissue regeneration. [DOI: 10.1117/1.JBO.18.12.128006]

Keywords: human osteoblast-like cells; low-level laser irradiation; osteoblasts proliferation; bone matrix deposition; osteogenic differentiation.

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1 Introduction

The identification of an amplification approach to improve and accelerate the modeling and remodeling of bone tissue is an intriguing challenge in the field of tissue engineering. Over the past 40 years, the importance of physical factors that modulate and accelerate biological processes has been highlighted. Physical factors, as well as biochemical factors, may induce cells to reprogram their functions and dynamically adapt to environmental conditions.[1–3] These factors may be applied in biomedicine and biotechnology in order to drive and modulate cell behavior for therapeutic purposes. In this context, studies focused on physical factors that promote tissue regeneration and that are therefore highly promising. Low-level laser therapy (LLLT) is a well-established clinical tool used to treat pathological tissue conditions, inflammatory processes, and promote wound healing.[4] LLLT refers to the use of lasers emitting a wavelength ranging from 600 to 1100 nm with an output power within 1 to 500 mW. A number of different laser light sources, including semiconductor diode lasers, He-Ne, and argon lasers, have been applied in different treatments.[5] LLLT has been found to modulate various biological processes, such as collagen production,[6] DNA synthesis,[7] mitochondrial respiration, and ATP synthesis.[8] Various studies have demonstrated that LLLT promotes both repair and regeneration.[9,10] It has been shown that LLLT induces cell proliferation,[11–13] promotes angiogenesis,[14] and allows the wound site to close more quickly.[10,15] Additionally, it has been demonstrated that LLLT irradiation results in an increase of circulating antioxidants and expression of heat shock protein. LLLT was also shown to stimulate the expression of multiple genes related to cellular migration, proliferation, anti-apoptosis, and prosurvival elements responsive to nuclear factor kappa-light-chain-enhancer of activated B cells,[14] besides modulating the production of growth factors and cytokines.[10–12]

Although the biochemical and cellular mechanisms behind the action of LLLT are not fully understood, it has been postulated that laser irradiation at low doses modulates cell activity by different mechanisms. It is proposed that its effect occurs through the absorption of red and near-IR light by chromophores, in particular, cytochrome c oxidase, which is contained in the respiratory chain located within the mitochondria.[16] It is

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supposed that this absorption of energy may cause the photodissociation of inhibitory nitric oxide from cytochrome c oxidase and lead to increased enzyme activity and electron transport.[17] Furthermore, it has been demonstrated that LLLT enhances cell proliferation by increasing the release of calcium into the cytoplasm, which triggers mitosis by causing a short-term rise in the intracellular pH.[18]

Increased proliferation after laser irradiation has been shown in cells of different origin, including fibroblasts, lymphocytes, mesenchymal and cardiac stem cells.[19] With respect to bone, LLLT has been applied in several clinical situations, such as orthodontic treatment, alveolar repair after tooth extraction, bone fracture healing, and osseointegration of dental implants as an adjuvant therapy.[20] *In vitro* laser irradiation improves proliferation and differentiation of human osteoblast cells,[21] and *in vivo* irradiation increases the functional attachment of biomaterial implants to bone.[22] This finding is also supported by *in vitro* observations that LLLT enhances the proliferation and osteogenic differentiation of murine mesenchymal stem cells.[23] The effects of LLLT should be more thoroughly investigated before low level laser therapy can be considered as a potential to for bone regeneration.[24]

Presently, optimal laser irradiation conditions for obtaining the maximum stimulating cell proliferation are still under debate. In several studies concerning the use of laser treatments to improve tissue repair, the authors highlight the difficulty in obtaining comparable results due to different laser sources, treatment protocols, and experimental models used. Some reports have clearly indicated that laser irradiation speeds up tissue repair and a consensus has been reached. (1) For a biological effect, laser wavelengths should be red or near-red (600 to 1200 nm). (2) For biostimulatory effects, the dose or energy density to induce cell proliferation should be between 0.05 and 10 J/cm^2, whereas energies greater than this value (>10 J/cm^2) may promote antiproliferative effects.[25]

Considering evidence that LLLT has a positive effect on bone healing, the aim of the present study was to study the *in vitro* effects of LLL irradiation (LLLI) by an aluminum gallium indium phosphide (AlGaInP) semiconductor diode laser at a wavelength of 659 nm on the proliferation and differentiation of Saos-2 cells.[26] With this aim, it was decided to first investigate the optimal stimulating parameters for Saos-2 proliferation and then evaluate the LLLI capacity under these parameters to induce osteogenic differentiation.

2 Materials and Methods

2.1 *Irradiation Protocol Settings*

The LLLI experiments were performed by using a semiconductor laser-diode, which emits a maximum output power of 130 mW at 659 nm (Table 1). The particular laser diode used for these experiments is based on an AlGaInP semiconductor (model ML101J27-01, produced by Mitsubishi Electric Corp., Tokyo, Japan, Fig. 1), which emits a single transverse-mode laser beam with a typical divergence of 10 and 17 deg in the two directions transverse to the beam propagation axis. After free propagation in air, the central portion of the beam was selected through an iris diaphragm in order to guarantee a constant beam intensity over the entire irradiated field (a circular well with radius $r = 8$ mm). The relative positions of the laser source, the mask, and the samples were appropriately chosen to obtain complete illumination of the well containing the test sample, while simultaneously avoiding illumination of adjacent wells. Both the operating temperature and current of the laser source were monitored by proper drivers (by Thorlabs GmbH, Germany). The value of the optical power incident on the samples, taking into account losses due to Fresnel reflection occurring on the cover, was set at 10 mW, corresponding to an intensity of 5 mW/cm^2. The optical power was periodically monitored to check for any laser performance degradation.

2.2 *Temperature Measurement*

In order to evaluate the possible thermal effects induced by the laser irradiation on cell cultures, a commercial temperature probe was used to measure the temperature in the culture dish before turning on the laser source, and also during laser irradiation. A T-type thermocouple (RS Components, code 621-2209, Mi, Italia) was used as the sensor; this has a good sensitivity (0.1°C) and a very broad operating range (from −200 to +350°C). The temperature in the culture dish before laser treatment was 22 ± 0.1°C. The samples were then irradiated for 600 s in a single exposition at dose of 3 J/cm^2. The final temperature was of 21.8 ± 0.1°C, indicating that the temperature remained unchanged during irradiation.

2.3 *Reagents*

The human osteosarcoma cell line Saos-2 was obtained from the American Type Culture Collection (HTB85, ATCC, Manassas, Virginia).[26,27] Unless otherwise specified, all reagents were from Sigma Aldrich (St. Louis, Missouri). Dr. Larry W. Fisher (National Institutes of Health, Bethesda, Maryland) provided us with the rabbit polyclonal anti type-I and III collagen, anti-decorin, anti-osteopontin, anti-osteocalcin, anti-osteonectin, and anti-alkaline phosphatase. Polyclonal antibody against human fibronectin (FN) was produced as previously described.[28] For the Western blot analysis, we used the anti-actin antibody and the phospho site-specific antibody against

Table 1 Laser irradiation parameters (Mitsubishi laser, model ML101J27-01, Tokyo, Japan).

Parameters	Value	Parameters	Value
Beam divergence (parallel)	10 deg	Beam divergence (perpendicular)	17 deg
Operating current	182 mA	Radiation wavelength	659 nm
Emitted power	97 mW	Power after the screen	11 mW
Power impinging on the sample	10 mW	Power density on the sample	5 $mW\,cm^{-2}$

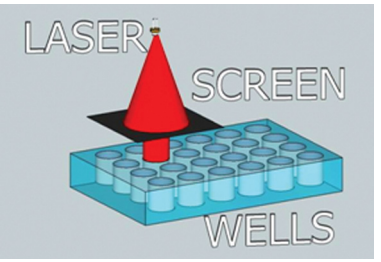

Fig. 1 Setup used for cell irradiation. Aluminum-gallium-indium-phosphide semiconductor diode laser emitting at 659 nm was perpendicularly positioned above the tissue culture plate containing the SAOS-2 cell monolayer.

Akt-P on Ser 473 and Akt from Cell Signaling Technologies®, Danvers, Massachusetts. Horseradish peroxidase (HRP)-conjugated secondary antibodies (HRP-conjugated from Dako) were used, and detection was performed by enhanced chemiluminescent substrate (ECL) solutions (Pierce Thermo Fisher Scientific, Rockford, Illinois). The TRAPeze™ kit used to detect telomerase activity was purchased from CHEMICON International (Millipore, Billerica, Massachusetts).

2.4 *Cell Culture*

Saos-2 cells were cultured in McCoy's 5A modified medium with L-glutamine and HEPES (Cambrex Bio Science, Baltimore, Maryland), supplemented with 15% fetal bovine serum, 1% L-glutamine, 0.4% antibiotics, 2% sodium pyruvate, and 0.2% fungizone. For osteogenic differentiation analysis, dexamethasone and β-glycerophosphate (both osteogenic factors) were added to the previously indicated medium at a concentration of 10^{-8} M and 10 mM, respectively. Ascorbic acid, another osteogenic supplement, is a component of McCoy's 5A modified medium. The cells were cultured at 37°C, 5% CO_2, routinely trypsinized after confluence, counted, and seeded at a density of 3×10^4 cells/well in 24-well plates.

2.5 *Cell Viability*

2.5.1 *MTT assay*

In order to establish an appropriate laser irradiation dose to induce proliferation, cell viability was evaluated after treatment with different doses and irradiation protocols: Saos-2 were divided into five groups: group I, dark control (not exposed); groups II and III, exposed to a single laser dose of 1 J/cm^2 (200 s irradiation) or 3 J/cm^2 (600 s irradiation) on day 0, and groups IV and V, exposed for three consecutive days to 1 or 3 J/cm^2 (multiple doses) on days 0, 1, and 2 (Table 2). The laser doses used have been previously reported to stimulate osteoblast growth *in vitro*.[25] The quantitative 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) test was used to assess cell viability following a single or multiple laser doses at days 1, 2, 3, and 7 of cell culture. The MTT solution (0.5 mg/mL, Sigma-Aldrich) was added to cells for 3 h. Absorbance was measured at 570 nm with a microplate reader (BioRad Laboratories, Hercules, California). The optical density value is directly proportional to the number of viable cells in the culture medium.

2.5.2 *Fluorescein diacetate assay*

At day 7 of culture, a qualitative viability assay [fluorescein diacetate (FDA) assay] was performed on each group. Briefly, cells were washed with phosphate-buffered saline (PBS, pH 7.4) and incubated with 100 μL of FDA working solution for 10 min.

Table 2 Experimental design.

	Scheme of irradiation	Energy density (J/cm^2)	Time (days)
Proliferation assays			
MTT test	Single and multiple doses	1 to 3	1, 2, 3, 7
Fluorescein diacetate assay	Single and multiple doses	1 to 3	7
Morphological observation	Single and multiple doses	1 to 3	7
Western blot	Single dose	3	10, 20, 30 min after irradiation
Telomerase activity	Multiple doses	3	7
Differentiation assays			
Alkaline phosphatase activity	Multiple doses	3 J/cm^2	14
Calcium quantification	Multiple doses	3 J/cm^2	14
Real-time polymerase chain reaction (PCR)	Multiple doses	3 J/cm^2	14
ELISA	Multiple doses	3 J/cm^2	14
Confocal laser scanning microscope analysis	Multiple doses	3 J/cm^2	14
SEM analysis	Multiple doses	3 J/cm^2	14

Note: Single irradiation was carried out at day 0; multiple irradiations were performed for three consecutive days at days 0, 1, and 2.

After washing with PBS, to stain the nuclei of dead cells, cells were incubated at room temperature (RT) for 3 min with 30 μL of propidium iodide (2 μg/ml) and then observed with a confocal fluorescence microscope (Leica TCS SPII Microsystems, Bensheim, Germany).

2.6 *Western Blot Analysis*

Saos-2 cells treated with a laser dose of 3 J/cm^2 were analyzed for Akt and Akt phosphorylation on Ser 473 by Western blot after 10, 20, and 30 min irradiation. Briefly, cells were scraped from the dish and lysed with ice-cold lysis buffer (50 mM Tris pH 7.5, 50 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 0.1% Triton, and 1 mM sodium sodium orthovanadate) for 30 min on ice. The lysates were centrifuged at 12,000 rpm for 5 min at 4°C, and supernatant protein concentrations were determined. Equivalent samples were subjected to SDS-PAGE on 8% gel. The proteins were then transferred onto nitrocellulose membranes and probed with primary antibodies anti-phospho-Akt on Ser 473 and anti-Akt diluted 1:2000, followed by secondary antibodies conjugated to HRP (1:1000). Detection was performed with ECL solution and revealed by autoradiography. Densitometry analysis of the band was performed using Image TM Software. Bands were then quantified by densitometric analysis.

2.7 *Telomerase Assay*

The Trapeze® gel-based telomerase detection kit was used to detect and evaluate telomerase activity in cells irradiated with multiple doses of 3 J/cm^2 and in control cells. This assay is a highly sensitive *in vitro* assay system for detecting telomerase activity and is based on an improved version of the original method described by Kim et al.[28] The assay is a one-buffer, two-enzyme system that uses a polymerase chain reaction (PCR) to enhance the sensitivity of telomerase detection in small samples. For visualization of the reaction products, we used a nonradioactive method: running 25 μl of the products on a 12.5% nondenaturing-page gel in 0.5x Tris/Borate/EDTA buffer. After electrophoresis, the gel was stained with SYBR® green according to the manufacturer's instructions (Life Technologies, ex Invitrogen, Carlsbad, California). The relative telomerase activity level is expressed as the total product generated (TPG), calculated by the following formula: $\mathrm{TPG\,(units)} = (x - x_o)/c/(r - r_o)/c_R \times 100$; x is the signal intensity of 6-bp ladders in the sample; x_o is the signal intensity in heat-treated sample; r is the signal intensity in TSR8 control; r_o is the signal intensity in lysis buffer; c is the signal intensity in 36-bp internal control in the sample; and c_R is the signal intensity in TSR8 control. The intensity of the TRAP product band and standard internal control bands were determined using Image™ Software.

2.8 *Confocal Laser Scanning Microscope Analysis*

Saos-2 cells (3×10^4) were seeded on glass coverslips in growth medium and then processed for confocal laser scanning microscope (CLSM) analysis. For morphological observation, cells were stimulated with a single or multiple laser doses (1 or 3 J/cm^2). On day 7 of culture, cells were washed with PBS, fixed with 4% (w/v) paraformaldehyde solution for 30 min at 4°C, permeabilized with 0.1% Triton X-100, and finally incubated with the primary antibody anti-α tubulin overnight at 4°C.

For osteogenic protein labeling, cells were irradiated with multiple doses at 3 J/cm^2 and stained on day 14 of culture both in proliferative medium (PM, without osteogenic factors) and in osteogenic medium (OM, with osteogenic factors). Paraformaldehyde-fixed samples were blocked with PAT [PBS containing 1% (w/v) bovine serum albumin and 0.02% (v/v) Tween 20] for 1 h at RT. Cells were then incubated with specific primary antibodies (anti-type-I collagen, anti-alkaline phosphatase, and anti-osteocalcin rabbit polyclonal antisera) diluted 1:1000 in PAT overnight at 4°C. Following incubation with the primary antibody, cells were washed once with PBS and incubated with Alexa-Fluor-488 conjugated secondary antibody (diluted 1:500 in PAT, Invitrogen, Carlsbad, California) for 1 h at RT. After extensive washes in PBS, nuclei were counterstained with propidium iodide (2 μg/mL) for morphological observation and Hoechst 33342 (2 μg/mL) for osteogenic protein labeling. Finally, samples were observed with a confocal fluorescence microscope (Leica TCS SPII Microsystems, Wetzlar, Germany).

2.9 *Osteogenic Differentiation*

To investigate the effect of LLLI on Saos-2 osteogenic differentiation, cells treated with three consecutive doses at 3 J/cm^2 and cultured in PM or OM were analyzed on day 14 of culture (Table 2). Control cell groups were unexposed and cultured in PM or OM, respectively.

2.10 *Alkaline Phosphatase Activity*

On day 14 of culture, the alkaline phosphatase (ALP) activity from LLLI stimulated and unstimulated samples cultured either in PM or OM was evaluated by a colorimetric end point assay as previously described.[2] The assay measures the conversion of the colorless substrate *p*-nitrophenol phosphate (PNPP) by the enzyme ALP to the yellow product p-nitrophenol, where the rate of the color change corresponds to the amount of enzyme present in the solution. Briefly, an aliquot (1 mL) of 0.3 M PNPP (dissolved in glycine buffer, pH 10.5) was added to each sample at 37°C. After incubation, the reaction was stopped by the addition of 100 mL 5 M NaOH. Standards of PNPP in concentrations ranging from 0 to 50 mM were freshly prepared from dilutions of a 500 mM stock solution and incubated for 10 min with 7U of ALP (Sigma-Aldrich) previously dissolved in 500 mL of ddH2O. The absorbance reading was performed at 405 nm with a microplate reader (BioRad Laboratories, Hercules, California) using 100 mL of standard or sample placed into individual wells of a 96-well plate. Samples were run in triplicate and compared against a calibration curve of p-nitrophenol standards. The enzyme activity was expressed as micromoles of p-nitrophenol produced per minute per milligram of enzyme.

2.11 *Calcium Quantification*

On day 14 of culture, the calcium deposition from LLLI stimulated and unstimulated samples cultured either in PM or OM was determined by calcein detection and calcium cresolphthalein complexone methods as previously described.[29]

2.11.1 *Calcein detection*

At the end of cell incubation, each sample was rinsed with sterile PBS and stained with a calcein solution (5 mM in PBS;

Invitrogen) for 30 min at 22°C. The samples were counterstained with a solution of Hoechst 33342 (2 μg/mL) to target the cellular nuclei and then washed with PBS. The images were taken by blue excitation (bandpass, 450 to 480 nm; dichromatic mirror, DM500; barrier filter, BA515) with a fluorescence microscope at 20× magnification.

2.11.2 *Calcium-cresolphthalein complexone method*

The calcium content of each sample was assayed to quantify the amount of mineralized matrix present and was measured using a Calcium Fast kit (Mercury SPA, Naples, Italy) according to the manufacturer's instructions. The colorimetric end point assay measures the amount of purple-colored calcium-cresolphthalein complexone complex formed when cresolphthalein complexone binds to free calcium in an alkaline solution. Briefly, an aliquot (1 mL) of 1 N HCl was added to each sample and incubated for 24 h at RT to release calcium into solution. The sample supernatant was diluted 1/10 with the Assay Working Solution previously prepared by mixing equal parts of calcium-binding reagent and calcium buffer reagent provided by the Kit. Ca^{2+} standards in concentrations ranging from 0 to 10 mg/mL were prepared from dilutions of a 100 mg/mL stock solution of Ca^{2+}. The absorbance reading was performed at 595 nm with a microplate reader (BioRad Laboratories) using 100 mL of standard or sample placed into individual wells of a 96-well plate. Samples were run in triplicate and compared against the standard solution calibration curve.

2.12 *Assay for Gene Expression*

Total RNA from LLLI stimulated and unstimulated samples cultured in PM or OM was extracted on day 14 of culture using the Trizol reagent, according to the manufacturer's protocol (Invitrogen). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed in order to evaluate the gene expression for bone sialoprotein (BOSP), decorin (DEC), fibronectin (FN), osteocalcin (OC), osteopontin (OP), type-I collagen (COL-I), type-III collagen (COL-III), alkaline phosphatase (ALP), osteonectin (OSN), and the housekeeping gene expression for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The reverse transcriptase reaction was performed with 300 ng total RNA using the iScript™ cDNA Synthesis kit from Bio-Rad. The primers (Primm s.r.l., Milan, Italy) were designed according to the published gene sequences, and the PCRs were performed with the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California) as previously reported.[29] The primers used are indicated in Table 3.

2.13 *Real-Time PCR*

Total RNA from LLLI stimulated and unstimulated samples cultured in PM or OM was extracted on day 14 of culture with the NucleoSpin® RNA XS kit (Macherey-Nagel, Duren, Germany) and retro-transcribed to c-DNA with the iScript cDNA Synthesis Kit (BioRad Laboratories, Marnes-La-Coquette, France). A quantitative RT-PCR analysis was performed in a 48-well optical reaction plate using a MiniOpticon® Real Time PCR System (BioRad Laboratories) as previously described.[2] Gene expression was analyzed in triplicate and normalized to GAPDH gene expression, using the Livak method.[30] Analysis was performed in a total volume of 20 μL amplification mixture containing 2x (10 μL) Brilliant® SYBR® Green QPCR Master Mix (Stratagene, La Jolla, California), 2 μL cDNA, 0.4 μL of each primer, and 7.2 μL H_2O. Thermal cycling was initiated by denaturation at 95 deg for 3 min, followed by 40 cycles at 95 deg for 5 s and 60 deg for 20 s. To perform the real-time analysis, we used Invitrogen™ (Carlsbad, California) primers for the genes listed in Table 3.

2.14 *Extraction of the Extracellular Matrix Proteins and ELISA Assay*

On day 14 of culture, in order to evaluate the amount of extracellular matrix proteins produced by LLLI stimulated and unstimulated samples cultured either in PM or OM, an ELISA assay was performed as previously described.[29] Briefly, the samples were washed extensively with sterile PBS to remove culture

Table 3 Primers used for qRT-PCR.

Genes	Upstream primer	Downstream primer	Amplicon size (bp)
ALP	5′- ACCTCGTTGACACCTGGAAG-3′	5′-CCACCATCTCGGAGAGTGAC-3′	189
BOSP	5′-TGAGGCTGAGAATACCACAC-3′	5′-GCCTAGTGGTGTGTTCTTAG-3′	380
Col-I	5′-TGTAAGCGGTGGTGGTTATG-3′	5′-GGTAGCCATTTCCTTGGAAG-3′	450
Col-III	5′-TGGATCAGATGGTCTTCCA-3′	5′-TCTCCATAATACGGGGCAA-3′	620
Dec	5′-CGAGTGGTCCAGTGTTCTGA-3′	5′-AAAGCCCCATTTTCAATTCC-3′	400
Fn	5′-TGGAACTTCTACCAGTGCGAC-3′	5′-TGTCTTCCCATCATCGTAACAC-3′	500
GAPDH	5′-TTCACCACCATGGAGAAGGC-3′	5′-GGCATGGACTGTGGTCATGA-3′	236
OC	5′-GGCAGCGAGGTAGTGAAGAG-3′	5′-CTGGAGAGGAGCAGAACTGG-3′	230
OSN	5′-CTTCAGACTGCCCGGAGA-3′	5′-GAAAGAAGATCCAGGCCCTC-3′	110
OP	5′-TCACTGATTTTCCCACGGAC-3′	5′-TCATAACTGTCCTTCCCACG-3′	280

Note: ALP, alkaline phosphatase; BOSP, bone sialoprotein; Col-I, type-I collagen; Col-III, type-III collagen; Dec, decorin; Fn, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OC, osteocalcin; OSN, osteonectin; and OP, osteopontin.

medium and then incubated for 24 h at 37°C with 1 mL of sterile sample buffer [20 mM Tris-HCl, 4 M GuHCl, 10 mM EDTA, 0.066% (w/v) sodium dodecyl sulphate (SDS), pH 8.0]. At the end of the incubation period, the total protein concentration in both culture systems was evaluated with the BCA Protein Assay Kit (Pierce Biotechnology Inc., Rockford, Illinois). Calibration curves to measure COL-I, COL-III, DEC, OP, OC, OSN, FN, and ALP were performed as previously described.[2] We have taken into consideration that an underestimation of the absolute protein deposition is possible because the sample buffer used for matrix extraction contains SDS, which may interfere with protein adsorption during the ELISA assay. The amount of extracellular matrix constituents from samples was expressed as pg/(cells per well).

2.15 *Scanning Electron Microscopy Analysis*

Saos-2 were seeded on plastic cell culture coverslip disks (Thermanox Plastic, Nalge Nunc International, Rochester, New York) and irradiated with multiple laser doses at 3 J/cm^2 in PM or OM. Control groups were treated under the same conditions. On day 14 of culture, cells were treated as previously described.[2] The specimens were sputter coated with gold and observed at 250× and 1000× magnification, respectively, with a Leica Cambridge Stereoscan 440 microscope (Leica Microsystems) at 8 kV.

2.16 *Statistical Analysis*

Each experimental condition was performed in triplicate in three separate experiments. Differences between groups were tested by one-way analysis of variance. Tukey's test was used to correct for multiple comparisons. Statistical significance was established at two-tailed $p \leq 0.05$. All calculations were generated using GraphPad Prism 5.0 (GraphPad Inc., San Diego, California).

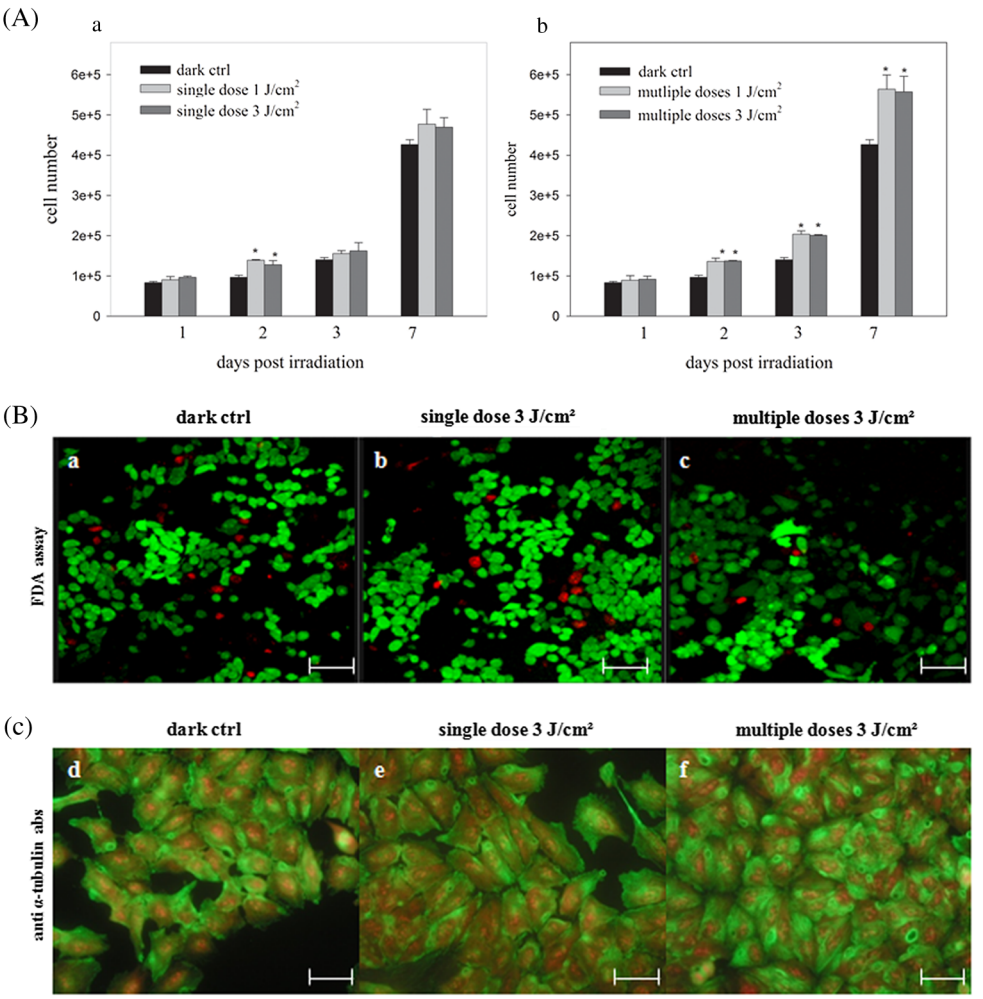

Fig. 2 Time course of cell Saos-2 proliferation following irradiation with a single and daily laser doses. (Aa) The viability of groups irradiated with a single dose at 1 or 3 J/cm^2 on day 0. (Ab) The viability of groups irradiated with a daily dose of 1 or 3 J/cm^2 on days 0, 1, and 2. The MTT test was performed to evaluate cell viability after a single dose or daily dose exposures at days 1, 2, 3, and 7 of cell culture. After seven days of culture, all experimental groups were treated with fluorescein diacetate (green cells alive) and propidium iodide (red cells dead) (B). Dark control and groups irradiated with one or three consecutive doses at 3 J/cm^2 are shown (20× magnification, the scale bar represents 50 μm). SAOS-2 cell morphological analysis after laser treatment: alfa-tubulin is shown in green and nuclei in red (40× magnification, the scale bar represents 50 μm) (C). The dark control and irradiated sample with one or three consecutive doses at 3 J/cm^2 are shown.

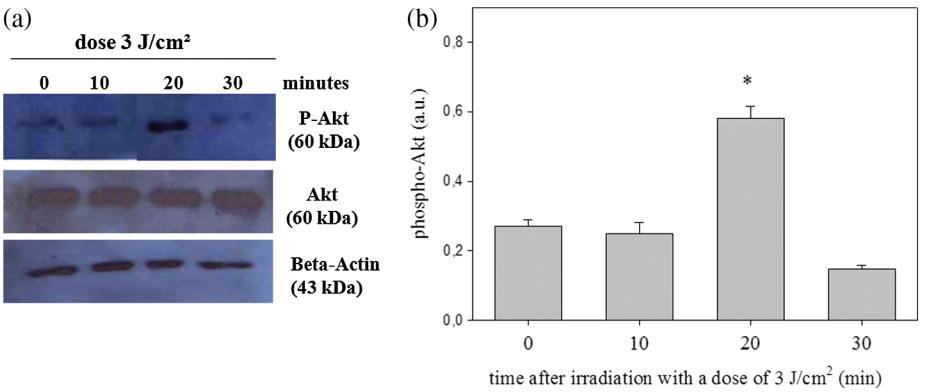

Fig. 3 Representative Western blot analysis for Akt phosphorylation up to 30 min after a single laser dose at 3 J/cm^2. SAOS-2 were cultured for 24 h in serum free medium, irradiated, and collected at the indicated time after laser treatment and then analyzed for Akt phosphorylation (Ser-473) by Western blot. Akt and b-actin were used as controls. Results represent one of three replicates (a). Bar graph represents the phosphorylation level of the signal protein calculated by the ratio between the phosphorylated and total protein obtained in three different experiments. *$p < 0.05$ versus time 0 (b).

3 Results

3.1 *Effect of Low-Level Laser Irradiation on Cell Proliferation and Morphology*

To test whether LLLI can act as a proliferative factor, the experimental setup was performed as indicated in Table 2. At all levels of applied irradiation and at each time interval, cell vitality and morphology were evaluated by MTT assay, FDA, and CLSM, respectively (Fig. 2). The results of cell viability after a single dose at 1 or 3 J/cm^2 is shown in Fig. 2(Aa). Significant differences were detected in cell proliferation after a single dose at 1 or 3 J/cm^2 with respect to the dark control ($p < 0.05$) on day 2. However, on days 3 and 7, these differences were not statistically significant. The results of cells treated with daily doses are also presented in Fig. 2(Ab). The viability analysis showed that repeated irradiation on three consecutive days with doses of 1 or 3 J/cm^2 resulted in a significantly higher proliferation when compared to the dark control group on days 2, 3, and 7 ($p < 0.05$). It is possible to hypothesize that single dose may exert an effect on cell proliferation in the first few days after exposure, but this effect may not last, as previously reported.[23]

The effect of irradiation was then qualitatively evaluated by FDA assay at day 7 of culture in untreated/laser-treated cells after single or multiple doses [Fig. 2(B)]. Results showed comparable cell viability between the samples. The Saos-2 cell morphology after laser exposure was evaluated by CLSM and no differences in the cytoskeletal organization of alpha tubulin between the experimental groups was observed [Fig. 2(C)]. To further complete the analysis on proliferative activity following laser treatment, the activation of mitogenic kinase Akt was analyzed (Fig. 3). This protein was previously reported to be involved in LLLI-induced cell proliferation.[31]

Western blot analysis showed that laser treatment with a dose of 3 J/cm^2 is able to induce a transient increase in Akt phosphorylation/activation, which gradually reached a maximum level of activation 20 min after stimulation [Figs. 3(a) and 3(b)]. The correlation between telomerase activity and cell proliferation after laser irradiation was also investigated (Fig. 4). Telomerase activity was measured quantitatively with the TRAPEZE Gel Based Telomerase Detection Kit. As shown in Fig. 4, both stimulated and unstimulated samples were telomerase positive, but telomerase activity was not significantly

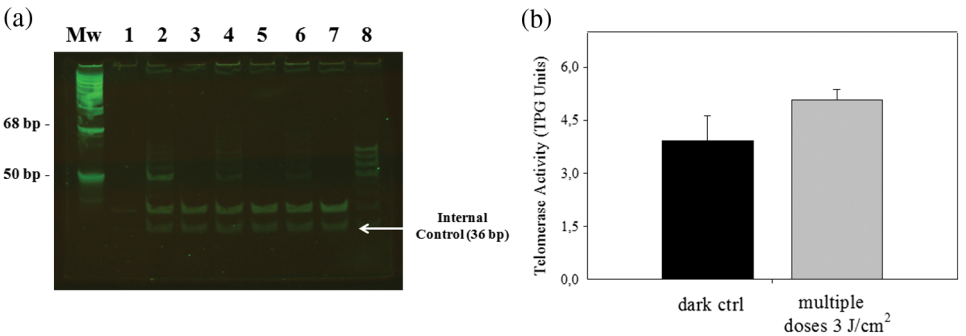

Fig. 4 Electrophoresis image of telomerase activity in Saos-2 after irradiation for three consecutive days at a dose of 3 J/cm^2 in proliferative medium using the TRAPeze™ kit. One representative telomerase assay out of three similar ones is presented (a). Lane 1: TRAPeze quality control cell lysate (HeLa cells) heat-treated; lane 2: non-heat-treated extract of control HeLa cells; lane 3: heat-treated dark control cells; lane 4: non-heat-treated dark control cells; lane 5: heat-treated irradiated cells; lane 6: non-heat-treated irradiated cells; lanes 7 and 8: TSR8 control and 1X CHAP lysis buffer, respectively. Bar graph represents the telomerase products quantification (b). Telomerase activity (in total product generated units) was calculated by comparing the ratio of telomerase products to an internal standard for each lysate, as described by CHEMICON International.

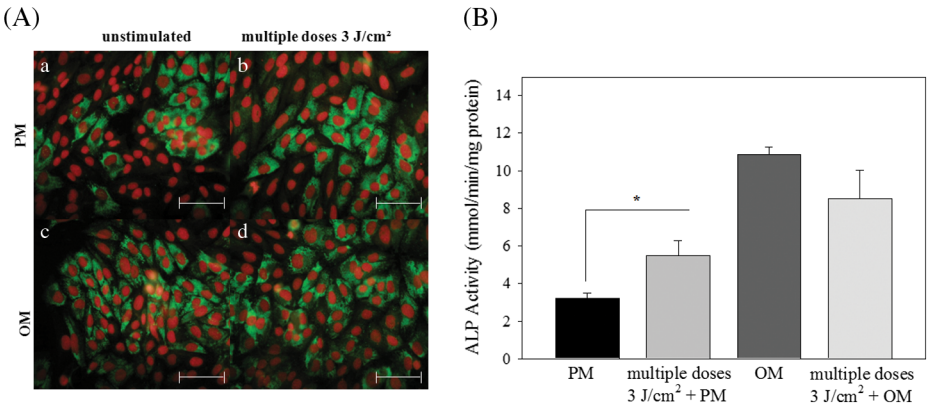

Fig. 5 Alkaline phosphatase (ALP) deposition and activity of Saos-2 cells exposed to three consecutive doses at 3 J/cm^2 and cultured in proliferative medium (PM) or in osteogenic medium (OM). ALP presence was determined by confocal laser microscopy (40× magnification, the scale bar represents 50 μm) (A), and ALP activity was measured colourimetrically, corrected for the protein content measured with the BCA Protein Assay Kit and expressed as millimoles of p-nitrophenol produced per minute per milligram of protein (B). Bars express the mean values ± SD of results from three experiments (*$p < 0.05$ versus PM).

increased in stimulated cells compared to the dark control ($p > 0.05$).

3.2 *Effect of Low-Level Laser Irradiation on Cell Differentiation*

To investigate whether low-level laser treatment was able to influence Saos-2 osteogenic differentiation (Table 2, for experimental setup), all analyses were performed on day 14 of culture on multiple-stimulated or unstimulated cells, with/without the addition of osteogenic factors in the culture medium. ALP activity, calcium deposition, gene expression, and bone extracellular matrix protein production were evaluated. The level of ALP activity was higher in cells in osteogenic medium (both unstimulated/laser stimulated) than cells in proliferative medium (both unstimulated/laser stimulated) [Fig. 5(B)]. The ALP activity was different between cells that were laser treated or untreated cultured in PM as well as in OM. Interestingly, the ALP activity of LLLI-exposed samples in PM was considerably higher when compared to unexposed samples in PM (*$p < 0.05$); on the contrary, ALP activity of laser-exposed samples in OM was slightly lower than that of unexposed samples in OM (#$p > 0.05$). These data are in accordance with the immunolocalization of ALP on the cell surface as observed by CLSM. A more intense green fluorescence signal was observed on laser-stimulated samples in PM and on stimulated/unstimulated samples in OM [Figs. 5(Ab), 5(Ac), and 5(Ad)] than on unstimulated cells in PM [Fig. 5(Aa)]. Figure 6 shows the calcium deposition detected by calcein assays: a more intense green fluorescence signal was observed on laser-stimulated cells in PM and laser-stimulated/unstimulated cells in OM [Figs. 6(Ab), 6(Ac), and 6(Ad)] than on unstimulated cells maintained in PM [Fig. 6(Aa)]. A significant difference was also observed between laser-exposed/unexposed cultures in PM. The results were quantitatively confirmed with the calcium creosolphatein complexone method [Fig. 6(B)].

In order to characterize bone-specific gene expression, an RT-PCR analysis was performed at 14 days culture with/without osteogenic factors on unstimulated/stimulated samples. The qualitative RT-PCR (qRT-PCR) showed differences for the transcripts specific for COL-I, BOSP, and OP (Fig. 7). To further expand these data, a qRT-PCR for the ALP,

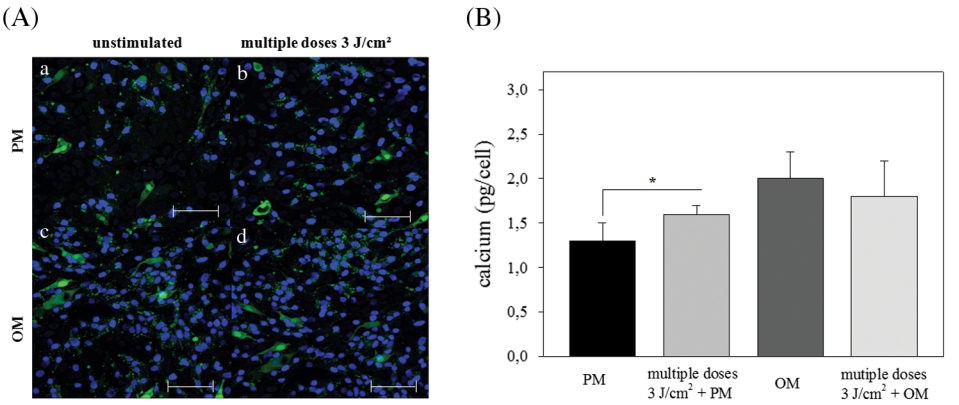

Fig. 6 Calcium matrix produced by Saos-2 cells irradiated with three consecutive doses at 3 J/cm^2 and cultured in PM or in OM. Calcium deposition was determined with a confocal laser scanning microscope [(A), 20× magnification, the scale bar represents 50 μm] and by quantification of calcium content as reported in the Materials and Methods section (B). Results are presented as an average ± SD (*$p < 0.05$ versus PM).

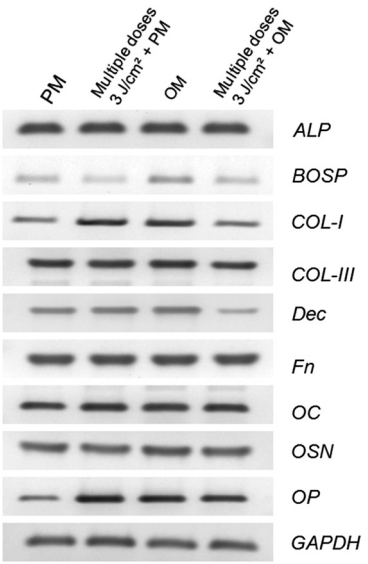

Fig. 7 Assay for gene transcription of cells cultured in PM, or irradiated with three consecutive doses at 3 J/cm^2 and cultured in PM, or cultured in OM, or irradiated with three consecutive doses at 3 J/cm^2 and cultured in OM. The indicated reverse transcriptase-polymerase chain reaction (RT-PCR) products were subjected to electrophoresis on 2% agarose gel and visualized by UV exposure. The level of specific bands was normalized for glyceraldehyde-3-phosphate dehydrogenase cDNA.

BOSP, COL-I, and OP in laser-stimulated/unstimulated cells cultured in PM or OM was performed at 14 days using the ΔΔCt method. These genes are involved in the osteogenic process, and they are the most characterized to evaluate bone differentiation of cells.[32–36] As shown in Fig. 8, the real-time PCR data revealed no significant differences between laser-treated/untreated cells cultured in PM in the expression of ALP and COL-I, while a statistically significant down regulation of BOSP and OP expressions ($p < 0.05$), which are genes related to the final phases of osteogenic differentiation, was observed [Fig. 8(a)]. No evident fold-increase was detected for these genes between laser-stimulated/unstimulated cells cultured in OM [Fig. 8(b)]. These findings were confirmed by the confocal analysis of COL-1 and osteocalcin (OCN) expression in laser-treated and untreated cultures in PM [Figs. 9(A) and 9(B)]. Instead, a more intense green fluorescence signal for COL-I and OCN in laser-stimulated/unstimulated cells was observed in osteogenic culture conditions compared with PM [Figs. 9(A) and 9(B)]. In Table 4, data are reported for the extracellular matrix protein deposition on day 14 of culture. An enhancement in COL-III, ALP, and DEC deposition was observed in laser-treated cells in PM. These were, respectively, 1.8-, 2-, and 1.8-fold greater when compared with unstimulated cells in PM ($p < 0.05$). On the contrary, the level of bone

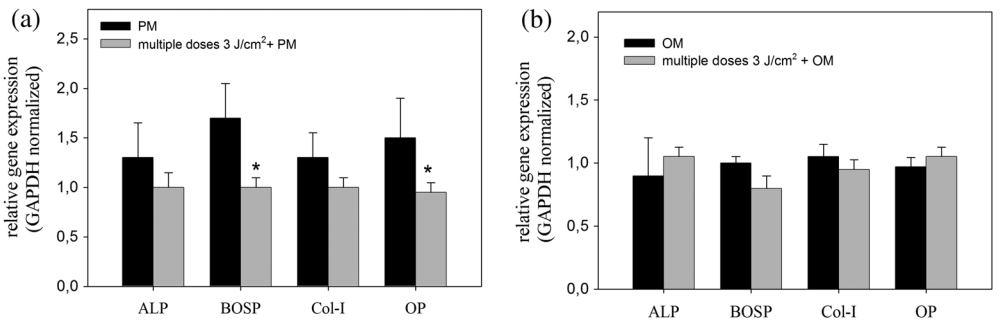

Fig. 8 Expression of the indicated bone-specific genes as determined by *q*RT-PCR. Saos-2 were stimulated for three consecutive days with a dose of 3 J/cm^2 and then cultured in PM (a) or in OM (b). The graph shows the fold induction of gene expression (arbitrary units), setting the expression of the indicated genes in cells grown in the absence of laser treatment at day 14 of culture. A $p < 0.05$ was considered statistically significant. Data are representative of one of the three experiments performed.

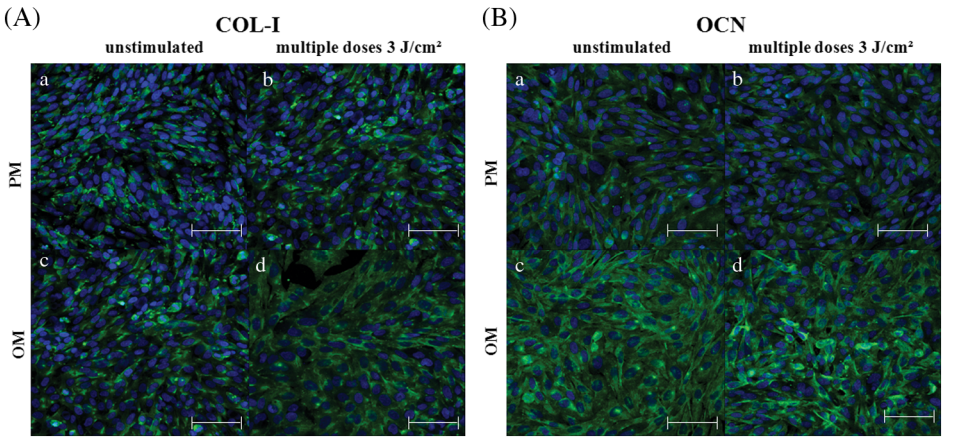

Fig. 9 Representative images of osteoblast protein markers after laser treatment by confocal laser scanning microscope. Saos-2 were treated for three consecutive days at 3 J/cm^2 and then cultured in PM [(Aa), (Ab), (Ba), and (Bb)] or in OM [(Ac), (Ad), (Bc), and (Bd)]. Immunofluorescence analysis was performed at day 14 of culture. Coll-1 (A) and osteocalcin (B) (in green), nuclei (in blue) (40× magnification, scale bar represents 50 μm).

Table 4 Normalized amount of extracellular matrix constituents secreted and deposited by Saos-2 with or without three doses laser of 3 J/cm^2 in proliferative medium (PM) or osteogenic medium (OM) after two weeks of cell culture (pg/cell × well). In comparison to unstimulated samples, a p value <0.05 was considered statistically significant (*).

Proteins	PM	Multiple doses of 3 J/cm^2 + PM	Multiple doses of 3 J/cm^2 + PM/PM	OM	Multiple doses of 3 J/cm^2 + OM	Multiple doses of 3 J/cm^2 + OM/OM
Alkaline phosphatase	3.8 ± 0.233	7.6 ± 1.06	2.0*	17.8 ± 1.2	10.7 ± 2.0	0.6
Decorin	11.7 ± 1.51	21.4 ± 2.14	1.8*	81.3 ± 3.3	50.3 ± 8.7	0.6
Fibronectin	3.8 ± 1.13	10.7 ± 1.57	2.8*	25.4 ± 2.2	15.2 ± 1.5	0.6
Osteocalcin	1.5 ± 0.27	1.8 ± 0.78	1.2	6.1 ± 0.7	3.6 ± 0.05	0.6
Osteonectin	0.9 ± 0.042	1.07 ± 0.036	1.2	1.8 ± 0.03	1.1 ± 0.06	0.6
Osteopontin	9.2 ± 1.76	12.2 ± 2.22	1.3	30.5 ± 2.0	19.8 ± 3.1	0.6
Type-1 collagen	11.7 ± 1.21	15.3 ± 1.03	1.3	40.6 ± 2.8	24.4 ± 1.3	0.6
Type-3 collagen	16.9 ± 2.06	30.16 ± 3.86	1.8*	91.5 ± 3.3	60.9 ± 11.2	0.7

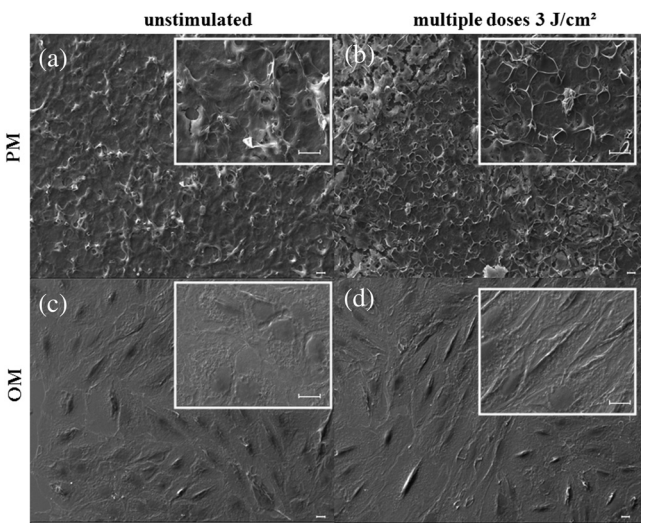

Fig. 10 SEM images of Saos-2 cells after laser treatment. Cells were irradiated with three consecutive doses at 3 J/cm^2 and cultured in PM [(a) and (b)] or in OM [(c) and (d)] at 250× magnification, scale bar represents 10 μm (insert at 1000×, scale bar represents 10 μm).

proteins was not statistically different when measured in cells stimulated with laser and cultured in the presence of osteogenic factors compared with osteogenic control (Table 3; $p > 0.05$). Moreover, two weeks after seeding, SEM revealed that cells formed a confluent multilayer with a more evident cellular density in laser-stimulated/unstimulated samples in PM [Figs. 10(a) and 10(b)] as compared with OM cultures [Figs. 10(c) and 10(d)]. At higher magnification, the characteristic cell morphology of PM and OM cultures following laser irradiation were observed: in PM, cells exhibited a round shape, while in OM, they were flat and elongated, typical of differentiated cells. These results suggested that the laser treatment did not interfere with proliferation and differentiation processes.

4 Discussion and Conclusions

In this study, we report the results obtained from the application of AlGaInP laser stimuli on adherent human osteoblastic Saos-2 cells. Through a systematic, analytical study, we provide evidence that, besides being able to modulate inflammation and the wound healing process in bone, LLLI is a positive proliferative factor in osteoblast-like cells. Within the parameters assessed in this study, we demonstrated that LLLI (1) enhances the proliferation potential of Saos-2 cells without increasing their tumorigenic characteristics and (2) does not induce morphological damage nor affect their osteoblastic phenotype. In the field of regenerative medicine, tissue engineering offers therapeutic alternatives for autologous bone grafts. The key to successful engineering of bone with optimal restoration of function lies in finding the optimal combination of biomaterial, biofactors, and cells.[37] A growing body of evidence shows that different laser systems can lead to enhanced proliferative potential in various cell lines, including stem cells, without compromising their innate characteristics and properties.[38] Many investigators have reported that low-level laser treatment positively affects bone regeneration both *in vitro* and *in vivo*.[39–41] Several *in vitro* experiments were performed to determine the optimum procedure and to elucidate the molecular mechanisms of LLLI. Recently, Yamamoto et al.[7] applied diode laser to mouse osteoblast culture and observed that laser treatment accelerated cell proliferation via mouse minichromosome maintenance genes, which are regulators of DNA replication. Moreover, Hirata and his colleagues[42] demonstrated that the pro-osteogenic effect of LLLI is related to the stimulation of bone morphogenetic proteins/Smad signaling pathway.

Our study focused on an evaluation of the effects of diode laser treatments on osteoblast-like cell proliferation and bone differentiation. The design of the LLLI protocol used in this study was based on an extensive review of previous data obtained *in vitro* in bone and rat calvarian cells.[21,43] The choice of the model cell line was based on Saos-2 osteoblastic features. To evaluate the effects of LLLI on cell proliferation and differentiation, the Saos-2 cell line was selected as it exhibits several fundamental osteoblast characteristics[27] and represents a widely

used model for *in vitro* osteoblast study. An ability to induce the formation of new bone at a specific site would represent a significant advance in bone repair and tissue engineering: this property seems to belong to Saos-2 cells. These osteoblasts uniquely contain an osteoinductive activity, whereas other human osteosarcoma cells, such as U-2 OS, cannot replicate that bone-inducing ability.[44] Devitalized Saos-2 cells, extracts, and secretions induced the formation of new bone when implanted subcutaneously in nu/nu mice.[26,45] These osteoblasts can be grown, virtually indefinitely, to produce large quantities of osteoinductive factors, such as BOSP and OSN.[44] The use of this cell line showed the potential of the LLLI stimulations; nevertheless, by appropriately tuning the LLLI parameters, a better result could be obtained with autologous bone marrow stromal cells instead of Saos-2 osteoblasts for total immunocompatibility with the patient. Initially, we focused on establishing the best irradiation protocol to obtain the maximum proliferation rate. An analysis was performed using red laser wavelength (659 nm) and considering different doses (1 or 3 J/cm^2) and different irradiation schemes (single irradiation and irradiation repeated for three consecutive days). Proliferative assays showed that an initial effect on cell proliferation was already evident 24 h after the first laser application of 1 or 3 J/cm^2. However, we found that the effect of a single laser dose on cell proliferation was transitory; in fact, following one laser treatment, after 96 h, no significant differences in proliferation were observed when compared with the dark control. Instead, when samples were irradiated for three consecutive days, the differences in growth rate between controls and treated samples were always statistically significant ($p < 0.05$), suggesting that this treatment was necessary to boost Saos-2 growth. LLLI is reported to stimulate cell proliferation through a wide network of signals. In order to explain our proliferation results, we analyzed the activation of the mitogenic protein Akt, which has been reported to play a key role in proliferation induction by LLLI.[31] These data confirmed the involvement of Akt in proliferation of treated cells. In agreement with Zhang et al.,[31] we observed, by Western blot analysis, that LLLI is able to induce a transient but strong increase of Akt phosphorylation, which reaches a maximum 20 min after treatment. It was reported that LLLI induces the expression of growth factors, such as insulin-like growth factor-1,[46] vascular endothelial growth factor,[47] and transforming growth factor-beta.[48] We speculate that the relatively prolonged effect on cell proliferation that we observed (up to seven days after multiple laser doses) may be attributed to the growth factors synthesized and secreted by the cells in response to irradiation. After observing that laser treatment promotes *in vitro* proliferation of these cells, we tested whether this effect was supported by an increase in telomerase activity, whose up regulation is considered to be responsible for the unlimited proliferation of cancer cells.[49] In our experiment, laser treatment did not increase the average level of telomerase activity, and these results led us to conclude that this treatment schedule induces proliferation of Saos-2 cells without changing their telomerase expression pattern. In addition, CLSM observations performed on laser-treated/untreated cells showed that irradiated cells did not change their morphology after laser application. After identifying the optimal laser protocol, we studied whether laser treatment influenced osteoblastic differentiation of Saos-2 cells. In most bone tissue engineering studies, bone-promoting factors are used to induce differentiation of Saos-2 along the osteogenic pathway. To screen laser effects on Saos-2 differentiation, we stimulated and cultured cells in the presence/absence of osteoinductive conditioned medium in comparison with unstimulated cultures in the same media conditions used as controls. The cultures were harvested on day 14, representing the almost completely differentiated and mineralized stage of Saos-2 cells. When this cell line is cultured with medium supplemented with osteogenic factors, osteoblasts will differentiate and form a calcified matrix.[50] It has been reported that an enhancement in bone formation depends on an increase in extracellular matrix synthesis.[51] In this osteogenic condition, Saos-2 cells showed a significant level of bone matrix constituents such as COL-I, COL-III, ALP, OP, OC, OSN, and DEC. In particular, COL-I is the most important and abundant structural protein of the bone matrix;[32] DEC is a proteoglycan that is considered to be a key regulator for the assembly and the function of many extracellular matrix proteins with a major role in the lateral growth of the collagen fibrils, delaying the lateral assembly on the surface of the fibrils;[33] OP is an extracellular glycosylated bone phosphoprotein secreted at the early stages of the osteogenesis before the onset of the mineralization; it binds calcium, is likely to be involved in the regulation of the hydroxyapatite crystal growth, and through specific interaction with the vitronectin receptor, promotes the attachment of the cells to the matrix;[34] OC is secreted after the onset of mineralization, and it binds to bone minerals.[35]

In this study, we consistently observed that during 14 days of culture in OM, Saos-2 cells exhibited the typical fully differentiated and mineralized stage of osteoblasts with respect to those maintained in PM as documented by ALP activity and calcium deposition. The same features were observed for laser-stimulated cells cultured in OM.

Furthermore, qRT-PCR analysis on osteoblastic differentiation markers showed comparable expression of BOSP, COL-I, and OP, and a slight increase in ALP and OP was visible in laser-treated cells cultured in OM. All together these data suggest that laser stimulation does not block the progression of osteoblast differentiation, as also directly confirmed by the Calcein green staining. More interesting were the results obtained in the unconditioned medium (PM). We demonstrated that when cells were treated with laser in PM, the expression of osteoblast markers, ALP activity, and the mineralization of Saos-2 were significantly enhanced, compared to unstimulated cells in PM (Figs. 5 and 6). Apparently, laser exposition by itself, without adding osteogenic factors, seems to direct cells toward the bone differentiation pathway. An increase in the secretion of proteins specific for differentiation toward bone was detected. An almost twofold increase in ALP production was observed in the laser-stimulated cells in PM (Table 4). It is generally believed that the matrix mineralization is initiated by the expression of membrane-bound glycoprotein ALP on osteoblasts. It has been previously reported that ALP is expressed in large amounts in osteoblasts *in vivo*[36] as well as *in vitro* differentiation studies with osteoblast-like cell lines.[52] The elevated expression of ALP and bone proteins in the samples exposed to multiple laser doses may be attributable to the ability of laser irradiation to accelerate cellular activity, for example, ATP synthesis,[53] early osteoblastic differentiation,[54] and release of growth factors.[55] Our data are in agreement with Dörtbudak et al.[56] who reported a marked increase in bone matrix production between days 12 and 16 after three laser irradiation treatments (diode laser, wavelength 690 nm). In contrast to data reported by Coombe et al.,[24] with our experimental conditions, a significant

increase in ALP activity in the irradiated group as compared with the unexposed control was detected. Unexpectedly, the qRT-PCR results showed no significant difference in the gene expression of ALP between laser-stimulated and unstimulated cells when cultured in medium without osteo-inductive factors for 14 days. Moreover, the late osteoblastic markers OP and BOSP were significantly down regulated. The early bone COL-I marker, which is known to be up regulated at the proliferation stage and down regulated at subsequent stages, was quite similar between stimulated/unstimulated samples. Hypothetically, bone protein transcription was immediately activated after laser exposition, allowing protein translation, and the effect was lost thereafter. On the other hand, protein transcription stopped earlier with respect to protein translation. Further experiments need to be performed to explain these results. In this study, we properly answered the question of whether laser stimulation could positively affect cell proliferation and activate the differentiation process: based on our findings performed in our experimental conditions, we conclude that laser treatment supports, in particular, the *in vitro* proliferation of Saos-2 cells. However, we also demonstrated that in the absence of osteostimulatory factors, the laser treatment can influence the activation of the osteogenic pathway, as demonstrated by the increased calcified bone extracellular matrix deposition.

On the other side, we showed that the cell differentiation due to laser exposition and in the presence of chemical osteostimulatory factors was not improved: the differentiation process was not significantly incremented when compared to unexposed cells cultivated in the presence of osteogenic factors. Our observations are in accordance with those of Ozawa and colleagues who suggested that a beneficial laser effect on bone could be achieved with a number of applications, but not after only a single application using the same parameters.[39]

In conclusion, our results demonstrate the effectiveness of laser treatment in modulating cellular functions *in vitro*. Saos-2 cells cannot be utilized in clinical applications, but they can be used as an *in vitro* model to further investigate the cellular mechanisms underlying laser treatment, which are as yet unknown. We further propose that this experiment design be utilized to stimulate the conversion of bone marrow mesenchymal cells to the osteogenic phenotype; the resulting data could lead to a potential application in regenerative medicine.

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