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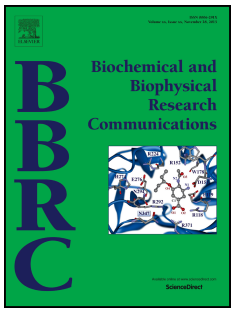

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SKELETAL STEM CELL AND BONE IMPLANT INTERACTIONS ARE ENHANCED BY LASER TITANIUM MODIFICATION

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Abstract

Purpose: To evaluate the osteo-regenerative potential of Titanium (Ti) modified by Light Amplification by Stimulated Emission of Radiation (LASER) beam (Yb-YAG) upon culture with human Skeletal Stem Cells (hSSCs[1]). **Methods:** Human skeletal cell populations were isolated from the bone marrow of haematologically normal patients undergoing primary total hip replacement following appropriate consent. STRO-1$^+$ hSSC[1] function was examined for 10 days across four groups using discs: i) machined Ti surface group in basal media (Mb[2]), ii) machined Ti surface group in osteogenic media (Mo[3]), iii) LASER-modified Ti group in basal media (Lb[4]) and, iv) LASER-modified Ti group in osteogenic media (Lo[5]). Molecular analysis and qRT-PCR as well as functional analysis including biochemistry (DNA, Alkaline Phosphatase (ALP[6]) specific activity), live/dead immunostaining (Cell Tracker Green(CTG[7]) / Ethidium Homodimer-1(EH-1[8])), and fluorescence staining (for vinculin and phalloidin) were undertaken. Inverted, confocal and Scanning Electron Microscopy (SEM) approaches

1.hSSCs(human Skeletal Stem Cells).
2.Mb(Machined Ti, basal media).
3.Mo(Machined Ti, osteogenic media).
4.Lb(LASER-modified Ti, basal media).
5.Lo(LASER-modified Ti, osteogenic media).
6.ALP(Alkaline Phosphatase).
7.CTG(Cell Tracker green).
8.EH-1(Ethidium Homodmer-1).

were used to characterise cell adherence, proliferation, and phenotype. **Results:** Enhanced cell spreading and morphological rearrangement, including focal adhesions were observed following culture of hSSCs[1] on LASER surfaces in both basal and osteogenic conditions. Biochemical analysis demonstrated enhanced ALP[6] specific activity on the hSSCs[1]-seeded on LASER-modified surface in basal culture media. Molecular analysis demonstrated enhanced ALP[6] and osteopontin expression on titanium LASER treated surfaces in basal conditions. SEM, inverted microscopy and confocal laser scanning microscopy confirmed extensive proliferation and migration of human bone marrow stromal cells on all surfaces evaluated. **Conclusions:** LASER-modified Ti surfaces modify the behaviour of hSSCs[1]. In particular, SSC[1] adhesion, osteogenic gene expression, cell morphology and cytoskeleton structure were affected. The current studies show Ti LASER modification can enhance the osseointegration between Ti and skeletal cells, with important implications for orthopaedic application.

Keywords: titanium surface, skeletal stem cell, tissue regeneration, bone formation, LASER.

Introduction

Research in the field of biomaterials has advanced significantly in recent years driven in part by the desire to develop biomaterials that will provide extended longevity and enhanced performance for an increasing ageing population [1]. Bone tissue engineering seeks to address the unmet need for new tissues lost as a consequence of disease, trauma or ageing, using a raft of interdisciplinary approaches including developmental biology, materials science, stem cells and bioengineering. Typically, the approach is to harness the therapeutic potential of stem cells together with an appropriate biomaterial [2,3]. Ti has long been the gold standard for orthopaedic given the excellent biocompatibility, low corrosion, wear resistance and to promote osseointegration at the bone-implant interface [4]. For the development of osseointegration the recruitment of cells with osteogenic potential is essential.

Subsequent colonisation by the cells is believed to occur through the release of growth factors and cytokines into the clot surrounding the site of implant placement, and it is widely accepted that SSCs[1] are the first cells recruited to such sites *in vivo* [5]. While Ti implants have found clinical utility for many decades, the process of osseointegration remains, to date, unclear. The process is time dependent and is dependent upon the close relationship between the bone quality and the Ti surface, although the bone structure is naturally difficult to change, the Ti surface can be relatively easily modified [6]. There are two accepted approaches to enhance the material bone response – the first is the development of a rough topography optimised for bone response [7], and the second is the establishment of a high surface energy (wettability) rendering the surface super-hydrophilic, thereby facilitating initial cell contact and adherence [8,9].

A number of approaches have been advocated to modify and improve the Ti surface, LASER treatment is an innovative approach that results in surfaces with increased surface area, enhanced wettability and, in preclinical (lapine) bone models, displays negligible corrosion and high removal torques of established implants [6]. As recently detailed in a number of studies, LASER treatment appears to provide a promising method for Ti implant generation, resulting in enhance and rapid onset of osseointegration [6,10,11,12,13].

Understanding how to control, manipulate, and enhance the intrinsic healing events modulated through osteogenic differentiation of SSCs[1] through the application of modified surfaces offers significant potential for the orthopaedic field. It is clear that an exquisite interplay exists between the cells and the microtexture of a material. *In vivo,* cells encounter a number of topographical features ranging from protein folding to collagen banding [14]. Due to the ease of manufacture, the development of materials with a range of surface roughness has been widely used to further examine the bone material interface. Such a strategy provides useful information regarding the bone cell response to structured materials [15]; either as a consequence of surface modification

that generates enhanced implant stability and/or indeed accelerated healing following implantation [16].

Based on the hypothesis that modified surfaces can modulate the initial osteo-inductive responses of cells, this study set out to examine the osteo-regenerative potential of Ti-modified by LASER beam (Yb-YAG) on hSSC[1] compatibility and subsequent cell function.

Methods: ***Cell culture*** Skeletal cell populations STRO-1$^+$ hSSCs[1] were isolated and cultured following previously described protocols [17] with the approval of the Local Research Ethics Committee (LREC 194/99).

2,500 STRO-1$^+$ hSSCs[1] derived from the same patient were cultured on titanium discs in non-tissue culture plastic multiwell dishes for 10 days across four groups: Mb[2], Mo[3], Lb[4] and Lo[5]. Basal media was DMEM with 10% FCS and osteogenic media included 10nM dexamethasone.

Ti discs were prepared at UNESP(Araraquara/Brazil). 180 Ti rods were cut into 8mm diameter by 2 mm long cylinders, and the surfaces of 90 discs were modified by LASER beam as described previously [6,11,18]. All samples were sterilized by ethylene oxide.

Analysis of hSSC[1] proliferation and viability cell number was determined using a standard DNA PicoGreen assay [18]. Cell lysate was measured for DNA content using PicoGreen (Molecular Probes, Paisley, UK) analysed using a BioTek FLx-800 microplate fluorescent reader.

Live / dead immunostaining CTG[7] was used to label viable cells and EH-1[8] for necrotic cell nuclei. Cell images were assessed for cell viability using Zeiss Axiovision software Ver 3.0 via an AxioCam HR digital camera on an Axiovert 200 inverted microscope (Carl Zeiss, Hertfordshire, UK) under fluorescent light.

Analysis of the osteogenic differentiation of hSSCs[1] ALP[6] activity within the cell lysate was measured using p-nitrophenyl phosphate as the substrate in 2-amino-2-methyl-1-propanol alkaline buffer solution (Sigma, Poole, UK), analysed using a BioTek

ELx-800 microplate reader to provide specific enzyme activity (ALP^6/DNA/hr) across samples.

Analysis of cell adherence and morphology, cytoskeleton structure and focal adhesion - Confocal laser scanning microscopy The FAK100 | Actin Cytoskeleton / Focal Adhesion Staining Kit (Millipore®) was used to analyse cytoskeleton modifications following culture of the $hSSCs^1$ on the different Ti samples. In brief, cells were fixed in 4% formaldehyde in PBS, permeabilised (0.1% Triton X-100 in PBS) and blocked (1% BSA in 1% PBS). Cells were incubated with a primary anti vinculin mouse monoclonal antibody (1:100) and then with FITC-conjugated goat anti mouse secondary antibody (1:100) and cultures were then stained simultaneously with TRITC-conjugated phalloidin (1:1000) (to enable labelling of actin filaments) and DAPI (1:1000 dilution of a 1mg/ml sotck). The secondary antibody alone was used as a negative control. Images were taken using a confocal laser scanning microscope (Leica TCS SP5, Leica Biosystems, Wetzlar, Germany).

SEM: samples were fixed whole in 3% gluteraldehyde and 4% formaldehyde in 0.1M PIPES buffer at pH 7.2. A post-fixative of 1% osmium tetroxide was applied prior to dehydration through a series of graded alcohols followed by critical point drying. The surface was putter-coated with gold-palladium and visualized with an FEI Quanta 200 SEM (FEI, Oregon, USA) to observe the morphology and attachment of cells on the Ti surfaces.

Molecular Analysis following incubation of $hSSCs^1$ on the different surfaces, samples were washed, incubated with collagenase IV, trypsinised and total RNA then extracted using RNeasy Plus Mini Kit (Invitrogen) to enable gene expression analysis. Extracted RNA was reverse transcribed using VILO cDNA synthesis kit (Invitrogen®) for RT-PCR. qRT-PCR was performed using a 96-well optical reaction plate and a 7500 Real Time PCR system (Applied Biosystems, Carlsbad, USA). Each sample was subjected to qRT-PCR against a panel of osteogenic gene primers (Table 1). Values were

calculated using the comparative threshold cycle (Ct) method, normalized to β-actin expression and expressed as the mean ± SD.

Statistics all experiments were run three times using four independent samples. Data was expressed as the mean ± SD. Statistical analysis was performed using SPSS v 17.0 (SPSS Inc, Chicago, IL/USA). The Wilcoxon's signed rank test was used to compare between groups. P values less than 0.05 were deemed significant.

Results:

hSSCs[1] cultured on LASER-modified Ti surface display enhanced cell growth and viability. No significant differences were observed after 10 days of culture of hSSCs[1] seeded on any of the Ti surfaces (Mb[2] versus Lb[4] (100% x 87.51%), Mo[3] versus Lo[5] (103% x 160.22%)) indicating cell survival and growth (Figure 1). Cell viability and an absence of cell necrosis were confirmed by live/dead staining with CTG[7]/EH-1[8] after 10 days culture. (Figures 1A-D).

hSSCs[1] cultured on LASER-modified Ti surface exhibit excellent biocompatibility, altered morphology, modified-cytoskeletal structures and focal adhesions

To analyse the effects of the Ti surfaces on the hSSC[1] cytoskeleton, fluorescence staining was performed with vinculin monoclonal antibody and TRITC-conjugated phalloidin. Enhanced cell spreading and cytoskeletal (actin) structure rearrangement was observed in cells cultured on the LASER-modified surface as analysed using confocal microscopy (Figures 2B, D, and F). Actin filaments (red) in hSSCs[1] grown on machined surface were observed to be organized parallel to the underlying surface topography (Figures 2A, C and E), while cells cultured on LASER-modified surfaces exhibited actin filaments arranged randomly (Figures 2B, D and F). hSSC[1] focal adhesion formation was evidenced by vinculin staining (green) and presented at the cell periphery on LASER modified surface (Figure 2F, J). In contrast, the actin filaments

of hSSCs[1] cultured on machined surface were oriented in a predominantly parallel manner (Figure 2A,C,E,G,I,K).

SEM: used for cell adherence and morphology following growth of hSSCs[1]. hSSCs[1] exhibited discrete differences in cell morphology as a function of profile surface and media. All groups displayed healthy adherent cells on Ti surfaces (Figure 3). On machined surface, cells presented as flattened structures, with a distinct spread morphology. On Mb[2] surfaces, hSSCs[1] displayed few protoplasmic processes attached to the surface (Figure 3I). Cells were observed to be distributed over the LASER surface and to form cytoplasmic bridges of variable thickness, suspended above the peaks and depressions of the LASER-modified surface (Figures 3B and D). On the LASER-modified surfaces, the hSSCs[1] presented numerous protoplasmic processes (Figures 3F, H, J and L), and filopodia (as a consequence of culture in osteogenic culture media) (Figures 3 K and L).

LASER-modified Ti surface enhance osteogenic differentiation of hSCCs[1]

To investigate the effect of hSSCs[1] differentiation on LASER-modified surfaces after 10 days in culture, the expression of osteogenic markers was analysed using biochemical and molecular approaches. ALP[6] specific activity was increased in hSSCs[1] cultured on LASER-modified surfaces compared to hSSCs[1] cultured on control (machined) surfaces, in basal culture media (154.28% vs 100% Mb[2] control) (Figure 1).

Molecular analysis showed that hSSCs[1] seeded on Lb[4] displayed enhanced osteogenic marker gene expression in comparison to hSSCs[1] cultured on Mb[2].

Specifically, *ALP*[6] and *OPN* mRNA levels in hSSCs[1] cultured on Lb[4] were respectively 2-fold and 3.6-fold higher than those cultured in Mb[2] (Figure 4).

Osteogenic conditions do not modulate hSSCs[1] cultured on LASER-modified Ti surface

The induction of proliferation and osteogenic differentiation of hSSCs[1] were assessed using standard osteogenic culture medium with samples from the same patients cultured on machined and LASER surfaces. Results revealed that the hSSCs[1] did not show any statistically significant increases in cell proliferation (mean ± SD 103 ± 20.94 versus 160 ± 8.27) (Figure 1), ALP[6] specific activity (mean SD 124.71 ± 33.78 versus 134.78 ± 23.11) and osteogenic marker gene expression *ALP*[6] (mean ± SD 4.82 ± 1.190 versus 8.814 ± 4.98) and *OPN* (mean ± SD 1.2 ± 0.84 versus 0.76 ± 0.09) (Figure 4) or any cytoskeleton modification (Figures 2).

Discussion

Ti surface modification to enhance implant function can be achieved using a variety of methods. In the current study we demonstrate the efficacy of LASER irradiation of titanium to generate a surface to improve skeletal stem cell function. LASER irradiation has been shown to be a promising method for Ti surface treatment, increasing the Ti surface area, wettability and, critically, offering a high degree of surface purity at relatively low cost [11,12,19]. Furthermore, studies with LASER-modified materials implanted in rabbit tibias and subsequently presenting high removal torques [11,12,19] and properties that favour cell adhesion and proliferation make this an attractive approach [10,20]. We have previously shown that the topography of a Ti LASER-modified surface displays distinct topographies including a surface roughness with an appearance comparable to a “cauliflower” morphology that provide enhanced wettability and surface area [6].

The biocompatibility of biomaterials is closely related to cell viability and proliferation with attachment**,** adhesion, and spreading in the early phase of the cell/material interaction being critical in modulating the capacity of a cell to proliferate and

differentiate [21]. The LASER treated surfaces in the current study, provide a topography that supported hSSC[1] viability and proliferation. A wealth of studies indicate rough Ti surfaces can enhance osseointegration in the clinic in comparison to smooth surfaces [6,7,12,22], although the cellular and molecular mechanisms that drive this process remain far from clear. ALP[6] activity is typically used as a marker to follow the differentiation of osteoblasts from non-calcium-depositing to calcium-depositing cells [23] , and as a marker of the early stages of osteogenic differentiation [24]. Studies suggest the ALP[6] activity of a cell is surface-dependent. Thus, if the Ti surface is modified elevated ALP[6] activity is a likely consequence [25]. In the current study, hSSCs[1] on LASER-modified Ti surfaces, displayed enhanced differentiation as assessed by ALP[6] activity. It is assumed this is a consequence of enhanced material surface reactivity [26] and enhanced physical–chemical properties [6]. In contrast, Takeuchi et al showed that a modified surface can reduce cell proliferation whilst initially driving the expression of specific cell markers except for ALP[6] [27]. The cell–biomaterial interface functions not only to define the boundary between tissue and implant, but also to act as a mediator of first stage protein interactions as well as later stage cell adhesion and orientation [28]. When blood cells arrive at the implant Ti surface, the blood cells express a variety of integrins, resulting in cytoskeletal changes. Changes in cytoskeleton tension have a direct effect on cell morphology as evidenced by actin staining. Alteration in cell morphology as a consequence of cytoskeletal tension has an indirect effect on mechano-transduction pathways, as demonstrated by expression changes in stem cell responses [14,29]. In the current studies cytoskeletal rearrangement were observed, including altered expression patterns of vinculin, a key structural component of focal adhesions [30]. On the LASER topography, vinculin displayed arrangement around the cell cytoplasm periphery. Interestingly, expression of the vinculin marker was spread distinctly revealing an extensive non focal distribution in the cytoplasm of hSSCs[1] on the machined surface. Furthermore, hSSCs[1] cultured on machine surfaces displayed a flat morphology, primarily orientated along

the discrete grooves, with relatively few protoplasmic processes attached to the Ti substrate. In contrast, cells cultured on LASER Ti discs displayed enhanced adherence to the modified surface, indicating the potential for modified cellular activity or tissue responses leading to greater osteogenesis [3].

We have previously reported that changes in cytoskeletal tension in response to topography may modify interphase nucleus organisation and hence directly influence cell gene expression profiles [14,31]. The pattern of five specific osteogenic markers, *RUNX2, ALP*[6], *COL1A1, OPN* and *OCN* and, the chondrogenic marker *SOX9*, in primary hSSCs[1] cells cultured on LASER Ti surfaces were compared with machined Ti surface substrates. *RUNX2* is essential for osteoblast maturation and osteogenesis [32] and is a key regulator of *OCN*, *COL1* and *ALP*[6] genes [33]. ALP[6] and COL1A1 are matrix-mineralizing proteins, and their expression has been shown to be important for bone matrix assembly [34]. In the present study *ALP*[6] gene expression showed a statistically significant increase from Mb[2] to Lb[4], in agreement with other studies (Stein and Lian, 1993). The mechanisms of bone remodelling underline the potential role of two non-collagenous matrix proteins, osteopontin and osteocalcin [35]. Osteopontin is a multifunctional phosphorylated glycoprotein secreted by osteoblasts, and has been suggested to be present at an early stage of bone development and to promote the attachment of osteoblasts to the extracellular matrix [36]. Osteopontin is involved in bone remodelling [37] whilst osteocalcin is a marker of primary bone formation and is produced by osteoblasts [38]. While the precise role of osteocalcin is still under examination, roles as an endocrine regulator of metabolism in the skeleton and as a regulator of mineralization have been proposed. Serum concentrations of osteocalcin have been shown to correlate with histo-morphometric indices of newly formed bone [39]. The present study show that hSSCs[1] cultured on LASER-modified Ti surfaces display enhancee osteopontin expression, indicating the possible osteogenic potential of LASER-modified Ti surfaces. Interestingly, no significant changes in osteocalcin

expression were observed in the current study, in agreement with previous studies. [40]. This may potentially be as a consequence of osteocalcin being a late marker of bone cell differentiation and osteopontin an early marker [41]. Although, *COL1A1*, *RUNX2* and *OCN* expression did not show statistical significant differences across the substrates, a trend of enhanced expression on LASER-modified surfaces was observed. As expected, *SOX9* expression, a chondrocytic marker was unaffected.

In conclusion, this study demonstrated the influence of the microtexture of LASER-modified Ti surfaces on the behaviour of hSSCs[1]. Cell proliferation, adhesion, osteogenic gene expression, cell morphology and cytoskeleton structure were all affected by the modified topography of Ti surfaces that resulted from LASER irradiation. These studies show the potential of Ti LASER modification to enhance the osseointegration at the material-bone cell interface with important implications for orthopaedic and dental application.

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Table 1. Human osteogenic gene primer sequences used for RT-PCR

Protein	Gene	Primer sequences
B-Actin (housekeeping gene)	*B-Actin*	F:5'- GGCATCCTCACCCTGAAGTA R:5'- AGGTGTGGTGCCAGATTTC
Alkaline phosphatase	*ALP*	F:5'-GGAACTCCTGACCCTTGACC R:5'-TCCTGTTCAGCTCGTACTGC
Collagen type 1A1	*COL1A1*	F:5'-GAGTGCTGTCCCGTCTGC R:5'-TTTCTTGTTCGGTGGGTG
Runt-related transcription factor 2	*RUNX2*	F:5'-GTAGATGGACCTCGGGAACC R:5'-GAGCTGGTCAGAACAAAC
Sex-determining region Y, box 9	*SOX9*	F:5'-CCCCAACAGATCGCCTACAG R:5'-GAGTTCTGGTCGGTGTAGTC
Osteopontin	*OPN*	F:5'-GTTTCTCAGACCTGACATCC R:5'-CATTCAACTCCTCGCTTTCC
Osteocalcin	*OCN*	F:5'-GGCAGCGAGGTAGTGAAGAG

		R:5'-CTCACACACCTCCCTCCTG

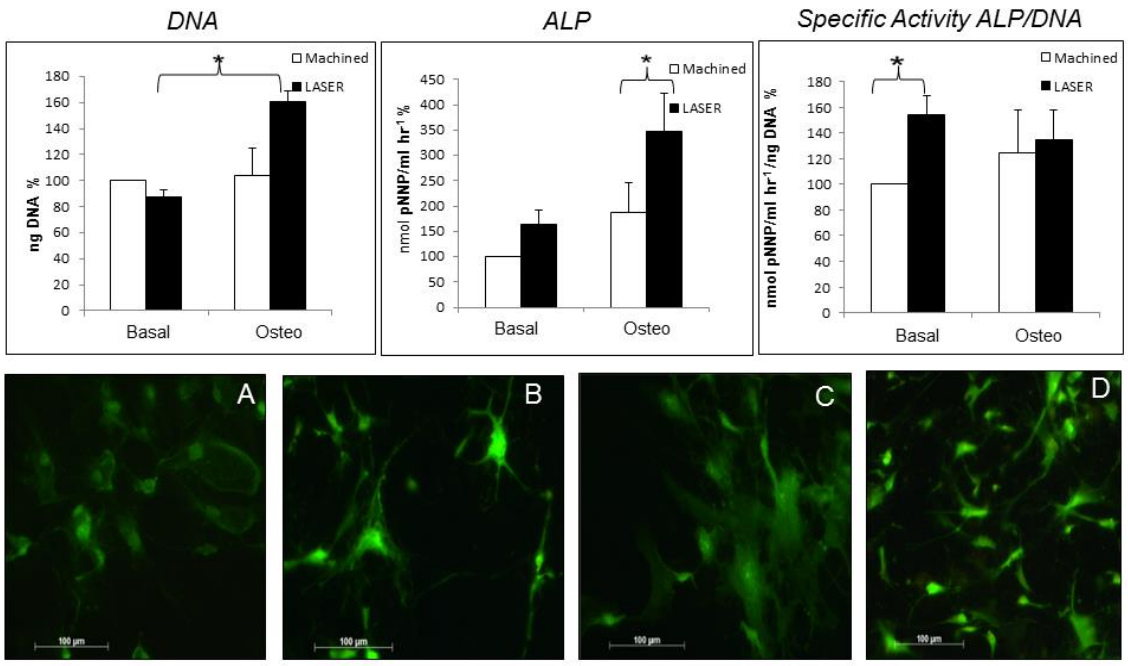
Figures:

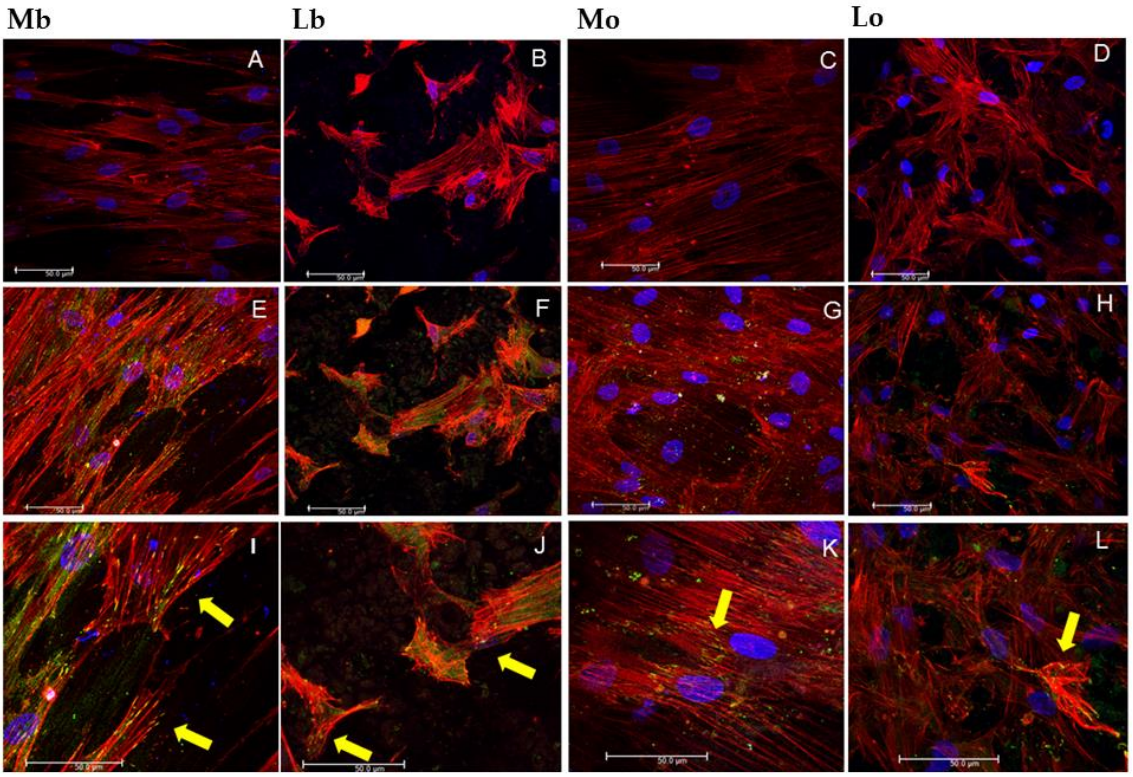
Figure 1. Biochemical analysis (DNA, ALP[6] and Specific Activity ALP[6]/DNA) of hSSCs[1] on Ti discs (10 days). Error bars denote Standard Deviation. *p<0.05. **A-D** Immunofluorescence (cell tracker green - inverted microscope 20X magnification, scale bar = 100µm) of hSSCs[1] (10 days) on Mb[2], Lb[4], Mo[3] and Lo[5].

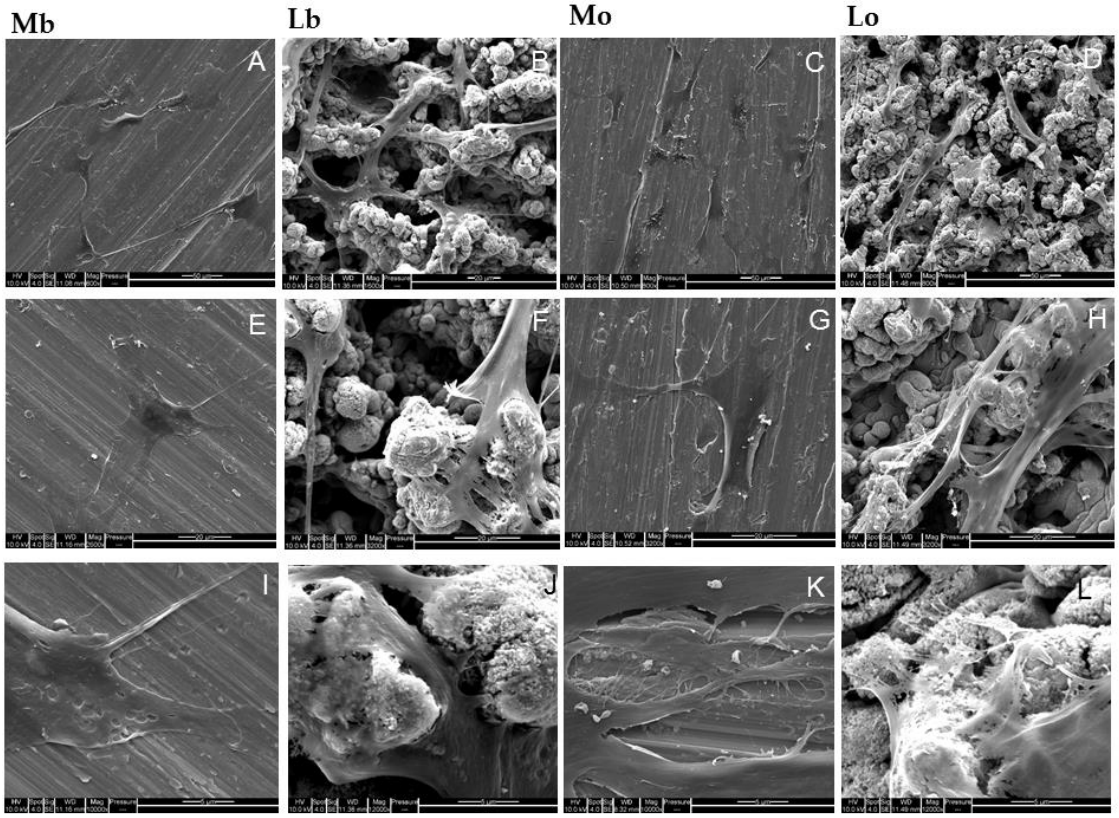
Figure 2: Confocal images: cytoskeletal structure hSSCs[1] (10 days) on Mb[2] (**A**,**E,I**), Lb[4] (**B**,**F,J**), Mo[3] (**C**,**G,K**) and Lo[5] (**D**,**H,L**). Red: actin. Blue: nucleus. Green: vinculin. Cell focal adhesion (arrows). Scale bar = 50µm.

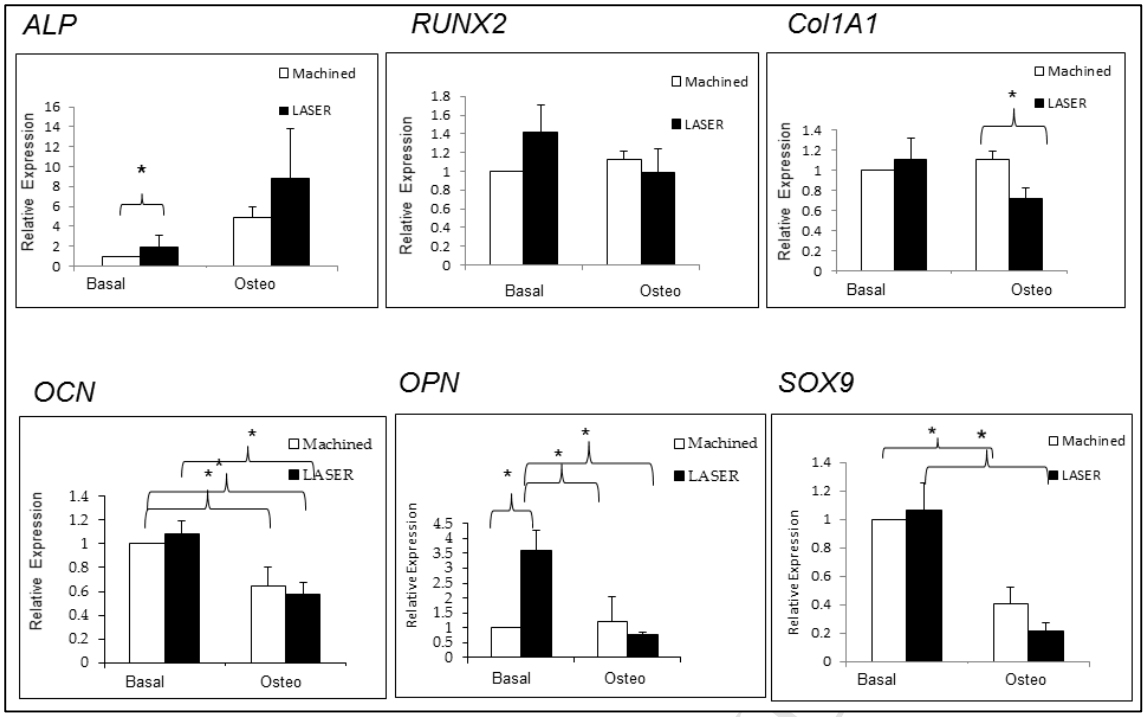
Figure 3. SEM: micrographs of hSSCs[1] adherence and morphology (10 days) on Mb[2] (**A**,**E** and **I**), Lb[4] (**B**,**F** and **J**), Mo[3] (**C**,**G** and **K**) and Lo[5] (**D**, **H** and **L**). **A-D** scale bar = 50µm, **E-H** scale bar = 20µm and **I-L** scale bar = 5µm.

Figure 4. hSSC[1] gene expression of osteogenic markers (*ALP[6], RUNX2, COL1A1, OCN, OPN*) and chondrogenic marker (*SOX9*) following culture for 10 days in basal and osteogenic conditions, on machined surface and LASER Ti surfaces (*β-actin* = internal control). Values are mean ± SD of 4 independent samples, *p < 0.05).









Research Highlights

- *Bone stem cells on LASER Ti surface display enhanced cell growth and viability.*
- *Bone stem cells on LASER Ti surface exhibit marked biocompatibility.*
- *Human bone stem cells on LASER Ti surface exhibit altered morphology.*
- *LASER Ti enhance osteogenic differentiation of human bone skeletal stem cells.*
- LASER Ti provides a unique approach to enhance osseointegration with the material.