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Comparison of osteogenic differentiation potential of human adult stem cells loaded on bioceramic-coated electrospun poly (L-lactide) nanofibres

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Abstract

Objectives: To compare potential of four types of stem cell in tissue engineering and regenerative medicine applications, osteogenic capacity of newly introduced mesenchymal stem cells (MSCs) derived from buccal fat pads (BFP) (an adipose-encapsulated mass of the oral cavity), was compared to those isolated from bone marrow (BM-MSCs), adipose tissue (AT-MSCs) and unrestricted somatic stem cells (USSCs). Cells were cultured on poly (L-lactide) (PLLA) nanofibres, Bio-Oss-coated PLLA (PLLA-Bio), and culture plates (TCPS) as control.

Materials and methods: Capacity of proliferation and osteogenic differentiation of the stem cells was investigated by MTT assay and common osteogenic markers, alkaline phosphatase activity, calcium mineral deposition and bone-related genes.

Results: Highest proliferation level was observed in cells cultured on PLLA-Bio, but with no significant difference between proliferation levels of the four types of stem cell. Over the period of study, BM-MSCs cultured on PLLA-Bio scaffolds exhibited greatest alkaline phosphatase (ALP) activity and mineralization with BFP-MSCs having the next closest results. However, AT-MSC had the lowest capacity for ALP activity and mineralization during osteogenic differentiation. Gene expression evaluation revealed that highest expression of three important bone-related genes was observed in stem cells cultured on bioceramic-coated nanofibrous scaffolds.

Conclusions: Results indicated Bio-Oss-coated PLLA to compose most appropriate substrates to support proliferation and osteogenic differentiation of stem cells *in vitro*. BFP-MSCs demonstrated the same osteogenic differentiation capacity as other stem cells tested and thus hold very promising potential for applications in bone tissue engineering and regenerative medicine.

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Introduction

Mesenchymal stem cells (MSCs) are adult stem cells which have been used, applied to cell-based therapies (1), and regenerative medicine (2) including for bone repair, heart disease reconstitution and neurodegenerative disease therapy (3). Although MSCs were originally isolated from bone marrow (4–6), they have also been isolated from other sites such as adipose tissue (7), peripheral blood (8), umbilical cord blood (9) and connective tissues of the dermis (10).

There are several methods for definition and confirmation of MSC phenotype, which include their growth and adherence on plastic, a pattern of certain non-specific surface antigens and also by *in vitro* and *in vivo* differentiation potentials (11–14). Osteogenic, adipogenic and chondrogenic differentiation potentials of MSCs, cultured under appropriate medium, have been extensively demonstrated (15,16). Phenotypic characterization of MSC has been performed by evaluation of proteins such as CD90, CD105, CD10, CD44, CD73 naturally expressed on surfaces of precursor stem cells,

and lack of haematopoietic lineage markers and HLA-DR (17–19).

There are many reports concerning the preference of using adipose-derived tissue than other MSC sources (20,21); adipose tissue-derived stem cells (ASCs) have been used in a number of experimental studies and are an interesting source, ahead of clinical therapies, in the field of bone tissue engineering (22).

Sierra-Johnson *et al.* have demonstrated that the oral cavity contains a mass of specialized fatty tissue, the buccal fat pad (BFP) or Bichat's fat pad, distinct from subcutaneous fat (23). Easy accessibility and rich vascularization make this adipose mass attractive for grafting, and it has been used widely in oral surgery for repair of bone and periodontal defects (24,25). Harvesting of BFP is a simple procedure, which requires minimal incision under local anaesthesia, causing minimal donor-site damage (26).

Bone tissue engineering has shown great promise for derivation of biological bone substitutes in patients with bone loss, fracture or osteoporosis, unable to acquire improvement by current orthopaedic methods. Bone is a specialized connective tissue that consists of cells and extracellular matrix (ECM) (27). Tissue engineering mimics this structure using 3D nanofibrous scaffolds and stem cells *in vitro* (28).

Over the past four decades, a variety of materials has been developed for tissue engineering applications and is still increasing. Nowadays, bioceramics are well known to be classic artificial bone substitution material. Furthermore, findings show that combination of stem cells and bioceramics has proven to be very efficient for *in vitro* osteogenesis (29) and also *in vivo* bone regeneration (30).

Bio-Oss is a deproteinized bovine bone material with unique features of condensed strength of 35 Mpa and high natural porosity (75–80% total volume) which provides large surface areas for scaffolds. Bio-Oss is one of the several bioceramics commonly used for treatment of bone lesions, periodontal defects and as dental implant (31,32).

In this study, we investigated osteogenic differentiation potential of BFP-MSCs compared to adipose tissue-MSCs (AT-MSCs), bone marrow-MSCs (BM-MSCs) and unrestricted somatic stem cells (USSCs) on a combination of Bio-Oss and PCL nanofibres, as scaffolds. For this purpose, after isolation and characterization of BFP-MSCs *in vitro*, osteogenic differentiation of these stem cells was investigated using ALP activity, calcium content assay and expression of bone-related gene markers. The study develops a good alternative to use of the more common stem cell types such as Ad-MSCs, BM-MSCs and USSCs.

Materials and methods

Scaffold preparation

Electrospinning methods were used for nanofibrous scaffold fabrication, as described previously (33). Briefly, 12% (w/v) solution PLLA – average molecular weight 58 000 Da and poly (ε-caprolactone) (M_w = 80 000 g/mol) (Sigma-Aldrich, St. Louis, MO, USA) in N-dimethylformamide (DMF; Merck, Darmstadt, Germany), was placed in a 5 ml syringe connected to a 21 gauge needle through an extension tube. A steel grounded collector was used to collect the electrospun nanofibres at a distance of 15 cm from the needle. Solution was fed through the tube into the needle by a syringe pump at 1 ml/h. Application of 20 kV voltage between needle and collector, forced solution droplets to leave the needle and be deposited on the cylinder, in the form of ultrafine fibres. Having reached thickness in the order of 200 μm, the mat was detached from the collector and placed in a vacuum for evaporation of residual solvent, for 24 h, and by constant vacuum pressure after this time we insure that DMF was removed.

Nanofibre surface modification

Oxygen plasma treatment was then performed using a low frequency plasma generator at 44 GHz with a cylindrical quartz reactor (Diener Electronics, Nagold, Germany). Pure oxygen was introduced into the reaction chamber at 0.4 mbar pressure, then glow discharge was ignited for 5 min. 1% (w/v) solution Bio-Oss particles (GeistlichPharma AG, Wolhusen, Switzerland) in deionized water was prepared after well dispersion of microparticles in an ultrasonic bath for 20 min. To deposit Bio-Oss on the surface of nanofibres, the plasma-treated mat was immersed in Bio-Oss aqueous solution overnight. Then, the mat was rinsed well with deionized water and dried under vacuum. As pristine PLLA nanofibres have very low capacity for cell attachment due to high hydrophobicity, plasma-treated PLLA were used in all experiments and are referred to as PLLA in this study.

Isolation and expansion of BFP MSCs

Buccal fat pads were collected during orthognathic surgical procedures from 10 donors (age 17–40, Taleghani Hospital, Tehran, Iran) with informed consent, according to the Medical Ethics Committee guidelines, Ministry of Health I.R.Iran. Samples were then treated with 0.075% collagenase I at 37 °C for 60 min. After centrifugation (400 ***g*** for 10 min), supernatant was discarded and cell pellets were treated with RBC lysis buffer (8.2 g/l NH_4Cl, 0.84 g/l $NaHCO_3$ and 0.37 g/l disodium ethyl-

ene-diamine-tetra-acetic acid, pH 7.4) at room temperature (RT) for 10 min. Then, samples were centrifuged at 400 ***g*** for 5 min and cell pellets were resuspended into 75 cm^2 culture flasks (Nunk) under Dulbecco's modified Eagle's medium (DMEM; Invitrogen Co., Carlsbad, CA, USA) with 10% foetal bovine serum (FBS; Invitrogen Co.), and incubated in 95% air and 5% CO_2 at 37 °C. After reaching 80–85% confluence after around 10 days, adherent cells were detached using 0.025% trypsin, for 2 min, in 5% CO_2, at 37 °C, and re-plated.

Stem cell characterization

For characterization of all MSCs, after two passages, expression of surface markers was evaluated using monoclonal antibodies phycoerythrin-conjugated anti-CD44, fluorescent isothiocyanate (FITC)-conjugated mouse anti-human CD45 (leucocyte common antigen), phycoerythrin (PE)-conjugated anti-CD105 (Endoglin or SH2) CD34, anti-human leucocyte antigen DR (HLA-DR) and anti-CD90. Cells were detached using trypsin/EDTA and incubated with the specific antibodies or isotype control antibodies (with FITC- or PE-labelled antibodies included in each experiment), in 100 μl 3% bovine serum albumin in PBS, for 1 h at 4 °C. Then, cells were fixed in 1% paraformaldehyde, and analysed using a Coulter Epics-XL flow cytometer (Beckman Coulter, Fullerton, CA, USA) and Win MDI 2.8 software (Scripps Institute, La Jolla, CA, USA).

Expansion of AT-MSCs, BM-MSCs and USSCs

These stem cells were isolated and characterized with BFP-MSCs by expression of mesenchymal-related surface markers. Prior to scaffold cell seeding, passage 2 cells were cultured and maintained in DMEM supplemented with 10% FBS and incubated in 95% air, 5% CO_2 at 37 °C.

Cell seeding

Prior to cell seeding, scaffolds were immersed for 24 h in the following solutions: (i) 70% ethanol for sterilization, (ii) penicillin, streptomycin and amphotericin B to prevent bacterial and fungal growth and (iii) culture medium to ensure sterilization and enhance cell attachment after seeding. Then, stem cells were seeded on PLLA loaded with Bio-Oss particles and were cultured under DMEM with 10% FBS in 95% air and 5% CO_2 at 37 °C for around 24 h for attachment. This investigation was categorized in five groups including Ad-MSCs-PLLA (with and without Bio-Oss), BM-MSCs-PLLA (with and without Bio-Oss), USSCs-PLLA (with and without Bio-Oss), BFP-MSCs-PLLA (with and without Bio-Oss) and control (TCPS). After 24 h incubation, osteogenic differentiation medium was added to all groups. This consisted of basal medium supplemented with 3 mM β-glycerophosphate (βGP), 50 μg/ml ascorbic acid and 10^{-9} M dexamethasone. Medium was replaced every 2 days.

Scanning electron microscopy (SEM)

Cell-polymer constructs were fixed in 2.5% glutaraldehyde, dehydrated through a graded series of ethanol, vacuum dried, mounted on aluminium stubs, and sputter coated with gold. Samples were examined using a scanning electron microscope (S-4500; Hitachi, Tokyo, Japan) at an accelerating voltage of 20 kV.

MTT assay

MTT assay was used for evaluation of scaffold biocompatibility and proliferation potential of the stem cells. Sterilized pristine and modified scaffolds were placed in 24-well culture plates, seeded with cells 4×10^3 cells per cm^2, and incubated at 37 °C in 5% CO_2. After 1, 2, 3, 4 and 5 days cell seeding, 50 μl MTT solution (5 mg/ml in DMEM) was added to each well ($n = 4$). For conversion of MTT to formazan crystals by mitochondrial dehydrogenases of living cells, each plate was incubated at 37 °C for 4 h. For dissolution of dark blue intracellular formazan, supernatant was removed and a constant amount of dimethyl sulphoxide solvent was added. Optical density was read in a micro-plate reader at 570 nm (BioTek Instruments, Winooski, VT, USA). The same procedure was performed for cultured cells in tissue culture polystyrene (TCPS) as control.

Alizarin red S histochemical staining

After 3 weeks, to evaluate mineralized matrix, alizarin red staining was performed. Medium was removed, and cells were washed twice in cold PBS, then fixed in cold 4% paraformaldehyde for 20 min at 4 °C. Cells were then washed twice again in PBS. Fixed samples were stained with 2% alizarin red S at pH 7.2 (Sigma, St. Louis, MO, USA). After 5–10 min at 37 °C, cells were washed three times again with PBS and observed by light microscopy. Morphology of stained cells was quantified using image J software (http://rsb.info.nih.gov/ij).

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was investigated *via* total protein extraction at 7 and 14 days, during

osteogenic differentiation, using 200 μl RIPA lysis buffer. For sedimentation of cell debris, lysate was centrifuged at 25 200 g at 4 °C for 15 min. Then, supernatant was collected and ALP activity was measured using *p*-nitrophenyl phosphate as phosphatase substrate. Enzyme activity level was normalized against total protein.

Calcium content assay

During osteogenic differentiation, amounts of calcium deposited on TCPS, PLLA and PLLA-Bio scaffolds were measured using the Cresolphthalein Complexone method. To extract calcium, scaffolds were homogenized in 0.6 N HCl (Merck) followed by shaking for 4 h at 4 °C. After addition of reagent (Calcium Content Kit, Pars Azmoon Iran) to calcium solutions, optical density of samples was measured at 570 nm in a micro-plate reader (BioTek Instruments). Calcium content values of samples were obtained from a standard curve of OD versus serial dilution of calcium concentrations.

Real-time RT-PCR

Relative quantification of Runt-related transcription factor 2 (*Runx2*), osteocalcin (*BGLAP*) and *osteonectin* gene expressions were carried out in the four stem cell types, on coated and non-coated scaffolds, compared to controls, at 7 and 14 days. Total RNA was extracted using RNeasy kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. RevertAid First Strand cDNA synthesis kit (Fermentas, Burlington, ON, Canada) was also used to synthesize complementary strands. PCR parameters included denaturation at 95 °C for 3 min, then 40 cycles at 95 °C for 20 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. For PCR amplification and real- time RT-PCR, primer sequences are illustrated in Table 1. Real-time RT-PCR was performed using SYBR Premix Ex Taq (TaKaRa Bio Inc., Otsu, Shiga, Japan). Relative quantification model was applied to calculate expression of target genes in comparison to β-actin used as endogenous control. Gene expression levels were quantified by Rotor Gene 6000 (Corbett, Concorde, NSW, Australia).

Table 1. Primers used in real-time RT-PCR

Gene	Primer sequence (F, R, 5′→3′)	Product length (bp)
HPRT1	CCTGGCGTCGTGATTAGTG TCAGTCCTGTCCATAATTAGTCC	125
Runx2	GCCTTCAAGGTGGTAGCCC CGTTACCCGCCATGACAGTA	67
Osteonectin	AGGTATCTGTGGGAGCTAATC ATTGCTGCACACCTTCTC	121 224
Osteocalcin	GCAAAGGTGCAGCCTTTGTG GGCTCCCAGCCATTGATACAG	80

Statistical analysis

All experiments were conducted at least three times. Data are reported as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare the results. *P*-value of <0.05 was considered statistically significant. All statistical analyses were conducted with SPSS software, version 11.0 (SPSS, Chicago, IL, USA).

Results

Characterization of nanofibres

Results of SEM analysis demonstrated that fabricated PLLA scaffolds had porous bead-free nanofiber with interconnected pores. Image analysis of SEM samples revealed aligned-oriented nanofibres with average diameter of 480 ± 119 nm (Fig. 1a,b). After BioOss coating, homogeneous distribution of BioOss micro- and nanoparticles, average size of 959 ± 346 nm, was observed on the surface of scaffolds (Fig. 1c,d). PLLA nanofibres had tensile strength of 2.125 (0.403 MPa) and an elongation break 92.964%, which did not significantly change after surface modification.

To evaluate potential of PLLA and BioOss-coated PLLA as tissue-engineered scaffolds, as well as to investigate their effects on stem cell proliferation, cells were cultured on surfaces of nanofibres and their proliferation, infiltration, and osteogenic differentiation were investigated *in vitro*. An example of scanning electron micrographs of scaffolds after 21 days stem cells culture, is shown in Fig. 2. According to MTT results (Fig. 3), an increasing pattern of cell population was detected during the period of study, for all stem cell types, even though no significant difference was observed between proliferation of the different cells until day 2. After that significant increase in pattern was observed in all stem cells seeded on TCPS, PLLA and PLLA-Bio at 24 and 48 h, although there was no significant difference between stem cell types; although all stem cells cultured on PLLA-Bio showed significant increase in proliferation level compared to PLLA and TCPS.

Characterization of stem cells

Adherent cells were observed 48 h after seeding of MSCs isolated from BFP samples. All non-adherent cells disappeared over time, by exchange of culture medium, and

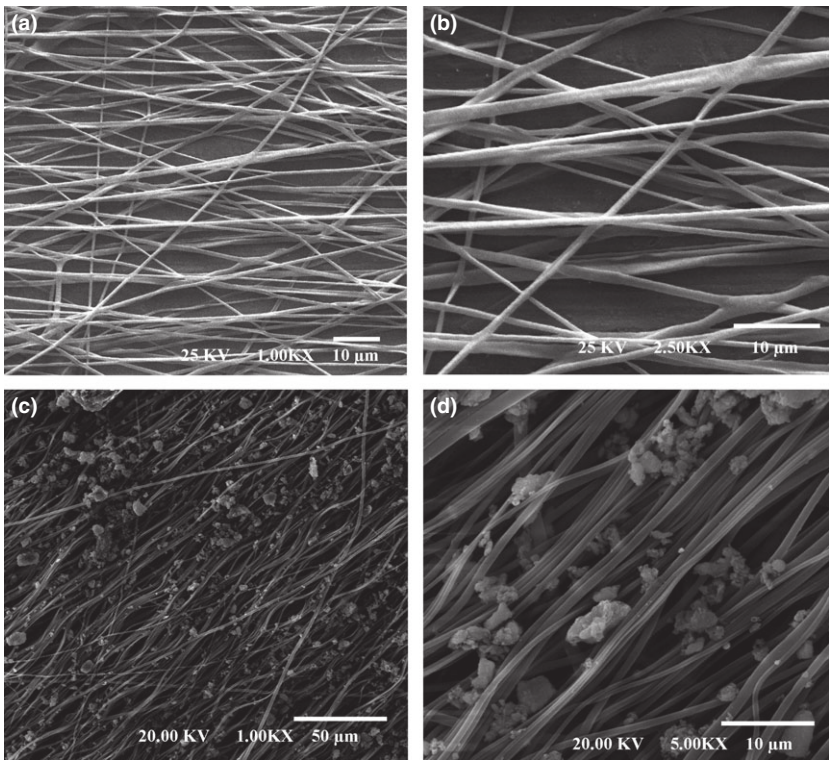

Figure 1. Morphology of fabricated scaffolds. Poly (L-lactide) (PLLA) at two magnifications (×1000, ×2500; a, b). PLLA after being coated with Bio-Oss at two magnifications (×1000, ×5000; c, d).

fibroblast-like cells expanded in radial directions. These growing colonies were trypsinized and re-plated when cells reached 90% confluence (approximately after 5 days). As demonstrated in Fig. 4, BFP-MSCs included spindle-shaped cells as did Ad-MSCs, BM-MSCs and USSCs.

To evaluate surface marker profiles of all stem cell types, passage 2 cells were found to be positive for CD44, CD90 and CD105, and negative for haematopoietic markers CD34, CD45 and HLA-DR (Fig. 5).

Osteogenic differentiation: alizarin red staining, ALP activity and calcium content

Mineralization of the four types of stem cell on TCPS was first visualized using alizarin red staining on day 14. Large quantities of mineralization on day 14 were qualitatively (Fig. 6a) and quantitatively (Fig. 6b) observed in BM-MSCs, compare to other stem cell types, after alizarin red staining. Calcium content assay was also performed to quantify calcium deposition. An increasing trend of ALP activity and calcium deposition was observed during the period of study. However, higher amounts of ALP activity were observed in stem cells on PLLA-Bio compared to other groups at days 7 and 14 of osteogenic differentiation (Fig. 7). On the other hand, these time points indicate significant differences between enzyme activities of BM-MSCs cultured on PLLA-Bio and PLLA compare to other the other stem cell types, with BFP-MSCs presenting closer ALP activity to BM-MSCs.

Total calcium content measurements were performed to further evaluate osteogenic differentiation in TCPS, PLLA and PLLA-Bio groups at days 7 and 14 (Fig. 8). At both time points, highest calcium deposition was observed on PLLA-Bio scaffolds. Again, BM-MSCs had greatest intracellular calcium concentration between all stem cell types cultured on scaffolds. Following on, BFP-MSCs cultured on PLLA scaffold at day 7 and also on PLLA and PLLA-Bio at day 14 presented highest concentrations.

Gene expression analysis

Relative expression of three important osteogenesis-related genes was evaluated during osteogenic differentiation of the stem cells under TCPS, PLLA and PLLA-Bio conditions (Fig. 9). Two of the three selected genes showed an increasing trends of expression, one of which was elevated in a time-dependent manner, the other whose increase was dependent on bioceramic coating of

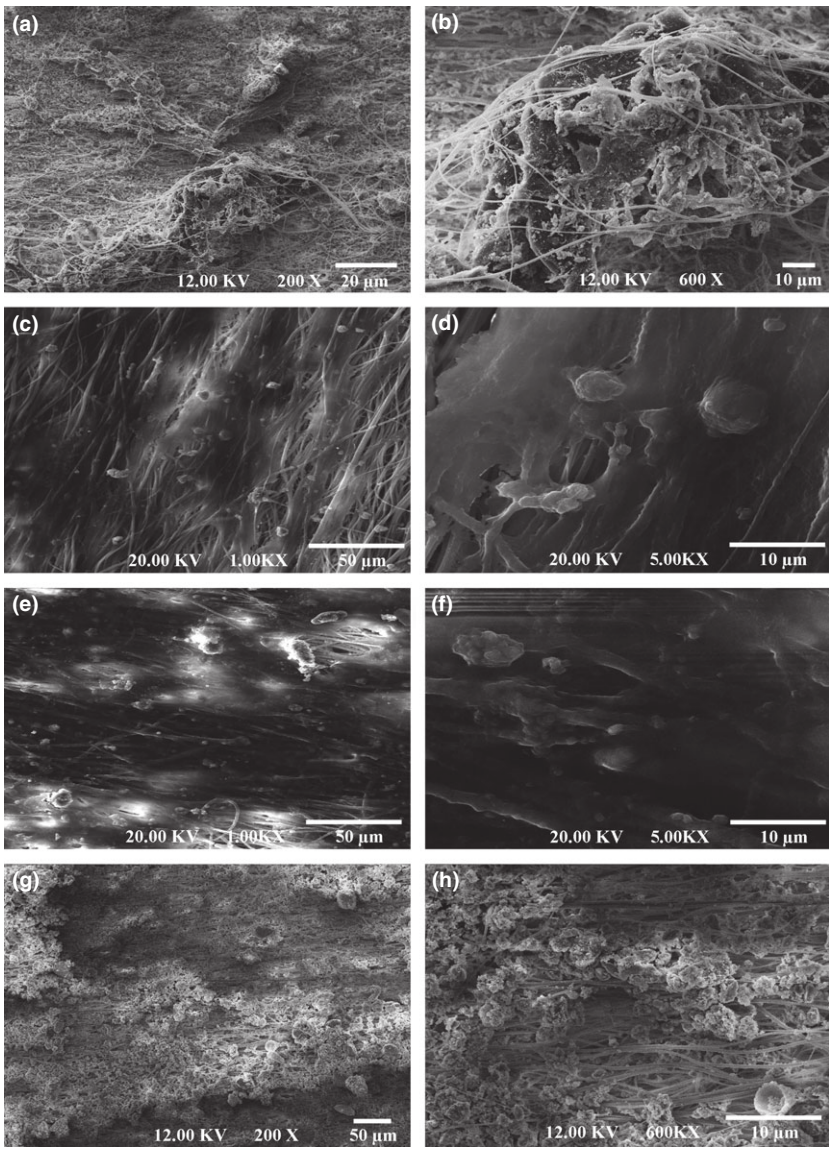

Figure 2. Micrograph of scaffolds after 21 days stem cells culture at two magnifications, unrestricted somatic stem cells (a, b), adipose tissue-mesenchymal stem cells (c, d), bone marrow-mesenchymal stem cells (e, f) and buccal fat pads-mesenchymal stem cells (g, h).

the scaffolds. This indicates that mRNA levels increased over time and coating of the bioceramics. Thus, on day 7, highest expression of *BGLAP*, *Runx2* and *osteonectin* was revealed in BFP-MSCs, BM-MSCs and USSCs cultured on PLLA-Bio scaffold respectively. However, this ratio changed after 14 days culture, where greatest expression of *Runx2* and *osteonectin* were observed in BFP-MSCs, and high *BGLAP* levels were detected in USSCs, all cultured on PLLA-Bio.

Discussion

In this study, we compared osteogenic potentials of four types of stem cell, knowing that choosing the appropriate one with best properties in preparation, proliferation and differentiation potential is an essential component for bone tissue engineering. Adult stem cells have been found in numerous organs and have critical roles in tissue maintenance and homoeostasis (34). An important goal for tissue engineers is to find new sources of stem cell procurement that can provide adequate numbers of stem cells with minimal patient morbidity, maximal proliferation rate and high differentiation potential. Despite other mentioned MSC sources, adipose tissue holds the greatest promise in stem cell therapy and regenerative medicine. This is due to its availability in large quantities as medical treatment biprodcut and also it contains high numbers of progeni-

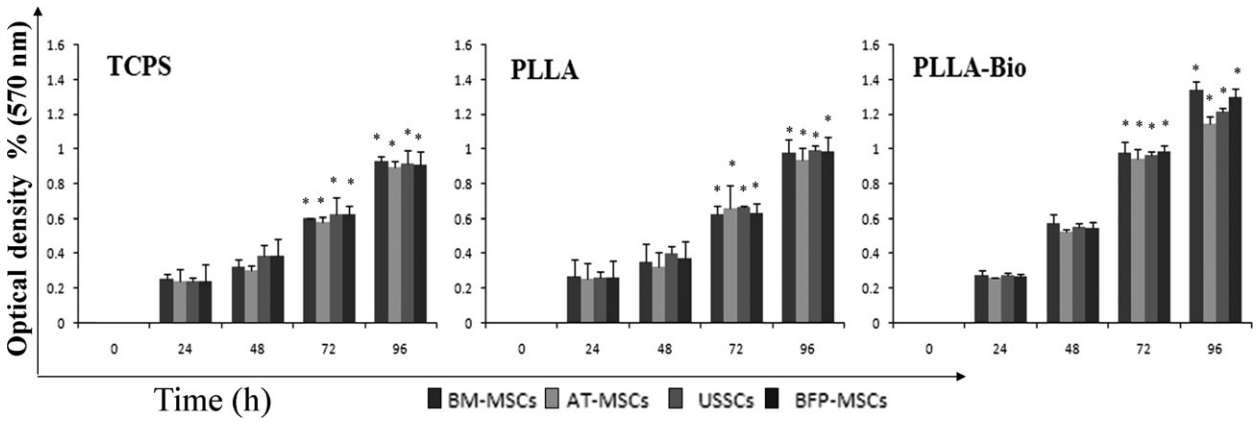

Figure 3. Proliferation of unrestricted somatic stem cells (USSC), adipose tissue-mesenchymal stem cells (AT-MSCs), bone marrow-mesenchymal stem cells (BM-MSCs) and buccal fat pads-mesenchymal stem cells (BFP-MSCs) on scaffolds (PLLA and Bio-Oss-coated PLLA) and tissue culture polystyrene (TCPS) over a 24, 48, 72 and 96-h culture period (asterisks indicate significant difference between the groups at $P < 0.05$).

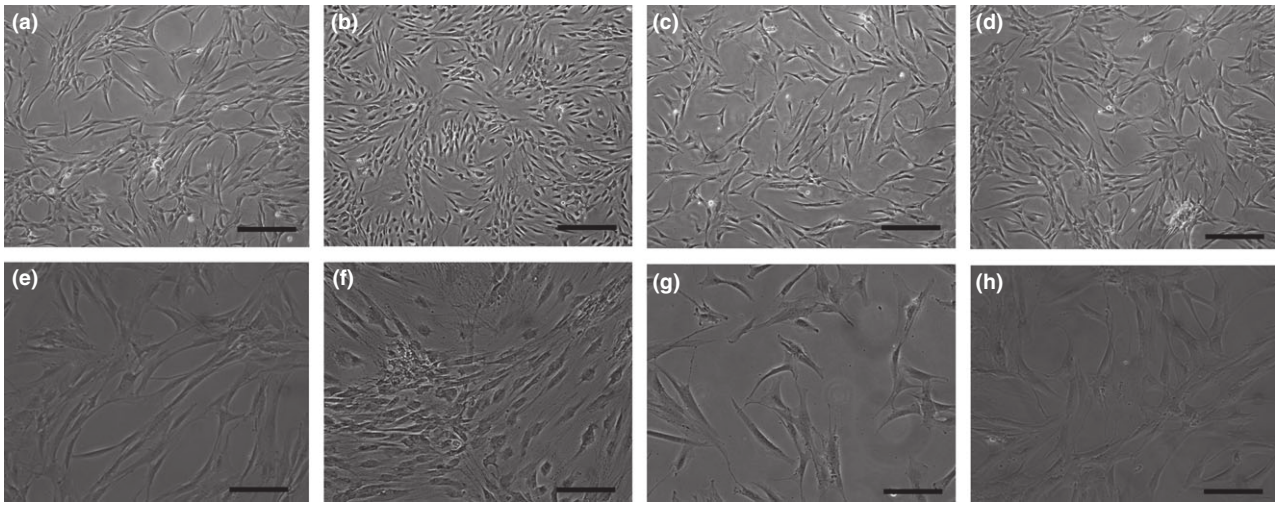

Figure 4. Optical micrographs of stem cells at two magnifications ×10 and ×40, adipose tissue-mesenchymal stem cells (a, e), buccal fat pads-mesenchymal stem cells (b, f), bone marrow-mesenchymal stem cells (c, g) and unrestricted somatic stem cells (d, h) in basal medium.

tor cells that give rise to different cell populations compared to other sources.

Several factors have led to preference of BFP rather than subcutaneous fat for MSC isolation and use for oral surgery. The most important of these factors are: as ease of harvesting, minimal donor-site damage, low complications and rich blood supply (35,36). Stuzin *et al.* have reported that unlike subcutaneous fat, weight and size of BFP in different individuals seems to be independent of their body weight and fat distribution, an advantage for isolation source (37). In this study, we aimed to assess osteogenic differentiation potential of MSCs derived from four different sites including bone marrow, cord blood, subcutaneous adipose tissue and BFP. As our ultimate goal was to use these cells in tissue engineering and regenerative medicine, osteogenic differentiation potentials of the cells were evaluated while they were cultured on TCPS, PLLA nanofibres and bioceramic-coated PLLA nanofibres.

We selected electrospinning to fabricate PLLA nanofibres, as several studies have demonstrated such nanofibres to adequately mimic the structure of ECM (33,38,39). Moreover, we confirmed the capability of our four types of stem cell for osteogenic differentiation on TCPS using alizarin red staining. Our fabricated nanofibres exhibited nanostructure with interconnected pores as critical requirements for ideal tissue engineering scaffolds if they are to mimic the ECM (40). One of the methods to increase similarity of scaffold to ECM and improve characteristics of biomaterials for tissue

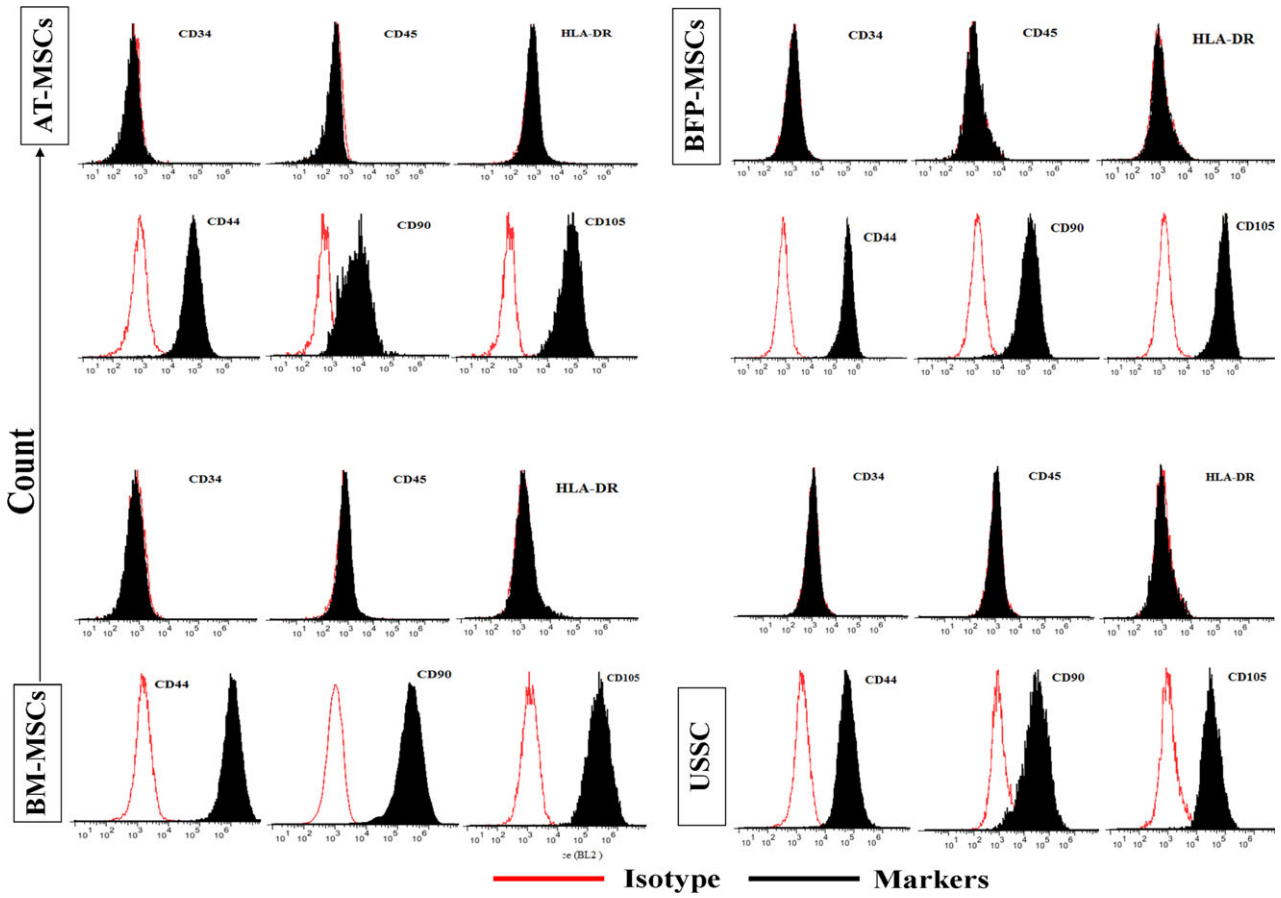

Figure 5. Flow cytometric analysis of passage 2 stem cells for surface marker profiles CD44, CD90, CD105, CD34, CD45 and HLA-DR.

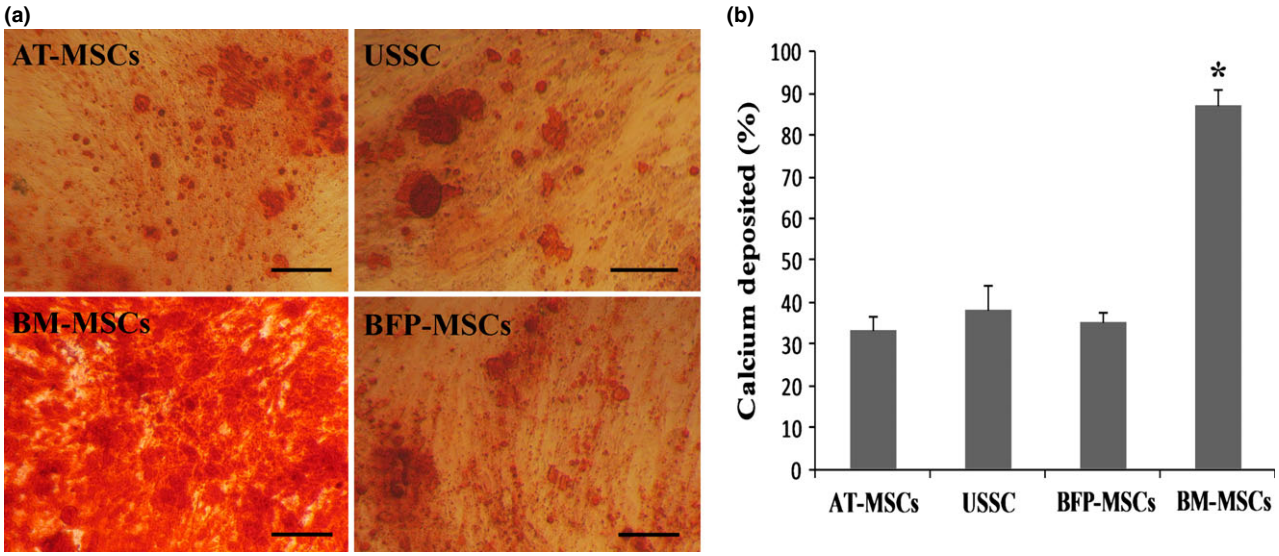

Figure 6. Photograph and quantified morphology of stem cells after alizarin red S staining at 14 days adipose tissue-mesenchymal stem cells (AT-MSCs), unrestricted somatic stem cells (USSC) (a), bone marrow-mesenchymal stem cells (BM-MSCs) (b) and buccal fat pads-mesenchymal stem cells cultured under induction medium (a). Magnification ×40. Quantified morphology of alizarin red S staining in all groups (asterisks indicate significant difference between the groups at $P < 0.05$) (b).

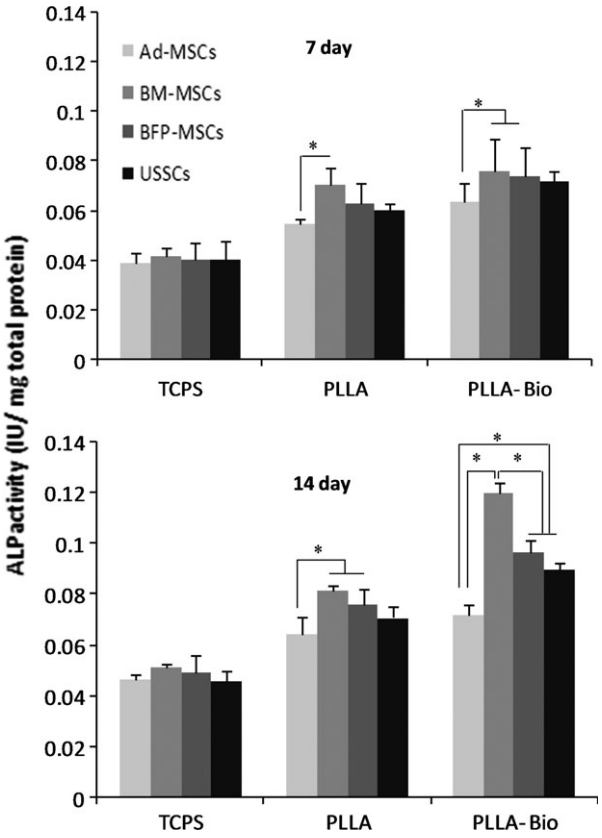

Figure 7. Alkaline phosphatase (ALP) activity in stem cells on scaffolds (PLLA and Bio-Oss-coated PLLA) and tissue culture polystyrene (TCPS) at 7 and 14 days, during osteogenic differentiation (asterisks indicate significant difference between the groups at $P < 0.05$).

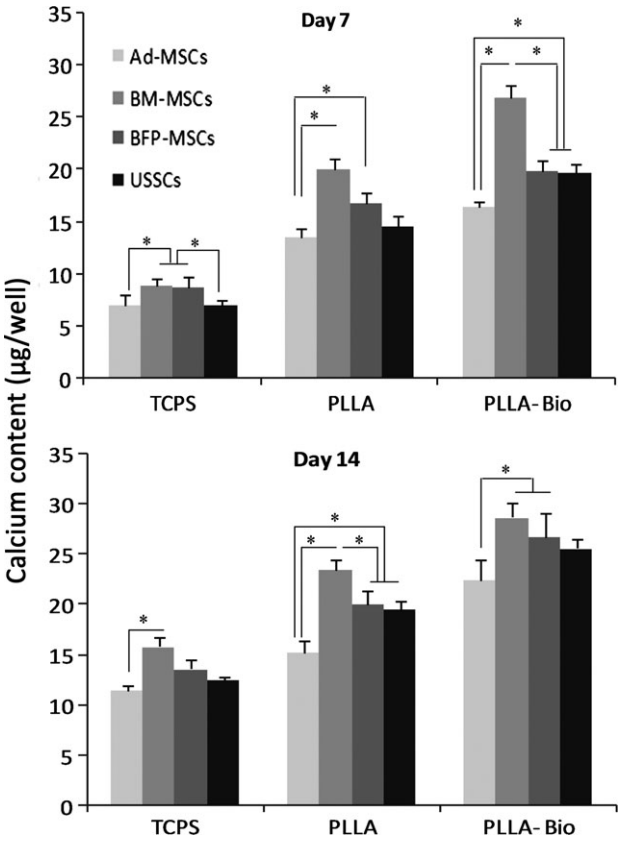

Figure 8. Calcium content of stem cells on scaffolds (PLLA and Bio-Oss-coated PLLA) and tissue culture polystyrene (TCPS) at 7 and 14 days during osteogenic differentiation (asterisks indicate significant difference between the groups at $P < 0.05$).

engineering, is to apply surface modifications (28,41). In the present study, plasma reaction was performed to treat surfaces of fabricated PLLA nanofibres, then coated with Bio-Oss bioceramics. During evaluation of the four stem cell types *in vitro*, increased level of proliferation was observed on both PLLA and Plasma-PLLA-Bio compared to TCPS. Higher cell viability on Plasma-PLLA-Bio compared to PLLA might also be attributable to increased hydrophilicity of the scaffold after plasma treatment and bioceramic coating. Proliferation level of the four stem cell types in all groups, was also different, but there was no significant difference between BM-MSCs, AT-MSCs, USSCs and BFP-MSCs.

All stem cells were demonstrated to differentiate efficiently into osteoblast-like cells on scaffold nanofibres, as revealed qualitatively by alizarin red staining, after 14 days culture in osteogenic medium. ALP, calcium content and bone-related genes were investigated as important osteogenic markers to evaluate differentiation of stem cells on PLLA, PLLA-Bio and TCPS. Several studies have reported that ALP plays a critical role in osteogenic differentiation. It cleaves calcium phosphate groups and enhances mineralization of calcium phosphate cement, the final osteogenic differentiation marker of stem cells *in vitro* (42). Highest ALP activity and calcium content was observed in BM-MSC culture on PLLA-Bio over our study period. Interestingly, BFP-MSCs had nearest characteristics to BM-MSCs in both ALP and calcium content potentials. Coating of PLLA scaffolds with bioceramic and plasma treatment was demonstrated to enhance osteogenic differentiation of all stem cell types *in vitro*, according to increased amounts of all markers on Plasma-PLLA-Bio compared to PLLA nanofibres. This indicates that bioceramic coated nanofibrous scaffolds were an ideal support for stem cells in our tissue engineering implants, and that BFP-MSCs had good osteogenic potential in comparison to well-known stem cells such as AT-MSCs, BM-MSCs and USSCs.

For more detailed comparison of osteogenic differentiation potential of the four stem cell types, expression

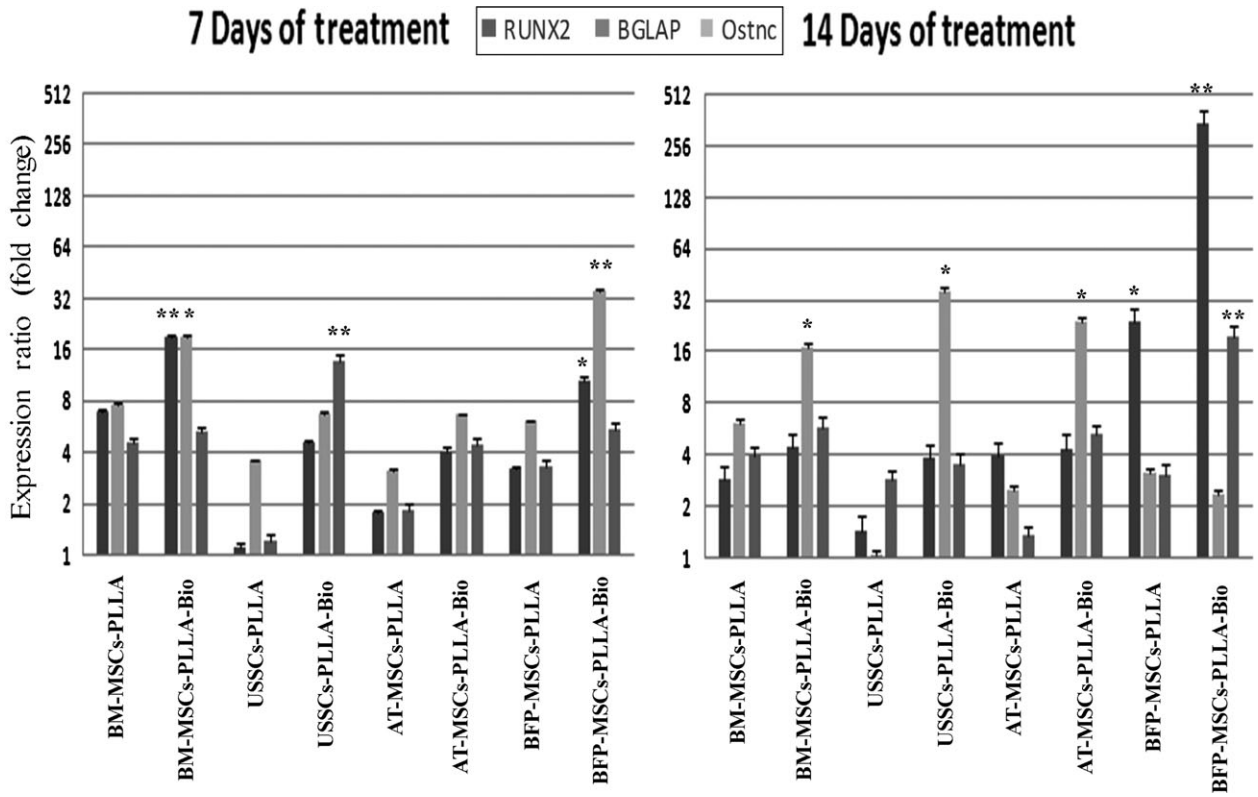

Figure 9. Relative expression of *Runx2*, *osteonectin* and *osteocalcin* (*BGLAP*) days 7 and 14, in stem cells on both scaffolds during osteogenesis (asterisks indicate significant difference between the groups at $P < 0.05$).

of three important bone-related genes was investigated *in vitro*. During the study, higher amounts of expression of *Runx2*, *osteocalcin* and *osteonectin* were detected on Plasma-PLLA-Bio compared to PLLA. *Runx2* is a fundamental regulator of osteoblast differentiation, but is not sufficient alone. *Osteocalcin* and *osteonectin* play essential roles in mineralization and initial nucleation of hydroxyapatite. All our stem cells cultured on PLLA-Bio expressed significantly higher levels of these genes compared to other groups, although at day 7, trends of gene expression were varied between the stem cell types. Highest expression of *BGLAP* was detected in BFP-MSCs, but on average, highest expression of all genes was found in BM-MSCs compared to other the stem cell types. For this reason, most ALP activity and calcium content levels were observed in these cells on day 7. By day 14, bone-related gene expression in BFP-MSCs was better than the other stem cells, as highest expression of *Runx2* and *osteonectin* was observed in cells cultured on PLLA-Bio. However, *BGLAP* gene expression level was detected in BM-MSCs and because of this, by day 14, ALP activity and calcium content levels were also observed in these cells, at higher levels than in the other stem cells. In our pervious study, human ASCs were cultured on bioceramic-collagen scaffolds and their effects were investigated on bone healing in a rat calvarial model (14). According to those results, combination of AT-MSCs, Bio-Oss and collagen gel, synergistically increased bone healing and reconstruction.

In conclusion, we have demonstrated that osteogenic differentiation potential of BFP-MSCs was almost similar to BM-MSCs and was better than AT-MSCs and USSC. We have also shown that bioceramic-coated nanofibrous scaffolds are suitable substrates to support proliferation and osteogenic differentiation of stem cells *in vitro*. Our results can be a cornerstone of following studies in the field of stem cell therapy and tissue engineering and may also lead to development of new stem cell combinations with nanofibrous scaffolds, applied as tissue substitutes and implants.

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References

1 Caplan AI (2005) Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. *Tissue Eng.* **11**, 1198–1211.
2 Tuan RS, Boland G, Tuli R (2003) Adult mesenchymal stem cells and cell-based tissue engineering. *Arthritis Res. Ther.* **5**, 32–45.
3 Ardeshirylajimi A, Hagh MF, Saki N, Mortaz E, Soleimani M, Rahim F (2013) Feasibility of cell therapy in multiple sclerosis: a systematic review of 83 studies. *Int. J. Hematol. Oncol. Stem Cell Res.* **7**, 13–30.
4 Koc ON, Peters C, Aubourg P, Raghavan S, Dyhouse S, DeGasperi R *et al.* (1999) Bone marrow–derived mesenchymal stem cells remain host-derived despite successful hematopoietic engraftment after allogeneic transplantation in patients with lysosomal and peroxisomal storage diseases. *Exp. Hematol.* **27**, 1675–1681.
5 DiGirolamo CM, Stokes D, Colter D, Phinney DG, Class R, Prockop DJ (1999) Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br. J. Haematol.* **107**, 275–281.
6 Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* **276**, 71–74.
7 Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H *et al.* (2002) Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell* **13**, 4279–4295.
8 Rieger K, Marinets O, Fietz T, Körper S, Sommer D, Mücke C *et al.* (2005) Mesenchymal stem cells remain of host origin even a long time after allogeneic peripheral blood stem cell or bone marrow transplantation. *Exp. Hematol.* **33**, 605–611.
9 Koch TG, Heerkens T, Thomsen PD, Betts DH (2007) Isolation of mesenchymal stem cells from equine umbilical cord blood. *BMC Biotechnol.* **7**, 26.
10 Hoogduijn MJ, Gorjup E, Genever PG (2006) Comparative characterization of hair follicle dermal stem cells and bone marrow mesenchymal stem cells. *Stem Cells Dev.* **15**, 49–60.
11 Fibbe W (2002) Mesenchymal stem cells. A potential source for skeletal repair. *Ann. Rheum. Dis.* **61**, ii29–ii31.
12 Battula VL, Treml S, Bareiss PM, Gieseke F, Roelofs H, de Zwart P *et al.* (2009) Isolation of functionally distinct mesenchymal stem cell subsets using antibodies against CD56, CD271, and mesenchymal stem cell antigen-1. *Haematologica* **94**, 173–184.
13 Curran JM, Chen R, Hunt JA (2006) The guidance of human mesenchymal stem cell differentiation in vitro by controlled modifications to the cell substrate. *Biomaterials* **27**, 4783–4793.
14 Daei-farshbaf N, Ardeshirylajimi A, Seyedjafari E, Piryaei A, Fathabady FF, Hedayati M *et al.* (2014) Bioceramic-collagen scaffolds loaded with human adipose-tissue derived stem cells for bone tissue engineering. *Mol. Biol. Rep.* **41**, 741–749.
15 Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD *et al.* (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143–147.
16 Ardeshirylajimi A, Soleimani M, Hosseinkhani S, Parivar K, Yaghmaei P (2013) A comparative study of osteogenic differentiation of human induced pluripotent stem cells and adipose tissue derived mesenchymal stem cells. *Cell J.* **16**(3): 5. [Epub ahead of print]
17 Rojewski M, Weber B, Schrezenmeier H (2008) Phenotypic characterization of mesenchymal stem cells from various tissues. *Transfus. Med. Hemother.* **35**, 168–184.
18 Djouad F, Bony C, Haupl T, Uzé G, Lahlou N, Louis-Plence P *et al.* (2005) Transcriptional profiles discriminate bone marrow-derived and synovium-derived mesenchymal stem cells. *Arthritis Res. Ther.* **7**, R1304–R1315.
19 Wagner W, Wein F, Seckinger A, Frankhauser M, Wirkner U, Krause U *et al.* (2005) Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. *Exp. Hematol.* **33**, 1402–1416.
20 de la Garza-Rodea AS, van der Velde-van Dijke I, Boersma H, Goncalves MA, van Bekkum DW, de Vries AA *et al.* (2012) Myogenic properties of human mesenchymal stem cells derived from three different sources. *Cell Transplant.* **21**, 153–173.
21 Ringdén O, Uzunel M, Rasmusson I, Remberger M, Sundberg B, Lönnies H, Dlugosz A, Szakos A, Hassan Z, Omazic B, Aschan J, Barkholt L, Le-Blanc K (2006) Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* **81**, 1390–1397.
22 Gimble JM, Bunnell BA, Casteilla L, Jung JS, Yoshimura K (2011) Phases I-III Clinical Trials Using Adult Stem Cells. *Stem cells international* **2010**, 1–2.
23 Sierra-Johnson J, Johnson BD (2004) Facial fat and its relationship to abdominal fat: a marker for insulin resistance? *Med. Hypotheses* **63**, 783–786.
24 Abuabara A, Cortez A, Passeri L, De Moraes M, Moreira R (2006) Evaluation of different treatments for oroantral/oronasal communications: experience of 112 cases. *Int. J. Oral Maxillofac. Surg.* **35**, 155–158.
25 Alkan A, Dolanmaz D, Uzun E, Erdem E (2003) The reconstruction of oral defects with buccal fat pad. *Swiss. Med. Wkly.* **133**, 465–470.
26 El Haddad SA, Abd El Razzak MY, El Shall M (2008) Use of pedicled buccal fat pad in root coverage of severe gingival recession defect. *J. Periodontol.* **79**, 1271–1279.
27 Weiner S, Wagner HD (1998) The material bone: structure-mechanical function relations. *Annu. Rev. Mater. Sci.* **28**, 271–298.
28 Ardeshirylajimi A, Dinarvand P, Seyedjafari E, Langroudi L, Adegani FJ, Soleimani M (2013) Enhanced reconstruction of rat calvarial defects achieved by plasma-treated electrospun scaffolds and induced pluripotent stem cells. *Cell Tissue Res.* **354**, 849–860.
29 Ardeshirylajimi A, Hosseinkhani S, Parivar K, Yaghmaie P, Soleimani M (2013) Nanofiber-based polyethersulfone scaffold and efficient differentiation of human induced pluripotent stem cells into osteoblastic lineage. *Mol. Biol. Rep.* **40**, 4287–4294.
30 Jamshidi Adegani F, Langroudi L, Ardeshirylajimi A, Dinarvand P, Dodel M, Doostmohammadi A *et al.* (2014) Coating of electrospun poly (lactic-co-glycolic acid) nanofibers with willemite bioceramic: improvement of bone reconstruction in rat model. *Cell Biol. Int.* **38**, 1271–1279.
31 Tadjoedin ES, De Lange GL, Bronckers A, Lyaruu D, Burger EH (2003) Deproteinized cancellous bovine bone (Bio-Oss) as bone substitute for sinus floor elevation. *J. Clin. Periodontol.* **30**, 261–270.
32 Hämmerle CH, Chiantella GC, Karring T, Lang NP (1998) The effect of a deproteinized bovine bone mineral on bone regeneration around titanium dental implants. *Clin. Oral Implant Res.* **9**, 151–162.
33 Dinarvand P, Seyedjafari E, Shafiee A, Babaei Jandaghi A, Doostmohammadi A, Fathi MH *et al.* (2011) New approach to bone tissue engineering: simultaneous application of hydroxyapatite and bioactive glass coated on a poly (L-lactic acid) scaffold. *ACS Appl. Mater. Interfaces.* **3**, 4518–4524.
34 Prockop DJ (2003) Further proof of the plasticity of adult stem cells and their role in tissue repair. *J. Cell Biol.* **160**, 807–809.
35 Amin M, Bailey B, Swinson B, Witherow H (2005) Use of the buccal fat pad in the reconstruction and prosthetic rehabilitation of oncological maxillary defects. *Br. J. Oral Maxillofac. Surg.* **43**, 148–154.

36 Baumann A, Ewers R (2000) Application of the buccal fat pad in oral reconstruction. *J. Oral Maxillofac. Surg.* **58**, 389–392.
37 Stuzin JM, Wagstrom L, Kawamoto HK, Baker TJ, Wolfe SA (1990) The anatomy and clinical applications of the buccal fat pad. *Plast. Reconstr. Surg.* **85**, 29–37.
38 Seyedjafari E, Soleimani M, Ghaemi N, Sarbolouki MN (2011) Enhanced osteogenic differentiation of cord blood-derived unrestricted somatic stem cells on electrospun nanofibers. *J. Mater. Sci. Mater. Med.* **22**, 165–174.
39 Dinarvand P, Hashemi SM, Seyedjafari E, Shabani I, Mohammadi-Sangcheshmeh A, Farhadian S *et al.* (2012) Function of poly (lactic-co-glycolic acid) nanofiber in reduction of adhesion bands. *J. Surg. Res.* **172**, e1–e9.
40 Liu C, Xia Z, Czernuszka J (2007) Design and development of three-dimensional scaffolds for tissue engineering. *Chem. Eng. Res. Des.* **85**, 1051–1064.
41 Barradas A, Lachmann K, Hlawacek G, Frielink C, Truckenmoller R, Boerman OC *et al.* (2012) Surface modifications by gas plasma control osteogenic differentiation of MC3T3-E1 cells. *Acta Biomater.* **8**, 2969–2977.
42 Golub EE, Boesze-Battaglia K (2007) The role of alkaline phosphatase in mineralization. *Curr. Opin. Orthop.* **18**, 444–448.

