CLINICAL ORAL IMPLANTS RESEARCH

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Evaluation of the osteoconductive potential of bone substitutes embedded with schneiderian membrane- or maxillary bone marrow-derived osteoprogenitor cells

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Abstract

Aim: Sinus augmentation procedures commonly employ osteoconductive scaffolding materials to stimulate and support bone formation. The aim of this study was to develop a simple screening methodology for the evaluation of the osteoconductive potential of various bone graft materials prior to clinical use.

Materials and methods: Materials tested were Bio-Oss, Bi-Ostetic, OraGraft, and ProOsteon. These Simple and composite bone substitutes were embedded with osteoprogenitor cells derived from either the human maxillary sinus schneiderian membrane (hMSSM) or from maxillary tuberosity bone marrow and then monitored both *in vitro* and *in vivo*.

Results: Cell adherence and proliferation was most pronounced in OraGraft, followed by ProOsteon. *In vivo* bone formation, within the bone graft, was also observed, with most marked results in OraGraft and ProOsteon grafts.

Conclusions: The proposed osteoconductivity testing method proved simple, informative, and reliable for the purpose of screening candidate biomaterials for sinus lifting or sinus augmentation.

In a well-established clinical procedure, osteoconductive materials of various origins (e.g., autograft, allograft, xenograft or alloplast) are surgically placed underneath the lifted membrane of the sinus to stimulate bone formation on the maxillary osseous floor (Tatum 1986; Hurzeler et al. 1997; Artzi et al. 2001a; Hallman et al. 2001; Haas et al. 2002a; Boyne et al. 2005; Cammack et al. 2005; Froum et al. 2006). The sinus lifting procedure offers structural support, necessary for expansion of bone volume and prevention of premature resorption at the reconstructed posterior area in the maxilla (Satow et al. 1997; Garg 1999). In this manner, the method provides a solution to inadequate alveolar bone height in the posterior maxilla, a critical impediment to effective anchorage of dental implants.

While the optimal osteoconductive and osteogenic properties of autografts have designated them the most prevalent source of bone substitute, their use is often associated with bone insufficiencies and secondary operations, which significantly increase risk factors and treatment costs. In addition, threat of disease transmission, as well as regulatory restrictions, limit application of allograft bone substitutes (Mastrogiacomo et al. 2005; Sousa et al. 2007). Such limitations have prompted a search for clinically relevant alternatives. Bone substitutes of various compositions, specialized characteristics and mechanisms of action (Giannoudis et al. 2005) have flooded the oral implantology market (De Leonardis & Pecora 2000; Maiorana et al. 2000, 2006; Cordioli et al. 2001; Haas et al. 2002a,b,c; Allegrini et al. 2003; Kaufman 2003; Mangano et al. 2003; Barone et al. 2005; Butz & Huys 2005; Boyapati & Wang 2006; Froum et al. 2006; Galindo-Moreno et al. 2007).The broad range of available osteoconductive bone substitutes and related clinical studies, call for an accurate and comparative screening methodology before broad clinical application of the proposed substitute.

The osteoconductive potential of bone substitutes is regularly assessed by evaluating the quality and quantity of the newly formed bone in the lifted area. Such assessment are performed using radiology and punch biopsy

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taken from the grafted sinus at the end of the healing process, which can often extend over 6 months (Hurzeler et al. 1997; Artzi et al. 2001a,b; Hallman et al. 2001; Haas et al. 2002a,b,c; Boyne et al. 2005; Cammack et al. 2005; Froum et al. 2006). The lengthy time lag between treatment and evaluation of its effectiveness hinders comparison of the bone forming kinetics and healing potential of the various substitutes in question (Browaeys et al. 2007). Although many studies have assessed bone substitute osteoconductivity *in vitro*, little information could be deduced with regards to the osteoconductive characteristics and clinical efficiency of bone substitutes in such experimental setups. Animal models have been proposed as a means of accelerating these comparative analyses, however, they pose challenges as well (Browaeys et al. 2007). Preclinical studies using orthotopic models have provided important information regarding the osteoconductivity of bone substitutes within their micro- and macroenvironments, however, failed to provide a true measure of the osteoconductive properties of the tested materials. Moreover, results obtained using non-human osteoprogenitors for evaluation of biomaterials characteristics often do not parallel those observed with human osteoprogenitors in the same experimental setup (Zannettino et al. 2010). Therefore, caution should be taken when translating such animal studies to human trials before performing appropriate comparative *in vitro* and *in vivo* analyses (Zannettino et al. 2010).

Our recent reports have demonstrated that osteoprogenitor cells isolated from the hMSSM can produce bone upon their ectopic transplantation in athymic nude mouse model (Srouji et al. 2009, 2010). At the same time, active osteoprogenitor cells derived from the maxillary tuberosity bone marrow (MTB) have been shown to generate bone within ectopically transplanted osteoconductive materials. The ectopic model is considered very beneficial; it enables true osteoconductivity evaluation of materials tested in osseous tissue free environment (Bruder et al. 1994; Doherty et al. 1998; Bianco & Robey 2001; Bianco et al. 2001; Zuk et al. 2002; Cicconetti et al. 2007; Srouji et al. 2009, 2010). As bone substitutes implanted during sinus lifting procedures are grafted between the Schneiderian membrane and the maxillary bone, an area which normally contains osteoprogenitor cells, it is tempting to assume that these osteoprogenitor cells are the main players in bone formation within these substitutes.

Osteoconductive substitutes placed between the maxillary host bone and the sinus membrane are intended to support the attachment, proliferation, and migration of local osteoprogenitor cells and thereafter initiate subsequent bone formation (Giannoudis et al. 2005; McKee 2006; Desai 2007). Herein, a simple non-invasive evaluation methodology, which includes *in vitro* and *in vivo* assays for assessing the biocompatibility and osteoconductivity of candidate bone substitutes is presented. The screening relies on osteoprogenitors isolated from hMSSM and MTB and embedded within bone substitutes of interest. Cell viability and proliferative capacities *in vitro* and their ability to form new osseous tissue *in vivo* are monitored in an ectopic athymic nude mouse model.

Materials and methods

Human MSSM sampling and cell isolation

hMSSM were obtained according to the ethical guidelines of the Carmel Medical Center, Haifa, Israel. The samples were obtained, following signed, informed consent, from five patients, aged 18–25, presenting posterior or total maxillary excess and were therefore candidates for orthognathic surgery. Smokers or patients with skeletal disorders and/or syndromatic diseases were excluded. Bone segments were removed from the lateral wall of the maxillary sinus prior to the impaction. MSSM from the medial side of the bone segments was separated and placed in PBS supplemented with antibiotics (100 U/ml penicillin, 100 U/ml streptomycin; hereby PBS-Abx). hMSSM samples were then cut into small pieces. The tissue fragments were incubated with dispase (Sigma-Aldrich, St. Louis, MO, USA, 37°C, 1 h) to separate the epithelial lining from the membrane, which was then discarded. Subsequently, the remaining tissue fragments were incubated in Hank's balanced salt solution supplemented with 150 U/ml collagenase (Collagenase type II; Sigma-Aldrich), for 2 h with constant rotation. 5×10^5 cells were counted and were plated in 10-cm diameter tissue culture dishes with αMEM medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin (Biological Industries, Beit Haemek, Israel), and hereby termed culture medium. To induce osteogenic differentiation, the cultured cells were passaged and P1 cultures were cultured for 14 days in induction medium prepared from culture medium with the addition of 100 μg/ml ascorbic acid and 10^{-8}M dexamethasone.

Bone marrow sampling from the maxillary tuberosity (MT) and cell isolation

Removal of the embedded tooth/odontogenic cyst was performed under general anesthesia, and a 1-cm^3-sized MTB sample was harvested as previously described (Cicconetti et al. 2007), using a U-shaped chisel. The harvested tissues were immediately placed in PBS-Abx. The MTB samples were gently scraped into culture medium, under sterile conditions, using a steel blade, and continually shaken to release marrow cells. Cell suspensions were passed through an 18-gauge needle to break up cell aggregates, and then filtered through a 70-μm pore-size cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA). 5×10^6 Nucleated cells were plated on 150-mm culture dishes in culture medium. To induce osteogenic differentiation in culture, the cells were passaged and P1 cultures were cultured for 14 days in induction medium.

Bone substitutes

The following bone substitutes were assessed:

- Bi-Ostetic containing 60% hydroxyapatite and 40% β-tri-calcium phosphate (Berkeley Advanced Biomaterials, Berkeley, CA, USA).
- Bio-Oss, a natural bone substitute material obtained from the mineral portion of bovine bone (Geistlich Pharma AG, Wolhusen, Switzerland).
- OraGraft, a freeze-dried bone allograft obtained from cortical or cancellous bone (LifeNet Health Inc., Virginia Beach, VA, USA).
- ProOsteon, a resorbable bone graft material derived from an abundant non-decorative coral (Interpore Cross, Costa Mesa, CA, USA).

Seeding the cells onto bone substitutes

Confluent cultures (P_0) were trypsinized and rinsed with PBS. 2.5×10^5 cells were suspended in 500 μl medium and carefully added to 40 mg of the various bone substitutes, in ventilated tubes (TPP, Innovation in plastic, Switzerland). The tubes were rotated (37°C, 1 h, 7.5 rpm) to allow cell adhesion to the construct. Additional culture medium (9.5 ml) was then supplied. The mixture was further rotated for up to 15 days. Half of the total medium volume was replaced twice a week.

Cell adhesion onto and proliferation within bone substitutes

The AlamarBlue™ Metabolic activity assay (Serotec, Raleigh, NC, USA) was used to determine the degree of cell adhesion (tested on day 1 post seeding) to the different bone

substitutes and to determine the impact of scaffold materials on cell proliferation.

Briefly, cell-embedded bone substitutes were incubated (37°C, 2 h) with 10% (v/v) AlamarBlue reagent in α-MEM. The medium was then collected and subjected to flourometric measurement using a fluorometer (Ex: 540 nm: Em: 580 nm). The assay was performed in replicates ($n = 6$) for each time point (1, 5, and 13 days post seeding).

Scanning electron microscopy

Samples of the different bone substitutes seeded with MSSM- or MTB-derived osteoprogenitors and cultured for 14 days, were taken for Scanning electron microscopy (SEM; $n = 3$) assessment. Samples were fixed in 0.1 M sodium cacodylate buffer, pH 7.2 containing 3% glutaraldehyde (24 h), followed by 1% OsO_4 and 2% tannic acid (1 h). They were then dehydrated in graded ethanol solutions followed by hydroxymethyl xylazine, sputter coated with gold palladium and examined using SEM (Jeol JSM-35 C operating at 15 kV, JEOL Ltd, Tokyo, Japan).

Subcutaneous transplant of cell-embedded bone substitute matrices in mice

Confluent cultures of osteogenically induced cells (14 days) were trypsinized, washed with PBS, and 2×10^6 cells were then mixed with 40 mg of the various bone substitutes and gently rotated (37°C, 1 h). Samples were further mixed with mouse fibrinogen and mouse thrombin (Sigma-Aldrich) to form fibrin clots, prior to subcutaneous transplantation in 8-week-old, athymic nude mice, each weighing ~25 g (Harlan Laboratories, Jerusalem, Israel). A midsagittal incision was made on the animal's back and the cell-embedded bone substitutes coated with fibrin were subcutaneously implanted ($n = 6$ for each tested material) under anesthetic conditions (Xylazine : Ketamin, 1 : 1), each mouse was implanted with four implants, one repeat of each biomaterial. Cell-free constructs were implanted into an additional six animals. After surgery, the skin was carefully sutured and topically dressed with 3% syntomycin. All mice recovered well from surgery, were housed separately in plastic cages and were monitored for up to 8 weeks, after which, they were sacrificed. Food and water were supplied *ad libitum*. The surgical protocols were approved according to institutional guidelines of the Animal Ethics Committee of the Technion–Israel Institute of Technology, Haifa, Israel.

Histological examination

The harvested transplants were fixed (5 days, room temperature) in 10% neutral buffered formalin (BioLab, Jerusalem, Israel), then decalcified in 20% Ethylene Diamine Tetraacetic Acid, dehydrated in graded ethanol solutions (70–100%) and embedded in paraffin. Serial sections (6-μm thick) were stained with Hematoxylin and Eosin (H&E), then used for histomorphometry measurements conducted using the Image Pro plus 6 computerized analysis system on five sections per material sample (Media Cybernetics, Rockville, MD, USA). The degree of bone formation is expressed as the mean percent of the bone substitute area within the image.

Statistical analysis

Data analysis was performed using the SPSS statistical package version 15 (SPSS, Chicago, IL, USA). Mann–Whitney test was used to compare the hMSSM- and MTB-derived cell groups. Pearson correlation coefficients were calculated between adhesion and the new bone formation in all bone substitutes. All *P*-values were two-sided, and statistical significance was defined as $P < 0.01$.

Results

Adhesion of osteoprogenitors to bone substitutes

Both hMSSM- and MTB-derived osteoprogenitor cells adhered more effectively to ProOsteon and OraGraft. More specifically, hMSSM-derived cells adhered better to ProOsteon and OraGraft, when compared to the Bio-Oss and Bi-Ostetic graft materials ($P < 0.05$, Fig. 1). MTB-derived cells adhered most strongly to ProOsteon and OraGraft ($P < 0.05$), when compared to Bio-Oss, but compared with Bi-Ostetic, the difference was less evident.

Proliferation of osteoprogenitors seeded on bone substitutes

As both hMSSM- and MTB-derived cells are involved in bone formation upon sinus augmentation, it was important to assess their growth rates on the various biomaterials. Proliferation profiles evaluated at various time points after cell seeding on the matrices (1, 5, and 13 days), demonstrated that both cell types were most proliferative, with linear growth rates on OraGraft. Growth rates measured on day 13 post seeding, were significantly lower (~2–4 fold, $P < 0.01$) on ProOsteon, Bio-Oss, and Bi-Ostetic matrices, when compared to those measured on OraGraft (Fig. 2a,b). However, both cell types failed to survive when cultured in the absence of matrices.

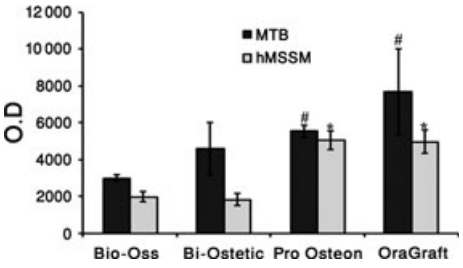

Fig. 1. Adhesion of hMSSM- and MTB-derived cells to the tested bone substitutes. *($P < 0.05$) difference between hMSSM-derived cell adhesion to OraGraft and ProOsteon vs. Bio-Oss and Bi-Ostetic. #($P < 0.05$) difference in MTB-derived cell adhesion to OraGraft and ProOsteon vs. Bio-Oss. $n = 6$ for each biomaterial.

SEM imaging of MTB- and hMSSM-derived cells seeded on the different biomaterials

Morphology of cells embedded within the test material for a period of 15 days was visualized using SEM imaging. SEM images corroborated the trends observed in the proliferation assays described above. Namely, cells cultivated on OraGraft were of normal morphology and homogenously covered the material surface, in contrast with the other biomaterials, in which the cells covered only a small surface area (Fig. 3). No difference was seen in the morphology of the two cell types.

In vivo transplantation of bone substitutes seeded with osteoprogenitors

In vivo bone formation is the most definitive test for evaluating the osteoconductive potential of the different biomaterials. Histological analysis of grafts embedded with hMSSM- or MTB-derived cells, subcutaneously transplanted in the backs of immunocompromised mice demonstrated new bone formation over the surface of the carrier particles (Fig. 4a). As generally seen in natural bone tissue, the newly formed bone contained osteocytes encased within the deposited matrix. No bone was observed in cell-free transplants.

Histological evaluation and histomorphometric quantification of resected mouse transplants revealed significant differences in the amounts of newly formed bone. Differences were mostly independent of the cell type and correlated more tightly with the biomaterial used. Both type of cells implanted with Bio-Oss and Bi-Ostetic resulted in significantly less new bone when compared with that formed after implantation of cell-embedded ProOsteon and OraGraft samples ($P < 0.01$) (Fig. 4b). As seen in the previous experiments, hMSSM- and MTB-derived cells combined with ProOsteon or OraGraft grafts were superior to the other tested materials (Fig. 4b). A marked difference in the amount of bone formed was

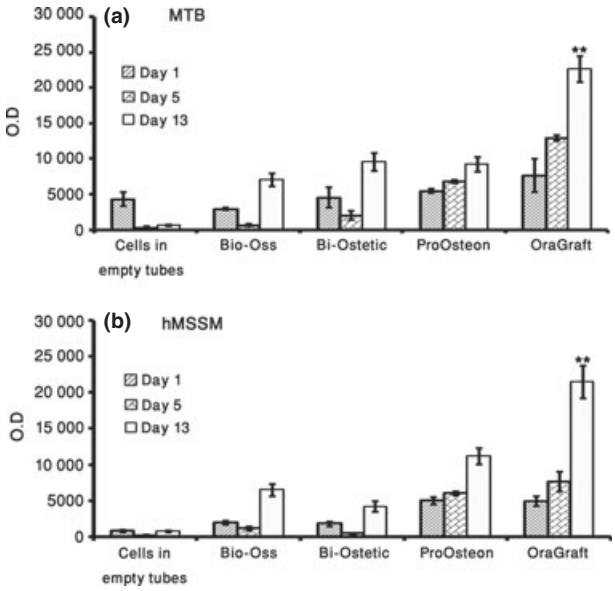

Fig. 2. Proliferation profiles of hMSSM- and MTB-derived cells embedded within different bone substitutes. (a) MTB-derived cells and (b) hMSSM-derived cells were cultured for 1, 5, and 13 days on various bone substitutes. Bars represent mean values (±SD) of six replicates samples tested on each evaluation day. **Significant ($P < 0.01$) difference of cell growth of both cell types after 13 days on OraGraft, compared with cells seeded on all other bone substitutes. $n = 6$ for each biomaterial at each time point.

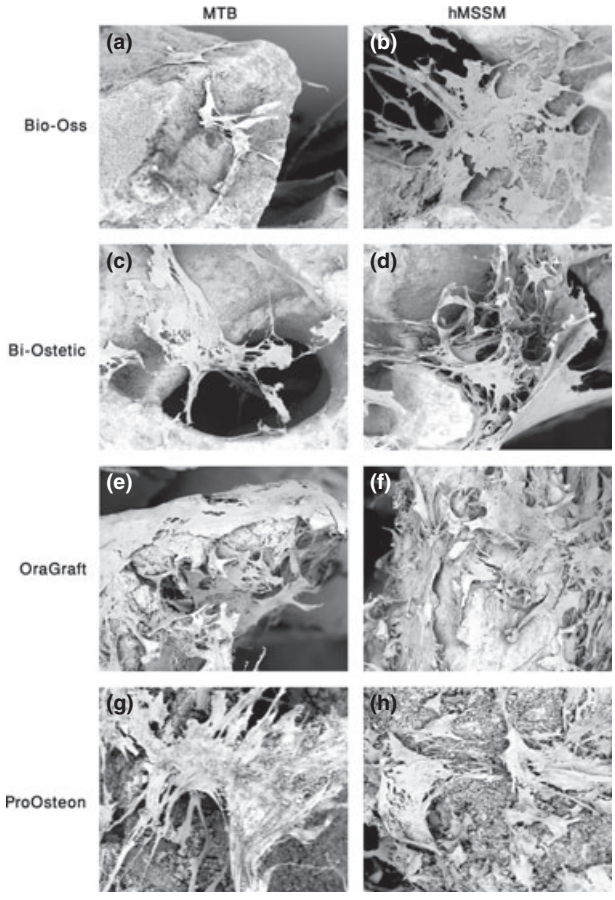

Fig. 3. SEM images of bone graft matrices embedded with hMSSM- and MTB-derived cells and cultivated for 15 days. (a, c, e, and g) MTB-derived cells. (b, d, f, h) hMSSM-derived cells.

evident between both cell types only when they were transplanted with Bi-Ostetic ($P < 0.05$). In this case, hMSSM-derived cells induced five-fold more bone formation than did MTB-derived cells embedded in the same scaffold material (Fig. 4b).

MTB-derived cells implanted with OraGraft generated bone marrow-like tissue within the newly formed bone (Fig. 4a). The ossification process took place on the external surface area of the different bone substitutes. No signs of inflammatory reactions to the implants were observed. Control samples showed massive infiltration of the fibrovascular tissue, but no osseous tissue. Regression analysis of adhesion to the different matrices vs. the amount of new bone formed upon implantation, calculated using Pearson's correlation coefficients, unequivocally demonstrated that improved adhesion leads to higher bone formation (the correlation coefficient was 0.634, $P < 0.08$ for hMSSM and 0.582, $P < 0.08$ for MTB-derived cells).

Discussion

In recent years, the market of oral implantology has been flooded with bone substitutes, mostly targeted toward maxillary sinus lifting and sinus augmentation procedures. To date, no single methodology for determination of the efficacy of these materials has been widely accepted. This study adopted a new approach to screen for the osteoconductivity of materials of interest. The presented technique mimics the natural environment of implanted grafts by taking advantage of local osteoprogenitor cells isolated from two distinct anatomical compartments of the maxillary sinus, namely, the MSSM and MTB. The viability and biofunctionality were examined by monitoring their growth on various commercial bone substitutes *in vitro*, as well as the degree of bone formation following their implantation *in vivo*.

Bone substitutes are commonly used in maxillary sinus lifting procedures, aimed at increasing the volume of the maxillary sinus osseous floor prior to insertion of dental implants (Boyne & James 1980; Tatum 1986; Hatano et al. 2004; Hallman et al. 2005). While autografts are still considered to be the gold standard for bone grafting, various alternatives have been developed and are in use, simplifying the procedure of bone grafting. In general, these osteoconductive materials are used in maxillary sinus lifting procedures and constitute scaffolds to which cells migrate, and on which they can proliferate and differentiate to form bone (Hurzeler et al. 1997; Artzi et al. 2001b; Hallman et al. 2001; Haas et al. 2002a; Boyne et al. 2005; Cammack et al. 2005; Froum et al. 2006). Many studies have unequivocally shown that certain bone substitute materials are osteoconductive

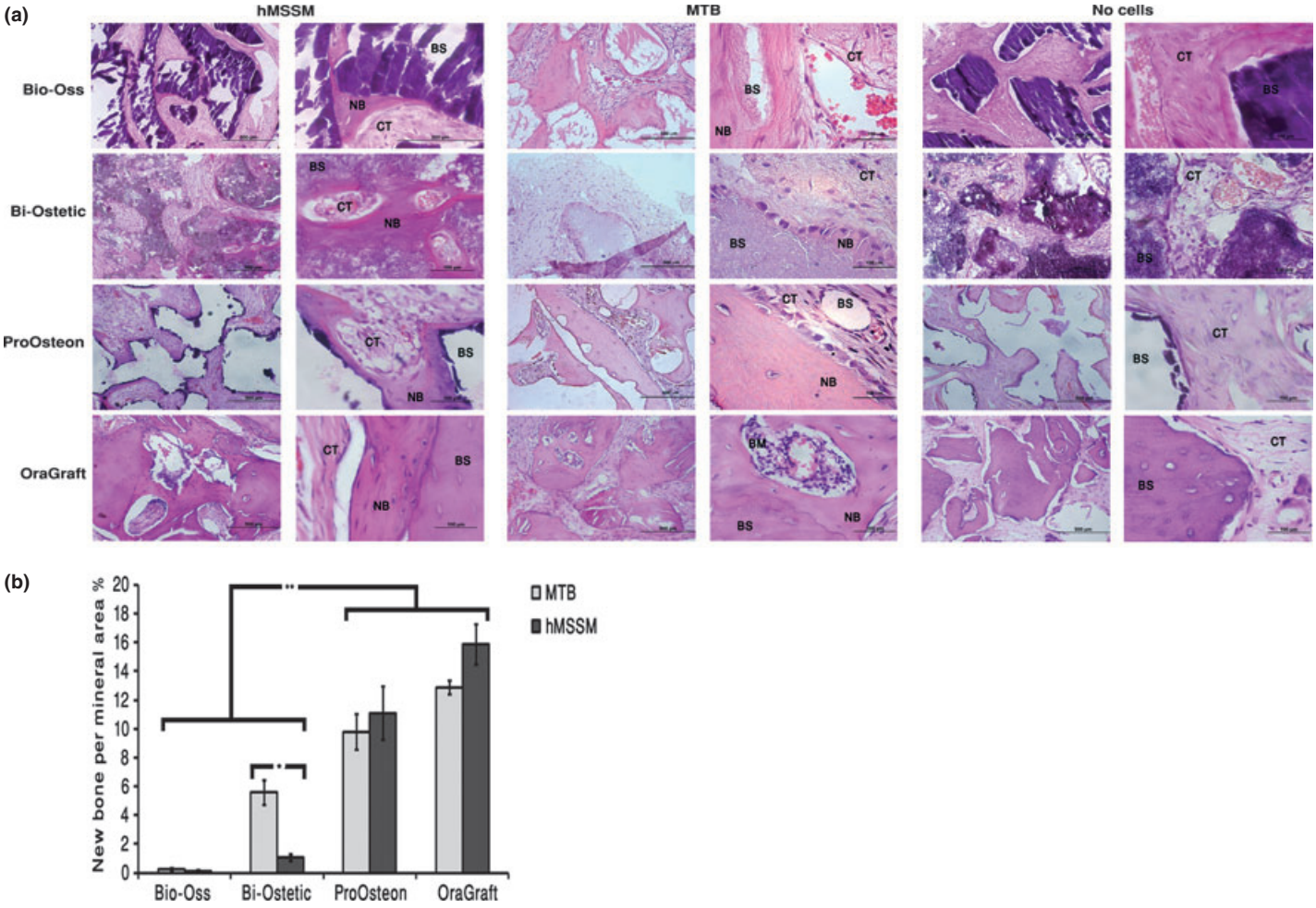

Fig. 4. In vivo bone formation generated by hMSSM- and MTB-derived cells mixed with the different bone substitutes and implanted in a subcutaneous nude mice model. (a) MTB-derived cells and hMSSM-derived cells (H&E stain). (b) Morphometric analysis of new bone formation as seen in histology. BS, bone substitute; NB, new bone; CT, connective tissue; BM, bone marrow. *($P < 0.05$) difference in the amount of bone formation observed for both cell types when seeded on Bi-Ostetic. **Significant ($P < 0.01$) difference in the amount of bone formation observed when cells were transplanted with OraGraft or ProOsteon vs. with Bio-Oss and Bi-Ostetic. $n = 6$ for each biomaterial.

and cell-supportive. However, reliable comparisons between the various studies cannot be drawn due to variability in study designs, the wide range of implant types, and variances in follow-up methodology (Browaeys et al. 2007). In addition, measure of implant osseointegration has been found to be a poor parameter for comparing osteoconductive materials, as integration was always present, regardless of the materials used (Ellegaard et al. 1997, 2006; Lundgren et al. 2004; Palma et al. 2006). Furthermore, radiological or punch biopsy analysis of new bone, provide only an approximate clinical picture when compared to histology of the entire graft, which is easily analyzed in ectopic animal model experiments.

In this study, *in vitro* and *in vivo* analysis of the osteoconductivity and biocompatibility of various bone substitutes seeded with osteoprogenitor cells were performed. Bone was formed within osteoconductive materials by osteoprogenitor cells originating from either hMSSM or the MTB, with variances originating from the inherent characteristics of the tested materials. Furthermore, the ectopic model utilized, herein, is highly reliable due to the fact that it is a bone-free environment and thus provides a genuine evaluation of the material's osteoconductivity. Bone formation was clearly detected within a short period (8 weeks) (McKee 2006; Desai 2007). The feasibility of the ectopic model in testing material osteoconductivity strongly indicated the orthotopic (bony environment) workability of the tested bone materials.

Xenogeneic grafts represented by Bio-Oss® (Wetzel et al. 1995) and ProOsteon are frequently used in sinus lifting procedures (Browaeys et al. 2007). Alloplastic materials, such as Bi-Ostetic, an inorganic, synthetic, biocompatible bone graft substitute (Browaeys et al. 2007), is also in regular use. Allogenic grafts, such as OraGraft, are also popular bone substitute matrices (Browaeys et al. 2007). Osteoprogenitor cells isolated from hMSSM and from MTB bone marrow adhered (Fig. 1) and proliferated (Fig. 2) to similar degrees when seeded on bone substitutes representative of these three graft subtypes. The similar behavior of both cell types *in vitro* on the same bone substitutes strengthens the reliability of the suggested approach. OraGraft (allograft) and ProOsteon (coral engraft) showed the highest levels of biocompatibility in terms of cell adhesion and proliferation *in vitro* (Figs 1 and 2). Similarly, all bone substitutes seeded with either cell type exhibited osteoconductivity *in vivo* and bone was formed, albeit, to various degrees. Intergroup comparisons demonstrated highest osteoconductivity for OraGraft and ProOsteon grafts, as manifested by higher levels of bone formation (Fig. 4). OraGraft not only provided the highest degree of cell adhesion and proliferation *in vitro* and bone formation *in vivo*, but

also generated bone marrow formation, a process considered a hallmark in the course of bone development. Cell-free bone substitutes did not induce bone formation in the ectopic model, indicating the importance of the combination of osteoprogenitor cells with the bone substitutes for the successful development of bone.

The different levels of bone formation seen *in vivo*, strongly emphasize the reliability of the presented approach in determining and defining the optimal bone substitute. Several studies have demonstrated that bone substitute characteristics directly influence the adhesion and proliferation of cells and the degree of bone formation when implanted *in vivo*. Mankani et al. demonstrated the effect of bone substitute topography in terms of shape, size and dispersion, on bone formation (Mankani et al. 2001). Other studies showed that incorporation of hydroxyapatite or Tri-calcium phosphate into scaffolds enhances bone formation, whereas wholly ceramic scaffolds are less supportive of cell adhesion and proliferation (Kon et al. 2000; Liu et al. 2008; Zannettino et al. 2010; Huang et al. 2011). Wettability and processing were also shown to influence results (Lavenus et al. 2011; Saranya et al. 2011). The results presented, herein, are in full concordance with these studies; when using cells with significant osteogenic potential, bone formation depended only on the bone substitute used and not on the cells.

Moreover, regression analyses unambiguously showed that bone substitutes that encourage adequate cell adhesion to its surface stimulate more bone formation *in vivo*.

The ectopic model introduced, herein, allows for rapid screening and determination of the osteoconductive potential of bone substitutes. The presented model may serve as an attractive tool for the evaluation of osteoconductivity and characterization of different bone substitutes used in clinical procedures, due to its non-invasiveness, lack of procedure-related complications, ease of material delivery, and the ability to rely on osteoprogenitor cells. Moreover, as both cell types gave fairly similar results, future studies using this screening model can be simplified by using only one of the two cell types. This model is expected to contribute to preclinical and clinical studies of new candidate bone substitutes and for further investigation of substitutes currently in use.

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The study made use of popular bone graft materials representing different graft families. The authors are indifferent to either one of the materials tested and the results obtained in this study were analyzed and described objectively.

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