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Skeletal site-specific characterization of orofacial and iliac crest human bone marrow stromal cells in same individuals

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Abstract

Autologous grafts from axial and appendicular bones commonly used to repair orofacial bone defects often result in unfavorable outcome. This clinical observation, along with the fact that many bone abnormalities are limited to craniofacial bones, suggests that there are significant differences in bone metabolism in orofacial, axial and appendicular bones. It is plausible that these differences are dictated by site-specificity of embryological progenitor cells and osteogenic properties of resident multipotent human bone marrow stromal cells (hBMSCs). This study investigated skeletal site-specific phenotypic and functional differences between orofacial (maxilla and mandible) and axial (iliac crest) hBMSCs in vitro and in vivo. Primary cultures of maxilla, mandible and iliac crest hBMSCs were established with and without osteogenic inducers. Site-specific characterization included colony forming efficiency, cell proliferation, life span before senescence, relative presence of surface markers, adipogenesis, osteogenesis and transplantation in immunocompromised mice to compare bone regenerative capacity. Compared with iliac crest cells, orofacial hBMSCs (OF-MSCs) proliferated more rapidly with delayed senescence, expressed higher levels of alkaline phosphatase and demonstrated more calcium accumulation in vitro. Cells isolated from the three skeletal sites were variably positive for STRO 1, a marker of hBMSCs. OF-MSCs formed more bone in vivo, while iliac crest hBMSCs formed more compacted bone that included hematopoietic tissue and were more responsive in vitro and in vivo to osteogenic and adipogenic inductions. These data demonstrate that hBMSCs from the same individuals differ in vitro and in vivo in a skeletal site-specific fashion and identified orofacial marrow stromal cells as unique cell populations. Further understanding of site-specific properties of hBMSCs and their impact on site-specific bone diseases and regeneration are needed.
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Keywords: Bone marrow stromal cells; Orofacial; Site-specific; Life span; Regeneration

Introduction

Autologous bone grafts used to stimulate new bone formation at sites of orofacial osseous defects are commonly obtained from several donor sites including orofacial, axial and appendicular bones. Bridging orofacial defects with grafts obtained from an orofacial donor site are usually more successful than those from non-orofacial sites, indicating anatomic skeletal site-specific differences affect graft integration [18,30,37]. Added evidence that orofacial bone development differs from that of axial and appendicular bone formation is suggested by the existence of skeletal diseases such as cherubism [42] and hyperparathyroid jaw tumor syndrome [41], which affect only jaw bones. In addition, craniofacial fibrous dysplasia is histologically and radiologically distinct from fibrous dysplasia in axial and appendicular bones [1,35]. The existence of site-specific variation in bone cell responses has been suggested based on skeletal site-dependent differences in the production of Insulin-like Growth Factor (IGF) system components by cultured human bone cells at various skeletal sites [24]. It is also noteworthy that the basic anatomy of axial and appendicular skeletons has been

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preserved despite habitat-specific adaptation, in sharp contrast to the craniofacial skeleton which has passed through different morphological modifications. Embryological development and amalgamations of the complex array of bones and cartilage in the craniofacial skeleton indicate that molecular mechanisms controlling skeletogenesis in the head are unique and different from those occurring in other body sites [17]. The cranial vault has a dual neural crest and mesodermal origin; the maxilla, mandible including alveolar bone, dentine, pulp and periodontal ligament are formed exclusively by neural crest cells [4], while axial and appendicular bones are formed by mesoderm. These clinical, laboratory and developmental differences imply the existence of site-specific properties of progenitor cells in bone marrow.

Within the bone marrow microenvironment are multipotent stromal cells that can differentiate into bone, fat, cartilage, myelosupportive stroma, and perhaps muscle and neural tissues [15,33,34]. Isolation and characterization of bone marrow stromal cells have focused on axial and appendicular bones with paucity of information from orofacial sites. Recent reports have identified unique cell populations with multipotent stem cell properties in dental tissues including dental pulp [14], exfoliated deciduous teeth [27], periodontal ligament [38] and dental extraction socket [26] all with diverse ontogeny and developmental potentials [39]. However, skeletal site-specific properties of human bone marrow stromal cells (hBMSCs) from the same individuals and their impact on site-specific bone diseases and tissue regeneration have not been clearly defined. In this study, we tested the hypothesis that hBMSCs from the same individuals have skeletal site-specific phenotypic and functional properties.

Materials and methods

Samples and cell culture

Trabecular bone isolated with a ronguer from four 3rd molar surgical sites (maxilla and mandible) and simultaneous iliac crest marrow aspirates were obtained from 7 normal volunteers (ages 18–27 years, 5 males, 2 females). Patients gave informed consent to participate in study protocols approved by the Institutional Review Board of the National Institutes of Health. Samples were obtained from the three skeletal sites in all 7 study subjects. Primary hBMSCs were established in α-modified Minimum Essential Medium (α-MEM) (Invitrogen, Life Technologies, Carlsbad, CA) by modifying a previously described method [21]. Briefly, maxilla and mandible trabecular bone samples were gently rinsed several times with α-MEM to eliminate residual oral fluids. Marrow contents were released with a #15 surgical blade into culture dishes containing α-MEM. Iliac crest marrow aspirates were centrifuged for 10 min at 135 × *g*, and the cell pellets were re-suspended in α-MEM. Marrow cells from all three sampling sites were processed separately, but similarly, by repeated pipetting and passage through 16G and 20G needles to disperse the cells followed by subsequent filtration through a 70 μm nylon cell strainer (BD Bioscience, San Jose, CA). To establish multi-colony-derived strains, nucleated cells (maxilla = 1.3×10^4 cells/cm^2, mandible = 1.3×10^4 cells/cm^2 and iliac crest = 4.7×10^4 cells/cm^2) were cultured in 25 cm^2 plastic flasks containing growth medium: α-MEM supplemented with 20% fetal bovine serum (Equitech Bio Inc, Kerville, TX), 100 U/ml penicillin, 100 mg/ml streptomycin sulfate and 2 mM glutamine (BioSource International Camarillo, CA). Osteogenically induced hBMSCs were established using osteogenic growth medium containing supplements of 10^{-8} M dexamethasone (Sigma-Aldrich, St. Louis, MO) and 100 μM L-ascorbic acid phosphate magnesium-hydrate (Wako Chemicals, Richmond VA). For these experiments, nucleated cells were plated in separate 25 cm^2 flasks using maxilla (0.7×10^4 cells/cm^2), mandible (0.7×10^4 cells/cm^2) and iliac crest (2.3×10^4 cells/cm^2). Plating cell density was higher for iliac crest cells due to the presence of a greater quantity of peripheral blood cells. Cells were incubated at 37°C, in a humidified atmosphere of 5% CO_2 and air. Growth medium was changed on day 1 for aspirate and day 7 for maxilla and mandible hBMSC cultures and once a week thereafter until 75% confluence. Subconfluent primary hBMSCs were released with trypsin–EDTA (Invitrogen, Life Technologies, Carlsbad, CA) and further characterized.

For all experimental methods described below, maxilla, mandible and iliac crest samples from each subject were compared simultaneously.

Colony forming efficiency assay

Nucleated cells were cultured in triplicate 25 cm^2 plastic culture flasks at 10^3, 10^4 and 10^5 cells/flask with non-osteogenic growth medium [20]. Cells were fixed on day 10 with 100% methanol, stained with methyl violet (Sigma-Aldrich, St. Louis, MO) and aggregates of 50 or more cells were counted as colonies.

Cell proliferation

The proliferation rate of first passage hBMSCs was assessed by growth curve analyses [10,36] after seeding 1×10^4 cells/well in triplicate 6-well plates (Corning Life Sciences, Acton, MA) containing growth medium with and without osteogenic inducers. Cells were released on days 1, 3, 5, 7, 9 and 14 and counted with a particle and cell size analyzer (Z1™ Coulter Counter®, Beckman-Coulter, Inc. Fullerton, CA) to plot a growth curve.

Life span measurements

The proliferative life span of hBMSCs was assessed by population doublings (PD) calculated from generation number after repeated cell passage at 1:10 split ratio until the cells attained replicative senescence [PD = product of passage number and split ratio] [16,19].

Telomerase activity

The presence of human telomerase reverse transcriptase (hTERT) was determined by Western blotting of nuclear extracts isolated with Nuclei EZ Prep® (Sigma-Aldrich, St. Louis, MO) and HeLa cell lysate as control. Nuclear extracts were derived from hBMSCs at two stages: PD20 and PD40, representing population doublings (PD) of 20 and 40. Equal protein amounts (10μg) of samples and control were tested, using a mouse monoclonal antibody to hTERT (ab5181, Abcam Inc, Cambridge, MA) as the primary antibody (1:250 dilution). The expression of telomerase in enzymatically active hBMSC (PD20) from the three sites was assessed by the PCR-based *T*elomerase *R*epeat *A*mplification *P*rotocol using the TRAPEZE® telomerase detection kit (S7700, Chemicon International, Temecula, CA) following manufacturer's recommendations. Briefly, pre-confluent PD20 hBMSCs were trypsinized, washed with PBS (pH 7.3), pelleted and re-suspended at 750 ng/μl in CHAPS lysis buffer supplied in TRAPEZE® kit. Samples tested included extracts and heat inactivated extracts of PD20 hBMSCs, positive telomerase control extract, primer–dimer/PCR contamination control and a quantitation control template. A 50 μl TRAPEZE® master-mix containing 1.5 μg cell extract was incubated at 30°C for 30 min followed by 2-step PCR at 94°C/30s and 59°C/30s for 33 cycles in a Perkin Elmer thermocycler (Perkin Elmer, Boston, MA). After amplification, 25 μl of the PCR products was loaded on a 10% non-denaturing polyacrylamide gel electrophoresis and the bands visualized by ethidium bromide staining. As part of the assay internal control, the TRAPEZE® primer mix also included PCR amplification internal control oligonucleotides that produced a band of 36 base pairs in each lane.

In vitro calcium accumulation

Mineralization potential of hBMSCs was assessed by in vitro calcium accumulation of first passage hBMSCs (1×10^4 cells/cm^2) in duplicate 60 mm dishes (Corning Life Sciences, Acton, MA). At confluence, growth medium was

changed to mineralization medium (α-MEM, 5% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, 10^{-8} M dexamethasone, 100 μM L-ascorbic-2-phosphate and 5 mM β-glycerophosphate) with medium change 2 times/week [13,22]. At week 6, hBMSCs were fixed with 70% ethanol and stained with 2% Alizarin Red S (pH 4.2). Unbound and nonspecifically bound stain was removed by copious rinsing with distilled water, and calcium-bound stain was extracted with 0.5 N HCl/5% sodium dodecyl sulfate. Absorbance of the extract was determined at 415 nm along with calcium standards (Cat #3605 Sigma-Aldrich, St. Louis, MO).

Alkaline phosphatase assay

Alkaline phosphatase production was assayed by a modification of an earlier described method [9]. Briefly, first passage hBMSCs (1×10^4 cells/cm^2) in triplicate 24-well plates (Corning Life Sciences, Acton, MA) were cultured for 15 days in α-MEM growth medium with and without osteogenic inducers and change of medium at 2-day intervals. The cell monolayer was lysed at 4°C on days 3, 7, 9, 11 and 15 with 0.2% cold NP 40 containing 1 mM $MgCl_2$ and denatured by repeated freeze–thaw on dry ice. Cells were scraped, sonicated three times in water/wet ice mixture and centrifuged at 800×*g* for 20 min. Supernatants were assayed for total protein [3] and alkaline phosphatase after 30 min incubation at 37°C using 0.075 M *p*-nitrophenyl phosphate substrate in 0.05 M barbital buffer, pH 9.3. Positive controls (Accutrol™ normal control, A2034, Sigma-Aldrich, St. Louis, MO) and standards (LS006130, Worthington Biochemical Corporation, Lakewood, NJ) were included to plot a standard curve. The reaction was stopped with 0.1 N NaOH and product measured at absorbance of 405 nm.

Adipogenesis

Adipogenic differentiation was induced as previously described [32]. Briefly, first passage hBMSCs (1×10^4 cells/cm^2) in duplicate 6-well plates (Corning Life Sciences, Acton, MA) were cultured with α-MEM growth medium without inducers until confluence before being exposed to adipogenic medium [containing supplements of 10^{-8} M dexamethasone, insulin (1 μg/ml), 1-methyl-3-isobutylxanthine (IBMX, 5×10^{-8} M) and indomethacin (10^{-4} M)] for 3 weeks; medium was changed twice weekly. Similar plates without exposure to adipogenic medium served as control. The cells were fixed with 4% paraformaldehyde, stained with 0.3% Oil Red O and counterstained with 1% Fast green dye. Lipid droplets were identified microscopically.

Marrow stromal cell transplantation and in vivo bone formation

Bone formation was assessed using the mouse model of in vivo bone formation as previously described [21] in accordance with an institutionally approved animal protocol (NIDCR #02-222). In two separate groups of 3 animals, non-induced and osteogenically induced cells were transplanted. From each skeletal donor site, 2×10^6 hBMSCs were attached to 40 mg spheroidal hydroxyapatite/tricalcium phosphate (particle size 0.5–1.0 mm, Zimmer, Warsaw, IN) and transplanted into 3 separate subcutaneous pockets aseptically created in 8-week-old immunocompromised nude female mice (NIH-III-nu, Charles River Laboratories, Wilmington, MA). Transplants were harvested at weeks 4, 6, 10 and 12, fixed in 4% formalin, decalcified in 10% EDTA (pH 8.0) and embedded in paraffin. Five-micrometer sections were deparaffinized, stained with hematoxylin/eosin and bone formation was scored microscopically by two blinded trained observers for semi-quantitative analysis as previously described [21]. The bone scores ranged from 0 (no bone evident within the transplant), 1 (minimal bone evident [1 trabecula]), 2 (weak bone formation occupying only a small portion of the transplant), 3 (moderate bone formation occupying a significant portion but less than 50% of the transplant) and 4 (abundant bone formation, occupying more than 50% of the transplant ± hematopoiesis). This bone scoring method has been validated with histomorphometric analysis [25]. The human origin of bone within transplants was established in deparaffinized unstained sections by reaction with primary rabbit anti-human osteopontin polyclonal antibody (Cat # 499265, EMD Biosciences, San Diego, CA) followed by enzymatic staining with broad-spectrum immunoperoxidase AEC kit (Zymed Laboratories, San Francisco, CA) [6].

Immunohistochemical localization of cell surface markers

hBMSCs were subcultured (2×10^4 cells/well) in 8-chamber slides (Nalge Nunc, Rochester, NY), fixed with 4% paraformaldehyde and non-specific binding was blocked before incubating with primary antibodies. Separate experiments were performed using the following primary antibodies: mouse monoclonal antibody to STRO-1 (provided by Dr. S. Shi, National Institute of Dental and Craniofacial Research (NIDCR), NIH, Bethesda, MD) and rabbit polyclonal antibodies against human bone sialoprotein (BSP, LF-120, from Dr. Larry Fisher, NIDCR/NIH), endostatin (Chemicon International, Temecula, CA), transforming growth factor receptor (TGFβ-R2, Santa Cruz Biotechnology, Santa Cruz, CA), fibroblast growth factor receptor 3 (FGF-R3, Novus Biologicals, Littleton, CO), vascular endothelial growth factor receptor 1 (VEGF-R1, R&D Systems, Minneapolis MN) and matrix extracellular phosphoglycoprotein (MEPE/LF 155, from Dr. Larry Fisher, NIDCR/NIH). STRO-1 antibody was undiluted, while other antibodies were incubated at dilutions between 1:25 and 1:100 [12,14,27,38]. Positive cells were visualized with enzymatic broad-spectrum immunoperoxidase AEC kit (Zymed Laboratories, San Francisco, CA).

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from second passage maxilla, mandible and iliac crest BMSCs using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA). First strand cDNA was prepared with first strand SuperScript™ Double-Stranded cDNA Synthesis Kit (Invitrogen Life Technologies, Carlsbad, CA) using an oligo-dT primer. Two microliters of first strand cDNA was added to a total volume of 50 μl PCR buffer containing: 1.5 mM $MgCl_2$, 200 μM dNTP, 2.5 U Taq DNA polymerase (Promega, Madison, WI) and 200 nM of each primer set. Primers sets used were: human endostatin (COL 18A1), GenBank Accession number AF018081, fragment size 240 bp, forward, 5′-ACAGAAGCCTGATCTGACAT-3′; reverse, 5′-TGCTAACAGGTCTGGGTTTTG-3′); human TGFβ-R2, GenBank Accession number M85079, fragment size 255 bp, forward, 5′-TGCCAACAACATCAACCACAA-3′; reverse, 5′-TCCGTCTTCCGCTCCTCA-3′ and human GAPDH, GenBank Accession number M33197, fragment size 816 bp, forward, 5′-AGCCGCATCTTCTTTTGCGTC-3′; reverse, 5′-TCATATTTGGCAGGTTTTTCT-3′. The PCR reaction was carried out in a Perkin Elmer thermal cycler (Perkin Elmer, Boston, MA) at 94°C for 2 min for 1 cycle then 94°C (1 min), 55°C (1 min) and 72°C (1 min) for 35 cycles and final extension at 72°C (10 min). Fifteen microliters of the amplified products were separated on 2% agarose gel, stained with ethidium bromide and visualized with Kodak ImageStation 440 (Eastman Kodak, Rochester, NY). Bands were analyzed with Scion Image® (Scion Corporation, Frederick, MD), and abundance of transcript in samples was semi-quantitatively compared with GAPDH expression.

Statistical analyses

All experiments were repeated at least three times, each cell type was tested in triplicates, and results were expressed as mean ± standard deviation (SD). Differences between iliac crest hBMSCs and OF-MSCs and between non-induced and osteogenically induced cells were compared with one-way analysis of variance (ANOVA) followed by post hoc comparisons with Turkey–Kramer test. In each analysis, differences were considered significant at $P < 0.05$. Inter-observer variability of bone score measurements was assessed with Kappa statistic.

Results

Isolation of hBMSCs from maxilla and mandible samples was limited to small (0.5 × 0.5 cm) tissue samples that yielded an average of 5×10^5 nucleated cells per sample. Sample size needed to determine phenotypic differences between orofacial and iliac crest hBMSCs was calculated using preliminary results of in vivo bone formation by osteogenically induced hBMSCs

at weeks 10 and 12. In these studies, the mean bone score for iliac crest hBMSCs was 2.18 (standard deviation = 0.2), and pooled maxilla and mandible (orofacial) mean bone score was 3.22 (standard deviation = 0.04). At a power of 0.8, α level of 0.05 and standard deviation of 0.35 (assuming more variability and equal standard deviation), the sample size needed to determine a difference is 4. However, a total of 7 subjects were enrolled and 21 samples analyzed.

hBMSCs isolated from the three sampling sites formed morphologically variable but discrete colonies with typical fibroblast appearance (Figs. 1 A, B, C and D). Mean colony forming efficiency (CFE) per 10^5 nucleated cells, determined using samples from three subjects, was 54, 17 and 37 for maxilla, mandible and iliac crest respectively. The percentage of immunoreactive STRO 1 positive cells (STRO 1^+), suggestive of early mesenchymal stem cells [13,40], ranged between 10 and 20% at all donor sites without appreciable difference between orofacial and axial sites (Fig. 1E). However, the low yield of nucleated cells from orofacial samples restricted the number of samples plated as primary cultures, thus limiting statistical analyses of differences among groups.

When first passage hBMSCs were plated at low density (10^3 cells/cm^2), significant differences in the proliferative and population doubling (PD) capacities were observed between orofacial (OF-MSCs) and iliac crest hBMSCs. Proliferation rates were much higher in OF-MSCs compared to iliac crest hBMSCs (Fig. 2A), and PDs over 225 days were consistently higher for OF-MSCs than for the iliac-crest-derived cells (Fig. 2B). Average PD sustained by OF-MSCs was PD180, while iliac crest hBMSCs were limited to PD40 (Fig. 2B). Expression of telomerase by hBMSCs was higher in OF-MSCs than in iliac crest cells by both Western blotting (Fig. 2C) and TRAPEZE® assay (Fig. 2D).

OF-MSCs were apparently more responsive to in vitro osteogenic differentiation than iliac crest hBMSCs as measured by amount of calcium accumulation and alkaline phosphatase activity. Retention of Alizarin Red S stain by OF-MSCs was higher (Figs. 3A–C). Quantitative analysis of calcium-bound stain (Fig. 3D) by the three cell types cultured for 6 weeks showed significant difference between iliac crest and maxilla ($P < 0.001$) and between iliac crest and mandible ($P < 0.001$) cells. Predictably, the three cell types demonstrated low levels of alkaline phosphatase activity when cultured in non-osteogenic medium (data not shown), but OF-MSCs produced higher levels of alkaline phosphatase activity than iliac crest hBMSCs in response to osteogenic induction (Fig. 3E). In contrast, differentiation to adipocytes, visualized by Oil Red O staining of lipid-containing cell clusters (Figs. 3F–H), was more pronounced in iliac crest hBMSCs after 3 weeks of culture in adipogenic medium. The iliac crest lipid clusters were more

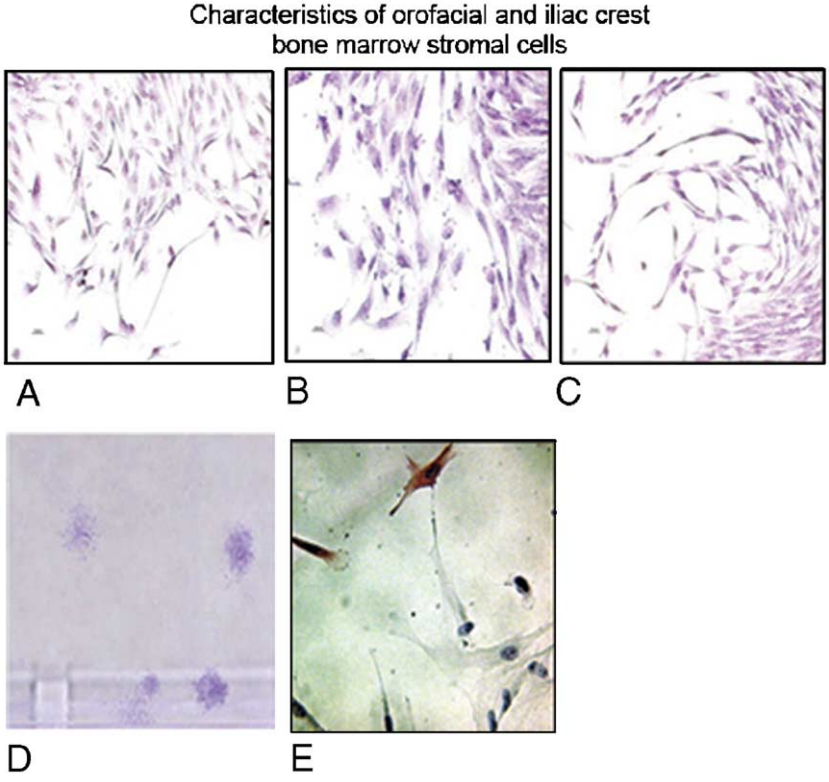

Fig. 1. Characteristics of orofacial and iliac crest primary human bone marrow stromal cells (hBMSCs). Single cell suspensions of nucleated cells isolated from three skeletal sampling sites ($n = 4$) were plated in 25 cm^2 plastic flasks for 14 days, fixed and stained with methyl violet. Top panel (A–C) shows fibroblast-like morphology of representative cell monolayers from primary hBMSCs derived from iliac crest (A), maxilla (B) and mandible (C). Macroscopic appearance of isolated colonies formed by mandible primary hBMSCs is shown in the lower panel (D) and is also representative of similar colonies formed by maxilla and iliac crest cells. Immunoreactivity of mandible hBMSCs to anti-STRO 1 monoclonal antibody (E) showed that 10–20% hBMSCs attached to chamber slides were STRO 1^+. Similar results were obtained for hBMSCs derived from iliac crest and maxilla ($n = 4$).

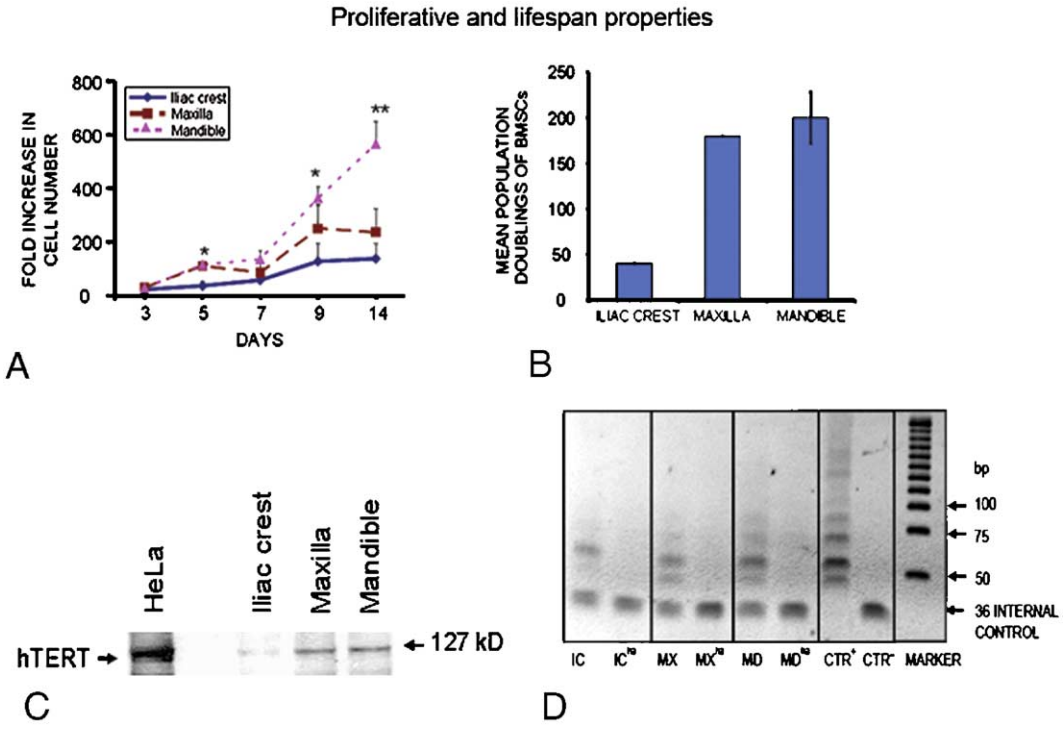

Fig. 2. Proliferative and life span characteristics of human bone marrow stromal cells (hBMSCs). A comparison of proliferative rates of first passage hBMSCs over 14 days (A) showed that maxilla and mandible cells sustained higher proliferative rate than iliac crest cells within 14 days of culture ($n = 4$; ANOVA, $P < 0.05$, days 5 and 9; $P < 0.01$, day 14). Similarly, maxilla and mandible hBMSCs (OF-MSCs) displayed higher population doublings (B) than iliac crest hBMSCs within 225 days ($n = 4$). Error bars indicate standard deviation. Production of telomerase by PD20 hBMSCs demonstrated by Western blot (C) showed higher telomerase levels in OF-MSCs than iliac crest cells ($n = 4$). Similar results were obtained at PD40. This was further corroborated by TRAPEZE® assay to evaluate telomerase expression (D). The 50-nucleotide product generated in the first step of the PCR reaction of telomerase extract is less abundant in iliac crest (IC) than in OF-MSCs; the levels in maxilla (MX) and mandible (MD) are comparable to those seen in the positive control (CTR^+). Repetitive PCR amplification by addition of 6 nucleotides produced a more pronounced step-ladder pattern of the PCR products of OF-MSCs [hTERT = human telomerase reverse transcriptase; HeLa = HeLa cell lysate telomerase control; ha = heat inactivated control samples; CTR^- = negative telomerase control].

numerous and larger in size than those of maxilla and mandible hBMSCs. Therefore, in vitro osteogenic differentiation of cultured OF-MSCs, as measured by both calcium accumulation and alkaline phosphatase activity, was more extensive than iliac crest cells, while the iliac crest cells responded better to adipogenic induction.

A panel of antibodies to 6 growth factors or matrix proteins identified some similarities and minor differences in hBMSC immunoreactivity (Table 1). Antibodies raised to human bone sialoprotein (BSP), matrix extracellular glycoprotein (MEPE), VEGF-R1 and FGF-R3 reacted with equal affinity in all three cell types, while antibodies to TGFβ-R2 and endostatin were less reactive with iliac crest hBMSCs (Table 1). A representative strong immunoreactivity of rabbit anti-human BSP (LF-120) and anti-human MEPE (LF 155) to hBMSCs from the three skeletal sites is shown in Fig. 4. Due to the higher immunoreactivity of TGFβ-R2 and endostatin to OF-MSCs compared with iliac crest hBMSCs, mRNA levels of TGFβ-R2 and endostatin were examined by semi-quantitative RT-PCR, but there were no significant differences between the cell types (data not shown).

Evaluation of ectopic bone formation in nude mice clearly demonstrated that iliac crest hBMSCs formed a complete bone/marrow organ (Fig. 5). However, based on semi-quantitative bone scoring [25], iliac-crest-derived cells produced quantitatively less bone (Fig. 7) than orofacial cells. Morphologic comparisons of sections of normal human trabecular bone (Figs. 5A, B, C) with new bone formed by non-induced (Figs. 5D, E, F) and osteogenically induced (Figs. 5G, H, I) hBMSCs transplanted into immunocompromised mice showed important differences. Dense bone with abundant adipose tissue was observed in sections of normal trabecular bone of iliac crest, maxilla and mandible (Figs. 5A, B and C), but additional appreciable hematopoietic tissues were observed only in iliac crest (Fig. 5A). Interestingly, the histological pattern displayed by bone formed in vivo by transplanted hBMSCs was similar to that seen in normal bone only when the hBMSCs were induced to undergo osteogenesis in culture. Both non-induced (Figs. 5D, E, F) and osteogenically induced (Figs. 5G, H, I) hBMSCs from the three skeletal sites formed abundant bone, but hematopoiesis was evident only in bone formed by osteogenically induced iliac crest cells (Fig. 5G). Panoramic microscopic evaluation at low magnification (10×) showed bone formed by maxilla, and mandible cells were isolated nodules, unlike iliac crest cells that formed more closely packed bone with histologically observable hematopoietic marrow (Figs. 5J, K, L). Immunoreactivity with human-specific anti-osteopontin polyclonal antibody [6] demonstrated that bone formed within transplants from all three skeletal hBMSCs donor sites were of human origin (Fig. 6). Further quantitative analysis of in vivo bone formation showed

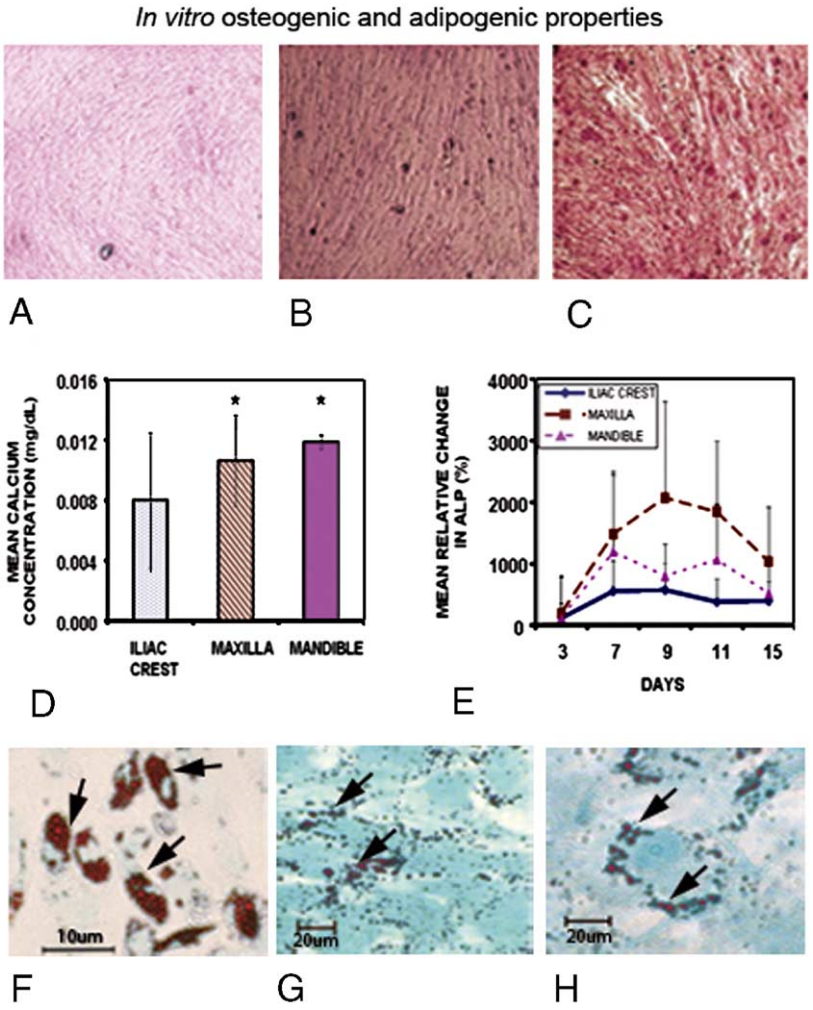

Fig. 3. In vitro osteogenic and adipogenic differentiation of human bone marrow stromal cells (hBMSCs). Culture of hBMSCs with mineralization medium for 6 weeks was followed by Alizarin Red S staining (A, B and C). Note that the differences in absorption of the stain by the three cell types are site-specific: iliac crest (A) < maxilla (B) < mandible (C). The calcium-bound stain was extracted, and absorbance at A_{415} was compared with calcium standards. Maxilla and mandible hBMSCs accumulated more calcium in vitro than iliac crest (D). The differences between iliac crest, maxilla and mandible were statistically significant at $P < 0.0001$ ($n = 4$; mean of 3–5 experiments). The effect of osteogenic induction on alkaline phosphatase activity of iliac crest, maxilla and mandible hBMSCs is shown in panel (E). All three cell types showed elevated alkaline phosphatase activity 7 days after addition of osteogenic media which was sustained until day 14, but maxilla and mandible hBMSCs (OF-MSCs) were more responsive to induction than iliac crest cells. The differences between iliac crest, maxilla and mandible were statistically significant at $P < 0.05$ respectively ($n = 4$; mean of 3 experiments). Site-specific response to adipogenic induction is shown by Oil Red O staining of hBMSCs cultured for 3 weeks in adipogenic medium. Iliac crest hBMSCs (F) demonstrated larger and more lipid filled cells than maxilla (G) and mandible (H) cells. Arrows point to lipid filled cell clusters (statistical analyses were by one-way ANOVA).

that OF-MSCs consistently formed more bone than iliac crest cells from weeks 4 to 12 (Fig. 7). Inter-observer agreement on bone score measurements was high based on a weighted Kappa statistic of 0.76. Unlike the in vitro osteogenic response displayed in Fig. 3, iliac crest cells pretreated with osteogenic inducer demonstrated more in vivo bone formation, with marked and sustained increase at each time point for 12 weeks (Figs. 7A and D) compared to gradual rise from weeks 4–12 by maxilla (Figs. 7B and D) and weak or negligible response by mandible cells (Figs. 7C and D).

Osteogenic induction of iliac crest hBMSCs resulted in early response to in vivo bone formation (week 4) that was sustained for 12 weeks (Fig. 7A). In contrast, OF-MSCs did not respond differently to osteogenic stimulation at week 4. However, osteo-induced maxilla cells formed relatively more abundant bone in 12 weeks (Fig. 7B) than mandible cells (Fig. 7C).

The sustained rate of in vivo bone formation over 12 weeks by osteogenically induced iliac crest hBMSCs (Fig. 7A) is distinct from the rapid response or “catch-up” effect displayed by non-induced maxilla (Fig. 7B, at week 12) and mandible (Fig. 7C, weeks 4–12) OF-MSCs, which formed bone comparable to that formed by osteogenically induced cells. In addition, iliac crest hBMSCs formed more dense bone containing distinct marrow-like cavities (Figs. 5G and J). These results indicate that in vivo response of hBMSCs to osteogenic induction is site-specific, and the iliac crest is most responsive while mandible is least responsive to induction (iliac crest > maxilla > mandible). Unlike iliac crest, it appears that

Table 1
Immunoreactivity of human bone marrow stromal cells to six growth factors or matrix proteins

Antibodies	Iliac crest	Maxilla	Mandible
BSP	++	++	++
MEPE	++	++	++
TGFβ-R2	+	++	++
Endostatin	+	++	++
VEGF-R1	+	+	+
bFGF-R3	+	+	+

Summary of comparative immunoreactivity of human bone marrow stromal cells (hBMSCs) to antibodies raised to 6 human cell surface markers. While immunostaining of maxilla and mandible hBMSCs was similar, iliac crest hBMSCs reacted with less affinity to anti-human TGFβ-R2 and anti-human endostatin (n = 4, mean of 3 different experiments; + = immunoreactivity; ++ = moderate immunoreactivity; BSP = bone sialoprotein; MEPE = matrix extracellular phosphoglycoprotein; TGFβ-R2 = transforming growth factor receptor 2; bFGF-R3 = basic fibroblast growth factor receptor 3).

OF-MSCs readily differentiated osteogenically in an in vivo model.

Discussion

Starting with limited amounts of maxilla and mandible bone marrow samples from third molar surgical sites, hBMSCs were successfully isolated, expanded and partially characterized. OF-MSCs displayed fibroblast-like morphology and formed isolated colonies in a manner similar to iliac crest hBMSCs. Both iliac crest hBMSCs and OF-MSCs contained STRO-1^+ cells which identify a cell surface antigen expressed by stromal elements in human bone marrow. STRO-1, a monoclonal IgM produced from mouse immunized with $CD34^+$ cells, recognizes only clonogenic and highly osteogenic progenitors. They share expression of CD34 with primitive hematopoietic precursors but are phenotypically distinct cell types. As these cells mature and differentiate, STRO-1 expression decreases while lineage-specific antigens increase [40].

Higher proliferation and osteogenic differentiation of OF-MSCs without direct stimulation indicate that OF-MSCs do not need induction to differentiate osteogenically, whereas iliac crest hBMSCs responded as well as OF-MSCs both in vitro and in vivo after osteogenic stimulation. In addition, iliac crest hBMSCs were more responsive to adipogenic stimulation.

The similar in vitro and in vivo characteristics of OF-MSCs from both maxilla and mandible sampling sites compared with iliac crest underscore site-specific differences between orofacial and iliac crest hBMSCs. This may be a reflection of the amount of bone marrow progenitor cells as well as site-specific ontogeny. Although age influences quantity of resident BMSC population and osteogenic differentiation [29], the impact of age on tissue samples was minimal because hBMSCs from the three donor sites were compared in same individuals without cross-matching samples, the age range of subjects was within a narrow limit and below 35 years, the upper age limit of peak bone mass following which dramatic decrease in bone composition and mineral density occurs [11].

Differences in histological appearance of in vivo bone formed by the three cell types may relate to biochemical strain associated with functional demands at each skeletal site. OF-MSCs formed isolated bone nodules, in contrast to the closely packed bone formed by iliac crest cells. Similarly, bone formed by OF-MSCs was separated by abundant fibrous tissue, unlike bone formed by iliac crest that contained appreciable hematopoietic components. These features are consistent with the different morphology of normal trabecular bone at the three sites and reflect site-specific functional demands. The ilium, a

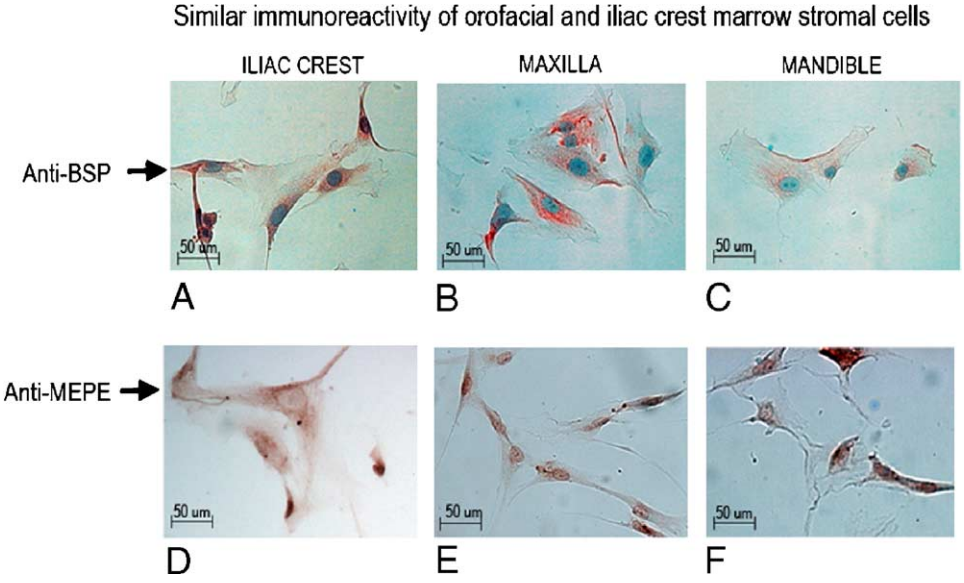

Fig. 4. Similar immunoreactivity of orofacial and iliac crest BMSCs. Iliac crest (left panel), maxilla (middle panel) and mandible (right panel) hBMSCs were attached to 8-well chamber slides and reacted with rabbit antibodies to bone sialoprotein (anti-BSP), top panel (A, B, C), and matrix extracellular phosphoglycoprotein (anti-MEPE), lower panel (D, E, F). Note the similarity in the strong immunoreactivity of hBMSCs from the three skeletal sites (n = 4; similar experiments obtained in 3 different experiments).

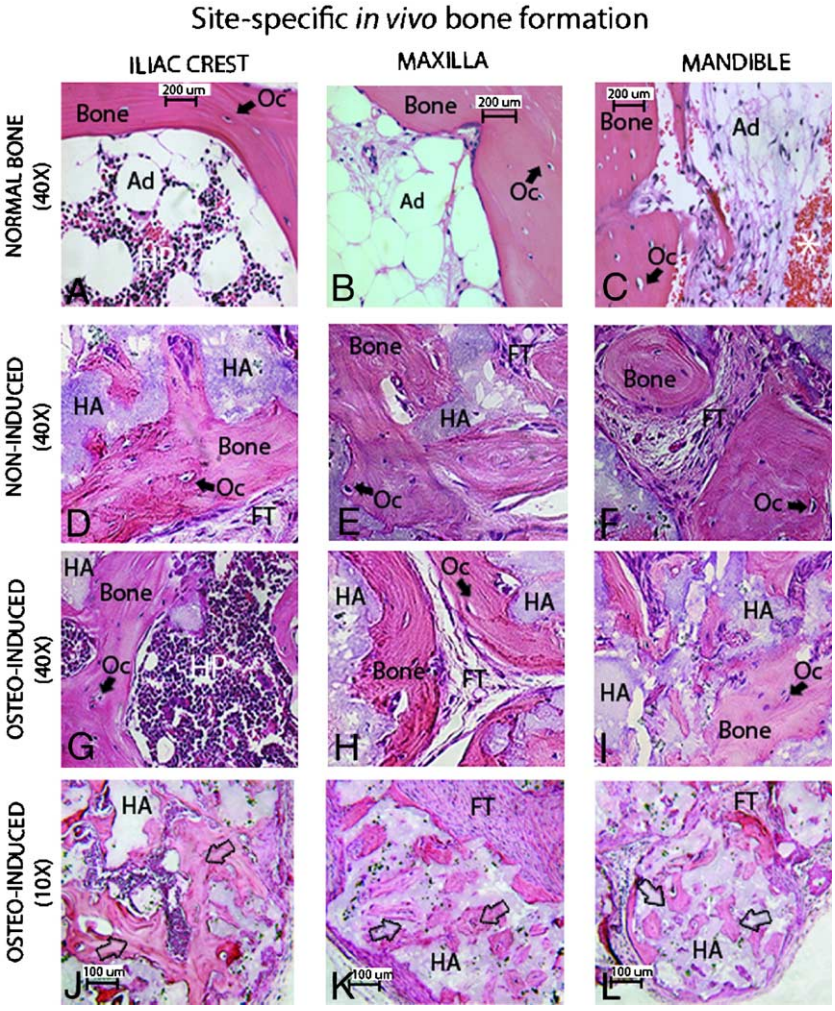

Fig. 5. Site-specific in vivo bone formation by human bone marrow stromal cells (hBMSCs). Sections of normal bone from iliac crest, maxilla and mandible were compared with in vivo bone formed by transplanted hBMSCs. First row shows microscopic sections of normal iliac crest (A), maxilla (B) and mandible (C) with dense bone and abundant adipose tissue. Note abundant hematopoietic tissue in iliac crest section. Second row shows representative sections of bone formed in vivo by non-induced iliac crest hBMSCs (D) and OF-MSCs [maxilla (E) and mandible (F)]. There is abundant bone with osteocytes in all three sections. When transplanted hBMSCs were cultured under osteogenic conditions, abundant bone was formed by all three cell types (third row, G, H and I); however, only bone formed by iliac crest hBMSCs contains appreciable amount of hematopoietic tissue (G). Fourth row shows panoramic view (10×) of in vivo bone formation. Note that the bone formed by iliac crest cells hBMSCs (clear arrows, J) was closely packed and contained hematopoietic tissue (HP), while OF-MSCs (maxilla, mandible) formed bone consisting of isolated nodules without hematopoietic tissue (clear arrows, K and L). Three separate transplants were performed in $n = 4$ subjects (HP = hematopoiesis; HA = hydroxyapatite/tricalcium phosphate carrier; Oc = osteocyte; FT = fibrous tissue; white * = hemorrhage resulting from surgical procedure).

part of the pelvic girdle, is physiologically adapted for support of body weight, contains more red marrow and contributes more to hematopoiesis [5]. In contrast, maxilla and mandible are parts of the craniofacial complex, contain less red marrow but offer protection for vital structures such as air sinuses, dentition and neurovascular bundle.

It is plausible that observed skeletal site-specific differences of hBMSCs are related to different embryological origins and adaptation to functional demands at each skeletal site. Other extrinsic and intrinsic factors that may lead to observed differences include local vascular supply, the cellular composition and genetic profile of the marrow microenvironment, hormonal effects and muscular attachments that directly accentuate biochemical strains of mechanical load [8,23]. While some of the observed differences between OF-MSCs and appendicular skeleton hBMSC may be attributed to environmental factors, it seems less likely that the vastly more sustained population doublings of OF-MSCs in comparison to iliac crest cells are environmentally regulated.

The higher in vitro activity of the early osteogenic marker, alkaline phosphatase, by OF-MSCs (Fig. 3E) is consistent with the increased tendency of these cells to differentiate osteogenically in vivo without prior osteoinduction (Fig. 7). It will be important to determine if bone-associated transcription factors such as Runx2 (Cbfa1) and osterix, which promote osteoblast differentiation [7,28], are more highly expressed in the OF-MSCs. The more marked PD property of OF-MSCs despite less response to osteogenic stimulation indicates more self-renewal ability than iliac crest cells and a reflection of cells in an earlier stage of stem cell differentiation. Since normal maxilla and mandible contain less hematopoietic marrow than ilium (Figs. 6A, B and C) [31], the higher proliferation and PD

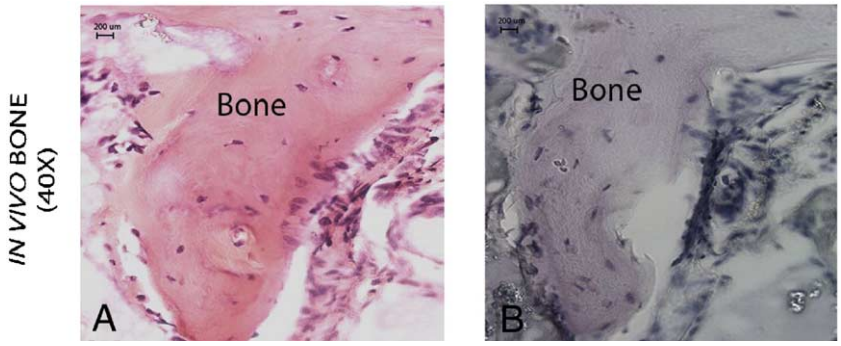

Fig. 6. A representation of immunohistochemical staining of in vivo bone with rabbit anti-human osteopontin to determine origin of bone formed. Adjacent 5-μm sections of decalcified paraffin-embedded bone formed by mandible OF-MSCs were stained with hematoxylin/eosin (A) or reacted with rabbit anti-human osteopontin (B). The positive immunoreactivity in panel (B) demonstrated that in vivo bone formed by mandible OF-MSCs was of human origin. Similar results were obtained for iliac crest and maxilla hBMSCs transplants.

are consistent with reports that stromal cells of non-hematopoietic marrow divide more actively unlike those of hematopoietic marrow that are usually mitotically quiescent while continuing to express the osteoblastic marker, alkaline phosphatase [2]. These reports and our current observations point to maxilla and mandible as important sources of hBMSCs for bone regeneration. Distinctive OF-MSCs phenotypes and differentiation support earlier reports of the unique nature of craniofacial-specific multipotent stem cells previously characterized from dental pulp [14], periodontal ligament [38], exfoliated deciduous teeth [27] and aspirate from dental extraction sockets [26]. The effects of these unique properties on orofacial bone grafting need further clarification. However, the observation that OF-MSCs need less induction than iliac crest hBMSCs to differentiate osteogenically in an in vivo model makes the maxilla and mandible prime donor sites for bone graft.

In summary, this study identified fibroblast-like, colony forming and STRO 1^+ hBMSCs in nucleated cells isolated from maxilla and mandible (orofacial) trabecular bone and compared them with those from iliac crest (axial bone) in same individuals. The findings point to existence of skeletal site-specific properties of orofacial and axial hBMSCs based on different embryological origins. However, the underlying mechanisms and clinical implications of their unique properties still need further clarification.

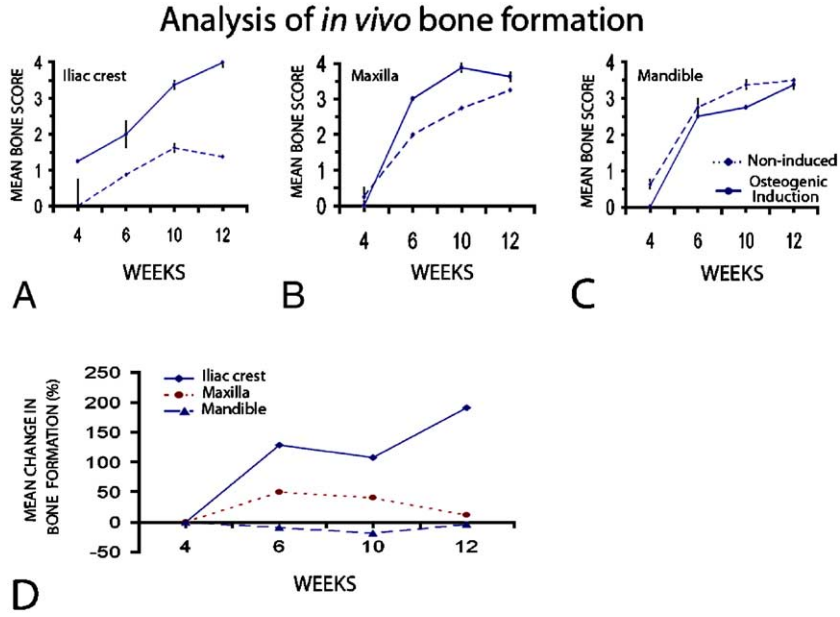

Fig. 7. Site-specific comparison and quantitative analyses of in vivo bone formation by iliac crest, maxilla and mandible human bone marrow stromal cells (hBMSCs). Bone formation by non-induced iliac crest hBMSCs (dashed lines) was quantifiable at week 6 but limited to bone score <3 during 12 weeks of transplantation (A). However, maxilla and mandible cells formed appreciable bone as early as week 4 and bone score >3 within 12 weeks of transplantation (B and C). When hBMSCs were induced osteogenically before transplantation (solid lines, A, B and C), iliac crest and maxilla cells responded by forming more bone, while there was negligible response by mandible cells as displayed by percent change in bone formation (D). The differences in bone formation between the three cell types were statistically significant at $P < 0.01$ ($n = 4$, results were mean of 3 transplantation experiments). Osteogenically induced iliac crest hBMSCs formed appreciable bone as early as week 4 (solid line, A). This indicates that in vivo response of hBMSCs to osteogenic induction is site-specific: iliac crest > maxilla > mandible.

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