Cytokine, Chemokine, and Growth Factor Profile
Characterization of Undifferentiated and Osteoinduced
Human Adipose-Derived Stem Cells

F. Mussano, 1 T. Genova, 1,2 M. Corsalini, 3 G. Schierano, 1 F. Pettini, 3 D. Di Venere, 3 and S. Carossa 1

1 CIR Dental School, Department of Surgical Sciences, UNITO, Via Nizza 230, 10126 Turin, Italy
2 Department of Life Sciences and Systems Biology, UNITO, Via Accademia Albertina 13, 10123 Turin, Italy
3 Dipartimento Interdisciplinare di Medicina, Università di Bari, Piazza Giulio Cesare 11, 70124 Bari, Italy

Correspondence should be addressed to F. Mussano; federico.mussano@unito.it

Received 18 September 2016; Revised 8 January 2017; Accepted 28 February 2017; Published 10 May 2017

Academic Editor: Dario Coletti

Copyright © 2017 F. Mussano et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Bone is the second most manipulated tissue after blood. Adipose-derived stem cells (ASCs) may become a convenient source of MSC for bone regenerative protocols. Surprisingly, little is known about the most significant biomolecules these cells produce and release after being osteoinduced. Therefore, the present study aimed at dosing 13 candidates chosen among the most representative cytokines, chemokines, and growth factors within the conditioned media of osteoinduced and undifferentiated ASCs. Two acknowledged osteoblastic cell models, that is, MG-63 and SaOs-2 cells, were compared. Notably, IL-6, IL-8, MCP-1, and VEGF were highly produced and detectable in ASCs. In addition, while IL-6 and IL-8 seemed to be significantly induced by the osteogenic medium, no such effect was seen for MCP-1 and VEGF. Overall SaOS-2 had a poor expression profile, which may be consistent with the more differentiated phenotype of SaOs-2 compared to ASCs and MG-63. Instead, in maintaining medium, MG-63 displayed a very rich production of IL-12, MCP-1, IP-10, and VEGF, which were significantly reduced in osteogenic conditions, with the only exception of MCP-1. The high expression of MCP-1 and VEGF, even after the osteogenic commitment, may support the usage of ASCs in bone regenerative protocols by recruiting both osteoblasts and osteoclasts of the host.

1. Introduction

Unlike the majority of adult tissues, bone is capable to self-repair without forming scars, as most fractures demonstrate by healing spontaneously [1] or through mild surgery. Notwithstanding this inherent regenerative capacity of bone, at least one tenth of the more than 6.2 million fractures [2] occurring yearly suffer from impaired healing. In addition, inborn malformations, alveolar resorption, and critical-size bone defects resulting from severe trauma or malignant tumor resection [3] make bone the second most transplanted tissue after blood [4]. Treatments include grafting with both autogenous and allogenic bone, which are not without limitations [5].

Autogenous bone is widely considered the gold standard of bone grafting materials. Nevertheless, there are still some limits to the use of autogenous bone due to the donor site morbidity, the difficulty in obtaining it, and the prolonged healing time [6, 7]. Recently, autologous bone has been used for the regeneration of bony structures and defects [8]. However, autologous bone administration has been highly associated with the risk of disease transmission and immune reaction [9]. Furthermore, synthetic bone grafting materials have been produced to mimic the bone structure and to promote osteoconduction. However, fabricating and manufacturing these graft materials preclude their extensive application due to the involved primary expenses [7, 10].
One of the major goals of tissue engineering [11] is to overcome the pitfalls traditional techniques face when applied to treat large bone defects [12]. Among the three key components of each regenerative protocol, besides scaffolds and signaling molecules, cells play a paramount role. To this end, primary multipotent stem cells, along with several immortalized cell lines, have been widely used for cytocompatibility testing and osteogenic potential evaluation of biomaterials in regenerative medicine [13]. However, the heterogeneity of these cells, too often simply defined as osteoblasts or osteoblastic precursors, should be carefully considered.

Albeit easy to obtain and handle, tumor-derived cell lines may present peculiar nonphysiological features [14]. For instance, osteosarcoma cell lines (SaOs-2, MG-63, and U-2 OS) differ significantly from primary osteoblasts as for immunocytochemical markers and matrix produced [15]. The most used human cell line SaOs-2 cells display a mature osteoblast phenotype and form a calcified matrix resembling woven bone [16]. SaOs-2 cells share with primary human osteoblasts a similar expression profile of cytokines, growth factors, and receptors for parathyroid hormone [17]. MG-63 cell line represents an immature osteoblast phenotype. Despite the inconsistencies about their mineralization capabilities [14], MG-63 cells have been used in long-term studies concerning cell behavior on biomaterials [18]. Notwithstanding the abovementioned pitfalls, SaOs-2 and MG-63 cells are the most studied osteoblasts.

On the other hand, primary stem cells are characterized by higher variability and are usually available in smaller amounts [19]. Although, mesenchymal stem cells deriving from bone marrow are somehow archetypic [20, 21], more recently, adipose-derived stem cells (ASCs) [22] have emerged as a viable alternative source of mesenchymal cells. As it has been exhaustively reviewed [23], ASCs are relatively abundant and easy to access and may therefore become the elective source of mesenchymal stem cells for bone regenerative protocols. Surprisingly, however, little is known about the most significant biomolecules osteo-committed cells produce and release. Therefore, the present study aimed at dosing 13 candidates chosen among the most representative cytokines, chemokines, and growth factors within the conditioned media of osteodifferentiated and undifferentiated ASCs. As a complimentary analysis, two acknowledged “osteoblastic” cell models were compared, based on their different maturation stage.

2. Materials and Methods

2.1. Cell Culture. ASCs were isolated from fat tissue obtained from three different donors as described previously [22] and maintained in Dulbeco’s minimum essential medium enriched with sodium pyruvate and supplemented with 10% foetal bovine serum (FBS, Gibco Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. The nonadherent cell population was removed after 48 h, and the adherent cell layer was washed twice with fresh medium; cells were then continuously cultured since their harvest until sixth passage. SaOs-2 (ATCC number: HTB-85) and MG-63 (ATCC number: CRL-1427) cells were, respectively, cultured in McCoy’s 5A (Gibco, Life Technologies) with 15% FBS (Benchmark, Gemini Bio-Products) and in Dulbecco’s modified eagle’s medium (DMEM, Gibco, Life Technologies) with 10% FBS. Both media were supplemented with 1% penicillin-streptomycin (MD Biomedicals, Thermo Fisher Scientific). Cells were always passaged at subconfluence to prevent contact inhibition and were kept under a humidified atmosphere of 5% CO2 in air, 37°C.

2.2. Detection of Interleukins, Chemokines, and Growth Factors Using Bio-Plex System. To analyze the profile of the biomolecules, cells were seeded in 96-well plates (10^3 cells/well) in their own maintaining medium for 1 day. Afterwards, cells were incubated in RPMI in the presence of 2% FBS and 2% FBS + osteogenic factors (50 μM ascorbic acid, 10 mM beta glycerophosphate, and 100 nM dexamethasone) either for 7 (T1) and 14 (T2) in the case of SaOs-2 or for 21 (T1) and 28 (T2) days in the case of MG-63 and ASCs. At the day of harvest, media were removed, cells washed twice in PBS, and fresh starving medium (RPMI 0.5% bovine serum albumin) was incubated for 2 hours. Conditioned media thus obtained were characterized, without adding any activation substances, by measuring the concentration of the following specific biomolecules: interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-12 (IL-12), granulocyte-colony stimulating factor (G-CSF), interferon-gamma (INF-γ), tumor necrosis factor-α (TNF-α), monocyte chemotactant protein-1 (MCP-1) (CCL-2), CXCL10 chemokine (IP-10), platelet-derived growth factor (PDGF), basic-fibroblastic growth factor (bFGF), and vascular endothelial growth factor (VEGF). The flexible Bio-Plex system (Bio-Rad Laboratories, Hercules, CA, USA) was employed as previously described [24]. All samples were analyzed following the manufacturer’s protocol. At least two independent replications in duplicate were made per sample. Concentrations of the analytes were expressed in pg/μl. A standard curve ranging on average from 0.15 pg/μl to 3700 pg/μl (High Photomultiplier Tube Setting—PMT setting) was prepared and then fitted by Bio-Plex Manager software.

2.3. In Vitro Osteogenic Differentiation Tests. In vitro osteogenic differentiation was performed at the same conditions described above to run a series of assays aiming at revealing established bone markers, as described elsewhere [25, 26].

2.3.1. Alkaline Phosphatase (ALP) Activity Assay. Alkaline phosphatase (ALP) activity was determined using a colorimetric end point assay [27, 28], which measures the conversion of the colorless substrate p-nitrophenol phosphate (PNPP) by the enzyme ALP to the yellow product p-nitrophenol. To measure ALP activity, cells were lysed with 0.05% Triton X-100 and incubated with the reagent solution containing phosphatase substrate (Sigma-Aldrich, Milan, Italy) at 37°C for 15 min. The rate of color change corresponds to the amount of enzyme present in solution. Optical density was measured at a wavelength of 405 nm (reference
620 nm). Samples were compared against the calibration curve of p-nitrophenol standards. The final alkaline phosphatase concentration was adjusted per total protein content, to avoid biases due to the cell number. Therefore, part of the cell lysates obtained for ALP quantification was incubated with BCA™ (Thermo Fisher Scientific, Waltham, MA, USA) protein assay, following to the manufacturer’s instructions. Optical density was measured at a wavelength of 570 nm, and results were adjusted to a calibration curve made by known number of cells. ALP values were determined and normalized on whole protein content at day 3 in SaOs-2 and at day 7 in MG-63 and ASCs.

2.3.2. Calcium Content Assay. Cell calcium content was determined at day 14 for SaOs-2 and at day 21 for MG-63 and ASCs by Calcium colorimetric assay kit (BioVision Research Products, Mountain View, CA, USA), according to the manufacturer’s protocol. The OD was measured at 575 nm within 20 minutes since preparation. A calibration curve was always made.

2.3.3. Collagen and Calcium Staining. At the established time points, cells grown in six-plate wells were washed once with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. The solution was removed and cells were washed with PBS. To stain collagen, Sirius Red dye (Direct Red 80, Sigma-Aldrich) dissolved (1 mg/ml) in a saturated aqueous solution of picric acid (Sigma-Aldrich), was added to the fixed cell cultures. After kept under mild shaking for 2 hours, samples were quickly rinsed in acid water (0.5% acetic acid in pure water) and then abundantly washed with distilled water. Calcium salts were stained after von Kossa following published protocols [15]. For both picro-Sirius Red and von Kossa stains, the cultures were observed under light microscopy and representative pictures captured by an Olympus camera.

2.4. Statistical Analysis. Data were analysed by GraphPad Prism6 (GraphPad Software, Inc., La Jolla, CA, USA). Each experiment was repeated at least three times. Statistical analysis was performed by using the nonparametric test Wilcoxon–Mann–Whitney test. A p value of <0.05 was considered significant.

3. Results

3.1. Detection of Interleukins, Chemokines, and Growth Factors. The concentrations of interleukin-2 (IL-2),
Figure 2: Cytokine quantification 2. Data from Bio-Plex analysis are reported as different histograms for each cytokine. In particular, the quantification of each molecule in ASCs, MG-63, and SaOs-2 is shown at T1 and T2 and in CM and OM conditions. For ASCs and MG-63, T1 = 21 days and T2 = 28 days; for SaOs-2, T1 = 7 days and T2 = 14 days. CM = control medium (DMEM 2% FBS); OM = osteogenic medium (see Materials and Methods).
interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-12 (IL-12), granulocyte-colony stimulating factor (G-CSF), interferon-gamma (INF-γ), tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1) (CCL-2), CXCL10 chemokine (IP-10), platelet-derived growth factor (PDGF), basic-fibroblastic growth factor (bFGF), and vascular endothelial growth factor (VEGF) are reported in Figure 1 for ASCs, MG-63, and SaOs-2 cells.

Interestingly, there is a big difference in the expression pattern of interleukins, chemokines, and growth factors among different cells. ASCs produce a considerable level of IL-6, IL-8, MCP-1, and VEGF without particular variations between osteodifferentiated and control condition (with the exception of IL-8). MG-63 shows high levels of expression of IL-12, IP-10, MCP-1, and VEGF. Importantly, in osteodifferentiating conditions, the expression of IL-12, IP-10, and VEGF decreases. SaOs-2 cells show very low expression levels of the considered molecules, except for the VEGF. Notably, the osteodifferentiating medium inhibits the expression of IL-12 and VEGF in SaOs-2, similarly to MG-63 cells. To further highlight the differential expression of the considered molecules among ASCs, MG-63, and SaOs-2 cells, a panel showing the expression values for each biomolecule is reported in Figure 2.

3.2. In Vitro Osteogenic Differentiation Tests. The osteogenic potential of the cells has been assessed at the early stage by quantifying ALP activity (Figure 3) and staining the collagen matrix through Sirius Red (Figure 4). Interestingly, the osteodifferentiating condition significantly increased the level of ALP activity for each cell type. At later stages, the extracellular calcium content was determined colorimetrically (Figure 5) and with the Von Kossa method (Figure 6). In osteodifferentiating condition, a significant increase of extracellular calcium content was found for each cell type. Collectively, the differentiating condition appeared more performing than the undifferentiated control, proving the effectiveness of the osteogenic medium.

4. Discussion

In the present study, the differential expression of signaling molecules among three different cell types under both osteodifferentiating and control conditions is shown for the first time. To achieve this, a highly sensitive method was used. In particular, the cellular models considered in this work are the ASC, the MG-63, and the SaOs-2 cells. Notably, the ASCs represent a particular type of mesenchymal stem cells of great potential applications in the context of bone regeneration. On the other hand, despite their ineligibility for clinical use owing to their tumor derivation [29–31], MG-63 and SaOs-2 cells were chosen to this study as they are a widely diffused and accepted in vitro model, in the field of bone biology [16, 17, 32–37]. This paper underlines also the differences in the expression variations of signaling molecules during differentiation among cells.
Figure 4: Collagen staining. Sirius Red dye staining was performed in order to show collagen deposition at day 3 in SaOs-2 and at day 7 in MG-63 and ASCs. In OM condition, the staining is more intense for each cell type. CM = control medium (DMEM 2% FBS); OM = osteogenic medium (see Materials and Methods). Images were taken at 100x magnification.
the therapeutic effect of ASCs in ischemic models as a result of the release of angiogenic factors such as HGF and VEGF. Human ASCs were proven to secrete both factors constitutively [45]. Kilroy et al. [46] reported that ASCs produce angiogenic (HGF and VEGF), proinflammatory (IL-6, IL-8, IL-11, LIF, and TNF alpha), and hematopoietic-supportive cytokines (G-CSF, M-CSF, GM-CSF, and IL-7) following exposure to common inductive factors including LPS. Ribeiro and colleagues characterized the secretome of ASCs in cell therapy of osteoarthritis [47]. Nevertheless, to the authors’ surprise, the scientific literature has lacked up to now a comprehensive description of a significant range of biomolecules secreted by ASCs subjected to osteogenic differentiation, the great interest being more focused on the intracellular dynamics.

Therefore, this study focused on the detection of a representative panel of signaling molecules that ASCs, SaOs-2 cells, and MG-63 cells produce when cultured in either maintaining or osteogenic medium. Each cell type behaved differently. It is noteworthy that IL-6, IL-8, MCP-1, and VEGF were highly produced and detectable in ASCs even in the absence of any stimulus. In addition, while IL-6 and IL-8 seemed to be significantly induced by the osteogenic medium, no such effect was seen for MCP-1 and VEGF. The multiplex immunological system here adopted called Luminex® is capable to simultaneously detect and quantify up to several hundreds of analytes across multiple samples, reducing time, cost, and sample requirements in comparison to ELISA assays [49]. The capture antibodies of Luminex recognize specific analytes and are attached to microbeads with defined spectral address. The technique sensitivity thus reaches concentrations even lower than 1 pg/ml, which explains, for instance, why we report on the presence of IL-12 in ASCs contradicting Kilroy and colleagues’ outcomes based on ELISA kits [46].

Overall SaOS-2 cells had a poor expression profile (only IL-12 and VEGF resulted greater than 10 pg/ml), which may be consistent with the more differentiated phenotype of SaOs-2 cells compared to ASCs and MG-63, as thoroughly reviewed elsewhere [14]. Instead, when kept in maintaining medium, MG-63 cells displayed a very rich production of IL-12, MCP-1, IP-10, and VEGF. This remarkable secretory activity was inhibited by the osteogenic conditions, except for MCP-1, a chemokine pivotal for macrophage activation and thus bone remodeling. Notably, MCP-1, which is known to be constitutively expressed in osteoblasts [50], was herein enhanced in osteodifferentiated MG-63 cells.

The high level of IP-10 quantified in MG-63 cells may be correlated to the tumor origin of the cell line [50, 51]. IP-10 was possibly produced in response to IFN-g, which was detected only in MG-63 (as shown in Figures 1 and 2). Compared to ASCs and SaOs-2, MG-63 produced also more FGF-b, although the overall level is generally low. Considering these results, it could be interesting to investigate the related TGF-b expression [52].

As noted above, contrary to MG-63 and SaOs-2 cells, VEGF did not trend downward when ASCs were osteoinduced, even though the inhibitory effect of dexamethasone, present in the osteogenic medium, is well known for endothelial and tumoral cells [53, 54]. Along with the constitutive high expression of MCP-1, the steady release of VEGF may underpin the usage of ASCs for bone regenerative protocols, where these biomolecules could contribute to recruit bone cells within the host [55–58]. Very interestingly, Hu and Olsen [55] studied bone repair in mice with a monocortical defect within the tibial cortex. Osteoblast-derived VEGF was proven to stimulate crosstalk between osteoblastic, endothelial, and hematopoietic cells in a paracrine manner, while directly affecting osteoblasts via autocrine mechanisms. The role of MCP-1 was instead investigated as for the PTH-induction during osteoclastogenesis by Li et al. [58], providing a rationale for increased osteoclast activity to initiate greater bone remodeling.

On these premises, it will be of great interest to study ASCs in a more physiologic context so as to provide more reliable and predictive results. A possible approach might consist in elucidating the behavior of ASCs in coculture systems, with endothelial cells that are known to be key players in bone formation and regeneration [5].

5. Conclusion

Currently, the amount of proposals for the use of ASCs in tissue repair and regeneration is impressive. The number of clinical trials evaluating the efficacy and safety of ASCs in the reconstruction and regeneration of tissues increases...
significantly every year. According to the clinical trials database (ClinicalTrials.gov database 2015), 122 studies are currently using ASCs [59, 60].

In particular, positive results have been obtained using autologous ASCs in clinical trials for craniofacial bone reconstruction by producing new, mature, vital, and vascularized

**Figure 6:** Calcium staining. Von Kossa staining was performed in order to show calcium deposition at day 14 for SaOs-2 and at day 21 for MG-63 and ASCs. In OM condition, the staining is more intense for each cell type. CM = control medium (DMEM 2% FBS); OM = osteogenic medium (see Materials and Methods). Images were taken at 100x magnification.
bone [40–42, 61–63]. To date, bone regeneration is the most promising field for clinical translation of experimental ASC protocols [62]. This study supports, once more, the viability of ASCs in bone tissue engineering based on the cytokines, chemokines, and growth factors detected.

Disclosure
No involvement is to be reported at any stage of the study or while preparing the paper.

Conflicts of Interest
The authors declare that there are no competing interests regarding the publication of this paper.

Authors’ Contributions
F. Mussano and T. Genova equally contributed to this work.

Acknowledgments
This research was partly funded by the Piedmont Region with the POLI D’INNOVAZIONE-III Annualità (research project acronym: BIOBONE).

References
[1] T. A. Einhorn, “The science of fracture healing,” *Journal of Orthopaedic Trauma*, vol. 19, no. 10, pp. S4–S6, 2005, August 2016, http://www.ncbi.nlm.nih.gov/pubmed/16479221.
[2] American Academy of Orthopaedic Surgeons, *Musculoskeletal Injuries Report: Incidence, Risk Factors and Prevention*, AAOS, Rosemont, IL, USA, 2000.
[3] M. D. McKee, “Management of segmental bone defects: the role of osteoconductive orthobiologics,” *The Journal of the American Academy of Orthopaedic Surgeons*, vol. 14, no. 10, pp. S163–S167, 2006, August 2016, http://www.ncbi.nlm.nih.gov/pubmed/17003191.
[4] P. Giannoudis, H. Dinopoulos, and E. Tsiridis, “Bone substitutes: an update,” *Injury*, vol. 36, Supplement 3, pp. S20–S27, 2005.
[5] T. Genova, L. Munaron, S. Carossa, and F. Mussano, “Overcoming physical constraints in bone engineering: “the importance of being vascularized”,” *Journal of Biomaterials Applications*, vol. 30, no. 7, pp. 940–951, 2016.
[6] M. E. Aichelmann-Reidy and R. A. Yukna, “Bone replacement grafts. The bone substitutes,” *Dental Clinics of North America*, vol. 42, no. 3, pp. 491–503, 1998, December 2016, http://www.ncbi.nlm.nih.gov/pubmed/9700451.
[7] G. Fernandes and S. Yang, “Application of platelet-rich plasma with stem cells in bone and periodontal tissue engineering,” *Bone Research*, vol. 4, article 16036, 2016.
[8] M. A. Brunsvoild and J. T. Mellong, “Bone grafts and periodontal regeneration,” *Periodontology 2000*, vol. 1, no. 1, pp. 80–91, 1993, December 2016, http://www.ncbi.nlm.nih.gov/pubmed/8401863.
[9] C. Delloye, “Bone grafts using tissue engineering,” *Bulletin et Mémoires de l’Académie Royale de Médecine de Belgique*, vol. 156, no. 7–9, pp. 418–425, 2001, December 2016, http://www.ncbi.nlm.nih.gov/pubmed/11995186.
[10] W. Li, L. Xiao, and J. Hu, “The use of enamel matrix derivative alone versus in combination with bone grafts to treat patients with periodontal intrabony defects,” *Journal of the American Dental Association* (1939), vol. 143, no. 9, pp. e46–e56, 2012.
[11] R. Langer and J. P. Vacanti, “Tissue engineering,” *Science*, vol. 260, no. 5110, pp. 920–926, 1993.
[12] C. Szpalski, F. Sagebin, M. Barbaro, and S. M. Warren, “The influence of environmental factors on bone tissue engineering,” *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, vol. 101, no. 4, pp. 663–675, 2013.
[13] V. Kartosgiannis and K. W. Ng, “Cell lines and primary cultures in the study of bone cell biology,” *Molecular and Cellular Endocrinology*, vol. 228, no. 1, pp. 79–102, 2004.
[14] E. M. Czekanska, M. J. Stoddart, R. G. Richards, and J. S. Hayes, “In search of an osteoblast cell model for in vitro research,” *European Cells and Materials*, vol. 24, no. 4, pp. 1–17, 2012, November 2015, http://www.ncbi.nlm.nih.gov/pubmed/22777949.
[15] C. Pautke, M. Schieker, T. Tischer et al., “Characterization of osteosarcoma cell lines MG-63, Saos-2 and U-2 OS in comparison to human osteoblasts,” *Anticancer Research*, vol. 24, no. 6, pp. 3743–3748, 2004, February 2015, http://www.ncbi.nlm.nih.gov/pubmed/15736406.
[16] S. B. Rodan, Y. Imai, M. A. Thiede et al., “Characterization of a human osteosarcoma cell line (Saoa-2) with osteoblastic properties,” *Cancer Research*, vol. 47, no. 18, pp. 4961–4966, 1987, January 2015, http://www.ncbi.nlm.nih.gov/pubmed/3040234.
[17] G. Bilbe, E. Roberts, M. A. Birch, and D. B. B. Evans, “PCR phenotyping of cytokines, growth factors and their receptors and bone matrix proteins in human osteoblast-like cell lines,” *Bone*, vol. 19, no. 5, pp. 437–445, 1996.
[18] J. Lincks, B. D. Boyan, C. R. Blanchard et al., “Response of MG63 osteoblast-like cells to titanium and titanium alloy is dependent on surface roughness and composition,” *Biomaterials*, vol. 19, no. 23, pp. 2219–2232, 1998, January 2015, http://www.ncbi.nlm.nih.gov/pubmed/9884063.
[19] C. Li, G. Wei, Q. Gu et al., “Donor age and cell passage affect osteogenic ability of rat bone marrow mesenchymal stem cells,” *Cell Biochemistry and Biophysics*, vol. 72, no. 2, pp. 543–549, 2015.
[20] M. F. Pittenger, A. M. Mackay, S. C. Beck et al., “Multilineage potential of adult human mesenchymal stem cells,” *Science*, vol. 284, no. 5411, pp. 143–147, 1999, July 2014, http://www.ncbi.nlm.nih.gov/pubmed/10102814.
[21] N. Jaiswal, S. E. Haynesworth, A. I. Caplan, and S. P. Bruder, “Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro,” *Journal of Cellular Biochemistry*, vol. 64, no. 2, pp. 295–312, 1997.
[22] P. A. Zuk, M. Zhu, H. Mizuno et al., “Multilineage cells from human adipose tissue: implications for cell-based therapies,” *Tissue Engineering*, vol. 7, no. 2, pp. 211–228, 2001.
[23] U. D. Wankhade, M. Shen, R. Kolhe et al., “Advances in adipose-derived stem cells isolation, characterization, and application in regenerative tissue engineering,” *Stem Cells International*, vol. 2016, Article ID 3208607, p. 9, 2016.
[24] F. Mussano, T. Genova, L. Munaron, S. Petrillo, F. Erovigni, and S. Carossa, “Cytokine, chemokine, and growth factor profile of platelet-rich plasma,” *Platelets*, vol. 27, no. 5, pp. 467–471, 2016.
[25] F. Mussano, K. J. Lee, P. Zuk et al., “Differential effect of ionizing radiation exposure on multipotent and differentiation-restricted...
bone marrow mesenchymal stem cells,” Journal of Cellular Biochemistry, vol. 111, no. 2, pp. 322–332, 2010.

[26] F. Mussano, A. Bartorelli Cusani, A. Brossa, S. Carossa, G. Bussolati, and B. Bussolati, “Presence of osteoinductive factors in bovine colostrum,” Bioscience, Biotechnology, and Biochemistry, vol. 78, no. 4, pp. 662–671, 2014.

[27] H. L. Holtorf, N. Datta, J. A. Jansen, and A. G. Mikos, “Scaffold mesh size affects the osteoblastic differentiation of seeded marrow stromal cells cultured in a flow perfusion bioreactor,” Journal of Biomedical Materials Research. Part a, vol. 74, no. 2, pp. 171–180, 2005.

[28] F. Mussano, T. Genova, P. Rivolo et al., “Role of surface finishing on the in vitro biological properties of a silicon nitride–titanium nitride (Si3N4–TiN) composite,” Journal of Materials Science, vol. 52, no. 1, pp. 1477–1477, 2017.

[29] H. Masuda, C. Miller, H. P. Koeffler, H. Battifora, and M. J. Cline, “Rearrangement of the p53 gene in human osteogenic sarcomas,” Proceedings of the National Academy of Sciences, vol. 84, no. 21, pp. 7716–7719, 1987.

[30] E. Murray, D. Provvedini, D. Curran, B. Catherwood, H. Sussman, and S. Manolagas, “Characterization of a human osteoblastic osteosarcoma cell line (SAOS-2) with high bone alkaline phosphatase activity,” Journal of Bone and Mineral Research, vol. 2, no. 3, pp. 231–238, 1987.

[31] H. Heremans, A. Billiau, J. J. Cassiman, J. C. Mulier, and P. De Somer, “In vitro cultivation of human tumor tissues II. Morphological and virological characterization of three cell lines,” Oncologia, vol. 35, no. 6, pp. 246–252, 1978.

[32] R. J. Fernandes, M. A. Harkey, M. Weis, J. W. Askew, and D. R. Eyre, “The post-translational phenotype of collagen synthesized by SAOS-2 osteosarcoma cells,” Bone, vol. 40, no. 5, pp. 1343–1351, 2007.

[33] L. G. Rao, M. K. Sutherland, G. S. Reddy, M. L. Siu-Caldera, M. R. Uskokovic, and T. M. Murray, “Effects of 1alpha, 25-dihydroxy-16ene, 23yne-vitamin D3 on osteoblastic function in human osteoblastic MG-63 osteosarcoma cells, differentiation-stage dependence and modulation by 17-beta estradiol,” Bone, vol. 19, no. 5, pp. 621–627, 1996, January 2017, http://www.ncbi.nlm.nih.gov/pubmed/8968029.

[34] J. Clover, R. A. Dodds, and M. Gowen, “Integrin subunit expression by human osteoblasts and osteoclasts in situ and in culture,” Journal of Cell Science, vol. 103, Part 1, 1992.

[35] R. Olivares-Navarrete, P. Raz, G. Zhao et al., “Integrin alpha2beta1 plays a critical role in osteoblast response to micron-scale surface structure and surface energy of titanium substrates,” Proceedings of the National Academy of Sciences of the United States of America, vol. 105, no. 41, pp. 15767–15772, 2008.

[36] A. Kumarasuriyar, S. Murali, V. Nurcombe, and S. M. Cool, “Glycosaminoglycan composition changes with MG-63 osteosarcoma osteogenesis in vitro and induces human mesenchymal stem cell aggregation,” Journal of Cellular Physiology, vol. 218, no. 3, pp. 501–511, 2009.

[37] L. Saldana, F. Bensiamar, A. Boré, and N. Vilaboa, “In search of representative models of human bone-forming cells for cytocompatibility studies,” Acta Biomaterialia, vol. 7, no. 12, pp. 4210–4221, 2011.

[38] P. A. Zuk, M. Zhu, P. Ashjian et al., “Human adipose tissue is a source of multipotent stem cells,” Molecular Biology of the Cell, vol. 13, no. 12, pp. 4279–4295, 2002.

[39] “Stem Cells From Fat Focus Of International Fat Applied Technology Society,” in Second Annual Meeting of the International Fat Applied Technology Society, Pittsburgh, PA, 2004.

[40] S. Lendeckel, A. Jödicke, P. Christophis et al., “Autoologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report,” Journal of Cranio-Maxillo-Facial Surgery, vol. 32, no. 6, pp. 370–373, 2004.

[41] T. Thesleff, K. Lehtimäki, T. Niskakangas et al., “Cranioplasty with adipose-derived stem cells and biomaterial: a novel method for cranial reconstruction,” Neurosurgery, vol. 68, no. 6, pp. 1535–1540, 2011.

[42] K. Mesimäki, B. Lindroos, J. Törnwall et al., “Novel maxillary reconstruction with ectopic bone formation by GMP adipose stem cells,” International Journal of Oral and Maxillofacial Surgery, vol. 38, no. 3, pp. 201–209, 2009.

[43] A. Miranville, C. Heeschen, C. Sengenès, C. A. Curat, R. Busse, and A. Bouloumié, “Improvement of postnatal neovascularization by human adipose tissue-derived stem cells,” Circulation, vol. 110, no. 3, pp. 349–355, 2004.

[44] V. Planat-Benard, J.-S. Silvestre, B. Cousin et al., “Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives,” Circulation, vol. 109, no. 5, pp. 656–663, 2004.

[45] J. Rehman, D. Traktuev, J. Li et al., “Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells,” Circulation, vol. 109, no. 10, pp. 1292–1298, 2004.

[46] G. E. Kilroy, S. J. Foster, X. Wu et al., “Cytokine profile of human adipose-derived stem cells: expression of angiogenic, hematopoietic, and pro-inflammatory factors,” Journal of Cellular Physiology, vol. 212, no. 3, pp. 702–709, 2007.

[47] C. A. Ribeiro, J. S. Fraga, M. Gräos et al., “The secretome of stem cells isolated from the adipose tissue and Wharton jelly acts differently on central nervous system derived cell populations,” Stem Cell Research & Therapy, vol. 3, no. 3, p. 18, 2012.

[48] P. Succar, E. J. Breen, D. Kuah, and B. R. Herbert, “Alterations in the secretome of clinically relevant preparations of adipose-derived mesenchymal stem cells cocultured with hyaluroran,” Stem Cells International, vol. 2015, Article ID 421253, p. 16, 2015.

[49] H. Kucpova Skalnikova, “Proteomic techniques for characterisation of mesenchymal stem cell secretome,” Biochimie, vol. 95, no. 12, pp. 2196–2211, 2013.

[50] D. T. Graves, Y. Jiang, and A. J. Valente, “The expression of monocyte chemotractant protein-1 and other chemokines by osteoblasts,” Frontiers in Bioscience, vol. 4, pp. D571–D580, 1999, August 2016, http://www.ncbi.nlm.nih.gov/pubmed/10393126.

[51] P. Proost, C. De Wolf-Peeters, R. Conings, G. Opdenakker, A. Billiau, and J. Van Damme, “Identification of a novel granulocyte chemotactic protein (GCP-2) from human tumor cells. In vitro and in vivo comparison with natural forms of granulocyte chemotactic protein (GCP-2) from human tumor cells,” Journal of Cellular Physiology, vol. 160, no. 3, pp. 1090–1096, 1994.

[52] T. Sobue, T. Gravely, A. Hand et al., “Regulation of fibroblast growth factor 2 and fibroblast growth factor receptors by transforming growth factor β in human osteoblastic MG-63 cells,” Journal of Bone and Mineral Research, vol. 17, no. 3, pp. 502–512, 2002.

[53] S.-H. Shim, J. H. Hah, S.-Y. Hwang, D. S. Heo, and M.-W. Sung, “Dexamethasone treatment inhibits VEGF production via suppression of STAT3 in a head and neck
cancer cell line,” Oncology Reports, vol. 23, no. 4, pp. 1139–1143, 2010, August 2016, http://www.ncbi.nlm.nih.gov/pubmed/20204302.

[54] J. J. Logie, S. Ali, K. M. Marshall, M. M. S. Heck, B. R. Walker, and P. W. F. Hadoke, “Glucocorticoid-mediated inhibition of angiogenic changes in human endothelial cells is not caused by reductions in cell proliferation or migration,” PloS One, vol. 5, no. 12, article e14476, 2010.

[55] K. Hu and B. R. Olsen, “Osteoblast-derived VEGF regulates osteoblast differentiation and bone formation during bone repair,” The Journal of Clinical Investigation, vol. 126, no. 2, pp. 509–526, 2016.

[56] Y.-Q. Yang, Y.-Y. Tan, R. Wong, A. Wendlen, L.-K. Zhang, and A. B. M. Rabie, “The role of vascular endothelial growth factor in ossification,” International Journal of Oral Science, vol. 4, no. 2, pp. 64–68, 2012.

[57] M. Ishikawa, H. Ito, T. Kitaori et al., “MCP/CCR2 signaling is essential for recruitment of mesenchymal progenitor cells during the early phase of fracture healing,” PloS One, vol. 9, no. 8, article e104954, 2014.

[58] X. Li, L. Qin, M. Bergenstock, L. M. Bevelock, D. V. Novack, and N. C. Partridge, “Parathyroid hormone stimulates osteoblastic expression of MCP-1 to recruit and increase the fusion of pre/osteoclasts,” The Journal of Biological Chemistry, vol. 282, no. 45, pp. 33098–33106, 2007.

[59] A. Bajek, N. Gurtowska, J. Olkowska, L. Kazmierski, M. Maj, and T. Drewa, “Adipose-derived stem cells as a tool in cell-based therapies,” Archivum Immunologiae et Therapiae Experimentalis (Warsz), vol. 64, no. 6, pp. 443–454, 2016.

[60] B. Péault, G. Asatrian, D. Pham, W. R. Hardy, and A. W. James, “Stem cell technology for bone regeneration: current status and potential applications,” Stem Cells and Cloning: Advances and Applications, vol. 8, p. 39, 2015.

[61] G. K. Sándor, V. J. Tuovinen, J. Wolff et al., “Adipose stem cell tissue–engineered construct used to treat large anterior mandibular defect: a case report and review of the clinical application of good manufacturing practice–level adipose stem cells for bone regeneration,” Journal of Oral and Maxillofacial Surgery, vol. 71, no. 5, pp. 938–950, 2013.

[62] M. Barba, C. Cicione, C. Bernardini, F. Michetti, and W. Lattanzi, “Adipose-derived mesenchymal cells for bone regeneration: state of the art,” BioMed Research International, vol. 2013, Article ID 416391, p. 11, 2013.

[63] B. E. Grottkau and Y. Lin, "Osteogenesis of adipose-derived stem cells," Bone Research, vol. 1, no. 2, pp. 133–145, 2013.