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Role of IGF1 and IGF1/VEGF on Human Mesenchymal Stromal Cells in Bone Healing: Two Sources and Two Fates

Concetta Ferretti, PhD,[1] Giovanni Vozzi, PhD,[2] Mirella Falconi, MD,[3] Monia Orciani, PhD,[1] Marco Gesi, MD,[4] Roberto Di Primio, MD,[1] and Monica Mattioli-Belmonte, PhD[1]

In the repair of skeletal defects one of the major obstacles still remains an efficient vascularization of engineered scaffolds. We have examined the ability of insulin growth factor-1, alone or in association with vascular endothelial growth factor, to modulate the osteoblastic or endothelial commitment of periosteum-derived progenitor cells (PDPCs) and skin-derived multipotent stromal cells (S-MSCs). A selected gene panel for endothelial and osteoblastic differentiation as well as genes that can affect MAPK and PI3K/AKT signaling pathways were investigated. Moreover, gene expression profile of Sox2, Oct4, and Nanog transcription factors was assessed. Our results showed that under growth factor stimulation PDPCs are induced toward an osteoblastic differentiation, while S-MSCs seem to move along an endothelial phenotype. This different commitment seems to be linked to a diverse MAPK or PI3K/AKT signaling pathway activation. The analysis of genes for stemness evidenced that at least in PDPCs multipotency and differentiation could coexist. These results open interesting perspective for the development of innovative bone tissue engineering approaches based on a good network of angiogenesis and osteogenesis processes.

Introduction

Bone is a dynamic tissue with a great healing ability. Indeed, critical-size defects caused by trauma, tumor resections, pathological degeneration, or congenital deformations could delay this process or generate an impaired healing. At present, because of the endogenous osseoconductive properties of bone that tend to promote integration with host tissue, grafting techniques are widely used in orthopedic practice. In this respect autografts have the best clinical outcome and represent the gold standard. Allografts and synthetic materials could also be used.[1,2]

Repair and integration of a bone graft are a well-orchestrated process in which, at first, the inflammatory response facilitates vascular invasion and mesenchymal stromal cell (MSC) recruitment at the injury site, in response to growth factors and/or cytokines.[3] A cell-based therapy promoting graft osseointegration is able to enhance this process.[4] The concept of using cells to restore damaged tissue seems intuitive, since it is based on their native role in tissue development and homeostasis. However, to date, the identification of the optimal cell sources for a successful cell-based skeletal treatment still remains the main challenge.[1]

The majority of bone tissue engineering purposes take advantage from bone-marrow-derived cells (BM-MSCs) that have been broadly studied. However, invasiveness of their harvesting and the decline in bone marrow activity with age have encouraged investigation of alternative sources of stem cells, including those derived from adipose tissue,[5] muscle,[6] dental pulp,[7] periodontal ligament,[8] bone,[9] pericytes,[10] skin,[11] and periosteum.[12]

Bone healing relies on skeletal progenitors from both local and systemic sources (i.e., bone marrow) and several studies highlight that during physiological bone repair the systemic recruitment of skeletal progenitors seems to be minimal, while the local environment contribution is predominant.[13,14] In this regard periosteum has a key role in this endogenous repair process.[15] Periosteum-derived progenitor cells (PDPCs) resident in its inner cambium layer[16] possess multipotency at single-cell level, proliferate faster than most MSCs, and retain the ability to differentiate *in vitro* into osteochondral cell types.[12] *In vivo* studies demonstrate that periosteum is essential for bone graft healing and remodeling[17] and that the beginning of graft repair requires activation, differentiation, and expansion of residing PDPCs.[3] Therefore, this tissue represents a smart cell source for skeletal tissue engineering applications.

[1]Department of Clinical and Molecular Sciences, Università Politecnica delle Marche, Ancona, Italy.
[2]Faculty of Engineering, Research Centre "E. Piaggio," University of Pisa, Pisa, Italy.
[3]Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy.
[4]Department of Translational Research and New Technology in Medicine and Surgery, University of Pisa, Pisa, Italy.

Another attractive stem cell reservoir that can meet bone tissue engineering criteria, being an accessible cell source with no-invasive procedures, is the skin basal layer.[18] The cells isolated from this site, called skin-derived multipotent stromal cells (S-MSCs), have multipotent differentiation capability, and are able to become adipocytes, osteoblasts, chondrocytes, neurons, and pancreatic cells. Their differentiation capability in combination with their immunosuppressive effect makes them an ideal challenger for assorted cellular transplantation therapies.[19]

Beside cells, to develop a flexible cellular construct suitable for bone graft applications, other two important components must be considered: growth factors able to induce cell differentiation and scaffolds to create a biocompatible environment.[3,20] Among cytokines involved in skeletal tissue regeneration, insulin growth factor 1 (IGF1) and vascular endothelial growth factor (VEGF) are particularly intriguing on account of their dual role in bone and endothelial development[21] and their potential importance in developing successful tissue engineering strategies.[22]

At last, *in vivo* construct abilities to replace bone are related to neovascularization processes. To obtain vascularized tissue-engineered bone grafts different experimental strategies (e.g., scaffold functionalization and cell-based techniques) have been proposed. Functionalization may be performed by increasing scaffold porosity or through different angiogenic-factor-loading techniques, such as VEGF and PDGF.[23,24] Cell-based approaches, instead, may be based on coculture of endothelial and osteoprogenitor cells[23–25] or on the use of cells transfected for the overexpression of angiogenic factors.[26] A further approach could be the use of a stem cell population able to differentiate toward an osteoblastic or endothelial phenotype. There is already evidence that stem cells isolated from bone marrow—MSCs and multipotent adult progenitor cells—may differentiate into endothelial cells.[27–29] Interestingly recent investigations showed that also S-MSCs are capable to acquire an endothelial phenotype under appropriate stimuli.[19]

On the basis of these considerations and of our previous experience on PDPCs and VEGF crosstalk,[12] in the present study we examined the ability of IGF1, alone or in association with VEGF, to modulate PDPC and S-MSC endothelial or osteoblastic differentiation. Our final purpose is the evaluation of the molecular mechanisms involved in cell modulation in order to realize an engineered structure in which proliferative and differentiation abilities of PDPCs and S-MSCs are optimized.

To this aim we selected a specific gene panel for endothelial and osteoblastic differentiation as well as genes that are engaged in MAPK and PI3K/AKT signaling pathways, which are activated upon IGF1- and VEGF-specific receptor binding. Endothelial phenotype was evaluated taking into account CD31 and CD34 mRNA expression. The designated genes for osteoblastic differentiation included runt-related transcription factor 2 (runx2), bone morphogenetic protein 2 (bmp2), β-catenin, matrix metalloproteinase 13 (mmp13), and osteocalcin (bglap). For MAPK and PI3K/AKT signaling pathways we analyzed mRNA expression for mitogen-activated protein kinase 3 (mapk3), phosphoinositide-3-kinase 5 (pi3k5), and Forkhead box O1 (FoxO1). At last we analyzed the gene expression profile of Sox2 (octamer-binding 4), Oct4, and Nanog transcription factors that are essential for the maintenance of the stem cell phenotype.

Materials and Methods

PDPC isolation

All patients provided their informed consent to participate in the study. Since the study not exposed the subjects to any risk, in lieu of a written consent form, a verbal authorization was obtained from all the recruited patients. To all subjects it was underlined that the tissue used for the study represents the typical discard during the surgical procedures and the voluntariness of their participation to the study (freedom from coercion or undue influence, real or imagined). Patients had sufficient opportunity to ask questions and consider their choice. PDPCs were obtained, as previously described, from periosteal tissue of five subjects undergoing surgery for orthopedic trauma (two men and three women; mean age 34 years; range 28–50).[12] Briefly, tissue was aseptically dissected, washed in Dulbecco's phosphate-buffered saline (D-PBS) lacking in Ca^{2+} and Mg^{2+}, cut into small pieces ($2–3 \times 2–3\ mm^2$), and placed in a 100-mm culture dish in Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (DMEM/F-12; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (100 U/mL), all from GIBCO® (Life Technologies Corporation). The use of DMEM-F12 supplies optimal and appropriate culture conditions for MSC isolation and *ex-vivo* expansion, allowing the maintenance of correct morphology, population doubling time, and immunophenotype for more than 25 passages.[30] The minced explants of periosteum were positioned with their cambium side placed against the dishes; cells were then allowed to adhere in standard cell culture conditions in a humidified atmosphere of 95% air and 5% carbon dioxide. Medium was changed twice a week.

Once cells migrating from the explants reached 50% of confluence, they were harvested by treatment with 0.25% trypsin/1 mM EDTA (Sigma-Aldrich) and replated at 1:3 dilution under the same culture condition. Cells were used at the 3rd passage for phenotypic characterization and differentiation.

S-MSC isolation

S-MSCs were obtained from skin biopsies of the mammary gland of three healthy female adult patients (mean age 41 years; range 31–50) undergoing cosmetic plastic surgery, as previously described.[31] Briefly, after patient's informed consent obtainment skin samples (10–15 mm in thickness) were collected postoperatively. Fat tissue was carefully removed, and the remaining dermal portion was finely minced into small pieces and transferred into six-well plates for culturing with the Mesenchymal Stem Cell Growth Medium Bullet kit (Lonza Group Ltd.). The cultures were maintained in a 5% CO_2 humidified incubator at 37°C. After 14 days of culturing, numerous mesenchymal colony-forming cells migrated from the explants and formed colonies. Non-adherent cells and dermal tissue were removed, and medium was changed twice a week. Cells were used at the 3rd passage for phenotypic characterization and differentiation.

Cell characterization

According to The International Society for Cellular Therapy for identification of human MSCs,[33] PDPCs and S-MSCs were analyzed by an FACSCalibur flow cytometry system (Becton Dickinson) and subjected to differentiation into mesenchymal lineages.

For immunophenotyping 2.5×10^5 cells were removed with D-PBS and then stained for 45 min with the following antibodies: fluorescein isothiocyanate (FITC)–labeled mouse anti-human CD90 (StemCell Technologies), CD105, CD14, and CD19 (Diaclone); R-phycoerythrin (PE)–labeled mouse anti-human CD34, CD45 (Diaclone), and CD73 (Becton Dickinson); and anti-HLA-DR purchased from Diaclone. Control for FITC- or PE-coupled antibodies was an isotypic mouse IgG1. For unconjugated antibodies secondary FITC-labeled goat anti-mouse IgG was used as control. Data were evaluated using CellQuest software (Becton Dickinson).

Cell differentiation into osteocytes, adipocytes, and chondrocytes was performed using STEMPRO® Osteogenesis, Chondrogenesis, and Adipogenesis kits (Life Technologies Corporation), respectively. Cells cultured in DMEM/F-12 with 10% FBS were used as negative controls.

For osteogenesis cells were seeded at a density 4.5×10^4 cells with appropriate medium for 10 days, changing the medium twice a week. Osteogenic differentiation was assessed by Von Kossa and alkaline phosphatase (ALP) stainings. For Von Kossa stain, cells were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature (RT) and incubated with 1% silver nitrate solution under UV light for 20 min at RT. Unreacted silver was removed with 5% sodium thiosulfate for 5 min. For ALP staining, cells were fixed in 4% PFA for 15 min at RT and washed in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 10 mM $MgCl_2$ buffer for 10 min at RT. Cells were then stained with fast 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium alkaline phosphate substrate (Sigma-Aldrich) for 10 min and rinsed in dH_2O. Reaction was observed with a light microscope (Nikon Eclipse 600; Nikon, Milan, Italy).

For adipogenic differentiation 9×10^4 PDPCs and S-MSCs were seeded and treated with the appropriate medium for 15 days, changing the media twice a week. Differentiation was assessed by Oil Red staining and CD36 immunoreaction. Briefly, cells fixed in 4% PFA were exposed to Oil Red O solution (0.5% in 100% isopropyl alcohol) for 20 min at RT, cleared with isopropanol 60%, and finally washed in dH_2O. For the detection of CD36 positivity, PDCPs were incubated with monoclonal anti-CD36 (ImmunoTools GmbH) diluted 1:100. The reaction was visualized using the streptavidin-biotin-peroxidase technique (DAKO LSAB$^+$/HRP peroxidase kit; Dako SpA). Cells were incubated with 3,3-diaminobenzidine [DAB; 10 mg diaminobenzidine in 15 mL of 0.05 M Tris buffer (pH 7.6) and 12 μL of hydrogen peroxide 30%] and counterstained with Mayer's hematoxylin (Bio-Optica SpA). Reaction was examined with a light microscope (Nikon Eclipse 600).

For chondrogenesis, PDPCs and S-MSCs were cultured in pellet culture system. For the preparation of each pellet, aliquots of 1×10^6 cells in 1 mL of appropriate medium were spun down at 1200 rpm for 5 min. Pellets were cultured for 20 days, changing the medium twice a week. Pellets were then fixed in 4% PFA, paraffin embedded, and sectioned. Sections were exposed to a solution of Alcian Blue (pH 1; Bio-Optica) for 20 min at RT or Safranin-O (0.1 g in EtOH 100%, working dilution 1: 2 dH_2O) for 5 min at RT and observed with a light microscope (Nikon Eclipse 600).

Growth factor treatment

PDPCs and S-MSCs were expanded in DMEM/F12 (Sigma-Aldrich) with 10% FBS (GIBCO) and then plated at the density of 2×10^4 cells/cm^2. After 24 h this medium was replaced with fresh DMEM/F12 containing rhIGF1 (R&D Systems, Inc.) or rhIGF1 and rhVEGF-A (Sigma-Aldrich) diluted to obtain a final concentration of 50 ng/mL. Media containing the growth factors were changed twice a week. Cells were cultured for 15 days.

RNA extraction and reverse transcription

Total RNA was isolated according to the manufacturer's instructions from PDPCs and S-MSCs with the Perfect Pure RNA Cultured Cell Kit (5-Prime GmbH) that provides DNase treatment. Quantification and evaluation of RNA quality were performed for all samples by spectrophotometric analysis (bioPhotometer plus; Eppendorf GmbH). One microgram of total RNA was reverse transcribed according to the manufacturer's instructions in a 20-μL reaction volume using go Script RT System (Promega Corporation). The neosynthesized cDNA was stored at −20°C.

Real-time PCR

Real-time assays were performed by the Mastercycler realplex2 (Eppendorf GmbH) using SsoFast™ EvaGreen® Supermix 1×, in a final volume of 10 μL. All PCRs contained 1 μL of cDNA (corresponding to 50 ng of total RNA template).

Each PCR assay was performed in white plasticware and comprised 30 s at 95°C for enzyme activation and 40 cycles of denaturation at 95°C for 5 s, annealing and extension at 60°C for 20 s. Every primer was used at 200 nM final concentration. Primer sequences were designed by Primer 3 (v. 0.4.0) software and their specificity was tested by BLAST in order to avoid any appreciable homology to pseudogenes or other unexpected targets. In each assay, both reference gene mRNA and each gene of interest mRNA were measured simultaneously under identical conditions. Primers showed the same amplification efficiency. Specificity of the PCRs was further confirmed by melt-curve analysis.

Oligonucleotide sequences for target genes are listed in Table 1.

Quantification of mRNA expression

Each assay was performed as triplicate and provided one reference gene whose Cq values were used to normalize cellular mRNA data. In this instance normalization involved the ratio of mRNA concentrations of specific genes of interest (as mentioned earlier) to those of Cq value of the reference gene. To compare the gene expression of PDPCs and S-MSCs that were treated with IGF1 and IGF1/VEGF with untreated cell cultures (i.e., cells cultured for 15 days in DMEM/F12 with 10% FBS), ΔΔCq method for the evaluation of fold-change was employed. Data in histograms are expressed as fold-regulation that represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicate an upregulation, and the

Table 1. Analyzed Gene Description

Gene	Detected transcript	Primer forward (5′->3′)	Primer reverse (5′->3′)	Amplicon length (bp)	Annealing T (°C)
CD31	NM_000442.4	GGATCGTGAGGGTCAACTGT	CCTAGCTTGACATCGGAAG	191	60°
CD34	NM_001025109.1	TGAAAAAGCACCAATCTGACC	AATAGCCAGTGATGCCCAAG	150	60°
RUNX2	NM_004348.3	CTCGTCCGCACCGACAGCC	TACCTCTCCGAGGGCTACCACC	111	60°
BMP2	NM_001200.2	CCAGCCGAGCCAACACTGTGC	TCTCCGGGTTGTTTTCCCACTCG	86	60°
Beta-catenin	NM_001904.3	CCAATGGCTTGGAATGAGAC	GTTCCATCATGGGGTCCATA	151	60°
MMP-13	NM_002427.3	AGTTCGGCCACTCCTTAGGT	GTCTGGCGTTTTTGGATGTT	179	60°
BGLAP	NM_199173	GACTGTGACGAGTTGGCTGA	GCCCACAGATTCCTCTTCTG	119	64°
FoxO1A	NM_002015.3	AAGAGCGTGCCCTACTTCAA	AGGCCATTTGGAAAACTGTG	209	60°
PI3K5	NM_014308.3	GGAGCAACCTGGAGAAGGTC	TGGTTAAAGCCCTTCTTGGA	151	60°
MAPK3	NM_002746.2	GCTACACGCAGTTGCAGTACA	GAAGGGGCTGATCTTCTTGA	107	60°
NANOG	NM_024865.2	TGAACCTCAGCTACAAACAG	CTGGATGTTCTGGGTCTGGT	248	60°
SOX	NM_003106.3	ACACCAATCCCATCCACACT	GCAAACTTCCTGCAAAGCTC	198	60°
OCT4	NM_203289.4	AGCGAACCAGTATCGAGAAC	GCCTCAAAATCCTCTCGTTG	199	60°
GAPDH[a]	NM_002046.3	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC	200	60°

[a]Reference gene.

fold-regulation is equal to the fold-change. Fold-change values less than one indicate a downregulation, and the fold-regulation is the negative inverse of the fold-change.

Statistical analysis

Mean and standard deviation of three different experiments are reported. Data were analyzed by one-way analysis of variance, Student-Newman-Keuls's, and Student's *t*-tests. Statistical significance was tested at $p < 0.05$.

Results

Cell characterization

In agreement with the criteria of the International Society for Cell Therapy,[32] PDPCs and S-MSCs were positive for CD73, CD90, and CD105 and negative for CD45, HLA-DR, and CD14 in agreement with the criteria of the International Society for Cytotherapy. Moreover, PDPCs and S-MSCs were able to differentiate into all the mesenchymal lineages tested (Fig. 1).

Growth factor treatment

PDPCs and S-MSCs display a different behavior to IGF1 treatment, either administered alone or in combination with VEGF.

After 15 days of treatment, S-MSCs showed a significant ($p < 0.05$) upregulation of CD31 and CD34 mRNA levels, even though the latter at a lower extent (Fig. 2a). On the contrary, PDPCs presented a less evident CD31 and CD34 mRNA upregulation.

Growth factor administration determined in S-MSCs a significant ($p < 0.05$) downregulation of the expression of mRNAs for mmp13, runx2, and BGLAP, while bmp-2 was significantly upregulated (Fig. 2b). No differences were detected between IGF1 treatment and its association with VEGF in the modulations of endothelial markers. On the contrary, IGF1/VEGF association seems to address S-MSCs to the osteoblastic phenotype (Fig. 2a, b). As far as β-catenin mRNA is concerned a decrease in its expression, although not significant, was observed when cells were treated with IGF1 alone, while the association of IGF1/VEGF determined its slight increase (Fig. 2b). PDPCs showed a clear upregulation ($p < 0.05$) of almost all genes involved in osteogenic differentiation after growth factor treatments, except for β-catenin mRNA expression (Fig. 2b). Overall in these cells the combination of IGF and VEGF seems to be more effective in their phenotypic commitment.

Interestingly, we also observed a different regulation of mapk3 and pi3k5, which are relevant genes of the two main IGF1 and VEGF pathways (Fig. 3). Mapk3 gene expression was faintly downregulated in S-MSCs treated with IGF1 alone, but it was significantly upregulated in cell culture treated with the IGF1/VEGF association. On the contrary it looked significantly ($p < 0.05$) downregulated in PDPCs, mainly in those treated with IGF1 alone (Fig. 2c). Pi3k5 mRNA transcription was weakly affected in S-MSC-treated cells, and it was greatly stimulated in PDPC-treated ones, mainly in those undergoing IGF1 treatment alone (Fig. 2c).

FoxO1 mRNA expression pattern was similar both in PDPCs and S-MSCs with no significant differences between the two treatments (Fig. 2c).

Considering the expression of genes involved in cell stemness—Sox2, Oct4, and Nanog—it was interesting to observe an upregulation of mRNA expression for Sox2 in both cell cytotypes. Oct4 and Nanog mRNA expression appears to be significantly ($p < 0.05$) increased in PDPCs treated with IGF1 and IGF1/VEGF association (Fig. 2d). In the latter IGF1 alone seems to work better than the association IGF1/VEGF. In S-MSCs subjected to both cytokine treatments Oct4 and Nanog, mRNA expression was downregulated (Fig. 2d).

Discussion

Even though decades of progress have been made, the treatment of damaged or diseased skeletal tissues, large-scale-size defects, and postinjury degeneration continues to be an unsolved issue in the field. Progresses in the understanding of cell and tissue biology have extended the focus of orthopedic medical research further than traditional graft transplantation to include more novel strategies for the *in vitro* or *in vivo* generation of biomimetic tissues as much as possible similar to the native tissue both in structure and

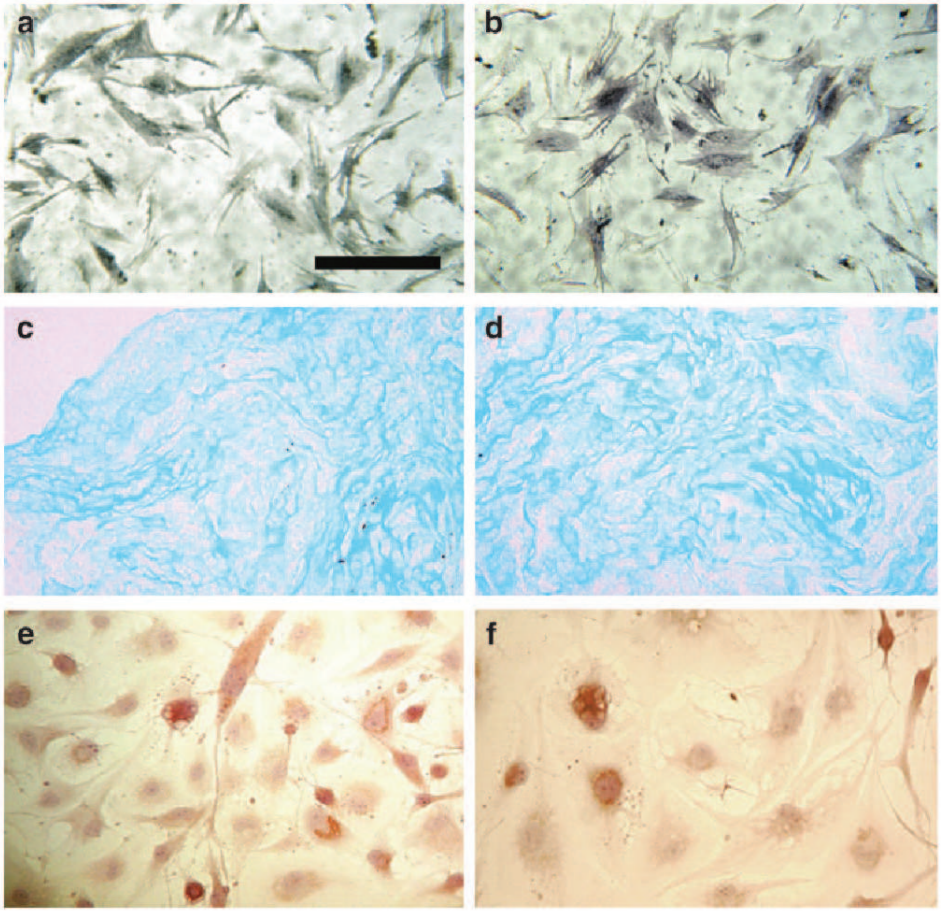

FIG. 1. Mesenchymal differentiation from PDPCs **(a, c, e)** and S-MSCs **(b, d, f)**. Osteogenic differentiation assessment by ALP staining **(a, b)**. Chondrogenic differentiation demonstrated by positive acid mucopolysaccharide coloration by Alcian blue **(c, d)**. Adipocyte differentiation confirmed by CD36 immunoreaction by streptavidin-biotin-peroxidase technique **(e, f)**. Scale bars = 50 μm. ALP, alkaline phosphatase; MSCs, mesenchymal stromal cells; PDPCs, periosteum-derived progenitor cells; S-MSCs, skin-derived multipotent stromal cells. Color images available online at www.liebertpub.com/tea

function. According to the interactive "diamond" concept of tissue engineering, regenerative medicine applications profit of correct cell selection, three-dimensional structure/architecture, mechanical/physical signals, and bioactive factors that collaborate to direct tissue repair and regeneration.[20]

As far as optimal cell sourcing is concerned, current options include autologous and allogeneic tissue-specific cells and adult multipotent stem cells. Tissue-specific cells are in principle the best cell types for cellular repair strategies and were in fact the first translated into clinical practice.[33] The limitations in tissue amount, morbidity at the harvest site, and their *in vitro* finite proliferative capacity have encouraged investigation of more readily available cell types. Indeed, stem and progenitor cells offer the advantages of harvest from other sites with potentially less tissue morbidity and a greater proliferation capacity.

Regarding bone tissue engineering purposes, periosteum can represent an available autologous source since PDPCs possess a good proliferation rate[34] and, moreover, their homing for *in situ* tissue regeneration could be an alternative to traditional tissue engineering approaches.[35] In addition, autologous S-MSCs can comply bone reconstruction criteria since these cells are easily obtained by skin biopsy as skin is the largest organ of the body.[18,19]

The success of bone replacement strategies is also related to the action of chemokines and growth factors that are able to optimize cell proliferative and differentiation abilities and/or to promote endogenous cell homing of adult MSCs.[35,36] Besides, the new frontiers of research should be directed toward a better mimicking of natural bone tissue regeneration, such as a coupling between osteogenesis and angiogenesis, crucial components in bone formation.[37] Several strategies for bone differentiation of stem cells have been developed, while there are still conflicting results for their endothelial commitment.

VEGF is a master regulator of vascular growth both in normal and pathological conditions, and it is the most attractive and well-characterized factor for the therapeutic induction of new blood vessel growth.[38] Recently, some studies provide new insights into IGF-1 signaling in angiogenesis, showing its ability to promote the development of mature functional endothelial cells also in combination with other growth factors (e.g., VEGF).[39] VEGF is secreted by different MSC populations and, via paracrine mechanisms, can influence endothelial cell behavior.[11,40]

To accomplish tissue engineering strategies based on connected osteogenesis and angiogenesis, in the present study we examined the effects of IGF1 supplementation, alone or in association with VEGF, on PDPC and S-MSC differentiation commitment. To this aim we analyzed changes in mRNA levels of specific target genes involved in endothelial (i.e., CD31 and CD34) or osteoblastic (i.e., runx2, bmp2, β-catenin, mmp13, and bglap) differentiation.

We observed that IGF1 plays an important role, either alone or in association with VEGF, in S-MSC endothelial commitment, as disclosed by a significant upregulation of

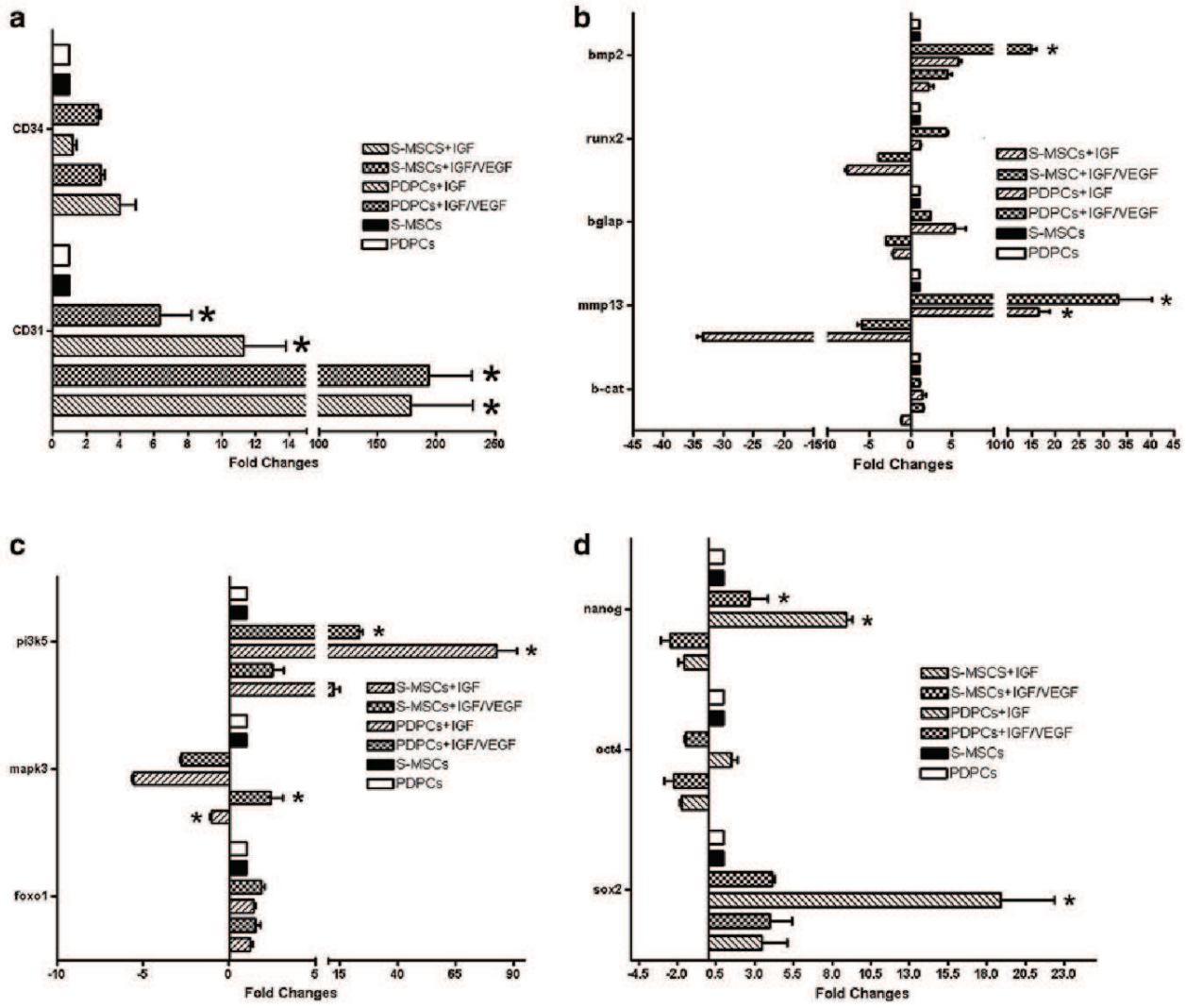

FIG. 2. Histograms depict changes in mRNA expression in S-MSCs and PDCPs after treatment with IGF1 and IGF/VEGF. **(a)** Endothelial markers: CD31 and CD34; **(b)** osteoblastic markers: bmp2, runx2, osteocalcin, mmp13, and β-catenin; **(c)** genes involved in MAPK and PI3K/AKT pathways: mapk3, pi3k5, and foxO1; **(d)** stemness indicators: sox2, oct4, and nanog. Data are expressed as fold-regulation that represents fold-change results in a biologically expressive manner. Fold-regulation is equal to the fold-change [2^(−ΔΔCt)] for fold-change values greater than one and indicate an upregulation. Fold-change values less than one indicate a downregulation; in this case the fold-regulation is the negative inverse of the fold-change. $*p < 0.05$, S-MSCs versus PDPCs. bmp2, bone morphogenetic protein 2; FoxO1, Forkhead box O1; IGF1, insulin growth factor 1; mapk3, mitogen-activated protein kinase 3; mmp13, matrix metalloproteinase 13; pi3k5, phosphoinositide-3-kinase 5; runx2, runt-related transcription factor 2; VEGF, vascular endothelial growth factor.

CD31 and CD34 mRNAs. Surprisingly this upregulation was more evident for CD31, which is the major constituent of endothelial intercellular junction and it is usually considered a late endothelial marker. PDPCs presented a less evident CD31 and CD34 mRNA upregulation, but also in this case CD31 upregulation was more evident than CD34.

In S-MSCs, the treatment with IGF1 alone determined a decrease in β-catenin mRNA expression, while the IGF1/VEGF association induced its weak increase. In PDPCs β-catenin mRNA levels were upregulated under both tested treatments. Either in S-MSCs or PDPCs this result correlates at least in part with the changes in bmp2 mRNA levels, which were upregulated in all treated cells, although at different extent. It is well known that IGF1 is one of the key factors during osteogenic differentiation, showing beneficial effect on bone development.[41] IGF1 signaling in bone enhances Wnt protein production and activates β-catenin. *In vivo*, a hyperactivated form of β-catenin synergizes with bmp2 to promote osteoblast differentiation and new bone formation.[42] Wnt signaling also activates bmp2 pathway and, in turn, bmp2 induces the expression of Wnt ligands, resulting in an increase of β-catenin transcriptional activity.[43] These observations support the idea that Wnt pathway and bmp-2 cooperate to drive osteoblastic differentiation.

The activation of bmp2 pathway usually triggers mRNA runx2 transcription. We observed a significant downregulation of runx2 in S-MSCs, while it was upregulated in PDPCs, especially after IGF1/VEGF treatment. Runx2 serves as an early

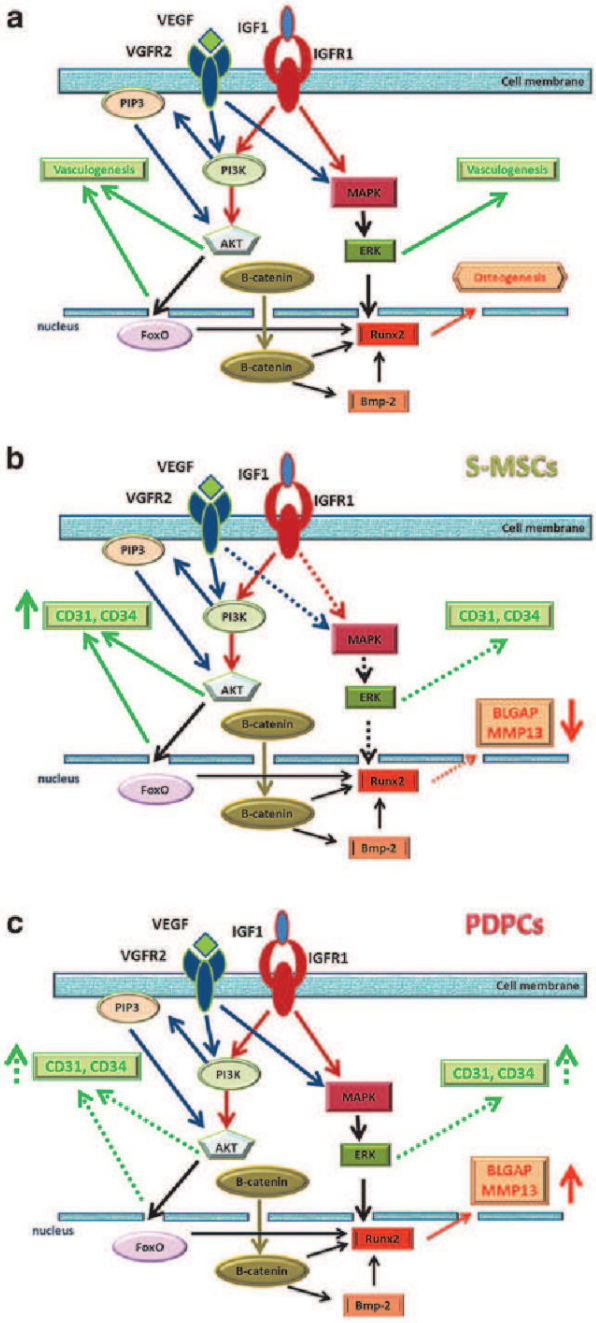

FIG. 3. Hypothesis of the different signaling mechanisms on PDPCs and S-MSCs. **(a)** Potential crosstalk between IGF1 and VEGF receptor signaling pathways. They both converge on ERK kinase, which can modulate the activity of the master osteogenic transcription factor Runx2. They also activate PI3/AKT signaling pathway that can induce vasculogenesis or modulate β-catenin phosphorylation. Unphosphorylated β-catenin can accumulate in the nucleus, where it can act as pro-osteogenic factor regulating Runx2 and other genes. **(b)** Effects of IGF1 or IGF1/VEGF treatment on S-MSC gene expression suggesting their commitment toward the endothelial phenotype. **(c)** Effects of IGF1 or IGF1/VEGF treatment on PDPCs showing their main commitment toward an osteoblastic phenotype. Continuous lines indicate a marked effect, while dotted lines pointed out a weaker regulation. Color images available online at www.liebertpub.com/tea

transcriptional regulator of osteogenic differentiation and its expression is essential for normal bone formation.[44] Runx2 directs the differentiation of mesenchymal stem cells into an osteoblastic lineage[41,45] and, in turn, it controls the expression of mmp13 and bgalp that are osteoblast-specific genes.[46]

A strong downregulation of mmp13 was detected in S-MSCs undergoing both treatments. Mmp13 has been considered essential in bone biology since it is exclusively expressed in the skeleton during development. It plays an important role in the degradation of the extracellular matrix components (i.e., collagens), it is expressed in hypertrophic chondrocytes and osteoblasts during embryogenesis, and it is involved in endochondral ossification and bone remodeling.[47] Similarly, in S-MSCs we observed a downregulation of bglap gene expression, which is generally considered another important marker in bone formation. On the contrary all genes involved in osteogenesis were upregulated in PDPCs, particularly in cells treated with the IGF1/VEGF association. We therefore hypothesize that the detected upregulation of bmp-2 in S-MSCs is associated to endothelial commitment. This hypothesis is strengthened by the recent observation that this gene, mainly devoted to osteogenic differentiation, could be potentially proangiogenic being involved in the regulation of vessel development.[48]

In the present study we also examined how IGF1 or IGF1/VEGF could affect MAPK and PI3K/AKT pathways, which are both critical for appropriate cell proliferation and differentiation.

Mapk3 gene was activated only in S-MSCs under IGF/VEGF treatment, while it was downregulated in these cells treated with IGF1 alone and in PDPCs. On the contrary, pi3k5 mRNA levels were significantly upregulated in S-MSCs treated with IGF1 and in PDPCs. IGF1 also induces changes in foxO1 expression, a transcriptional factor that acts as a downstream mediator of IGF1 signaling in the PI3K/AKT pathway. To date few data on the role of foxO1 in osteoblastogenesis and in the regulation of skeletal homeostasis are available.[44] In the current study we detected for the first time foxO1 expression in PDPCs and S-MSCs and the induction of its upregulation after growth factor supplementation. The stronger increase in PDPCs than in S-MSCs, especially after IGF1/VEGF treatment, further stresses the role of PDPCs in bone cell development. Moreover, the expression analysis of mapk3, pi3k5, and foxO1 suggested that in the two tested cell types stimulation with IGF1 or IGF1/VEGF involves a different activation of the signaling pathway mediating osteoblast or endothelial commitment (Fig. 3a); in S-MSCs the growth factor treatment mainly activates PI3K/AKT pathway (Fig. 3b), favoring in turn endothelial differentiation, while in PDPCs it activates MAPK only (Fig. 3c).

When using MSCs for regenerative medicine purposes it is important to obtain a sufficient number of cells as well as maintain their multipotency. Typically MSCs will gradually lose their potency during proliferation in culture and, otherwise, a diminished proliferation rate is related to differentiation stimuli.[49] For this reason we analyzed the gene expression profile of Sox2, Oct4, and Nanog transcription factors that are essential to preserve stem cell phenotype. We observed a modulation of these genes in all culture conditions, showing that at least in PDPCs pluripotency and differentiation could coexist.[17] Sox2 mRNA levels were upregulated in both S-MSCs and PDPCs. S-MSCs showed a

downregulation of Oct4 and Nanog, while these genes were upregulated in PDPCs. It must be stressed that sox2/Oct4 interaction is essential for nanog activation[50] and the latter is able to promote osteogenic differentiation by means of BMP signaling components, such as runx2, bmp2, and β-catenin.[49,51] This trend is confirmed by our results in PDPC culture treated with IGF1 or IGF1/VEGF association. On the contrary the downregulation of Oct4 in S-MSCs could be responsible for a downregulation of nanog. In these cells the detected Sox2 upregulation concomitant with the overexpression of bmp2, further reinforces our supposition on the S-MSC commitment toward endothelium that Sox2 may interact with bmp signaling[52] that could be possibly proangiogenic.[48]

In conclusion our results evidenced a possible different commitment of the tested MSCs within a bone microenvironment; PDPCs are induced toward an osteoblastic differentiation, while S-MSCs seem to be brought in the direction of an endothelial phenotype. The evaluation of the expression of genes involved in MAPK and PI3K/AKT pathways, both activate under IGF1 or IGF1/VEGF stimuli, suggested that differences in cell commitment could be related to a main activation of PI3K/AKT pathway in S-MSCs respect to the activation of MAPK pathway in PDPCs. We also detect for the first time foxO1 expression in PDPCs and S-MSCs and its upregulation by growth factor supplementation. Finally, we observed that genes involved in the stemness of PDPCs and S-MSCs could be differently modulated by the growth factor treatment. This aspect needs surely to be better investigated to better understand the crosstalk between proliferation and differentiation in stem cells. All these observations open intriguing perspective for the development of innovative strategies for bone tissue engineering approaches based on the possibility to concomitantly favor osteogenesis and angiogenesis; S-MSCs seeded onto appropriate scaffolds, differentiating in endothelial cells, would favor nutrient support within the engineered structure, while PDPCs, which could also be recruited from the surroundings, would furnish bone-forming cells.

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Disclosure Statement

Each author certifies that he or she has no commercial associations (e.g., consultancies, stock ownership, equity interest, patent/licensing arrangements, etc.) that might pose a conflict of interest in connection with the submitted article.

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Address correspondence to:
Monica Mattioli-Belmonte, PhD
Department of Clinical and Molecular Sciences
Università Politecnica delle Marche
Via Tonto 10/a
60126 Ancona
Italy

E-mail: m.mattioli@univpm.it

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