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Human Periosteal Derived Stem Cell Potential: The Impact of age

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Abstract There is a great deal of interest in the understanding of possible age-related changes in Mesenchymal Stem Cells in view of their use for regenerative medicine applications. Given to the outmost standing of periosteum in bone biology and to probe data for a cell-based therapy promoting graft osseointegration in the elderly, we tried to identify specific aging markers or pattern of expression in human periosteal precursor cells. Immunohistochemical detection of Ki67 and p53, Nitric Oxide production and qRT- PCR of a selected gene panel for osteoblastic differentiation, bone remodeling and stemness were evaluated. We confirmed that both Ki67 and p53 are noteworthy indicators of senescence in human periosteal precursor cells and their expression significantly correlate with cell NO production. Moreover, cell age affects genes involved in bone remodeling, with a significant increase in interleukin-6 mRNA expression and receptor activator of nuclear factor kappa-B ligand/osteoprotegerin ratio. The analysis of mRNAs of genes involved in pluripotency regulation and self-renewal of stem cells, evidenced changes at least in part related to bone remodeling. We believe that this is the first study taking on age-related changes in human periosteal precursor cells, and paving the way toward new regenerative medicine strategies in bone aging and/or bone metabolic diseases.

Keywords Periosteal cells · Age-related · Nitric Oxide · Ki67 · p53 · qRT-PCR · Mathematical modelling

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Introduction

Human aging is a complex phenomenon defined by a progressive function deterioration of several organs including the skeletal system. Bone is a dynamic tissue that undergoes continuous regeneration. This tissue consists of highly specialized cells, mineralized and unmineralized connective tissue matrix, and spaces that include the bone marrow cavity, vascular canals, canaliculi, and lacunae. During development and growth, the skeleton is sculpted to achieve its mature shape and size by the removal of bone from one site and deposition at a different one (*modeling*). Once the skeleton has reached maturity, regeneration continues in the form of a periodic replacement of old bone with new one at the same location (*remodeling*) [1]. This process is responsible for the complete regeneration of the adult skeleton every 10 years.

The bone loss due to aging is the consequence of several factors that influence the bone remodeling process and alter the balance between bone resorption by osteoclasts and bone formation by osteoblasts. These processes are finely regulated to maintain both a constant bone mass, replacing old with new bone, and bone strength and elasticity [2, 3]. Indeed, decreased bone formation observed in the elderly can result from impaired osteoblast recruitment, defective proliferation/differentiation of progenitor cells, and decreased activity of mature osteoblasts [4]. It remains unclear which of these processes has a causal role in mediating the decreased bone formation with age.

Mesenchymal Stem Cells (MSCs) have a direct role in the maintenance of bone balance, since they act as a source of progenitors for osteoblasts, responsible for the anabolic half of the homeostatic balance, and as regulators for osteoclastogenesis. Moreover, MSCs constitutively secrete a distinct set of cytokines, suggesting that these cells serve specific supportive functions in the bone marrow microenvironment [5]. A large effort of aging research has focused on genes and signaling pathways that control the rate of aging and several factors

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have been reported as causing age-related changes in MSCs, even though with conflicting results [6].

Cell senescence occurs when normal cells stop dividing. This phenomenon was initially described more than 40 years ago during studies on cultured human fibroblasts [7]. Antigen Ki67 is a nuclear protein that is necessary for cellular proliferation and it is an excellent marker to determine the growth fraction of a given cell population [8]. Several authors found that its missing or low expression is associated with senescence. For instance, Lawless et al. demonstrated that Ki67 negativity is a good criterion to quantify cell senescent state in mixed human fibroblast populations [9]; Yan et al., in a study for bone regeneration, reported that the expression of Ki67 was significantly lower in adipose-derived mesenchymal stem cells (Ad-MSCs) isolated from aged donors than Ad-MSCs isolated from younger ones [10].

Senescence in MSCs has also been associated with increases in p53 expression and reduced defenses against reactive oxygen species (ROS) [11]. P53 is a cell-cycle regulator and senescence marker that has a role in senescence-inducing apoptotic responses to stress and cell dysfunction [12–14]. As concerns the oxidative damage, the increased nitric oxide (NO) and oxidizing species levels during aging induced MSC apoptosis and senescence and inhibited osteoblastic differentiation [14].

Other intrinsic changes in MSC features during aging are relative to gene expression modifications. Aging is known to cause a decrease in the number of bone-forming osteoblasts and an increase in marrow adipocytes [15]. Bone-forming osteoblasts and marrow adipocytes both originate from common MSCs and there is a reciprocal relationship between the two differentiation pathways [16]. Jiang and co-workers suggested that, with aging, the lineage of MSCs is modified, with remarkable changes in decreased osteoblastogenesis [17]. In addition, they observed a higher Receptor Activator of Nuclear factor kappa-B Ligand (RANKL) mRNA level in MSCs with age, which might contribute to the gradual loss of bone. RANKL is in fact essential for the differentiation and maintenance of osteoclasts and it is highly expressed on osteoblast/stromal cells.

At last, with regard to cytokines, an age-related increase in MSC constitutive secretion of the osteolytic cytokines interleukin-6 (IL-6) and interleukin-11 (IL-11) and Insulin-like Growth Factor (IGF)-binding protein-3 and a decrease in the expression of osteoprotegerin (OPG), the osteoclastogenesis-inhibitory factor, were observed [13]. These cytokine pattern modifications contributed to the increase in osteoclastogenesis detected during aging.

On the basis of these considerations and to deepen knowledge for cell-based therapies promoting graft osseointegration in the elderly, the aim of the present work was the identification of specific aging markers or pattern of expression in a population of MSCs derived from periosteum, i.e. Periosteum Derived Progenitor Cells (PDPCs). These cells are resident in its inner cambium layer [18] and may be considered an attractive MSC reservoir for bone tissue engineering perspective [19, 20]. Indeed, periosteum plays a key role in bone endogenous repair process [21], and in vivo studies demonstrate that the residing PDPCs are essential for bone graft healing and remodeling [22]: the beginning of graft repair effectively requires their activation, differentiation and expansion [23]. Understanding if there could be differences in PDPC potential during aging, paves the way to innovative regenerative medicine strategies during bone aging and/or bone metabolic diseases.

To these purposes immunohistochemical detection of Ki67 and p53, NO production and qRT- PCR of a selected gene panel for osteoblastic differentiation and bone remodeling as well as of genes engaged in the maintenance of the stem cell phenotype were assessed. The designated genes for osteoblastic differentiation included runt-related transcription factor 2 (runx2), bone morphogenetic protein 2 (bmp2), whilst for bone remodeling they were IL-6, RANK-L and OPG. Stemness was assessed analyzing the gene expression profile of Sex-determining region Y (SRY)-Box2- Sox2, octamer-binding-4 (Oct4) and Nanog transcription factors.

At last, a systems approach was employed by combining the biological results obtained with mathematical modeling and in silico simulations. The ultimate aim of this phase was to unveil the possible underlying quantitative dynamics of PDPC biochemical behavior. Indeed, the methodologies and techniques of systems biology are strongly demanded for analyzing the molecular mechanisms of complex biological networks, both in physiological and pathological conditions [24]. Firstly, numerical values and trends were examined for immunohistochemical markers, NO production and gene expression levels in differently aged subjects, looking for possible quantitative correlations. Then, the elucidated relationships were exploited to define a mathematical model mimicking the metabolic profile of a PDPC culture and focusing on age-related increase in oxidative stress level.

Material and Methods

Human Periosteal Derived Precursor Cells (PDPCs) Harvesting

Periosteal tissue was obtained from 8 differently aged healthy subjects, gender matching, undergoing surgery for orthopedic trauma: two subjects had a mean age of 16 years, two of 28, two of 63 and at last two of 92 years. All patients provided their informed consent to participate in the study. Since the study did not expose 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 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. As previously described [25, 26], periosteal tissue was aseptically dissected, washed three times in Dulbecco's Phosphate-Buffered Saline (D-PBS) lacking in Ca^{2+} and Mg^{2+}, cut into small pieces (2–3×2-3 mm) and placed in a 100 mm culture dish in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10 % Fetal Bovine Serum (FBS) and 1 % penicillin-streptomicin (100 U/mL). Both culture media and supplements were purchased from GIBCO® (Invitrogen, Milan, Italy). The medium was changed twice a week and prior to further investigations PDPCs mesenchymal stem cells phenotype, cells were characterized by FACSCalibur flow cytometry system (Becton Dickinson, CA, USA), using antibodies against the following surface antigens: HLA-DR, CD34, CD105, CD14, CD19 and CD45 (Diaclone, Besancon, France); CD73 and CD90 (StemCell Technologies, Inc. Vancouver, BC, Canada) [27].

For the immunohistochemical and qRT-PCR analyses cells at the 3rd passage of subculture were seeded at a density of $1 \times 10^4/cm^2$ in 6/well tissue culture plates or on glass slides (Lab-Tek II Chamber Slide, Nalgene Nunc International, Denmark) and cultured for 48 h at 37 °C in a humified 5 % (*v*/*v*) CO_2 incubator. At the same endpoint culture media were collected and, after centrifugation (1,000×g, 10 min), the supernatants filtered through a 0.22 μm membrane (Millipore, Bedford, MA, USA) and stored at – 80 °C until use.

Immunohistochemistry

For immunohistochemical staining PDPCs were fixed in 4 % paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 20 min at room temperature and permeabilized in 0.1 % Triton X100 in PBS for 10 min. The cells were then incubated overnight at 4 °C with mouse monoclonal antibody against Ki67 antigen (MIB-1, prediluted, Dakocytomation, Dako, Carpinteria, USA) and mouse anti-human p53 monoclonal antibody (clone DO-7, Dako Cytomation, Milan, Italy) (dil. 1:50). PDPCs were then immunostained using the streptavidin–biotin peroxidase technique (Envision peroxidase kit, Dako Cytomation, Milan, Italy). Ki-67 and MIB-1 monoclonal antibodies are directed against different epitopes of the same proliferation-related antigen [28]. After incubation with 0.05 % 3,3′diaminobenzidine (Sigma–Aldrich, Milan, Italy) in 0.05 M Tris buffer, pH 7.6 with 0.01 % hydrogen peroxide, sections were counterstained with Mayer's hematoxylin (BioOptica, Milan, Italy), dehydrated in ethanol and coverslipped with Eukitt mounting medium (Electron Microscopy Sciences, PA, USA). Positive controls for antibody against Ki67 and 53 were represented by samples of breast cancer. For negative controls primary antibody was replaced with non-immune serum. Each experiment was carried out three times in duplicate.

The immunohistochemical expression of Ki67 and p53 was evaluated under a Nikon Eclipse E600 light microscope (Nikon Instruments, Europe BV, Kingston, Surrey, England) by two independent observers. Agreement between the observers was always >95 %. Images were captured with a Nikon DSVi1 digital camera (Nikon Instruments, Europe BV, Kingston, Surrey, England) and NIS Elements BR 3.22 imaging software (Nikon Instruments, Europe BV, Kingston, Surrey, England) was used. Stained cells were counted in at least 10 fields / sample (field 0.7 mm^2, magnification: ×400) and quantified as a percentage of the total counted cells. The fields were randomly selected evaluating the most positive, moderate and less positive areas. Mean±standard deviation (SD) was considered for each value.

NO Production

NO released by PDPCs was measured using a commercially available kit (DetectX® Nitric Oxide Colorimetric Detection Kit, Arbor Assays, Ann Arbor, MI, USA). All measurements were carried out according to the manufacturers' instructions with a microplate reader (Multiskan GO, Thermo Fisher Scientific Inc., Waltham, MA, USA). Blank (background) was determined in each experiment utilizing the same volume of DMEM-F12 placed in the incubator under the same conditions and for the same time, but without cells. NO production was expressed as nanomoles per milliliters (nmol/mL).

RNA Extraction, Quantitation and Reverse Transcription

Total RNA was isolated from PDPCs cultured onto the different scaffolds with TRIzol® Reagent (Invitrogen, Milan, Italy), according to manufacturer's instructions. Quantification and evaluation of RNA quality were performed by spectrophotometric analysis (bioPhotometer plus, Eppendorf GmbH, Germany). One microgram of total RNA was reverse transcribed in a 20 μL reaction volume using the GoScript™ Reverse Transcription System (Promega, Milan, Italy). Neosynthesized cDNA was stored at −20 °C.

Realtime PCR (RT-qPCR) Assay

Real-time assays were performed using a Mastercycler Realplex2 thermocycler (Eppendorf GmbH, Germany) with SsoFast™ EvaGreen® Supermix 1×, in a final volume of 10 μL. All PCR reactions contained 1 μL of cDNA (corresponding to 50 ng of total RNA template). Each PCR assay was performed in white plastic-ware and comprised 30 s at 95 °C for enzyme activation, 40 cycles of denaturation at 95 °C for 5 s, annealing and extension at 60 °C for 20 s. Each primer was used

at a 200 nM final concentration. Primers' sequences were designed by Primer 3 (v. 0.4.0) software. Their specificity was tested by BLAST Assembled RefSeq Genomes in order to avoid any appreciable homology to pseudo-genes or other unexpected targets. The oligonucleotide sequences for target genes are listed in Table 1.

In each assay, both reference genes and each gene of interest mRNA were measured simultaneously under identical conditions. Primers showed the same amplification efficiency. Specificity of the PCR reactions was furthermore confirmed by melt curve analysis.

Quantification of mRNA Expression

Each assay was performed as triplicate and reference genes' Ct 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 above) to that of GAPDH and GUSB Cq medium values [29]. Data were expressed as gene relative expression ($2^{-\Delta Ct}$). Moreover, in order to highlight the effect of aging on PDPCs, $\Delta\Delta Ct$ method for the evaluation of Fold-Change was employed comparing values of the elderly (i.e. 92 or 63 years) with the younger ones (i.e. 16 and 28 years) as well as 92 vs 63. The relative amount of each mRNA was calculated using the comparative threshold (Ct) method with $\Delta Ct = Ct(mRNA) - Ct(GAPDH)$ and relative quantification of mRNA expression was calculated with the $2^{-\Delta\Delta Ct}$ method [30]. The qPCR efficiency in all our experiments was more than 90 %. The difference between the actual and theoretical (100 %) efficiencies would result in a underestimation of the mRNA concentration of all analyzed samples.

Data in histograms are expressed as fold-regulation that represents fold-change results in a biologically meaningful way. In particular, fold-change values greater than one indicate an up-regulation, and the fold-regulation is equal to the fold-change. Fold-change values less than one indicates a down-regulation, and the fold-regulation is the negative inverse of the fold-change.

Mathematical Analysis and Modeling

An analytical approach was used to investigate about possible correlations among immunohistochemical results, NO production levels, gene expression profiles based on fold-change values and age of PDPC donors. The most visually similar numerical trends as functions of age were selected for further analysis and, at last, the best candidate factors for an interesting quantitative relationship were focused on. The values of the NO production for differently aged PDPCs were divided by the corresponding values of percentage about Ki67: the results were plotted against the corresponding age of donors and Curve Fitting Toolbox™ (MATLAB, The MathWorks, Inc.) was employed to perform an exploratory fitting of these data. Once the fit was created, its analytical expression was implemented in silico as a sort of "senescence index" affecting NO production along with the change of the age of simulated PDPCs. The in silico model applied to simulate PDPCs behavior derived from other virtual single-cell models (for hepatic, adipose and endothelial cells), previously defined [31]. This kind of computational model relates on-line metabolic pathway databases to the systems theory language. It is based on a modular approach and on a set of nonlinear differential equations implemented in Simulink® applying Michaelis-Menten kinetic laws and energy balance considerations for different metabolic pathways. Cell proliferation model is also treated.

Statistical Analysis

Statistical analyses were performed using the SAS statistical package (Statistical Analysis System Institute). All experiments were carried out in triplicate. Results are expressed as mean±SD. ANOVA and Bonferroni test were used to analyze the differences between groups. Correlation studies were performed by linear regression analysis, using Pearson's correlation coefficient r. Statistical significance was set at $p<0.05$.

Results

Immunohistochemistry

Our immunohistochemical analysis was performed evaluating immunopositivity with respect to negative controls that showed no staining. The expression of Ki67 and p53 is summarized in Table 2.

Ki67 protein, which is expressed in all cycling cells, was studied as an index of cell proliferation and determined by labelling with the MIB-1 monoclonal antibody against the Ki67 antigen. Positive cells showed a dark nuclear staining. PDPCs isolated from patients aged 16, 28, 63 and 92 years exhibited a statistical different expression within all the considered age classes ($p<0.005$), in particular PDPCs from patients aged 92 years showed the lowest values, whilst PDPCS from subjects aged 16 years showed the highest ones (Fig. 1).

Regarding p53, immunohistochemical staining was detected in nucleus: its expression was statistically higher in PDPCs from patients aged 92 years ($p<0.05$, 41.36±4.07) compared to PDPCs from subjects aged 28 (22.49±1.86) and 16 years (23.34±1.82). We found no difference between the other groups (Fig. 1).

NO Production

Our results showed that NO release was significantly higher in PDPCs isolated from subjects aged 92 years compared to

Table 1 Analysed gene description

Genes	Detected Transcript	Primer Forward (5′->3′)	Primer Reverse (5′->3′)	Amplicon length (bp)
Bmp2	NM_001200.2	CCAGCCGAGCCAACACTGTGC	TCTCCGGGTTGTTTTCCCACTCG	86
Runx2	NM_004348.3	CTCGTCCGCACCGACAGCC	TACCTCTCCGAGGGCTACCACC	111
Il-6	NM_000600.2	CCAGAGCTGTGCAGATGAGT	CATTTGTGGTTGGGTCAGGG	150
Opg	NM_002546	TGATGGAAAGCTTACCGGGA	CAGGATCTGGTCACTGGGTT	270
Rankl	NM_003830	TAATGCCACCGACATCCCAT	ATGTTGGAGATCTTGGCCCA	200
Sox2	NM_003106.3	ACACCAATCCCATCCACACT	GCAAACTTCCTGCAAAGCTC	198
Oct4	NM_203289.4	AGCGAACCAGTATCGAGAAC	GCCTCAAAATCCTCTCGTTG	199
Nanog	NM_024865.2	TGAACCTCAGCTACAAACAG	CTGGATGTTCTGGGTCTGGT	248
Gusb[a]	NM_000181.2	AAACGATTGCAGGGTTTCAC	TCTCGTCGGTGACTGTTCA	81
Gapdh[a]	NM_002046.3	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC	200

Bmp2 bone morphogenetic protein 2, *Runx2* runt-related transcription factor 2, *Il-6* interleukin-6, *Opg* osteoprotegerin, *Runkl* receptor activator of nuclear factor κB ligand, *Sox2* sex-determining region Y (SRY)-Box2), *Oct4* octamer-binding transcription factor 4, *Gusb* beta glucuronidase, *Gapdh* glyceraldehyde-3-phosphate dehydrogenase

[a] Reference genes

those obtained from the other subjects (being 16 years = 2.82 ±0.82 nmol/mL, 28 years = 1.98±0.98 nmol/mL, 63 years = 10.67±3.03, 92 years = 27.94±7.94 nmol/mL; $p<0.05$). Moreover we found a significant negative correlation between NO release and Ki67 expression ($p<0.05$, $r=-0.87$; Fig. 2a), whilst it was positive with p53 immunostaining ($p<0.05$, $r=0.82$; Fig. 2b).

qRT-PCR Assay

Table 3 summarized data on mRNA relative expression in the differently aged subjects. No changes were detected for Bmp2 mRNA expression in 16 and 28 years old cells, while an increase in its expression was detected in the elderly with the highest value in the 63 years old PDPCs. Runx2 mRNA was significantly increased in 16 and 92 years old samples in comparison with both 28 and 63 years old ones. As for as genes involved in bone remodeling, a significant increase in IL-6 mRNA expression was detected only in 92 years old PDPCs, whilst OPG was increased at the same extent both in the minor and the elderly in comparison with 28 and 63 years old samples. A similar behavior was detected for RANKL mRNA. Finally, gene involved in stemness showed significantly lower expression of Oct4 mRNA in 16 years old PDPCs in comparison with the other samples, while Nanog and Sox2 mRNA expression was higher both in 28 and 92 years cells.

Age-related changes resulted more evident using the ΔΔCt method for the comparison of values detected in 92 and 63 years old subjects with those measured in 16 and 28 years old ones, respectively.

Bmp2 mRNA expression was 9-fold up-regulated in 63 years old specimens respect to the youngest, whilst no significant changes were detected for 92 years old samples (Fig. 3a, b and c). On the contrary changes in Runx2 mRNA regulation were not significantly different among the experimental groups (Fig. 3a, b and c).

Interesting results were detected for gene involved in bone remodeling (Fig. 3d, e and f). A significant increase in IL-6 mRNA was detected in 92 years old samples respect to 16 years old (10-fold) and 28 years old (199-fold) ones, while no significant changes in OPG and RANKL mRNAs were noticed. On the contrary a significant increase of these mRNAs were detected in the comparison with the 28 years old samples (28-fold for OPG and 300-fold for RANKL). A similar behavior was detected in 63 years old PDPCs compared to 28 years old ones even though at a lower extent (IL-6 20-fold, RANKL 11-fold), whilst a significant down regulation of OPG and RANKL was present when compared 63 years old cells with the youthful ones (Fig. 3d, e and f). The up-regulation of all genes involved in bone remodeling was much more evident comparing fold regulation of 92 years vs 63 years, where the increase in IL-6 mRNA expression goes with a significant increase of OPG and, mainly, of RANKL (Fig. 3f).

Table 2 Immunohistochemical analysis

	KI67	p53
16 years	67.19±4.98	23.34±1.82
28 years	46.34±3.21	22.49±1.86
63 years	36.81±7.33	32.00±14.96
92 years	16.74±3.24	41.36±4.07
ANOVA	$P=0001$	$P=0.005$
Bonferroni $p<0.05$	$P<0.005$ 92 vs 16,28,63 63 vs 16,28 28 vs 16	$P<0.05$ 92 vs 28,16

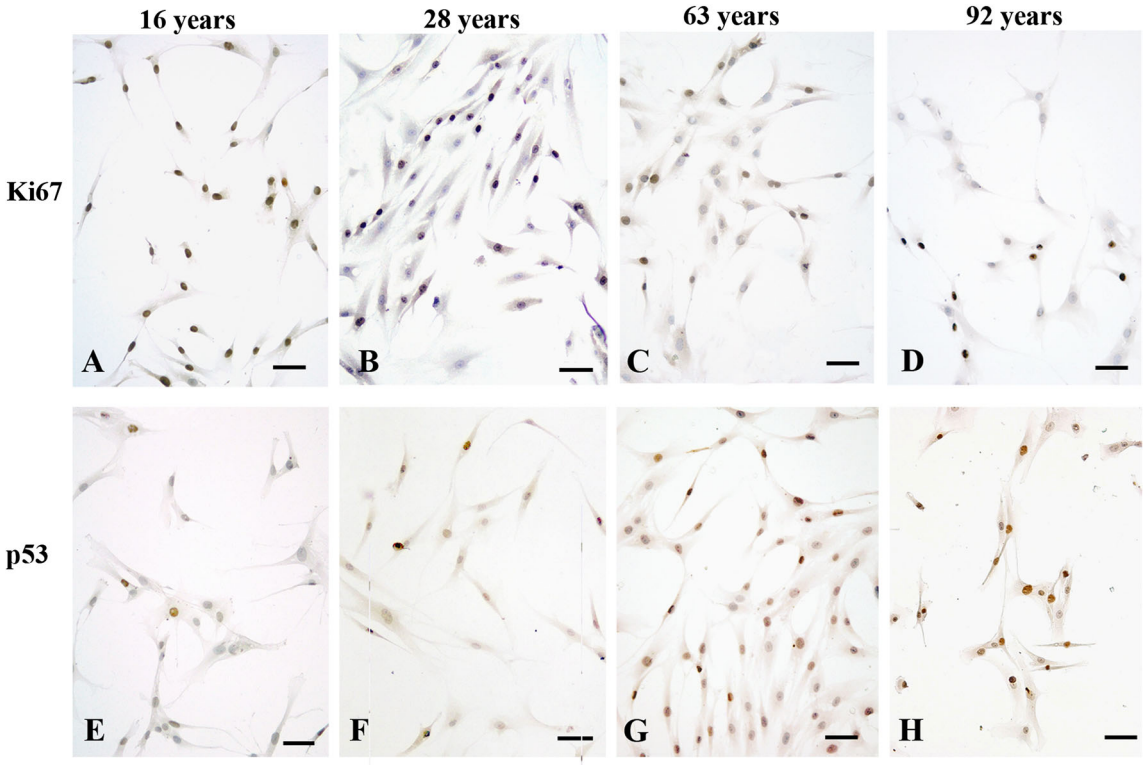

Fig. 1 Immunohistochemical expression of Ki67 and p53 in PDPCs isolated from patients aged 16, 28, 63 and 92 years. Positive cells showed a dark nuclear staining. Ki67 expression was higher in the patients aged 16 (**a**) and 28 (**b**) years compared to 63 (**c**) and 92 (**d**) years; in particular PDPCs from patients aged 92 years showed the lowest staining, whilst PDPCS from subjects aged 16 years showed the highest one. P53 expression was lower in PDPCS from patients aged 16 (**e**) and 28 years (**f**) compared to PDPCs from subjects aged 63 (**g**) and 92 years (**h**). (Immunoperoxidase reaction, scale bars 18 μm)

It was worthy of mention that RANKL/OPG ratio was significantly higher and at the same extent in 16 and 92 years PDPCs in comparison with both 28 and 63 years old ones (Fig. 4), evidencing the high level of remodeling of these youthful PDPCs.

In the end, the comparison of genes involved in stemness showed up-regulation in Sox2 and Nanog mRNA expression when compared 92 years old with 16 years old cells (Fig. 5a), while no significant changes were detected for 63 years old PDPCs. In the comparison with the 28 years old cells Sox2 mRNA appeared down-regulated for both 92 and 63 years old cells (Fig. 5b). For the latter a down-regulation was detected also for Oct4 and Nanog, while in 92 years old PDPCs these genes remained almost unchanged. The comparison of the two oldest populations underlined the up-regulation of Nanog mRNA expression in 92 years old cells (Fig. 5c).

Mathematical Analysis and Modeling

Our mathematical approach for the analysis of biochemical results unveiled a very interesting quantitative correlation when the NO production/Ki67 percentage ratio was considered vs the corresponding age of PDPC donors. Figure 6 shows the relative plot and fitting procedure result.

A clear exponential relationship exists between the two physical quantities defined. The curve fitting process returned the subsequent analytical expression to us:

$$\frac{[NO]}{Ki67(\%)} = a \cdot e^{(b \cdot age)}$$

where a and b are the coefficients estimated with 95 % confidence bounds by means of the fitting procedure. These parameters were found to be equal to 0.005871 and 0.06142, respectively. The goodness of fit was firstly evaluated through a visual examination of the fitted curve and of the residual plot; then, numerical measures like the ones of statistics for parametric models were examined. In both cases, results were good: for example, the sum of squares due to error (SSE) was equal to 0.0008168 and R-square was equal to 0.9996. R-square can take only value between 0 and 1, with a value closer to 1 indicating that a greater proportion of variance is accounted for by the model.

Based on these values we defined an in silico model aimed at mimicking PDPCs behavior in terms of NO production. We exploited the exponential correlation described above to implement a sort of "senescence index" changing as a function of the age of donors and influencing NO production. Given the static in vitro culture, NO synthesis was modeled with an

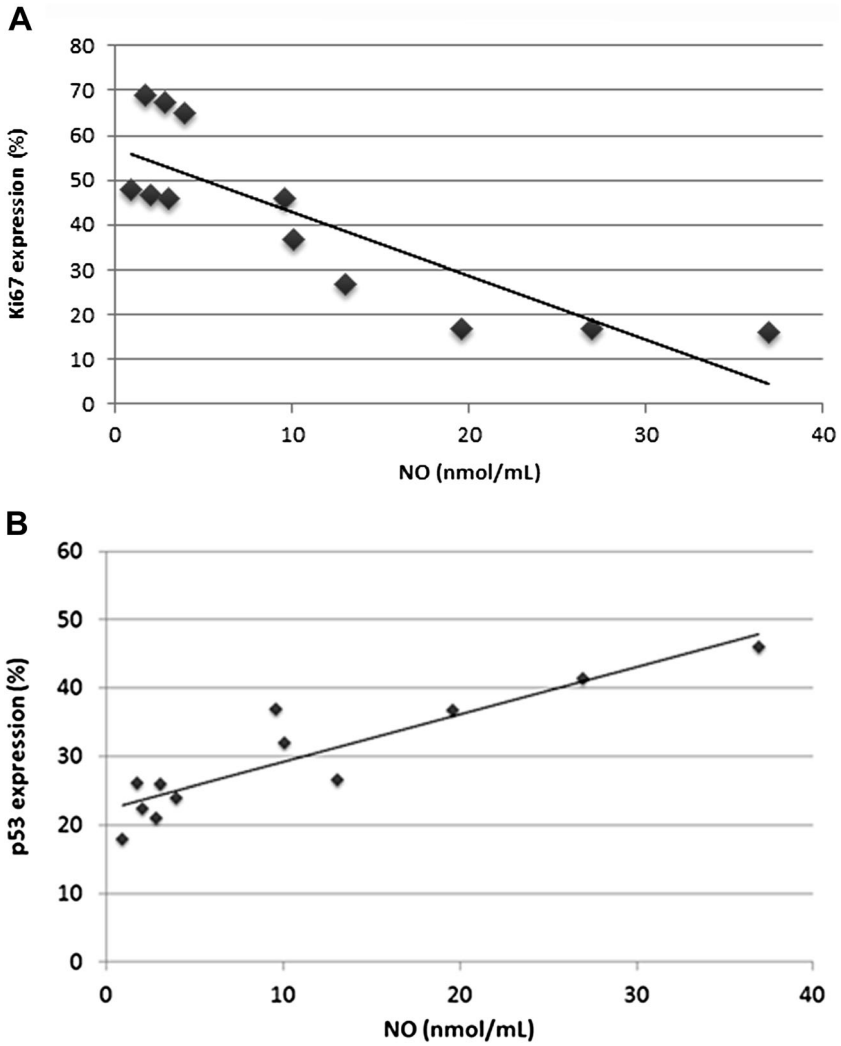

Fig. 2 **a** Negative correlation ($p<0.05$, r:-0.87) between nitric oxide (*NO*) production and Ki67 immunostaining; **b** Positive correlation ($p<0.05$, r: 0.82) between nitric oxide (*NO*) production and p53 expression

irreversible reaction catalyzed by endothelial nitric oxide synthase (eNOS, E.C.: 1.14.13.39) which is constitutively expressed [32]. The number of cells seeded was set equal to 9.5×10^4 and modeled PDPCs were considered to be at the 3rd passage with a consequent doubling time of 13 h (this value was experimentally obtained in our laboratory, but corresponding data are not yet published). Cell proliferation growth was modeled through the classical exponential function, based

Table 3 mRNA relative expression

	16y		28y		63y		92y	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
bmp2	2.14E-04	4.08E-05	2.04E-04	7.07E-06	2.04E-03	5.65E-05	5.57E-04	4.03E-05
runx2	1.89E-03	4.52E-04	5.74E-04	3.97E-05	1.11E-03	2.38E-04	2.25E-03	1.40E-04
Il-6	6.93E-01	2.40E-03	3.82E-02	3.30E-03	8.13E-01	2.89E-01	7.60E+00	3.16E-01
opg	3.20E+00	2.22E-01	1.25E-01	3.02E-03	1.18E-01	3.65E-02	3.04E+00	2.94E-01
Rankl	1.61E-04	5.84E-07	3.54E-07	8.21E-08	1.86E-06	6.59E-07	1.66E-04	5.76E-06
sox2	4.64E-08	1.38E-08	1.65E-07	0.00E+00	5.68E-08	0.00E+00	1.62E-07	4.02E-08
oct4	9.04E-04	9.46E-05	1.90E-03	9.05E-04	1.58E-03	5.46E-05	2.00E-03	1.09E-04
nanog	7.32E-05	3.35E-06	2.66E-04	6.07E-05	6.95E-05	4.57E-06	6.53E-04	6.30E-05

Bmp2 bone morphogenetic protein 2, *Runx2* runt-related transcription factor 2, *Il-6* interleukin-6, *Opg* osteoprotegerin, *Rankl* receptor activator of nuclear factor κB ligand, *Sox2* sex-determining region Y (SRY)-Box2), *Oct4* octamer-binding transcription factor 4

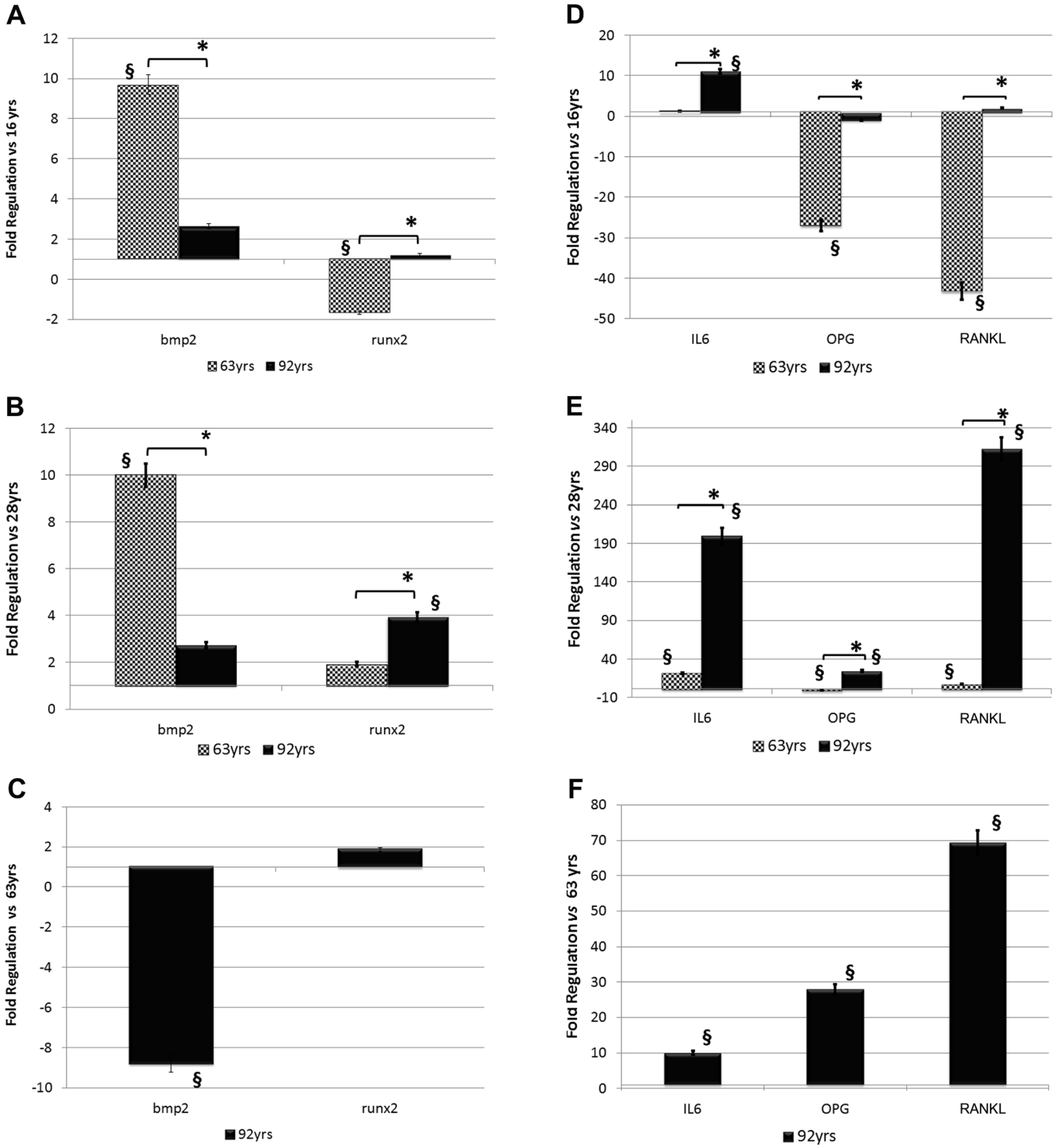

Fig. 3 Histograms depict changes in bmp2, runx2 IL-6, OPG and RANKL mRNA expression in PDPCs of 92 years and 63 years old subjects compared to PDPCs of 16 years old subjects (**a**, **d**) or 28 years old patients (**b**, **e**). In C and F bmp2, runx2 OPG and RANKL mRNAs of 92 years old PDPCs are compared to 63 years old subjects. Data are expressed as Fold-regulation which represents fold-change results in a biologically expressive manner. Fold-regulation is equal to the fold-change ($2^{-\Delta\Delta Ct}$) for fold-change values greater than one, which indicate an up-regulation. Fold-change values less than one indicate a down-regulation; in this case the fold-regulation is the negative inverse of the fold-change ($-1/2^{-\Delta\Delta Ct}$). *=$p<0.05$ 63 vs 93; §=$p<0.05$ vs CTRL

on doubling time. Simulation time unit was considered equivalent to 4 h and simulations stopped after 12 time units in order to reproduce experimental cell culture duration (48 h). Figure 7 shows the comparison between experimental values of NO production and corresponding simulation results. There is a good agreement between them.

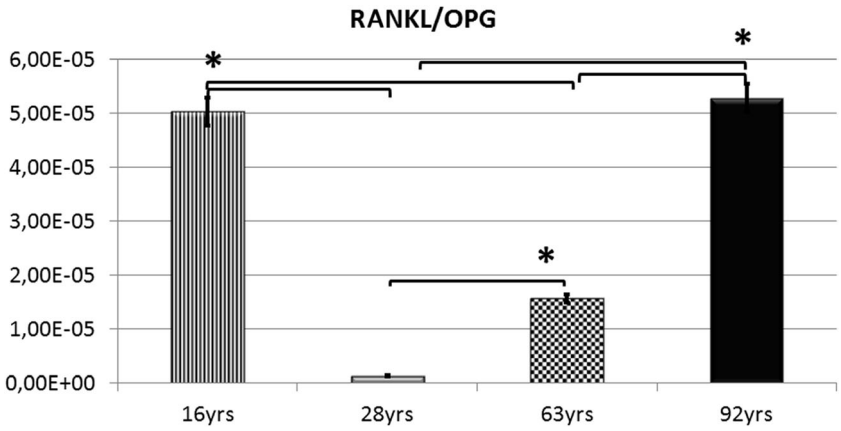

Fig. 4 Histogram depict changes in RANKL/OPG ratio in the differently aged PDPCs. *=$p<0.05$

Discussion

Increased aging populations pose new challenges and emphasize the need for innovative approaches to repair tissue lost through trauma or disease. Stem cell-based therapies have emerged as the likely candidates for bone repair and regeneration in non-union fractures, healing of critical-sized defects and regeneration of tissues in degenerative joint diseases. Even though there is a growing interest in the transplantation of MSCs, which can be isolated from various tissues, the decrease in their viability and impaired function with age could hamper the therapeutic efficacy of engrafted cells.

Periosteum cambium layer is host to osteochondroprogenitor cells with unique tissue-building capacity [18]. It recently has renewed scientific interest due to its inherent regenerative power and stimuli-responsive material properties [33]. Several studies describe mechanical, permeability, and regenerative properties of periosteal tissue and periosteum-derived cells mainly in animal models [34, 35], yet little is known on the structural and cellular characteristics of human periosteum [25, 26] and on its changes related to donor age [36].

By the way, the few investigations on the expression of human MSC cell-cycle regulators and senescence markers, coupled in vivo or in vitro with aging, are mainly related to bone marrow sources [37] and, as far as we know, no data on possible markers or pattern of senescence are available for human PDPCs.

To this purpose we first analyzed the immunohistochemical expression of Ki67 antigen and p53. We detected Ki67, a reliable commonly used marker present only in proliferating cells, instead of proliferating cell nuclear antigen, since the latter may also be localized in cells which have recently undergone arrest [38]. Our data evidenced a low percentage of proliferating PDPCs isolated from aged patients (63 and 92 years) compared to the younger ones (16 and 28 years). Our observations are in agreement with previous works on proliferation rate in senescent cells [38] and with the reduced expression of Ki67 in MSCs derived from different sources [39–41]. Recently, Correia-Melo and his group [42] showed that the lack of Ki67 proliferation marker is one of the best quantitative indicator of cellular senescence and, similarly, we believe that our data disclose for the first time that low Ki67 expression represents a good marker to identify aged PDPCs.

In contrast to Ki67, we found that p53 expression was statistical higher in PDPCs from patients aged 92 years compared to PDPCs from subjects aged 28 and 16 years. Depending on the cell types, p53 activation can result in apoptosis, reversible (quiescence) and irreversible (senescence) cell cycle arrest. Indeed, the mechanisms by which p53 regulates aging remain unclear and its role in the choice between cell quiescence and senescence has been only recently investigated [43]. Several potential processes have been proposed, including the regulation of the insulin/insulin-like growth factor, mTOR pathway, stem cells, and oxidative stress and reactive oxygen species. For instance, constitutive p53 activation may lead to the decline of self-renewal function of stem/progenitor cells and therefore contribute to aging [44], moreover its activation affects mTOR pathway, which drives conversion of "young" cells to senescent phenotype [45]. P53 levels increase with age, mirroring cumulative levels of stress [46], and inhibition of p53 results in an extension of fibroblast lifespan and osteoblastic differentiation [47]. Zhou S and co-workers suggested that the upregulation of p53 pathway with age may have a critical role in mediating the reduction in both proliferation and osteoblastogenesis of human MSCs [13]. Moreover, Stolzing and co-workers [14] showed that in aged MSCs all indicators of aging, including p53, were increased, and suggested that a loss of MSC fitness with age leads to a reduction in MSC number and differentiation capacity. Our results are in agreement with these finding confirming p53 as another efficient marker for the detection of senescence in PDPCs.

As far as NO release is concerned, our data showed higher levels in PDPCs isolated from the elderly and a good correlation with the immunohistochemical analysis. These results are

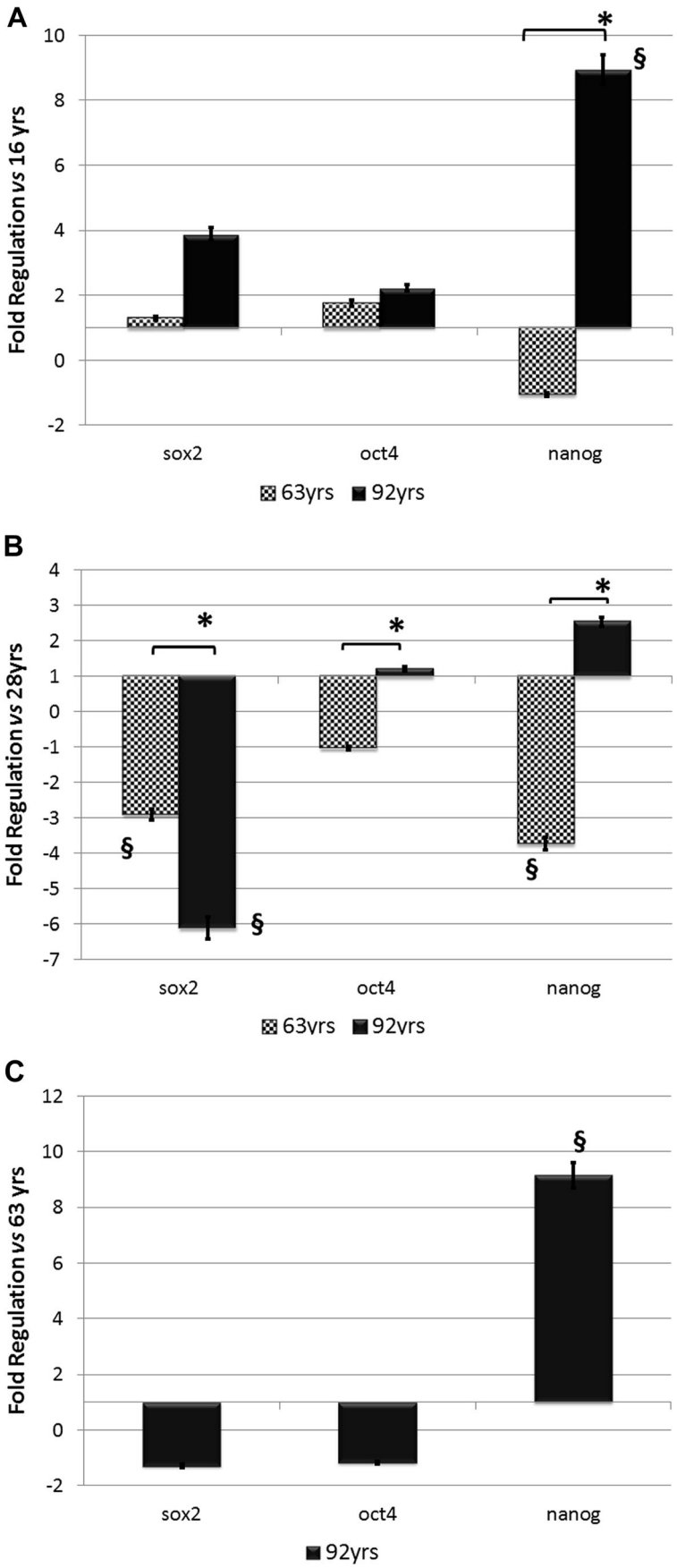

Fig. 5 Histograms depict changes in Sox2, Oct4 and Nanog mRNA expression in PDPCs of 92 years and 63 years old subjects compared to PDPCs of 16 years old subjects (**a**) or 28 years old patients (**b**). In (C) Sox2, Oct4 and Nanog mRNAs expression in PDPCs of 92 years is compared to 63 years old subjects. Data are expressed as Fold-regulation. *=$p<0.05$ 63 vs 93; §=$p<0.05$ vs CTRL

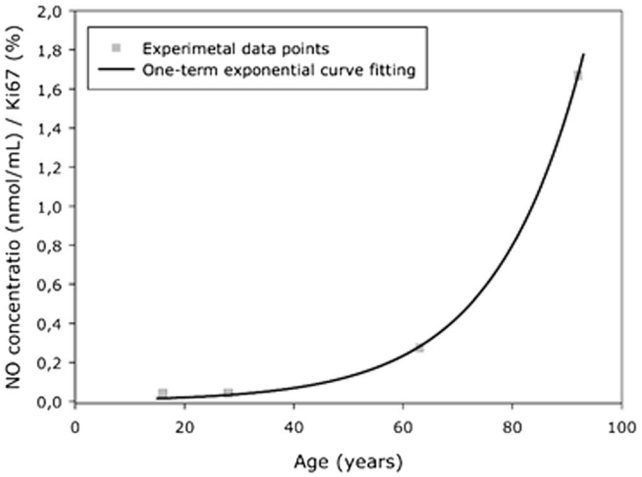

Fig. 6 Experimental Nitric Oxide concentration in the culture medium (nmol/mL) / Ki67value (%) ratio plotted against the corresponding age of PDPC donors (data points) and resulting curve from the fitting procedure with a one-term exponential model by means of Curve Fitting Toolbox (Goodness-of-Fit-statistics: SSE=0.0008168; R-square=0.9996; Adjusted R-square=0.9993; RMSE (Root mean squared error) = 0.02021)

in agreement with previous studies, in which an increase in NO and ROS levels was detected in bone marrow MSCs from old subjects. The accumulation of this oxidative damage during aging can lead to MSC apoptosis and senescence, as well as inhibits their differentiation toward the osteoblastic lineage [13, 14]. In this study, a further analysis was performed on experimental data about NO production and senescence markers employing a systems approach. Mathematical modeling, in silico simulations and biochemical experiments were combined to investigate about possible underlying quantitative correlations. A clear one-term exponential relationship emerged from a comparison of involved marker trends against

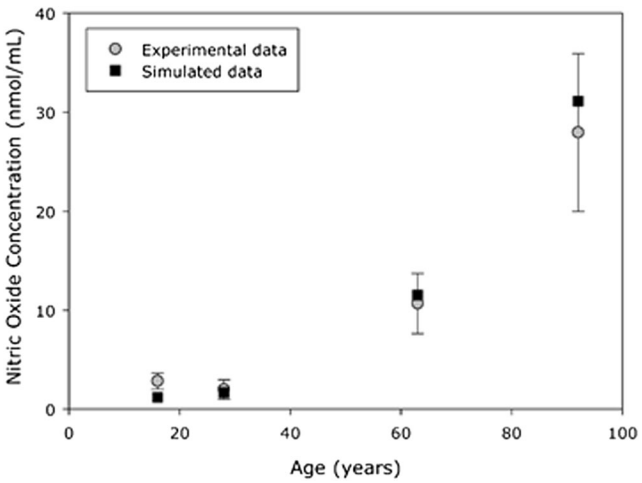

Fig. 7 Measured and simulated Nitric Oxide (*NO*) concentration in the culture medium for PDPC static on-plate cultures. Squares represent experimental data points, while circles represent the corresponding simulated values. Measured values are expressed as means±standard deviation for experiments run in triplicate: numerical values are reported in section "Results - NO production" and error bars represent the standard deviation

age of donors concerning measured NO concentration / Ki67 ratio. This analytical approach confirmed Ki67 as a senescence marker to be focused on.

NO can promote apoptosis (pro-apoptosis) in some cells, whereas it inhibits apoptosis (anti-apoptosis) in other cells. This complexity is a consequence of the rate of NO production and the interaction with biological molecules such as metal ion, thiol, protein tyrosine, and reactive oxygen species [48]. Based on literature data [49], we suggest additional biochemical tests to unveil pro-apoptotic or anti-apoptotic NO role in the context of PDPC senescence. For example, an interesting study [50] shows that pretreatment with a source of NO can protect human osteoblasts hydrogen peroxide-induced apoptotic insults possibly via Runx2-involved regulation of bcl-2 gene expression. We then exploited the mathematical correlation described above to define an in silico model mimicking PDPCs biochemical behavior from NO production perspective with aging. To sum up, the proposed systems biology approach showed to be an useful framework for understanding unknown molecular mechanisms and providing novel insights.

In addition, in order to get in depth the matter, we compared the mRNA expression of early genes involved in osteogenesis, as well as in bone remodeling, between elderly (i.e. 63 and 93 years old) and young (i.e. 16 and 28 years old) PDPCs. Indeed this comparison gave raise to different argumentation when using 16 years old instead of 28 years old cells as control.

Osteogenesis depends on an ordered sequence of gene activation, starting with bmp2 pathway which usually triggers mRNA runx2 transcription, one of the first factors directing the differentiation of precursor stem cells towards an osteoblastic lineage [51]. We found a sequential activation of both genes in comparison with the 28 years old subjects, whilst basically no significant changes were detected in comparison with 16 years cells.

As far as bone remodeling is concerned a significant increase in IL-6 and RANKL mRNAs was detected in comparison of the elderly with 28 years old samples. On the contrary, respect to 16 years, IL-6 was up-regulated only in 92 years old subjects, whilst RANKL was deeply down-regulated in 63 years old PDPCs. At last OPG, the RANKL decoy receptor, showed an up-regulation in its mRNA expression only when comparing cells from 92 years subjects old with 28 years old ones; on the contrary it was down-regulated when comparing cells from 63 years old patients with 16 years old ones. Interestingly, RANKL/OPG ratio resulted higher than 1 in 16 and 92 years old PDPCs. Overall these data indicates that periosteal cell populations modify their potential with age, with a trend toward bone resorption. Moreover aging-related remodeling result more evident in the comparison with 28 years old subjects, since in the teenager bone modeling and remodeling act in concert to maintain the bone shape during skeletal development.

Transcription factors that regulate the maintenance of the pluripotent state in embryonic stem cells, Oct4, Nanog and Sox2, have been proposed to play a similar role also in adult stem cells, although with conflicting results. Indeed, few studies investigated their expression in PDPCs [26, 20]. In our cells we found a significantly lower expression of Oct4 mRNA in 16 years old subjects in comparison with the differently aged patients, while an increase in Nanog and Sox2 expression was detected in both 28 and 92 years cells, with a higher expression for the latter. Recent studies on Oct4 expression in somatic stem cells, including MSCs, concluded that its expression is not required for self-renewal and differentiation ability of these cells [52, 53]. NANOG expression has been also investigated, even though at lesser extent than Oct4 [54, 55]. It has been proposed that the activation of NANOG in MSCs is merely associated with the transition from in vivo quiescence to adaptation to in vitro growth conditions [56]. Sox2 has a role in maintaining pluripotency and self-renewal of stem cells; it is also expressed in immature osteoblasts/osteoprogenitors and is important for their self-renewal [57], suggesting its importance in supporting the differentiation of the MSCs along the osteoblast lineage. Interestingly, Yoon et al. showed that the lineage commitment of primitive multipotent cells is strongly induced in the presence of cytokines secreted by senescent-like cells in a cell culture insert system [58]. Senescent-like cells secreted higher levels of interleukin-6 (IL-6) than primitive multipotent cells, bringing the lineage commitment and stemness loss in multipotent cells by a decreasing in Sox2 expression. This observation is corroborated by our data in which the increase of IL-6 mRNA expression in old PDPCs (i.e. 63 and 92 years) is concomitant with the down-regulation of Sox2 mRNA. Overall these results, although providing evidence that OCT4, NANOG and SOX2 are expressed in PDPCs, were not sufficient to identify role for these genes during aging.

In conclusion, over the last years important progresses have been made, on the understanding of possible age-related changes in MSCs in view of their use for regenerative purposes. To the best of our knowledge this is the first work facing this problem in human periosteal precursor cells (PDPCs). We found that both Ki67 and p53 represent striking markers of cell-cycle arrest in these cells and that their expression correlates with NO production. In addition, age affects genes involved in bone remodeling, with a significant increase in IL-6 mRNA expression as well as RANKL/OPG ratio. As far as stemness genes (Oct4, Nanog and Sox2) our results fall short from identifying their role during aging.

Given to the crucial importance of periosteum in bone regeneration and remodeling, these data represent a further step toward innovative strategies for bone regenerative medicine approaches in the elderly. It seems however obvious that a much greater knowledge of the molecular mechanism that govern these processes are still necessary.

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