Human Periosteum-Derived Cells Maintain Phenotypic Stability and Chondrogenic Potential throughout Expansion Regardless of Donor Age

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Objective. **To assess the in vitro chondrogenic potential of adult human periosteum-derived cells (PDCs) with regard to the number of cell passages and the age of the donor.**

Methods. **Cells were enzymatically released from the periosteum of the proximal tibia obtained from adult human donors and expanded in monolayer. PDCs were harvested at multiple passages for total RNA extraction and semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) gene expression analysis. For the chondrogenesis assay, cells were plated in micromass and treated with transforming growth factor β1 (TGFβ1) in a chemically defined medium. At different time points, micromasses were either harvested for RT-PCR analysis for cartilage and bone markers or fixed, paraffin-embedded, and stained for cartilage matrix, and immunostained for type II collagen.**

Results. **At the first 2 passages, human PDCs from young donors formed chondrogenic nodules. This spontaneous chondrogenic activity was lost upon passaging, and it was not observed in donors older than 30 years. Using a panel of marker genes, PDCs were shown to be phenotypically stable during cell expansion. Regardless of donor age or cell passage, chondrogenesis could be induced consistently by combining micromass culture and TGFβ1 treatment. Histochemical and immunohistochemical analyses demonstrated the hyaline-like cartilage phenotype of the tissue generated in vitro.**

Other TGFβ superfamily members, such as growth differentiation factor 5/cartilage-derived morphogenetic protein 1, and bone morphogenetic proteins 2, 4, and 7, were poorly chondrogenic under the same culture conditions.

Conclusion. **Adult human PDCs have the potential to differentiate toward the chondrocytic lineage in vitro, retaining this property even after extensive subculture. Human PDCs are easily accessible, expandable, and maintain their chondrogenic potential, and are therefore promising progenitor cells for use in the repair of joint surface defects.**

Articular cartilage has a limited capacity for repair. Whatever their etiology—whether acute or chronic processes—joint surface defects rarely heal completely. Most commonly, fibrocartilage or scar tissue forms. Eventually, the clinical picture can evolve into osteoarthritis and functional disability. For end-stage osteoarthritis, the usual treatment is prosthetic joint replacement, which is satisfactory for pain relief, but carries significant morbidity and a limited lifespan of the prosthesis. This poses a challenge in younger individuals. Therefore, joint surface defects represent a major health and social problem, which has spurred the search for techniques by which articular cartilage can be regenerated.

Repair of joint surface defects by transplantation of autologous chondrocytes has been described (1). Major drawbacks of this procedure are the source of the cells and the in vitro expansion of the chondrocytes. The donor tissue consists of articular cartilage specimens obtained from an "uninvolved" and minor load-bearing area of the same joint. This, however, results in additional injury to the joint surface, creating a potential *locus minoris resistentiae* and increased risk for developing osteoarthritis, especially in genetically predisposed individuals. Moreover, in some cases, there may be very little unaffected articular cartilage tissue available.

Chondrocyte expansion is essential for obtaining a sufficient number of cells. Unfortunately, the in vitro cell culture results in chondrocyte dedifferentiation (2), i.e., chondrocytes lose their phenotype and, potentially, are no longer able to organize and produce appropriate matrix, as in normal articular hyaline cartilage. The use of progenitor cells may circumvent these problems.

Studies of limb development indicate that the perichondrium may be embryologically related to the joint interzone and may contribute to the process of formation and differentiation of the joint-associated tissues, including articular cartilage (3–7). In addition, in postnatal fracture healing, mesenchymal progenitor cells in the periosteum form hyaline cartilage during endochondral ossification (8). Therefore, the periosteum can be a source of chondroprogenitor cells.

The in vitro and in vivo chondrogenic potential of periosteum-derived cells (PDCs) as reported in the literature appears to be dependent on the age of the donor and the number of cell passages (9–18). In most of the in vitro studies, cells are released from the periosteum of very young animals, in which the probability of obtaining progenitor cells is higher than in adult and elderly donors (11,19,20), and tested in chondrogenesis assays only at very early passages, when the cells have an inherent chondrogenic potential (12–14). The differentiation toward a chondrocyte phenotype is achieved in high-density culture and occurs spontaneously (12,13,15). The addition of growth factors seems to accelerate chondrogenesis, rather than induce it de novo (14,15).

In the present study, we confirmed the existence of a spontaneous chondrogenic activity at very early passages in PDCs from young donors. We then characterized the PDCs obtained from adult donors of various ages. We show that the PDCs can be expanded in vitro over at least 15 passages without loss of their phenotypic traits. We provide data demonstrating that adult human PDCs can be induced consistently into the chondrogenic differentiation pathway in vitro, regardless of donor age or cell passage number.

SUBJECTS AND METHODS

Harvest of periosteum and isolation of PDCs. A sample of periosteum measuring 1 cm^2 was harvested aseptically from the proximal medial tibia of human donors of various ages (mean 57 years, range 24–95 years); samples were obtained either postmortem (within 12 hours after death) or at the time of surgical knee replacement. Periosteum samples were rinsed twice with Hanks' balanced salt solution (Life Technologies, Merelbeke, Belgium) supplemented with antibiotic–antimycotic solution (100 units/ml penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B; Life Technologies), minced finely, and digested with 0.2% collagenase (Life Technologies) in high-glucose Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 10% fetal bovine serum (FBS; BioWhittaker, Verviers, Belgium) and antibiotics.

Following overnight incubation at 37°C, periosteal cells were collected by centrifugation, washed twice, resuspended in high-glucose DMEM supplemented with 10% FBS and antibiotics (growth medium), plated in a T25 culture flask, and allowed to attach for 4 days. Nonadherent cells were removed by changing the medium.

Cell expansion and cryopreservation. For expansion, cells were cultured in monolayer in growth medium at 37°C in a humidified atmosphere of 5% CO_2. The medium was replaced every 3 days. After 10–20 days of primary culture, when the sparsely attached cells reached confluence, they were washed twice with calcium- and magnesium-free phosphate buffered saline (PBS), harvested by treatment with trypsin–EDTA (0.25% trypsin, 1 mM EDTA; Life Technologies), and replated at a 1:4 dilution for the first subculture. Cell passages were continued in the same way with a 1:4 dilution every 6–12 days when cells reached confluence.

At passage 3 (P3), aliquots of trypsin-released primary PDC cultures in DMEM with 20% FBS and 10% DMSO (Sigma, Bornem, Belgium) were cryopreserved in liquid nitrogen, thawed after various times (range 1–18 months), replated, and expanded for the in vitro chondrogenesis assay.

Treatment with growth factors. Recombinant human bone morphogenetic protein 2 (BMP-2) and BMP-4 were obtained from Genetics Institute (Cambridge, MA); recombinant human BMP-7 and growth differentiation factor 5 (GDF-5)/cartilage-derived morphogenetic protein 1 (CDMP-1) were from Creative BioMolecules (Hopkinton, MA). These proteins were dissolved in 45% acetonitrile, 0.1% trifluoroacetic acid and added to the culture medium at final concentrations of 100–300 ng/ml. For all experiments, vehicle controls were performed in parallel cultures. The induction of alkaline phosphatase activity in ATDC5 and C2C12 cells was used to assess the biologic activity of the growth factors.

Recombinant human transforming growth factor $\beta1$ (TGF$\beta1$; R&D Systems, Abingdon, UK) was dissolved in 4 mM HCl containing 1 mg/ml of bovine serum albumin (BSA; Serva, Heidelberg, Germany) and added to the culture medium at a final concentration of 10 ng/ml. Identical amounts of 4 mM HCl containing 1 mg/ml of BSA were added to parallel cultures as treatment controls.

Treatments were carried out in triplicate or quadruplicate for at least 6 days, both in growth medium and in a chemically defined serum-free medium (21).

In vitro chondrogenesis assay. Micromass cultures were performed as described elsewhere (22). Briefly, expanded PDCs were released by trypsin treatment, counted, tested for viability by trypan blue exclusion, and resuspended in growth medium at a density of 2.0×10^7 viable cells/ml. Micromass cultures were obtained by pipetting 20-μl droplets of cell suspension into individual wells of 24-well plates. After cells were allowed to attach without medium for 3 hours, the chemically defined serum-free medium was added. The day of plating in micromass culture was designated as day 0.

Table 1. Primers used for RT-PCR analysis and expected sizes of PCR products*

Gene	Sense primer	Antisense primer	Product
β-actin	5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′	5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′	661 bp
α1(II) collagen	5′-CCCTGAGTGGAAGAGTGGAG-3′	5′-GAGGCGTGAGGTCTTCTGTG-3′	511 bp
Sox9	5′-GAACGCACATCAAGACGGAG-3′	5′-TCTCGTTGATTTCGCTGCTC-3′	631 bp
Link protein	5′-CCTATGATGAAGCGGTGC-3′	5′-TTGTGCTTGTGGAACCTG-3′	618 bp
β1 integrin	5′-AATGAAGGGCGTGTTGGTAG-3′	5′-ATCTGTCCGTTGCTGGCTTC-3′	344 bp
TGFβRI	5′-TTCAGCCACAGATACCATTG-3′	5′-TTTGATGCCTTCCTGTTGAC-3′	430 bp
α1(I) collagen	5′-CGTGGTGACAAGGGTGAGAC-3′	5′-TAGGTGATGTTCTGGGAGGC-3′	827 bp
Osteocalcin	5′-TCACACTCCTCGCCCTATTG-3′	5′-GAAGAGGAAAGAAGGGTGCC-3′	362 bp

* RT-PCR = reverse transcription–polymerase chain reaction; TGFβRI = transforming growth factor β receptor type I.

Starting on day 1, when the culture medium was changed, TGFβ1 was added to the culture medium every other day, at a final concentration of 10 ng/ml. As a negative control, human dermal fibroblasts (kindly provided by S. Tejpar, Centrum Menselijke Erfelijkheid [CME], Leuven, Belgium) were kept under identical conditions. Micromass cultures were harvested after 6 days for Alcian blue staining and reverse transcription–polymerase chain reaction (RT-PCR) gene expression analysis.

Alcian blue staining in vitro. Cultures were rinsed twice with PBS, fixed with methanol for 30 minutes at −20°C, washed with distilled water, and covered with Alcian blue at pH 0.2 (0.5% Alcian blue 8 GS [Carl Roth, Karlsruhe, Germany] in 1N HCl). After overnight staining, cultures were washed extensively with distilled water. For quantitative analyses, Alcian blue–stained cultures were extracted with 200 μl of 6M guanidine HCl in Milli-Q water for 6 hours at room temperature. The optical density of the extracted dye was measured at 630 nm using a Spectronic 2000 spectrophotometer (Bausch & Lomb, Rochester, NY).

The specificity of Alcian blue staining at pH 0.2 in vitro and its correlation with the expression of type II collagen are described elsewhere (manuscript in preparation).

Total RNA extraction and semiquantitative RT-PCR analysis. Total RNA was extracted using a commercially available kit (SNAP kit; Invitrogen, Groningen, The Netherlands), according to the manufacturer's instructions. Complementary DNA (cDNA) was obtained by RT of 1 μg of total RNA (Thermoscript; Life Technologies) with oligo(dT)$_{20}$ as primer. PCR was performed in a volume of 10 μl; cDNA samples were added to the following PCR mixture: 0.5 units of *Taq* polymerase (Eurogentec, Seraing, Belgium), 0.2 mM dNTPs, 0.5 μM specific primers, and 1.5 mM MgCl$_2$. Negative controls were either RT without enzyme or PCR with Milli-Q water instead of cDNA.

PCR reactions were carried out in a Perkin Elmer Thermal Cycler 9600 (Applied Biosystems, Lennik, Belgium). After 1 minute of denaturation at 95°C, cycles (19 for β-actin, 33 for type II collagen, 34 for osteocalcin, and 30 for the other genes) were 10 seconds at 94°C, 10 seconds at the optimal annealing temperature, and 30 seconds at 72°C. Cycling was followed by 10 minutes of elongation at 72°C. Primer pairs were designed using Vector NTI software (InforMax, North Bethesda, MD). The sequences of the primers and the sizes of the PCR products are shown in Table 1.

Complementary DNA samples were equalized for the expression of the housekeeping gene β-actin. PCR products were electrophoresed in 1.5% agarose gel in Tris–borate–EDTA electrophoresis buffer, stained with ethidium bromide, visualized by ultraviolet transillumination, and analyzed by densitometry using the Image Master software (Amersham Pharmacia Biotech, Roosendaal, The Netherlands).

Senescence-associated β-galactosidase (β-gal) staining. Staining for senescence-associated β-gal was performed as described (23). Early-passage (before P6) and late-passage (after P10) PDCs from young and old donors were plated in chamber slides and allowed to attach in growth medium. Cells were washed in PBS, fixed for 5 minutes at room temperature in 3% formaldehyde, washed, and incubated overnight in a humid chamber at 37°C with fresh β-gal staining solution: 1 mg of X-Gal per ml (Duchefa, Haarlem, The Netherlands), 40 mM citric acid/sodium phosphate at pH 6, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl$_2$. Senescent human dermal fibroblasts were used as a positive control.

Histology and immunohistochemistry. For histologic and immunohistochemical analyses, 100 μl of PDC suspension was plated in micromass in individual wells of a 12-well plate. Micromasses were treated with 10 ng/ml of TGFβ1 or with the same amount of TGFβ1 carrier solution in the chemically defined serum-free medium. After 7 and 15 days, micromasses were peeled off the wells, fixed with 10% formalin, embedded in paraffin, and sectioned at 5 μm.

For histologic evaluation, sections were deparaffinized and stained with toluidine blue or Alcian blue at pH 2.5, according to standard protocols. In the Alcian blue–stained sections, neutral red was used for counterstaining the nuclei.

For immunohistochemical analysis, sections were deparaffinized and predigested with chondroitinase ABC (Sigma) at 50 mU/ml at room temperature for 1 hour to facilitate antibody access. Nonspecific antibody binding was blocked by incubating the slides for 1 hour in 5% BSA in PBS. Sections were then incubated for 1 hour with a mouse anti-human type II collagen monoclonal antibody (Chemicon, Hofheim, Germany) diluted 1:5 in 0.5% BSA in PBS. Reactivity was detected with fluorescence microscopy after incubation for 1 hour with a Cy3-conjugated secondary antibody (goat anti-mouse IgG; Jackson ImmunoResearch, West Grove, PA) that had been diluted 1:500 in 0.5% BSA in PBS. Nuclei were counterstained
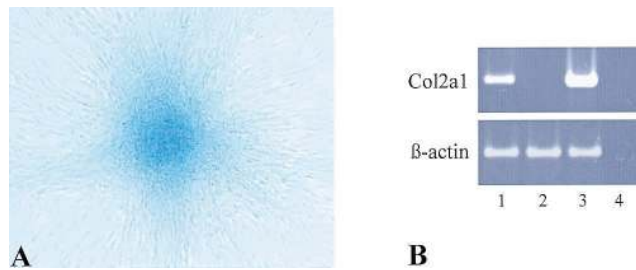
Figure 1. Spontaneous chondrogenic activity of periosteum-derived cells (PDCs) from young adult donors at very early passages. **A,** Nodule stained with Alcian blue. **B,** Semiquantitative reverse transcription–polymerase chain reaction (RT-PCR), showing the expression of $\alpha1$(II) collagen (Col2a1) by PDCs at passage 1 (P1) (lane 1) but not at P4 (lane 2). Lane 3, Freshly isolated human articular chondrocytes (positive control); lane 4, RT-negative control of lane 1. Templates are equalized for the expression of β-actin.

with 4′,6-diamidino-2-phenylindole (ICN, Asse-Relegem, Belgium).

RESULTS

Loss of spontaneous chondrogenic activity on serial passaging and with increasing age of donor. At very early passages (P0 and P1), human PDCs from donors younger than 30 years formed densely packed nodules, with round or polygonal cells. The process of nodule formation from the adherent fibroblastic cell population was not uniform. Some cells appeared to give rise to nodules soon after adherence to the culture plate, whereas other cells did not contribute to nodule formation until they reached confluence.

A substantial number of nodules stained with Alcian blue, indicating the presence of proteoglycans (Figure 1A). Fibroblast-like cells were spreading between the nodules. The focal chondrogenic differentiation was confirmed by the detection of type II collagen messenger RNA (mRNA) in P0 and P1 monolayer cultures (Figure 1B). With the subsequent subculturing, PDCs lost the potential to form Alcian blue–positive nodules and appeared microscopically to be a relatively homogeneous population of fibroblast-like cells. PDCs from donors older than 30 years never formed chondrogenic nodules nor expressed type II collagen, having a fibroblast-like morphology from the beginning of the culture.

Phenotypic stability of PDCs through serial passaging and cryopreservation, independent of donor age. To define the effects of donor age and in vitro cell expansion on the molecular phenotype of PDCs, cells from donors of various ages were passaged serially. At multiple passages, gene expression analysis was performed by semiquantitative RT-PCR. The marker analysis included genes known to have a role in skeletal development and maintenance (3). The first 2 passages (P0 and P1) of PDCs obtained from donors younger than 30 years were not included in this experiment. The expression of 40 selected genes was analyzed (results not shown). Figure 2 shows a representative panel of the genes tested. The molecular profile of the cells was stable during serial passaging and independent of the age of the donors. No phenotypic differences were observed between samples obtained postmortem and samples obtained during surgical knee replacement (Figure 2).

To test whether cryopreservation could affect the phenotype of PDCs, the same panel of markers was tested for expression by cells that had been cryopreserved compared with passage-matched cells that had never been frozen. No phenotypic differences were detected between cryopreserved and noncryopreserved cells (results not shown).

Cell senescence during PDC expansion. Cell senescence, a common phenomenon occurring to various degrees in many somatic cells during in vitro culture expansion, is known to induce phenotypic and functional changes (24). Therefore, we analyzed senescence-associated β-gal activity in early-passage and late-passage PDCs from donors of different ages. Senescence-associated β-gal activity was not detected in PDCs (expanded up to P15) from donors younger than 30 years. PDCs from donors older than 60 years were largely β-gal negative at early passages, whereas weak-
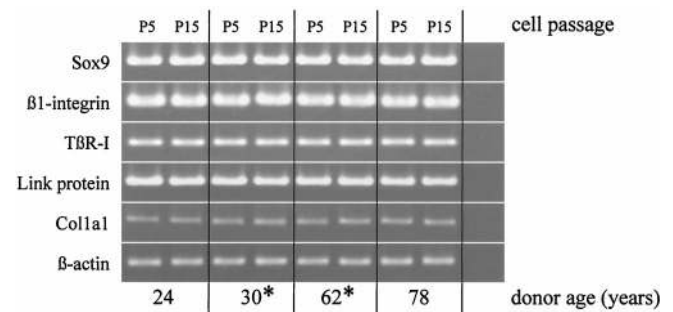
Figure 2. Stability of PDC phenotype independent of cell passage number or donor age. PDCs at P5 and P15 from 4 donors of different ages show a comparable molecular profile, as assessed by semiquantitative RT-PCR. A representative panel of the genes tested is shown here. In the last lane is Milli-Q water (negative control). Templates are equalized for β-actin expression. Col1a1 = $\alpha1$(I) collagen. Asterisk indicates samples obtained postmortem. TβR-I = transforming growth factor β receptor type I (see Figure 1 for other definitions).
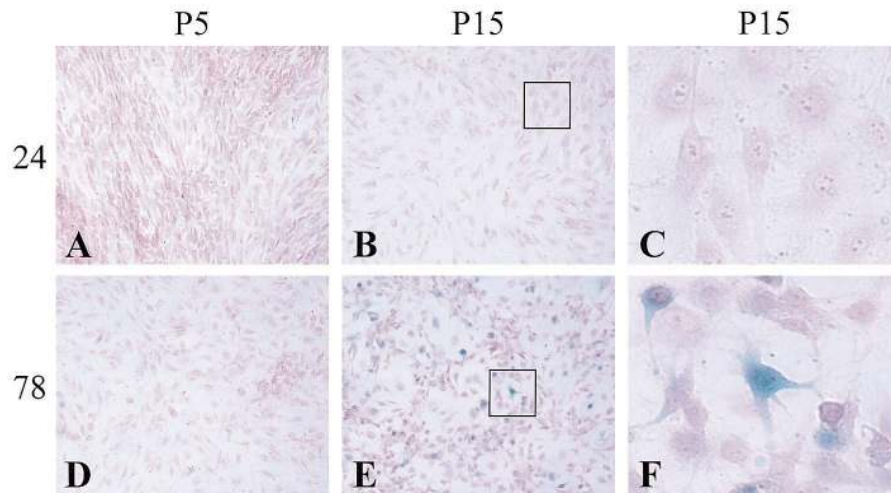
Figure 3. Senescence-associated β-galactosidase staining. PDCs at P5 and P15 from a 24-year-old donor (**A–C**) and from a 78-year-old donor (**D–F**) are shown. **C,** High-magnification view of the boxed area in **B**. **F,** High-magnification view of the boxed area in **E**, showing the detail of a senescent cell. PDCs from younger donors could be expanded for many passages without senescence. Few positive cells are visible in PDCs at P15 from the 78-year-old donor. (Neutral red counterstained; original magnification × 100 in **A**, **B**, **D**, and **E**; × 630 in **C** and **F**.) See Figure 1 for definitions.

to-moderate β-gal staining of a few scattered cells (5–10%) was observed at late passages (Figure 3).

Requirement for both micromass culture and TGFβ1 treatment for induction of chondrogenesis in vitro. We wished to determine whether it was possible to induce chondrogenesis in late-passage PDCs and in PDCs from older donors by using different culture systems and treatments. Supplementing the medium with growth factors, members of the TGFβ superfamily (TGFβ1, GDF-5/CDMP-1, and BMPs 2, 4, and 7), failed to induce chondrogenesis in confluent monolayer PDCs, as evaluated by Alcian blue staining and RT-PCR for
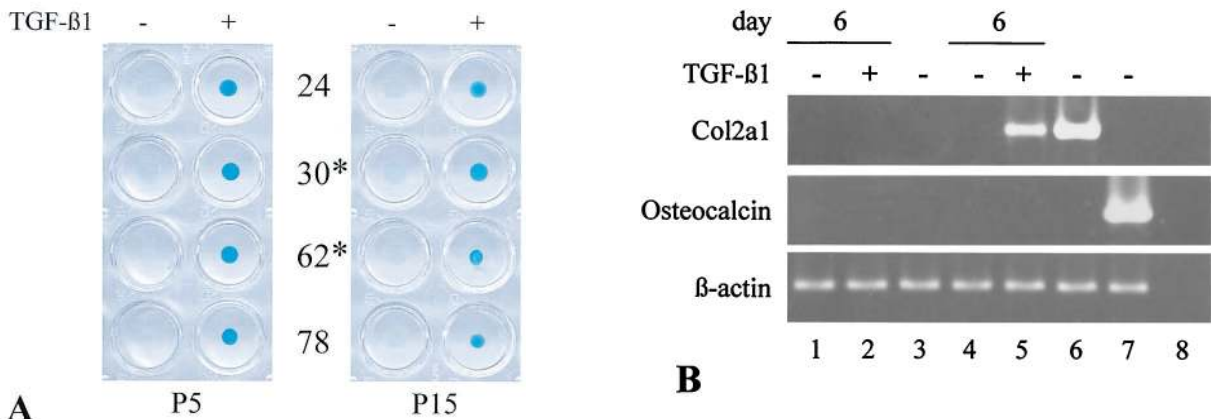

Figure 4. Stability of the chondrogenic potential of adult human PDCs over many passages and lack of impairment in older donors. **A,** PDCs at P5 and P15 from donors of different ages were plated in micromass culture and either treated or not treated with transforming growth factor β1 (TGF-β1). After 6 days, micromasses were fixed and stained with Alcian blue. The ages of the donors are shown in between the culture plates; asterisk indicates periosteum samples obtained postmortem. **B,** Semiquantitative RT-PCR for type II collagen and osteocalcin. Lanes 1 and 2, Human dermal fibroblasts in micromass; lane 3, PDCs at P16 in monolayer; lanes 4 and 5, PDCs at P16 in micromass; lane 6, freshly isolated human articular chondrocytes; lane 7, human trabecular osteoblasts; lane 8, RT-negative control of lane 5. All cDNA samples are equalized for β-actin expression. See Figure 1 for other definitions.
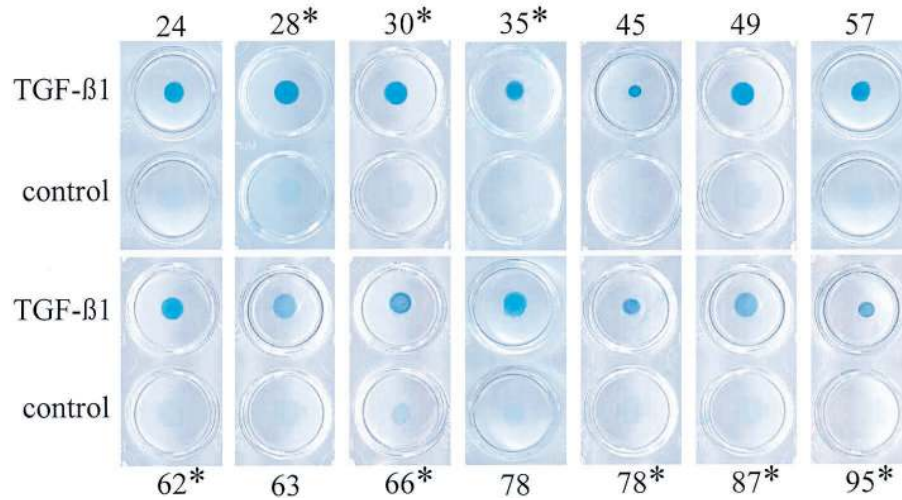
Figure 5. Induction of chondrogenesis in periosteum-derived cells (PDCs) from adult donors of various ages. PDCs were plated in micromass, treated or not treated with transforming growth factor β1 (TGF-β1) for 6 days, fixed, and stained with Alcian blue. The ages of the donors are shown across the top and the bottom; asterisk indicates periosteum samples obtained postmortem. For each donor, the chondrogenesis assay was carried out in triplicate.

type II collagen (results not shown). In contrast, differentiation toward the chondrocyte phenotype could be elicited reproducibly and consistently by combining the micromass culture system with TGFβ1 treatment. This treatment induced chondrogenesis regardless of the number of cell passages or the age of the donors. No difference was observed between samples obtained postmortem and samples obtained during surgical knee replacement (Figure 4). The expression of type I collagen remained unchanged during chondrogenic differentiation at all time points examined up to 21 days (results not shown). Human dermal fibroblasts under the same
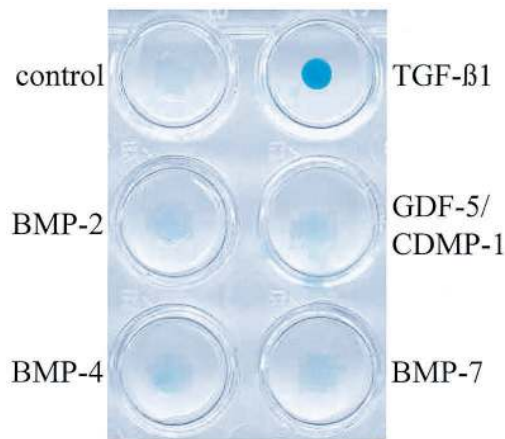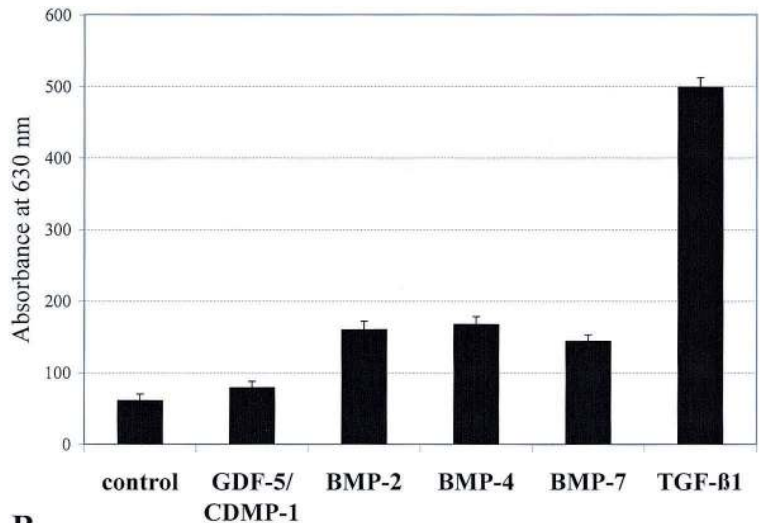
Figure 6. Chondrogenic potential of bone morphogenetic proteins (BMPs) compared with transforming growth factor β1 (TGF-β1). **A,** Periosteum-derived cells at passage 5 were plated in micromass culture and treated with growth factors, members of the TGFβ superfamily. Final concentrations were 10 ng/ml for TGF-β1 and 300 ng/ml for growth differentiation factor 5 (GDF-5)/cartilage-derived morphogenetic protein 1 (CDMP-1) and the BMPs. After 6 days, cultures were fixed and stained with Alcian blue. **B,** Stained micromasses were extracted with 6M guanidine HCl. The absorbance of the extracted dye was measured at 630 nm. Values are the mean and SD of 4 wells.

culture conditions and treatments failed to undergo chondrogenesis (Figure 4).

The chondrogenic differentiation could be reproduced in all donors tested (Figure 5). Importantly, no apparent impairment of the chondrogenic potential of PDCs was evident after cryopreservation. Under the same culture conditions, GDF-5/CDMP-1, BMP-2, BMP-4, and BMP-7 at concentrations of 100 or 300 ng/ml were poorly chondrogenic in comparison with TGFβ1 (Figure 6). The cell number was not affected by the treatments (results not shown). As proven by RT-PCR, PDCs expressed BMP receptors (results not shown). In the absence of TGFβ1, chondrogenesis did not take place even after 20 days of micromass culture, either in growth medium or in chemically defined serum-free medium (results not shown). As opposed to serially passaged PDCs, P0 and P1 cells in micromass cultures from donors younger than 30 years underwent chondrogenesis independent of TGFβ1 treatment (results not shown).

Histochemical and immunohistochemical characterization of periosteum-derived TGFβ1-induced neocartilage. Histochemical analysis of the micromass cultures treated with TGFβ1 displayed cartilage-specific metachromasia with toluidine blue dye and intense staining with Alcian blue, features which are highly suggestive of chondrogenic differentiation. Positive staining was evident only after 2 weeks. Untreated micromass cultures were all negative (Figures 7A–F). The detection of type II collagen protein by immunostaining further confirmed the cartilaginous nature of the tissue generated in vitro (Figures 7G and H). The extracellular matrix appeared to be homogeneous in all the histologic preparations. Hematoxylin and eosin staining as well as Masson's trichrome staining did not demonstrate the presence of dense fibrous bundles as in fibrocartilage tissue. This finding was confirmed by polarized light microscopy (results not shown). Alizarin red staining for calcium deposits (results not shown) and RT-PCR analysis for osteocalcin (Figure 4B) were negative at all time points examined up to 21 days.

The overall characteristics identified in the findings described above allow us to define the neocartilage as hyaline-like.

DISCUSSION

In the present study, we demonstrate that expanded adult human PDCs under appropriate culture conditions can be induced to form cartilage in vitro, independent of the number of cell passages or the age of the donors (within the ranges examined). We provide further characterization of a population of progenitor cells derived from human periosteum that possess high self-renewal capacity and retain their phenotype throughout expansion. We present additional data demonstrating full retention of the chondrogenic potential of this cell population following cryopreservation.

The chondrogenic potential of PDCs has been the subject of extensive investigation (9–18) and has been reported to be a phenomenon that is dependent on donor age and cell passage in culture (11). In most of the in vitro studies, PDCs were harvested from young animals, in which the probability of obtaining progenitor cells is very high (11,19,20), and were used in chondrogenesis assays at very early passages, when chondrogenesis seems to occur spontaneously (12–14).

Consistent with the results reported in the literature, we found that very early–passage PDCs from young adult human donors undergo spontaneous chondrogenesis in vitro. PDC cultures are initiated as primary cultures of fibroblast-like cells that grow out of PDC suspensions by selectively attaching to plastic tissue culture plates. In monolayer cultures, P0 and P1 PDCs from young donors express type II collagen and form nodules that stain with Alcian blue, indicating that at least a subpopulation of these cells has inherent chondrogenic potential. This phenomenon is rapidly lost upon cell passaging and is not observed in PDCs from donors older than 30 years.

The loss upon cell passaging of the spontaneous chondrogenic activity of PDCs from young donors may be due to factors related to the in vitro culture. Another possible explanation is that during the subculture period, a cell selection process takes place, so that PDCs responsible for the formation of chondrogenic nodules are overgrown by other cell subsets.

Not surprisingly, the decline in the in vitro chondrogenic potential of human PDCs is related to the age of the donor. In rabbit periosteum, it has been associated with a decrease in the size of the precursor pool in the cambium layer, where the progenitor cells reside (20). The observation that the in vitro chondrogenic potential of periosteum correlates directly with the thickness of the cambium layer and inversely with the age of the donor further supports this hypothesis (20,25). Alternatively, the age-related decline can also be attributable to a reduction in the inherent potential of the progenitor cells to undergo chondrogenic differentiation.

Regardless of the age, periosteum in vivo participates in bone remodeling and contributes to fracture healing by generating hyaline cartilage, which further
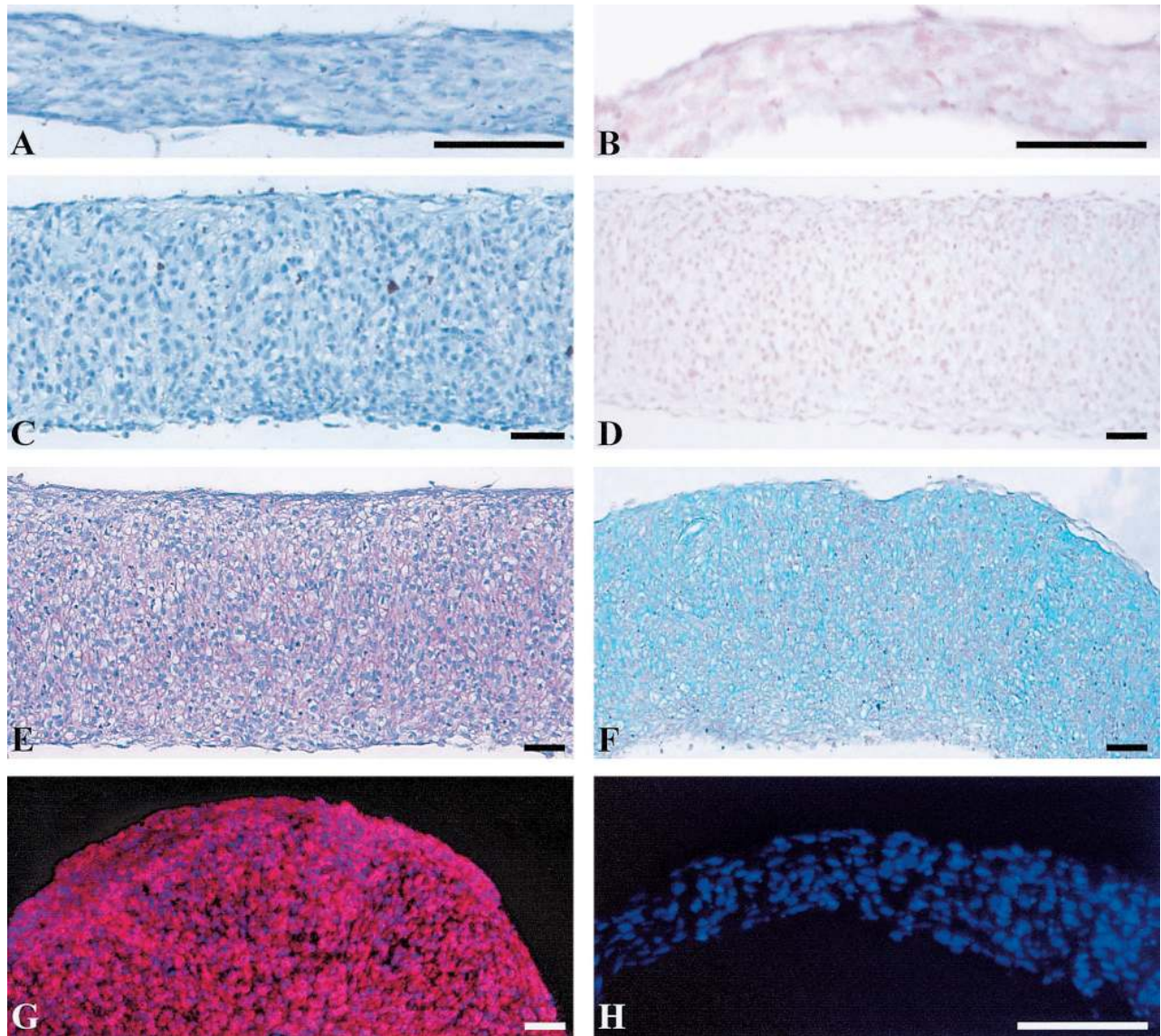
Figure 7. A–F, Toluidine blue (**A, C,** and **E**) and Alcian blue (**B, D,** and **F**) staining of paraffin sections of micromasses either treated (**C–F**) or not treated (**A** and **B**) with transforming growth factor $\beta1$ (TGFβ1) for 7 days (**C** and **D**) or 15 days (**A, B, E,** and **F**). **G** and **H,** Immunohistochemical analysis of paraffin sections with anti–type II collagen antibody. **G,** Micromass treated with TGFβ1 for 15 days. **H,** Untreated micromass. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue). Findings indicate the presence of cartilage differentiation in the micromasses treated with TGFβ1. Note the difference in scale between TGFβ1-treated and control micromasses. Bar = 50 μm.

undergoes endochondral ossification (26). With this observation in mind, we hypothesized that, independent of the age of the donor, an inducible chondrogenic potential of PDCs could be retained in vitro.

Treatments with growth factors, members of the TGFβ superfamily, which are known to be involved in morphogenesis, differentiation, and maintenance of cartilage (3–5,27), failed to induce chondrogenesis in con-fluent monolayer PDCs. Independent of the age of the donors or the number of cell passages, the differentiation toward the chondrocyte phenotype could be elicited reproducibly and consistently only when micromass culture and TGFβ1 treatment were combined. In contrast, neither untreated micromasses nor TGFβ1-treated monolayers could undergo chondrogenesis.

These results may appear to be at odds with the

previous literature reporting enhancement of in vitro chondrogenesis in PDCs using either the micromass culture system or TGFβ1 treatment alone (12–14). In those studies, however, PDCs were obtained from very young animals and assessed for chondrogenic potential at very early passages. Our data and data from previously published studies (14–16) demonstrate that in these settings, both culture conditions only enhance and accelerate an already spontaneous process.

We speculate that PDCs from young donors display spontaneous chondrogenesis that is lost with age or during in vitro cell expansion. Nevertheless, competence to respond to at least 1 chondrogenic signal is retained even in very elderly individuals or extensively passaged cells. The cytology, the presence of a metachromatic-staining matrix, and the detection of type II collagen mRNA and protein demonstrate the cartilage phenotype of the tissue generated in vitro. The positivity of the staining is evident only after 2 weeks, suggesting that, despite the early response to TGFβ1 treatment, time is required for the full maturation of PDCs to the cartilaginous phenotype.

The requirement for high cell density demonstrates the importance of cell–cell interactions for in vitro chondrogenic differentiation (28). In this respect, the micromass culture system reproduces cell interactions analogous to those occurring in precartilage mesenchymal condensation during limb development (3). However, in our system, this cell configuration alone was not sufficient for the induction of chondrogenesis; TGFβ1 treatment was also required. We hypothesize that PDCs in micromass culture would acquire competence to TGFβ1 signaling for chondrogenic differentiation.

Interestingly, under the same culture conditions, GDF-5/CDMP-1, BMP-2, BMP-4, and BMP-7 displayed a poor chondrogenic effect compared with TGFβ1. Our results are consistent with the observation that BMP-2 treatment does not affect chondrogenesis in high-density PDC cultures (16). However, BMPs have been shown to stimulate chondrogenesis in vitro (29–31). This discrepancy may be due to differences in the culture systems, BMP molecules, and concentrations used and/or the target cells. Specifically in human PDCs, the poor chondrogenic effect of BMPs as compared with that of TGFβ1 may depend on the relative levels of expression of TGFβ/BMP receptor subfamilies and related downstream factors or on distinctive regulation of their signaling pathways. These possibilities are currently under investigation.

For a potential use in tissue engineering proto-

cols, PDCs should retain a stable phenotype as well as chondrogenic potential throughout extensive expansion and, possibly, after cryopreservation. Gene expression analysis by semiquantitative RT-PCR for a panel of genes known to be involved in skeletal development and maintenance (3) showed that, regardless of donor age, PDCs can be cryopreserved and largely expanded, retaining a stable phenotype and chondrogenic potential. Expanding further the number of marker genes, the analysis of PDC phenotype can lead to the identification of a set of molecular markers that is predictive of the chondrogenic potential of PDCs and other chondroprogenitor cell populations.

Cell senescence is known to alter the functional properties of somatic cells during in vitro expansion (23,24). We demonstrate that cell senescence is a limited event restricted to late-passage PDCs from elderly donors. Taking into consideration the high number of cells already obtainable after a few passages (about 1 billion cells after 6 passages, starting from a 1-cm^2 periosteum sample from the proximal medial tibia), senescence does not represent a significant factor limiting cell expansion.

A recent study showed a progressive decline in the chondrogenic potential of rabbit periosteum harvested postmortem (as soon as 4 hours after death) that was related to the viability of the tissue (32). In our study, no differences were observed in the molecular profile and chondrogenic potential of expanded cells obtained from periosteum harvested postmortem (within 12 hours) and at the time of surgical knee replacement. This apparent discrepancy can be explained by the different experimental settings. O'Driscoll et al (32) used an ex vivo organ culture of periosteum as a tissue. In our procedure, cells were first released and then selected based on their capacity to adhere to plastic. Adherent cells were a minority of the total pool of cells that were plated. The subsequent expansion of this selected cell subpopulation most probably corrected the possible differences in the number of viable chondroprogenitor cells present in the original tissue samples.

The characteristic poor capacity for repair of articular cartilage has prompted great interest in cartilage tissue engineering and regeneration. Autologous chondrocyte transplantation (1) is limited by the availability of cells, particularly in elderly individuals, and the well-known dedifferentiation events associated with chondrocyte expansion. In the selection of the optimal cells for repairing joint surface defects, the following criteria should be considered: the chondrogenic potential, the autologous source, the relative ease of access, the cell expandability, the phenotypic stability of the

cells throughout the expansion procedures, and the least possible surgical morbidity. The potential use of human embryonic stem cells (33,34) for tissue regeneration therapy is very attractive, but ethical issues need to be resolved. Less controversial, but equally promising, is the perspective of using progenitor cells derived from adult tissues (35). These adult stem cells seem to retain a remarkable plasticity, since they have much wider differentiation potential than previously thought (36–43). This plasticity, however, presents the potential risk of cell differentiation toward an undesired direction(s) upon transplantation. It would be more reassuring, therefore, to use cells that have restricted differentiation potential.

We show that regardless of their age, adult human PDCs are easily accessible, expandable, and phenotypically stable. Moreover, they maintain their chondrogenic potential up to at least 15 passages and after cryopreservation. It has also been reported that human bone marrow–derived mesenchymal cells can be largely expanded in culture with retention of their chondrogenic potential, independent of donor age (44). However, in contrast to the broad plasticity of bone marrow–derived mesenchymal cells (37,41–43), PDCs are known to have bilineage potential, since they can give rise to cartilage and bone (11). At least in vitro, expanded PDCs can, under appropriate conditions, form a tissue close to that of hyaline cartilage, with no evidence of mineralization or bone differentiation. These characteristics make PDCs promising precursor cells for use in the repair of joint surface defects. If required in the in vivo environment, modern gene transfer technology and carrier systems can provide the means to deliver bioactive factors, such as TGFβ1, to prime chondrogenesis.

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