Human mastoid periosteum-derived stem cells: Promising candidates for skeletal tissue engineering

ARTICLE *in* JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE · MARCH 2008

Impact Factor: 5.2 · DOI: 10.1002/term.75 · Source: PubMed

JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med 2008; **2**: 136–146.
Published online 31 March 2008 in Wiley InterScience (www.interscience.wiley.com) **DOI:** 10.1002/term.75

RESEARCH ARTICLE

Human mastoid periosteum-derived stem cells: promising candidates for skeletal tissue engineering

J. Ringe[1#*], I. Leinhase[2#], S. Stich[1], A. Loch[3], K. Neumann[4], A. Haisch[5], T. Häupl[1], R. Manz[6], C. Kaps[4] and M. Sittinger[1]

[1]*Tissue Engineering Laboratory and Berlin-Brandenburg Center for Regenerative Therapies, Department of Rheumatology and Clinical Immunology, Charité-University Medicine Berlin, Tucholskystrasse 2, 10117 Berlin, Germany*
[2]*Department of Trauma and Reconstructive Surgery, Charité-University Medicine Berlin, Hindenburgdamm 30, 12200 Berlin, Germany*
[3]*Department of Otorhinolaryngology, Charité-University Medicine Berlin, Schumannstrasse 20/21, 10117 Berlin, Germany*
[4]*TransTissue Technologies GmbH, Tucholskystrasse 2, 10117 Berlin, Germany*
[5]*Department of Otorhinolaryngology, Charité-University Medicine Berlin, Hindenburgdamm 30, 12200 Berlin, Germany*
[6]*Humoral Immunology, German Rheumatism Research Centre, Schumannstrasse 21/22, 10117 Berlin, Germany*

Abstract

Currently, mesenchymal stem cells (MSCs) are considered as the most eligible cells for skeletal tissue engineering. However, factors such as difficult stimulation and control of differentiation *in vivo* hamper their clinical use. In contrast, periosteum or periosteum-derived cells (PCs) are routinely clinically applied for bone and cartilage repair. PCs have often been named MSCs but, although cells of osteochondrogenic lineages arise from MSCs, it is unclear whether periosteum really contains MSCs. Our aim was to investigate the MSC-like character of PCs derived from the periosteum of mastoid bone. Harvesting of periosteum from mastoid bone is easy, so mastoid represents a good source for the isolation of PCs. Therefore, we analysed the MSC-like growth behaviour and the expression of embryonic, ectodermal, endodermal and mesodermal markers by microarray and FACS technology, and the multilineage developmental capacity of human PCs. Regarding clinical relevance, experiments were performed in human serum-supplemented medium. We show that PCs do not express early embryonic stem cell markers such as *Oct4* and *Nanog*, or the marker of haematopoietic stem cells CD34, but express some other MSC markers. Osteogenesis resulted in the formation of calcified matrix, increased alkaline phosphatase activity, and induction of the osteogenic marker gene *osteocalcin*. Staining of proteoglycans and deposition of type II collagen documented chondrogenic development. As shown for the first time, adipogenic stimulation of mastoid-derived PCs resulted in the formation of lipid droplets and expression of the adipogenic marker genes *aP2* and *APM1*. These results suggest MSC-like PCs from mastoid as candidates for therapy of complex skeletal defects. Copyright © 2008 John Wiley & Sons, Ltd.

Received 13 November 2007; Accepted 13 February 2008

Keywords human periosteal cells; mastoid; surface marker expression; multilineage potential; microarray; chondrogenesis; osteogenesis; adipogenesis; tissue engineering

1. Introduction

Regenerative medicine approaches using autologous cells for bone and cartilage repair have progressed 'from bench to bedside' (Brittberg *et al*., 1994; Ossendorf *et al*., 2007; Schmelzeisen *et al*., 2003). Stem cells have become very prominent candidates for such cell therapy approaches

*Correspondence to: J. Ringe, Tissue Engineering Laboratory, Department of Rheumatology, Charité-University Medicine Berlin, Tucholskystrasse 2, 10117 Berlin, Germany.
E-mail: jochen.ringe@charite.de
# These authors contributed equally to this work.

because of their pronounced expansion capacity, their remarkable plasticity and, therefore, their potential to regenerate complex tissue defects. For connective tissue repair, adult stem cells derived from sources such as adipose tissue (Zuk *et al*., 2001), synovial membrane (De Bari *et al*., 2003), fetal membranes (Soncini *et al*., 2007), trabecular bone (Noth *et al*., 2002) and bone marrow (Caplan *et al*., 1994; Reyes *et al*., 2002) have been extensively characterized but are not routinely used in the clinic so far. Despite many cell and molecular biological approaches characterizing mesenchymal stem cells (MSCs), their multilineage differentiation pathways, their immunoprivileged status and their *in vivo* applicability (Barry and Murphy, 2004), very limited clinical studies have been performed (Picinich *et al*., 2007). Factors such as failure of stable ectopic tissue formation, difficult stimulation and control of differentiation *in vivo* and occasionally insufficient cell survival after seeding on a construct and subsequent transplantation hamper their application.

In marked contrast to MSCs, periosteum as a whole or periosteum-derived progenitor cells (PCs) have been used in thousands of surgeries, e.g. as a covering layer to prevent chondrocyte leakage during autologous chondrocyte implantation (ACI; Brittberg *et al*., 1994), as a graft for reconstruction of the patellar articulation (Hoikka *et al*., 1990) and as tissue-engineered bone transplant for maxillary sinus floor augmentation (Schmelzeisen *et al*., 2003). Interestingly, in contrast to MSCs, PCs are barely characterized at the cellular and molecular level.

Periosteum plays a major role in bone growth, bone development, bone fracture healing and in cortical blood supply (Decker *et al*., 1996). The periosteum of endochondral bones (bones formed by endochondral ossification), and also of membranous bones (bones formed by intramembranous ossification in which bone formation is not preceded by cartilage formation in normal development) consists of two layers. The outer layer is fibrous, contains fibroblasts and abundant collagen fibres and provides attachment of muscles, tendons and ligaments to the bone. The inner cambial layer contains progenitor cells with osteogenic and chondrogenic capacity and is involved in bone healing (Buckwalter and Cooper, 1987; Nakahara *et al*., 1991; O'Driscoll and Fitzsimmons, 2001). Since the pioneer work in the 1990s of A. Caplan, who has investigated the osteogenic potential of PCs in the field of bone repair, the capacity of PCs to develop into bone and cartilage has been evaluated in several studies (De Bari *et al*., 2001; Fukumoto *et al*., 2003; Groger *et al*., 2003; Nakase *et al*., 1993).

On the basis of their osteochondrogenic developmental potential, PCs have often been named 'multipotent MSCs'. Really, although cells of osteogenic and chondrogenic lineages generally arise from mesenchymal stem cells (Marks and Popoff, 1988), it is unclear whether periosteum contains such stem cells. The fact that even periosteum from membranous bones expresses cartilage markers and differentiates into chondrocytes has been interpreted in different ways, based on distinct experimental results: chondrocytes may arise from bi- or multipotent stem cells, from a particular chondroprogenitor cell population, or from osteogenic cells (Fang and Hall, 1997). So far it has not been shown that PCs have the same potential as MSCs to form all varieties of connective tissue cells. For instance, their adipogenic development is not well characterized on the cellular and molecular level. Furthermore, very few data regarding the expression profile of embryonic, ectodermal, endodermal, mesodermal and MSC markers have been reported until now.

The aim of this study was to investigate the MSC-like character of human periosteal cells derived from periosteum of the endochondral mastoid bone. Harvesting of periosteum from mastoid bone is relatively easy and so, this tissue represents a good cell source for the isolation of PCs for regenerative medicine. We have studied their expansion capacity using clinically applied cell culture techniques (e.g. human serum), their stem cell-related surface marker expression profile applying genome-wide microarray and FACS analysis, their osteogenic and chondrogenic potential, and for the first time on the cellular and molecular level their time course of adipogenesis. We hypothesize, that periosteal cells in general and thus, also from the mastoid bone, are even more MSC-like then known so far and therefore, are very promising candidates for cell therapies of complex skeletal tissue defects such as osteochondral defects.

2. Materials and methods

2.1. Cell isolation and expansion

Periosteal autografts (0.5 cm^2) were harvested from the human mastoid of 15 patients undergoing mastoidectomy. PCs were isolated as described previously (Zheng *et al*., 2006). Briefly, periosteal tissues were rinsed with PBS (Biochrom, Berlin, Germany) and Hanks' solution (Biochrom), minced and digested in DMEM/Ham's F12 medium (Biochrom) containing 10 000 U/ml collagenase II (Biochrom), 10% human allogeneic serum (German Red Cross, Berlin) and 1% antibiotic–antimycotic solution (Biochrom). The tissues were digested for 3 h at 37 °C and the cells were subsequently harvested, resuspended in DMEM/Ham's F12 medium containing 10% human allogeneic serum, plated and allowed to attach for 4–10 days. Adherent growing PCs were cultured under standard cell culture conditions and the medium was replaced every 2–3 days. On reaching 90% confluence, the PCs were subcultured by treatment with 0.5% trypsin–EDTA (Biochrom) and subsequently replated at a density of 6000 cells/cm^2. The study was approved by the ethical committee of the Charité-University Medicine Berlin.

DOI: 10.1002/term

2.2. Genome-wide gene expression profiling

To analyse the expression of marker genes, total RNA from passage 2 (P2) PC cultures ($n = 6$) was isolated as described previously (Chomczynski, 1993) and was used for genome-wide microarray analysis with the Affymetrix HG-U133 plus 2.0 array (Affymetrix, Santa Clara, USA) according to the manufacturer's recommendations. Briefly, cDNA was synthesized from 1 µg total RNA and submitted to *in vitro* transcription (ENZO Biochem, New York, USA) to generate biotin-labelled complementary RNA (cRNA); 15 µg of fragmented cRNA were hybridized to gene chips for 16 h at 45 °C. The gene chips were washed and stained as recommended (fluidics station) and scanned using the GeneArray scanner. Affymetrix GCOS 1.4 software was used to generate DAT, CEL and EXP files and to process and normalize the raw data for signal and detection call calculation. We were exclusively interested in genes that were already published as stem cell markers. Out of the huge number of such candidates, we selected those genes whose detection call was 'present' or 'absent' on all six microarrays (100% 'present' or 100% 'absent'). From these selected candidates the mean signal values and mean standard deviations (SD) of the signal values were calculated.

2.3. Flow cytometric analysis (FACS)

Single cell suspensions of PCs ($n = 3$, P4) were washed in PBS/0.5% BSA. The cells were incubated with titrated primary staining reagents for 15 min on ice (2.5×10^5 cells/0.1 ml in PBS/0.5% BSA). Primary staining reagent unlabelled monoclonal mouse anti-human SH2 (CD105) was provided by the German Rheumatism Research Center. Fluorescein isothiocyanate (FITC)-labelled mouse anti-human CD44, CD45 and CD90, and R-phycoerythrin (PE)-labelled mouse anti-human SH3 (CD73), CD14 and ALCAM (CD166) were purchased from Pharmingen (Heidelberg, Germany). Monoclonal mouse anti-human CD34 labelled to PE was purchased from Miltenyi (Bergisch Gladbach, Germany). FITC-labelled monoclonal mouse anti-human Stro-1 was a kind gift from Dr Pierre Charbord (Inserm, Tours, France). For double staining with SH2, the cells were incubated with unlabelled SH2, washed with PBS/0.5% BSA and incubated with biotinylated goat anti-mouse IgG for 10 min on ice. After washing again and incubation on ice with streptavidin coupled to cytochrome 5 (Cy5) for an additional 10 min, the cells were washed again and finally incubated with PE or FITC antibodies specific for a second surface molecule, such as SH3, CD14, CD34, CD44, CD45 CD90 or CD166. Prior to analysis in a LSR I cytometer (Heidelberg), the cell samples were washed. Dead cells and debris were stained with PI (Sigma, Taufkirchen, Germany) and excluded. Data were evaluated using CellQuest software (Becton-Dickinson).

2.4. Cell differentiation studies

The differentiation potential of human PCs ($n = 3$, P4) was demonstrated by culturing these cells under conditions that promote mesenchymal stem cell differentiation (Pittenger *et al.*, 1999). To induce osteogenesis, 6000 PCs/cm^2 were seeded and induced in DMEM/Ham's F12 (5% human serum) containing 100 nM dexamethasone (Dex; Sigma), 0.05 mM L-ascorbic acid 2-phosphate (AsAP; Sigma), 10 mM β-glycerophosphate (Sigma) and 1.5 mM calcium chloride dihydrate (Sigma). For chondrogenesis, PCs were centrifuged to form a micromass and were cultured in a defined medium consisting of DMEM (Biochrom), ITS+1 (Sigma), 100 nM Dex, 0.17 mM AsAP and 10 ng/ml transforming growth factor-β1 (TGFβ1) or TGFβ3 (R&D Systems, Wiesbaden, Germany). To induce adipogenesis the PCs were treated with induction medium, consisting of DMEM supplemented with 10% human serum, 1 µM Dex, 0.2 mM indomethacin (Sigma), 10 µg/ml insulin (Novo Nordisk, Mainz, Germany), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), and maintenance medium containing DMEM, human serum and 10 µg/ml insulin, within three cycles (3 days induction, 2 days maintenance).

2.5. Polymerase chain reaction

To demonstrate osteogenesis and adipogenesis at the mRNA level, total RNA was isolated (Chomczynski, 1993). Subsequently, 5 µg total RNA were reverse-transcribed after annealing with 500 ng oligo-(dT) primers (Gibco, Karlsruhe, Germany) and 5 U Superscript reverse transcriptase (Gibco) in 70 µl reaction mixture. The housekeeping gene *GAPDH* was used to normalize marker gene expression in each sample in different concentrations. Real-time PCR applying the BioRad iCycler (BioRad, Munich, Germany) was performed with 1 µl cDNA sample, using the SYBR Green PCR Core Kit (Applied Biosystems, Darmstadt, Germany). Relative quantitation of marker genes (Table 1) was performed as described (ABI Prism 7700, 1997) and the results given as percentages of the *GAPDH* product.

2.6. Histological methods and immunohistochemistry

During osteogenesis, cells express high levels of alkaline phosphatase, which was visualized by staining with Sigma fast BCIP/NBT (Sigma). von Kossa staining demonstrated the deposition of a bone-specific mineralized matrix. Chondrogenesis was documented by histological staining of cartilage proteoglycans with Alcian blue 8GS (Roth, Karlsruhe) as well as by immunohistochemical methods using the EnVision™ + System, Peroxidase Mouse Kit (Dako, Hamburg, Germany). Cryosections (6 µm) were incubated for 1 h with primary antibodies (rabbit anti-human types I and II collagen; DPC Biermann,

DOI: 10.1002/term

Table 1. Oligonucleotide sequences

Gene	Accession No.	Oligonucleotides (5′ → 3′) (up/down)	Product size (bp)
GAPDH	XM_006959	GGC GAT GCT GGC GCT GAG TAC TGG TCC ACA CCC ATG ACG A	149
Collagen type Ia1	NM_000088	CGA TGG CTG CAC GAG TCA CAC CAG GTT GGG ATG GAG GGA GTT TAC	180
Osteocalcin	X_51699	GAG CCC CAG TTC CCC TAC CC GCC TCC TGA AAG CCG ATG TG	103
aP2	NM_000134	CCT TAG ATG GGG GTG TCC TGG TA AAT GTC CCT TGG CTT ATG CTC TC	156
APM1	NM_004797	GGC CTG CCC AGC TCT CGT AT CTC TCC TCT TTG GGC ATC ACC	83

bp, base pairs.

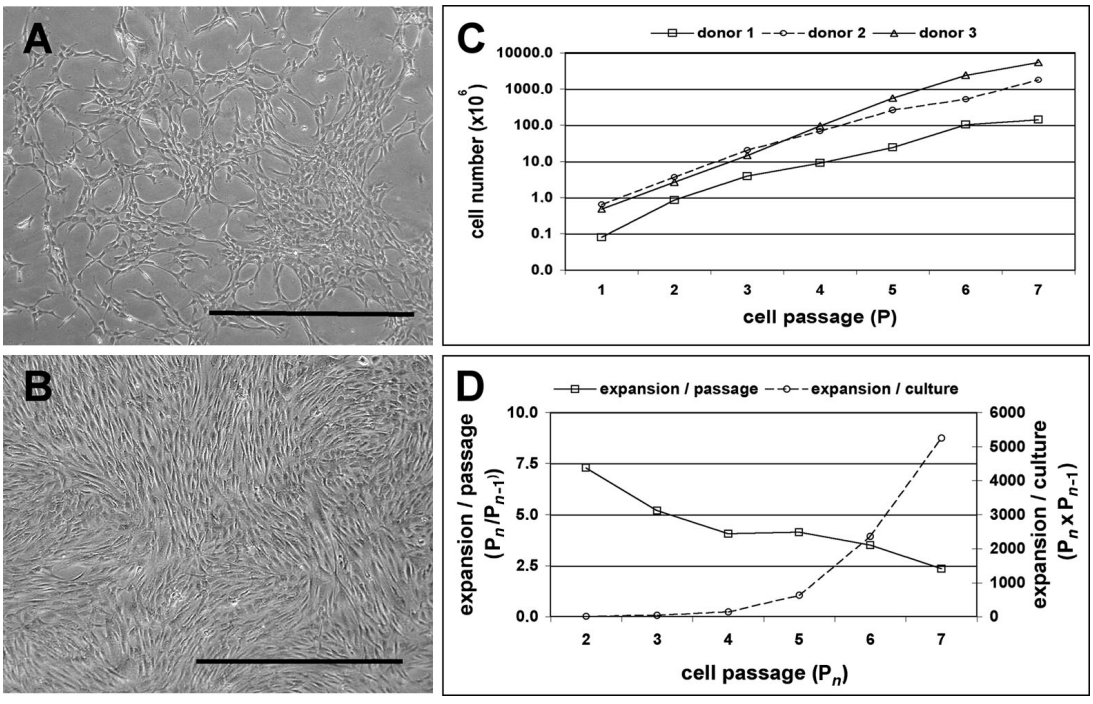

Figure 1. Morphological appearance and expansion capacity of human mastoid PCs. (A) By day 14, human mastoid-derived periosteal cells (PCs) showed a fibroblast-like morphology when cultured in medium supplemented with 10% human allogenic serum. (B) During subculture, the PCs appeared slightly larger and had a more stretched morphology. (C) Although donor-dependent, primary cultures starting with <100 cells could be expanded to >2.5 x 10^9 cells in P7. (D) During *in vitro* expansion the proliferative activity of PCs (cell passage P_n/cell passage P_{n-1}) decreased. The expansion factor of PCs (cell passage P_n x cell passage P_{n-1}) was 5200. Original magnifications: (A, B) x100

Bad Nauheim, Germany). Subsequently, samples were treated according to the manufacturer's protocol and counterstained with haematoxylin (Merck, Darmstadt). Adipocytes were identified by staining with oil red O (Sigma).

3. Results and discussion

3.1. Culture of human mastoid-derived periosteal cells in human serum

Human periosteal cells were isolated from the mastoid bone and expanded in medium containing allogenic human serum. Within 5 days, PCs attached to the culture plates. By day 14, the PCs became adherent and round-shaped erythrocytes were removed by exchange of medium (Figure 1A). Primary PCs exhibited an MSC-like fibroblastic morphology. In higher cell passages, the PCs appeared slightly larger (Figure 1B). To investigate their expansion potential, the PCs were cultured up to passage 7 (P7) and cell numbers (mean of $n = 3$ donors) were calculated in each cell passage following subconfluence (Figure 1C). Single primary cultures starting with <100 adherent cells and expanded up to 4×10^5 cells at subconfluence (P1), could be expanded to over 13×10^6 PCs in P3, and 2.5×10^9 cells in P7 (Figure 1C, D). Dividing the number of PCs obtained in passage P_n through those obtained in P_{n-1} (expansion/passage; Figure 1D) demonstrates a decreasing proliferative activity during expansion. Because of their high expansion capacity, PCs represent promising candidates for the regeneration of

DOI: 10.1002/term

large defects. In our own studies, we have shown that the osteogenic potential of PCs is reproducibly stable until cell passage 6 (data not shown). In addition, in *in vitro* (Zheng *et al.*, 2006) and *in vivo* (Schmelzeisen *et al.*, 2003) studies, we observed that bone transplants of 0.5 cm^2 containing 2.5×10^6 PCs are suitable for the regeneration of bone defects. From 0.2 cm^2 small biopsies, at P4 we isolated about 60×10^6 PCs, at P5 280×10^6. Extrapolated, after P4 theoretically a defect area of 12 cm^2 and after P5 of 56 cm^2 might be filled. However, vascularization of the repair tissue has to be assured, which is still a main problem in most approaches regarding the regeneration of large defects. As expected for primary cells, the number of PCs obtained strongly varied from donor to donor. Circumstances such as the periosteum harvesting procedure, cell isolation by enzymatic digestion and donor age and state influence the number and also the developmental potential of PCs and other mesenchymal cells (Brownlow *et al.*, 2000; O'Driscoll *et al.*, 1999). From about 15% of the biopsies, no cells could be isolated. This may be partly due to the remaining PCs containing cambial layer on the bone during mastoidectomy. Enhanced techniques for tissue harvest, such as hydraulic elevation (Marini *et al.*, 2004) or the use of a raspatory, ensures the harvest of the cambial layer. Nevertheless, P7 mastoid-derived PCs showed a 6000-fold increased cell number compared to primary cultures. Interestingly, for human bone marrow-derived MSCs, a similar expansion potential has been reported (Haynesworth *et al.*, 1992). In contrast, Agata *et al.* (2007) reported a significantly higher proliferation potential of mandibular-derived PCs than that of MSCs. However, the average age of the periosteum donors was 20 years and of the bone marrow donors 37 years, indicating an age-related effect. A progressive age-associated decline in the growth rate of PCs is known from other studies (De Bari *et al.*, 2006). Regarding cell therapy applications, besides their impressing expansion capacity it is important that PCs, as shown in our study, grow in allogenic human serum, maintaining their developmental capacity. In contrast, MSCs adherence and growth strongly depends on proper serum batches (Lennon *et al.*, 1996).

3.2. Mastoid PCs express mesenchymal stem cell-related surface marker

Genome-wide Affymetrix HG-U133 plus 2.0 array analysis was performed ($n = 6$ donors) to investigate whether PCs express early embryonic, ectodermal, endodermal and mesodermal genes and haematopoietic and mesenchymal stem cell markers. In summary (see Table 2 and Supplementary Material), they do express embryonic stem cell marker genes such as *CD9* (100% detected as present), but do not express the most prominent early markers, such as the transcription factor *Oct4/POU5F1, NANOG, SSEA4/SIAT6* and *TERT* (100% detected as absent). In contrast, subpopulations of human bone marrow MSCs express *Oct4/Pou5F1, TERT* and distinct other ES genes (Pochampally *et al.*, 2004), indicating a more 'primitive' character of MSCs. MSCs obtained from amniotic fluid also express *Oct4/Pou5F1* (Tsai *et al.*, 2004) and human term placenta-derived MSCs express *SSEA4/SIAT6* (Yen *et al.*, 2005). Like human MSCs (Tremain *et al.*, 2001), PCs also express marker genes of cells present in the three germ layers. This implies a broad developmental potential of mastoid-derived PCs. Nevertheless, most of the prominent ectodermal,

Table 2. Marker genes expressed by human PCs (microarray data, $n = 6$ donors)

Marker genes expressed by	PC expression profile
Pluripotent embryonic stem cells (ES)	*Present:* CD9, COMMD3, DIAPH2, GJA1, IFITM1, IFITM2, IL6ST, NUMB, PTEN, sFRP2
	Absent: CLDN6, CFC1, FGF4, GABRB3, GDF3, Lefty1, Lefty2, LIN28, NANOG, NR5A2, Oct-4/POU5F1, REX1, SOX1, SSEA4/SIAT6, TERT, UTF1
Ectoderm	*Present:* CRABP2, nestin, TUBB3, vimentin
	Absent: ISL1, MAP2, MSI1, NeuroD1, Olig2, PAX6, SOX18, synaptophysin
Endoderm	*Present:* decorin, fibronectin1, GATA6, laminin-γ1, PDHX, Smad2
	Absent: α-fetoprotein, FGF8, FOXA2, GATA4, glucagon, HHEX, HNF4A, IAPP, insulin, laminin-α1, nodal, Otx2, PAX4, SOX17, TAT, Wnt3
Mesoderm	*Present:* Col I, MSX1, CBFA1
	Absent: α-actin, Col II, desmin, haemoglobin-β, haemoglobin-ξ, HLXB9, NPPA, WT1
Multipotent haematopoietic stem cells (HSCs)	*Absent:* CD14, CD34, CD45, CDCP1, ITGAL, ITGAM
Multipotent mesenchymal stem cells (MSCs)	*Present:* Extracellular matrix components: Col-I, Col-III, Col-IV, Col-V, Col-VI, Col-VII, Col-VIII, Col-XI, Col-XII, Col-XIII, Col-XIV, Col-XVI, perlecan, versican, syndecan, hyaluronan and proteoglycans, link protein 3, fibronectin, laminin Growth factors and cytokines: IL-6, IL-7, IL-8, M-CSF, LIF, SCF, TGFβ1, TGFβ2 Matrix receptors: ICAM3, VCAM1, LFA3 Growth factors and cytokine receptors: IL-1R, IL-9R, IL-10R, IL-11R, IL-13R, PDGFR, TNFRSF-1, TNFRSF-10, TNFRSF-11, TNFRSF-12, FAS, TGFβ1R, TGFβ2R, TGFβ3R, IFNγR1, IFNγR 2, EGFR Surface antigens: CD44, CD58, CD71, CD73, CD90, CD105, CD166, HLA-ABC Integrins: α4, α5, α7, α10, β1, β3, β5 chains

Further details of ES, ectodermal, endodermal, mesodermal, HSC and MSC expression can be found, for example, in the following references: ES, ectodermal, endodermal, mesodermal: Adewumi *et al.*, 2007; Brandenberger *et al.*, 2004; Chiu and Rao, 2003; Ginis *et al.*, 2004: HSCs, Wognum *et al.*, 2003; MSC: Barry and Murphy, 2004; Haynesworth *et al.*, 1996; Majumdar *et al.*, 1998; Pittenger *et al.*, 1999.

DOI: 10.1002/term

endodermal and mesodermal marker genes could not be detected. PCs showed no expression of typical haematopoietic stem cell surface antigens such as *CD11a*, *CD11b* and the early haematopoietic stem cell marker *CD34*. This is in concordance with the expression profile of these markers by MSCs (Pittenger *et al*., 1999). Moreover, PCs showed a similar but not identical expression of marker genes also expressed by mesenchymal stem cells (see Table 2 and Supplementary Material): they express a broad pattern of: (a) cell surface antigens, such as the hyaluronan receptor *CD44*, the transferrin receptor *CD71*, the ecto-5′-nucleotidase (*SH3*, *CD73*), the Thy-1 cell surface antigen *CD90*, the TGFβ1 and -β3 receptor endoglin (*SH2*, *CD105*) and the activated leukocyte cell adhesion molecule (*ALCAM*, *CD166*); (b) growth factors, cytokines and their receptors; (c) extracellular matrix components, such as collagens, fibronectin and laminin; and (4) integrins. However, compared with MSCs, PCs clearly expresses fewer interleukins, integrins, growth factors and their receptors. The expression of such molecules underlines the role of MSCs in the bone marrow for formation and function of the stromal microenvironment, which produces inductive and regulatory signals not only for MSCs but also for the development of haematopoietic progenitors and other non-mesenchymal stromal cells (Majumdar *et al*., 1998). This implies a more restricted function of PCs limited to bone remodelling and repair. Interestingly, both PCs and MSCs express CD44, a receptor that plays an important role in the organization of the extracellular matrix in bone marrow or in bone, respectively (Yamazaki *et al*., 1999).

To verify some of the microarray data and to further characterize mastoid-derived PCs with respect to their MSCs resemblance, cells were FACS analysed ($n = 3$ donors) for the presentation of typical human MSC marker epitopes (Pittenger *et al*., 1999). Analysis of P4 PCs showed a homogeneous cell population (Figure 2). They were uniformly negative for cell epitopes of the lipopolysaccharid receptor CD14 (Figure 2A), the leukocyte common antigen CD45 (Figure 2A, D), and CD34 (Figure 2B). In addition, they were negative for Stro-1 (95%) (Figure 2F). In contrast, periosteal cells showed a high presentation of the MSC markers CD44 (hyaluronan receptor; Figure 2C), CD73 (SH3, ecto-5′-nucleotidase; Figure 2E, F), CD90 (Thy-1; Figure 2G), CD166 (ALCAM; Figure 2H) and CD105 (SH2; Figure 2B–E, G, H). A similar FACS profile has also been reported for periosteal cells, e.g. derived from periosteum of the distal insertion of a dissected semitendinosus tendon (Sakaguchi *et al*., 2005), for mesenchymal progenitor cells derived from trabecular bone (Tuli *et al*., 2003), from term placenta (Yen *et al*., 2005) and from adipose tissue (Lee *et al*., 2004). The frequency of Stro-1, originally suggested as marker for human MSCs (Gronthos *et al*., 1999), was <5%. Low PC expression of this marker was shown in one current investigation (Sakaguchi *et al*., 2005) and was also observed in some studies using human MSCs (Colter *et al*., 2001). However, Stro-1 antigen is progressively lost during cell culture (Stewart *et al*., 1999).

3.3. Multilineage potential: osteogenic and chondrogenic development

To address the question of whether mastoid-derived periosteal cells, like bone marrow MSCs, can develop into the osteochondrogenic and adipogenic lineage, PCs ($n = 3$, P4) were cultured under conditions that were favourable for osteogenic, chondrogenic and adipogenic development of human MSCs (Pittenger *et al*., 1999). Osteogenesis was documented by visualization of alkaline phosphatase (AP) activity (Figure 3A, C, E), by matrix deposition of calcium (Figure 3B, D, F) and by real-time PCR analysis (Figure 4). During osteogenic stimulation, the cells remained confluent and formed multilayer clusters of mineralized matrix. AP and von Kossa staining of induced PCs demonstrated AP activity and an early

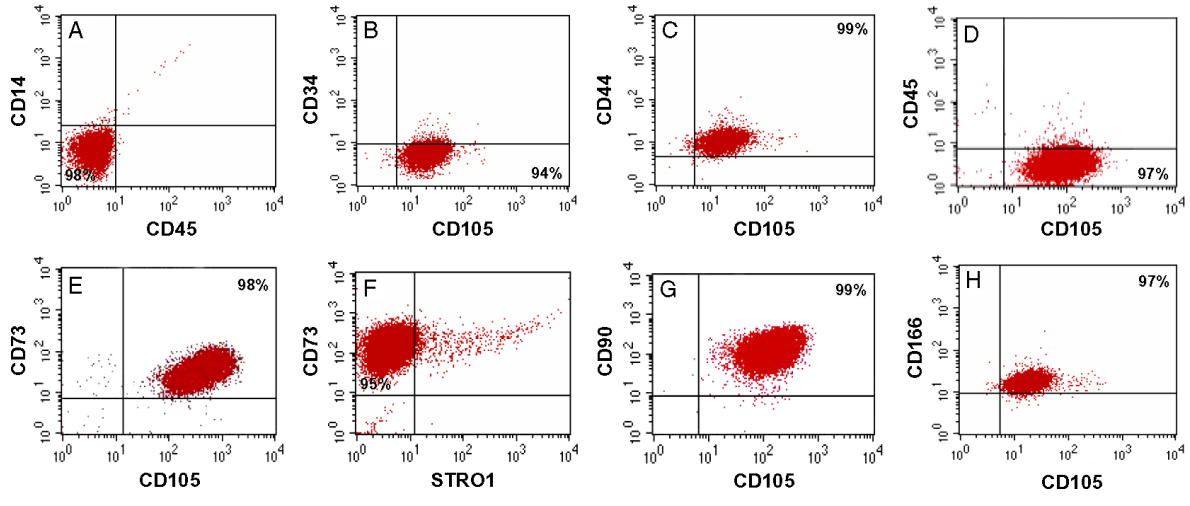

Figure 2. Flow cytometric analysis of human mastoid-derived periosteal cells. (A–H) Analysis of surface marker routinely used for the characterization of human bone marrow-derived mesenchymal stem cells (MSCs) demonstrated that, similar to MSCs, human mastoid-derived PCs were negative for reactivity to antigens CD14, CD34 and CD45 but positive for reactivity to antigens SH2 (CD105), CD44, SH3 (CD73), CD90 and ALCAM (CD166). Unlike MSCs, PCs were more or less negative for Stro-1

DOI: 10.1002/term

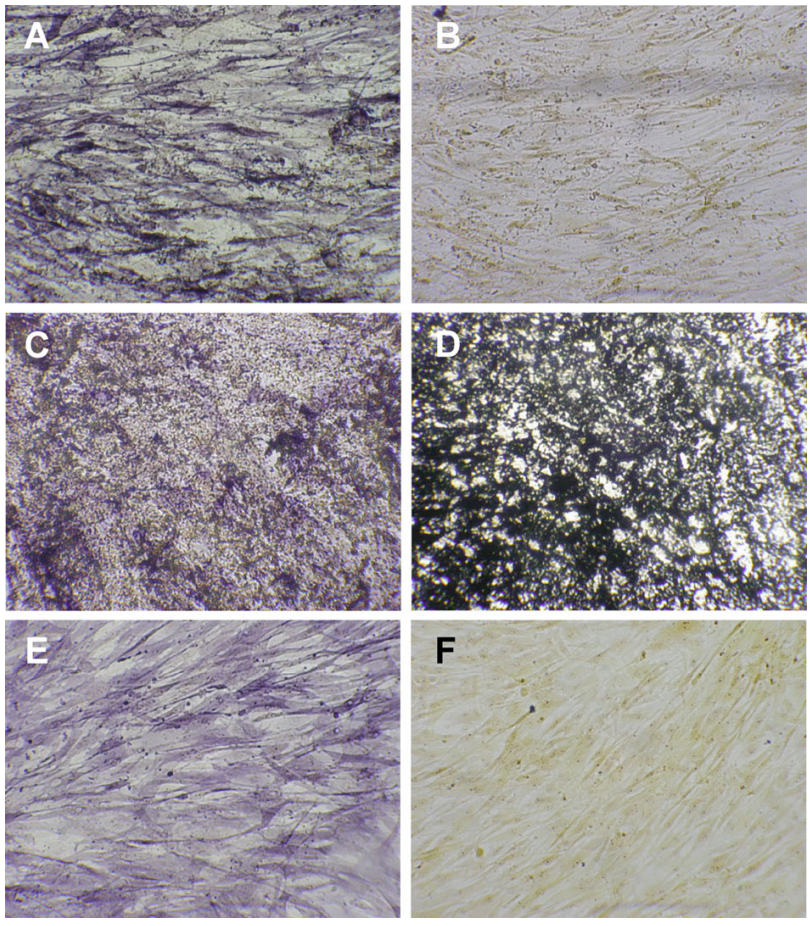

Figure 3. Histochemical analysis of human PCs undergoing osteogenic differentiation. (A, C, E) Visualization of alkaline phosphatase activity (AP) in cultures of osteogenic-induced human PCs. (B, D, F) von Kossa staining of mineralized bone tissue. (A) By day 3, AP staining demonstrated AP activity and (B) von Kossa staining an early formation of a mineralized bone matrix. (C) By day 9, the AP staining was partially overlaid with bone matrix and (D) almost the whole cell layer was covered with a calcified matrix. (E) Control cultures showed a stable amount of AP expression over the whole culture period and (F) no formation of a mineralized bone matrix. Original magnifications: (A–E) ×100

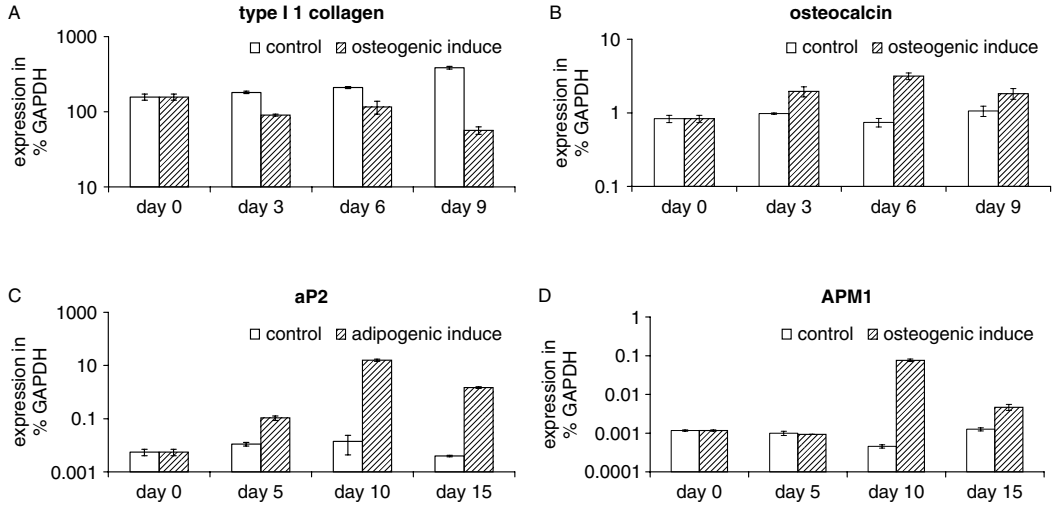

Figure 4. Gene expression analysis of osteogenic and adipogenic marker genes. The expression of osteogenic marker genes (A) *type I collagen* and (B) *osteocalcin* was analysed to show osteogenic differentiation. Adipogenic differentiation was assessed by gene expression of (C) *aP2* and (D) *APM1*. The expression of marker genes was calculated as the percentage of the expression of the housekeeping gene *GAPDH*. The mean of each triplicate well is plotted and the error bars represent SD

DOI: 10.1002/term

production of a calcified bone matrix starting at day 3 in small layering spots (Figure 3A, B), which formed increasing nodules at day 6 (data not shown). At day 9, the AP staining (Figure 3C) was partially overlaid with bone matrix. At this time, almost the whole cell layer of PCs was heavily covered with mineralized matrix (Figure 3D). In contrast to induced PCs, untreated control cultures showed a stable amount of AP-positive cells over the whole culture period and no deposition of mineralized bone matrix (Figure 3E, F). Gene expression analysis was performed for the characteristic osteogenic marker genes *type Ia1 collagen* and *osteocalcin* (Figure 4). The relative expression level was calculated as a percentage of the expression of the housekeeping gene *GAPDH*. Over the culture period, expression of *type Ia1 collagen* increased in the controls and decreased upon osteogenic induction (Figure 4A). Already on day 3, induced and non-induced cultures showed different *osteocalcin* expression patterns. While *osteocalcin* was induced on day 6 up to 4-fold and on day 9 up to 2.5-fold in the osteogenic stimulated cells, the controls showed a nearly stable level (Figure 4B). An osteogenic development of PCs has often been described. However, in contrast to our observations using human MSCs (data not shown), it has been reported that bone marrow MSCs are more osteogenic than PCs (Agata *et al*., 2007). Factors like different donors and cell isolation protocols may explain these differences. Many molecular data are known regarding the determination MSCs into the osteogenic lineage and their subsequent maturation to bone. For instance, the transcriptional factors Runx2 and Osterix are required for osteoblast differentiation during both endochondral and intramembranous bone formation (Kobayashi and Kronenberg, 2005). Interestingly, despite endochondral and intramembranous differentiation pathways generally having been thought to be two different and not overlapping processes, a cartilage formation during fracture healing of membranous bone and a chondrogenic potential of PCs from membranous bones have been reported (Nah *et al*., 2000).

In the present study, chondrogenic development of PCs was induced with TGFβ1 (data not shown) and TGFβ3 (Figure 5). Chondrogenesis was documented by Alcian blue staining of cartilage proteoglycans and by immunohistochemistry using type I and type II collagen-specific antibodies (Figure 5). PCs exposed to both TGFs displayed a compact and homogeneous pellet structure. Alcian blue staining showed an increasing deposition of proteoglycans, with a homogeneous distribution by day 21 within the whole section of the pellet culture (Figure 5A). Staining of cartilage-specific type II collagen was not detectable at day 7, but displayed an increasing amount of cartilage-specific collagen at day 21 (Figure 5B). At that time, a concentration of type II collagen in the border areas of the pellets was observed (data not shown), whereas the central part of the pellet cultures stained almost negative. Instead, type I collagen was concentrated in the central parts of the pellets (data not shown). Control PCs exhibited a fibrous pellet structure with marginal staining of Alcian blue (Figure 5C) and no type II collagen staining (Figure 5D). Distinct possibilities have been proposed to explain the origin of chondrocytes appearing in membrane bones (Fang and Hall, 1997): chondrocytes may develop from stem cells, from particular chondroprogenitor cells or osteogenic cells. However, all these possibilities have pros and cons. In a recent study,

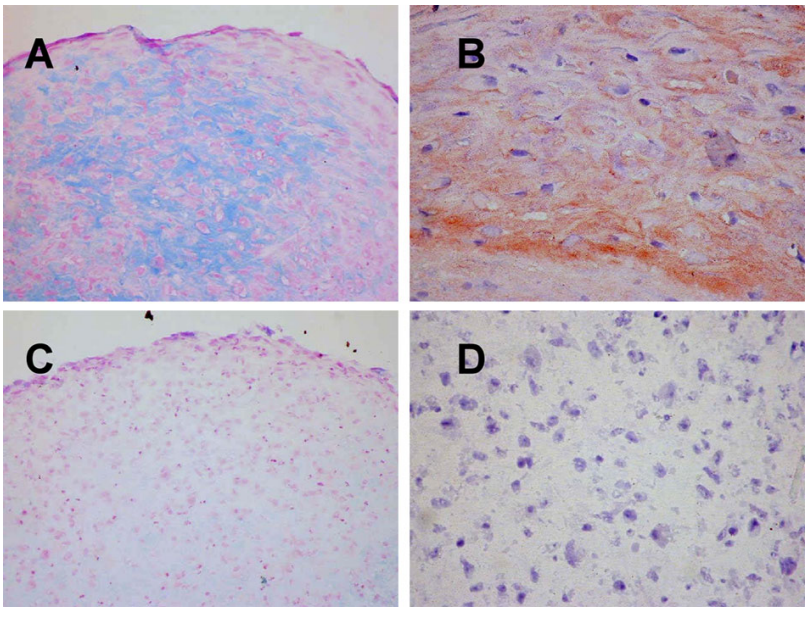

Figure 5. Histological analysis of PCs undergoing chondrogenic differentiation. Transforming growth factor-β3 induced PCs displayed a compact round pellet structure. (A) By day 21, Alcian blue staining showed a homogeneous distribution of cartilage proteoglycans over the pellets. (B) At that time, antibody staining of cartilage-specific type II collagen demonstrated a type II collagen expression focused on the border areas. (C, D) Control PCs exhibited a fibrous structure, with marginal staining of proteoglycans and no staining of cartilage-specific type II collagen. Original magnifications: (A, C) ×200; (B, D) ×400

DOI: 10.1002/term

Nah and colleagues have proposed a further explanation, that the normal intramembranous pathway includes a so-far unrecognized transient chondrogenic phase, and that cells in this phase retain their chondrogenic potential that can be expressed in specific *in vitro* and *in vivo* environments (Nah *et al.*, 2000). On the other hand, based on data of a rib fracture model, it has been reported that periosteal osteoblasts have the potential to differentiate into both osteogenic and chondrogenic lineages and that osteocytes play an important role in these processes (Li *et al.*, 2004).

3.4. Human mastoid-derived PCs also differentiate into adipocytes

An adipogenic potential of mastoid bone-derived PCs has not so far been documented. However, the adipogenic capacity of PCs isolated from other periosteal tissues was shown recently, based on histological oil red O staining (Sakaguchi *et al.*, 2005) and aP2 mRNA expression (De Bari *et al.*, 2006). In the present study, to our knowledge for the first time, the time course of adipogenesis of PCs was analysed on the cellular and molecular level. Adipocytes were easily identified by cell morphology, by oil red O staining (Figure 6), and by real-time PCR mRNA expression analysis (Figure 4). During the early phases of adipogenesis, PCs changed their morphology from regularly stretched cells to a disordered monolayer culture. By day 5, the cells started to form lipid droplets (Figure 6A), which increased in number, constituting almost 50% of all cells by day 10 (Figure 6B). By days 10 and 15, the lipid droplets accumulated, which was demonstrated by oil red O staining (Figure 6B, C). Non-adipogenic controls displayed only a negligible amount of lipid droplets (Figure 6D). Gene expression analysis of the adipogenic marker genes adipocyte-specific fatty acid-binding protein 2 (aP2), and adipose most abundant gene transcript 1 (APM1) also documented the potential of PCs to undergo adipogenic development (Figure 4C, D). aP2 was induced up to 2850-fold, showing a maximum at day 10 (Figure 4C), and APM1 showed an about 60-fold induction (Figure 4D). In controls, expression of both adipogenic genes was stable during the whole culture period. As described earlier (De Bari *et al.*, 2006), no apparent influence of the donor age on adipogenesis was observed. Adipogenesis can be viewed as proceeding along a two-step path in which MSCs are first determined to become pre-adipocytes and then differentiated into mature adipocytes. The factors involved in the determination of the multipotent cell to the adipogenic lineage are still unknown. Cell–cell, cell–matrix and hormonal interactions are suggested to be important in early adipogenesis. Several markers are known to be involved in the development of pre-adipocytes to adipocytes. Of particular interest are transcription factors of the CCAAT/enhancer binding protein (C/EBP) family, such as C/EPBβ and C/EPBδ (Yeh *et al.*, 1995). These factors induce C/EPBα and the nuclear hormone receptor PPARγ. C/EPBα is sufficient to drive the whole programme of adipogenesis in pre-adipocyte *in vitro* models, and PPARγ plays a crucial role in the function of several fat cell-selective genes, such as *aP2*, and plays a dominant role in the induction and regulation of terminal differentiation of pre-adipocytes into adipocytes (Wu *et al.*, 1995). It is also known that

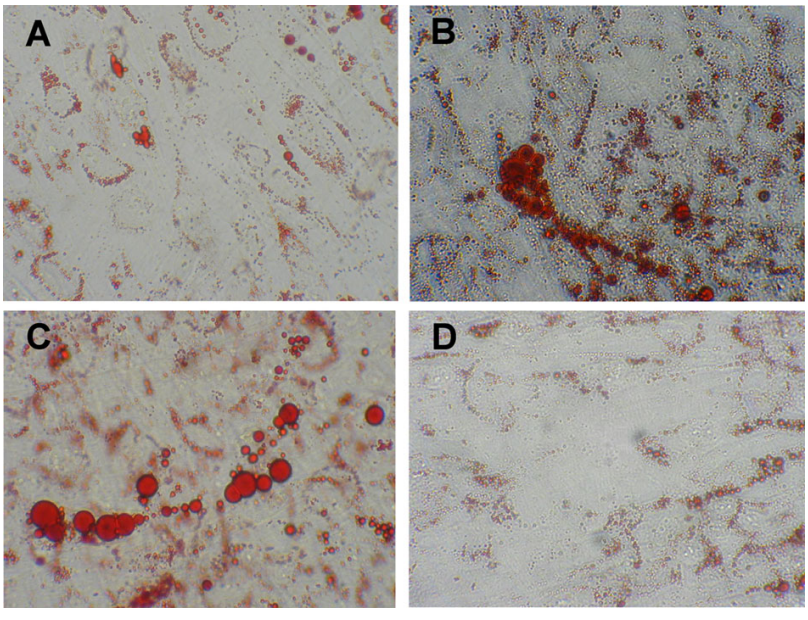

Figure 6. Morphological appearance and oil red O staining of human mastoid bone-derived PCs undergoing adipogenic differentiation. Adipocytes were identified by their cell morphology and by oil red O staining of lipid droplets. During adipogenesis, human PCs changed their morphology from regular stretched cells to a disordered monolayer culture. (A) They started filling with lipid droplets by day 5; (B) which increased in number at day 10; (C) and accumulated at day 15. (D) Controls showed only a negligible development of lipid droplets. Original magnifications: (A–D) ×400

DOI: 10.1002/term

PPARγ insufficiency enhances osteogenesis by stimulating osteogenic development of MSCs, and that the activation of PPARγ inhibits Runx2 expression and osteoblast differentiation, indicating that the expression level of PPARγ and Runx2 is involved in the regulation of balance between osteo- and adipogenesis (Lecka-Czernik *et al*., 1999). C/EPBβ is also involved in the regulation of balance between osteo- and adipogenesis, since it promotes osteogenesis in cooperation with Runx2 (Hata *et al*., 2005).

Although we have shown the multilineage potential of mastoid-derived PCs, it is still not proven that really multipotent stem cells exist in the periosteum. This question has to be addressed using clonal assays. In an initial study, seven PCs clones were prepared from four donors by limiting dilution and showed a multilineage (osteogenic, chondrogenic, adipogenic, myogenic) potential (De Bari *et al*., 2006). For MSCs, such assays have been performed and resulted in inconsistent data. One model for stem cell differentiation is that lineage progenitor cells are directly derived from MSCs (Caplan, 1994). According to this model, one would expect to see differentiation into a random combination of phenotypes upon stimulation of clonal MSCs. To test this model, Muraglia *et al*. (2000) analysed 185 non-immortalized human MSC clones and found that 184 clones differentiated into the osteogenic lineage, about 80% showed an osteo-chondrogenic potential, 30% developed in all three lineages, and no clone had a differentiation potential limited to the adipo-osteogenic, adipo-chondrogenic, adipogenic or chondrogenic phenotype. These data suggest the existence of a hierarchy in the MSC differentiation pathway, in which the adipogenic lineage diverges and becomes independent earlier than osteo-chondrogenic lineages, which proceed together and divide later. In contrast, clonal analysis of immortalized human MSCs has resulted in all kinds of clones except of adipo-chondrogenic phenotypes, indicating the problems but also the necessity of such studies (Okamoto *et al*., 2002).

4. Conclusions

Our data demonstrate a mesenchymal stem cell-like growth behaviour of human mastoid bone-derived periosteal cells and, on the basis of oligonucleotide microarray and FACS technology, for the first time the expression of embryonic, ectodermal, endodermal and mesodermal marker genes. They do not express markers of haematopoietic stem cells but express several MSC markers. Moreover, and to the best of our knowledge also for the first time, at the cellular and molecular level we showed the time course of adipogenesis of these cells. Regarding the clinical relevance, experiments were performed in medium containing human serum. These results clearly support our hypothesis that mastoid-derived PCs show an MSC-like character and strongly enhance the basic knowledge about PCs in general. Therefore, our data indicate a comparable cell therapy capacity of mastoid-derived PCs and periosteal cells derived from sources, such as mandible or tibia. Since biopsy of periosteum from mastoid bone is relatively easy, the results encourage the use of mastoid-derived PCs within the scope of advanced tissue-engineering applications, such as the treatment of complex skeletal tissue defects.

Acknowledgements

The authors would like to thank Johanna Golla, Samuel Vetterlein and Anja Wachtel for excellent technical assistance. This work was supported by grants from the Investitionsbank Berlin (IBB) and the European Regional Development Fund (Grant Nos 10020666 and 10128098), and the 6th European Union Framework Programme (GENOSTEM consortium, Grant No. LSHB-CT-2003-503161). C.K. and K.N. work for TransTissue Technologies GmbH. TransTissue works in the field of cell-based bone and cartilage repair. In this regard, the company has developed bone transplants for augmentation of the edentulous posterior maxilla based on mandibular-derived periosteal cells. J.R. and M.S. work as consultants for TransTissue Technologies.

Supplementary material

The supplementary electronic material for this paper is available in Wiley InterScience at: http://www.interscience.wiley.com/jpages/1932-6254/suppmat/term.75.html

References

ABI Prism. 1997; User Bulletin No. 2, ABI Prism 7700 Sequence Detection System. Applied Biosystems: 1–36.

Adewumi O, Aflatoonian B, Ahrlund-Richter L, *et al*. 2007; Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol* **25**(7): 803–816.

Agata H, Asahina I, Yamazaki Y, *et al*. 2007; Effective bone engineering with periosteum-derived cells. *J Dent Res* **86**(1): 79–83.

Barry FP, Murphy JM. 2004; Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol* **36**(4): 568–584.

Bhattacharya B, Miura T, Brandenberger R, *et al*. 2004; Gene expression in human embryonic stem cell lines: unique molecular signature. *Blood* **103**(8): 2956–2964.

Brittberg M, Lindahl A, Nilsson A, *et al*. 1994; Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med* **331**(14): 889–895.

Brownlow HC, Reed A, Joyner C, *et al*. 2000; Anatomical effects of periosteal elevation. *J Orthop Res* **18**(3): 500–502.

Buckwalter JA, Cooper RR. 1987; Bone structure and function. *Instr Course Lect* **36**: 27–48.

Caplan AI. 1994; The mesengenic process. *Clin Plast Surg* **21**(3): 429–435.

Chomczynski P. 1993; A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* **15**(3): 532–534, 536–537.

Colter DC, Sekiya I, Prockop DJ. 2001; Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. *Proc Natl Acad Sci USA* **98**(14): 7841–7845.

De Bari C, Dell'Accio F, Luyten FP. 2001; Human periosteum-derived cells maintain phenotypic stability and chondrogenic potential throughout expansion regardless of donor age. *Arthritis Rheum* **44**(1): 85–95.

De Bari C, Dell'Accio F, Vandenabeele F, *et al*. 2003; Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. *J Cell Biol* **160**(6): 909–918.

De Bari C, Dell'Accio F, Vanlauwe J, *et al*. 2006; Mesenchymal multipotency of adult human periosteal cells demonstrated by single-cell lineage analysis. *Arthritis Rheum* **54**(4): 1209–1221.

Decker JD, Marshall JJ, Herring SW. 1996; Differential cell replication within the periosteum of the pig mandibular ramus. *Acta Anat (Basel)* **157**(2): 144–150.

Fang J, Hall BK. 1997; Chondrogenic cell differentiation from membrane bone periostea. *Anat Embryol (Berl)* **196**(5): 349–362.

Fukumoto T, Sperling JW, Sanyal A, *et al*. 2003; Combined effects of insulin-like growth factor-1 and transforming growth factor-beta1 on periosteal mesenchymal cells during chondrogenesis *in vitro*. *Osteoarthritis Cartilage* **11**(1): 55–64.

Ginis I, Luo Y, Miura T, *et al*. 2004; Differences between human and mouse embryonic stem cells. *Dev Biol* **269**(2): 360–380.

Groger A, Klaring S, Merten HA, *et al*. 2003; Tissue engineering of bone for mandibular augmentation in immunocompetent minipigs: preliminary study. *Scand J Plast Reconstr Surg Hand Surg* **37**(3): 129–133.

Gronthos S, Zannettino AC, Graves SE, *et al*. 1999; Differential cell surface expression of the STRO-1 and alkaline phosphatase antigens on discrete developmental stages in primary cultures of human bone cells. *J Bone Miner Res* **14**(1): 47–56.

Hata K, Nishimura R, Ueda M, *et al*. 2005; A CCAAT/enhancer binding protein beta isoform, liver-enriched inhibitory protein, regulates commitment of osteoblasts and adipocytes. *Mol Cell Biol* **25**(5): 1971–1979.

Haynesworth SE, Baber MA, Caplan AI. 1996; Cytokine expression by human marrow-derived mesenchymal progenitor cells *in vitro*: effects of dexamethasone and IL-1α. *J Cell Physiol* **166**(3): 585–592.

Haynesworth SE, Goshima J, Goldberg VM, *et al*. 1992; Characterization of cells with osteogenic potential from human marrow. *Bone* **13**(1): 81–88.

Hoikka VE, Jaroma HJ, Ritsila VA. 1990; Reconstruction of the patellar articulation with periosteal grafts. 4-year follow-up of 13 cases. *Acta Orthop Scand* **61**(1): 36–39.

Kobayashi T, Kronenberg H. 2005; Minireview: transcriptional regulation in development of bone. *Endocrinology* **146**(3): 1012–1017.

Lecka-Czernik B, Gubrij I, Moerman EJ, *et al*. 1999; Inhibition of Osf2/Cbfa1 expression and terminal osteoblast differentiation by PPARγ2. *J Cell Biochem* **74**(3): 357–371.

Lee RH, Kim B, Choi I, *et al*. 2004; Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. *Cell Physiol Biochem* **14**(4–6): 311–324.

Lennon DP, Haynesworth SE, Bruder SP, *et al*. 1996; Human and animal mesenchymal progenitor cells from bone marrow: identification of serum for optimal selection and proliferation. *In Vitro Cell Dev Biol* **32**: 602–611.

Li M, Amizuka N, Oda K, *et al*. 2004; Histochemical evidence of the initial chondrogenesis and osteogenesis in the periosteum of a rib fractured model: implications of osteocyte involvement in periosteal chondrogenesis. *Microsc Res Tech* **64**(4): 330–342.

Majumdar MK, Thiede MA, Mosca JD, *et al*. 1998; Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. *J Cell Physiol* **176**(1): 57–66.

Marini RP, Steven MM, Langer R, *et al*. 2004; Hydraulic evaluation of the periosteum: a novel technique for periosteal harvest. *J Invest Surg* **17**(4): 229–233.

Marks SC Jr, Popoff SN. 1988; Bone cell biology: the regulation of development, structure, and function in the skeleton. *Am J Anat* **183**(1): 1–44.

Muraglia A, Cancedda R, Quarto R. 2000; Clonal mesenchymal progenitors from human bone marrow differentiate *in vitro* according to a hierarchical model. *J Cell Sci* **113**(7): 1161–1166.

Nah HD, Pacifici M, Gerstenfeld LC, *et al*. 2000; Transient chondrogenic phase in the intramembranous pathway during normal skeletal development. *J Bone Miner Res* **15**(3): 522–533.

Nakahara H, Goldberg VM, Caplan AI. 1991; Culture-expanded human periosteal-derived cells exhibit osteochondral potential *in vivo*. *J Orthop Res* **9**(4): 465–476.

Nakase T, Nakahara H, Iwasaki M, *et al*. 1993; Clonal analysis for developmental potential of chick periosteum-derived cells: agar gel culture system. *Biochem Biophys Res Commun* **195**(3): 1422–1428.

Noth U, Osyczka AM, Tuli R, *et al*. 2002; Multilineage mesenchymal differentiation potential of human trabecular bone-derived cells. *J Orthop Res* **20**(5): 1060–1069.

O'Driscoll SW, Fitzsimmons JS. 2001; The role of periosteum in cartilage repair. *Clin Orthop* **391**: (suppl): S190–207.

O'Driscoll SW, Meisami B, Miura Y, *et al*. 1999; Viability of periosteal tissue obtained postmortem. *Cell Transplant* **8**(6): 611–616.

Okamoto T, Aoyama T, Nakayama T, *et al*. 2002; Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. *Biochem Biophys Res Commun* **295**(2): 354–361.

Ossendorf C, Kaps C, Kreuz PC, *et al*. 2007; Treatment of posttraumatic and focal osteoarthritic cartilage defects of the knee with autologous polymer-based three-dimensional chondrocyte grafts: 2-year clinical results. *Arthritis Res Ther* **9**(2): R41.

Picinich SC, Mishra PJ, Glod J, *et al*. 2007; The therapeutic potential of mesenchymal stem cells. Cell- and tissue-based therapy. *Expert Opin Biol Ther* **7**(7): 965–973.

Pittenger MF, Mackay AM, Beck SC, *et al*. 1999; Multilineage potential of adult human mesenchymal stem cells. *Science* **284**(5411): 143–147.

Pochampally RR, Smith JR, Ylostalo J, *et al*. 2004; Serum deprivation of human marrow stromal cells (hMSCs) selects for a subpopulation of early progenitor cells with enhanced expression of OCT-4 and other embryonic genes. *Blood* **103**(5): 1647–1652.

Reyes M, Dudek A, Jahagirdar B, *et al*. 2002; Origin of endothelial progenitors in human postnatal bone marrow. *J Clin Invest* **109**(3): 337–346.

Sakaguchi Y, Sekiya I, Yagishita K, *et al*. 2005; Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. *Arthritis Rheum* **52**(8): 2521–2529.

Schmelzeisen R, Schimming R, Sittinger M. 2003; Making bone: implant insertion into tissue-engineered bone for maxillary sinus floor augmentation-a preliminary report. *J Craniomaxillofac Surg* **31**(1): 34–39.

Soncini M, Vertua E, Gibelli L, *et al*. 2007; Isolation and characterization of mesenchymal cells from human fetal membranes. *J Tissue Eng Regen Med* **1**(4): 296–305.

Stewart K, Walsh S, Screen J, *et al*. 1999; Further characterization of cells expressing STRO-1 in cultures of adult human bone marrow stromal cells. *J Bone Miner Res* **14**(8): 1345–1356.

Tremain N, Korkko J, Ibberson D, *et al*. 2001; MicroSAGE analysis of 2353 expressed genes in a single cell-derived colony of undifferentiated human mesenchymal stem cells reveals mRNAs of multiple cell lineages. *Stem Cells* **19**(5): 408–418.

Tsai MS, Lee JL, Chang YJ, *et al*. 2004; Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. *Hum Reprod* **19**(6): 1450–1456.

Tuli R, Tuli S, Nandi S, *et al*. 2003; Characterization of multipotential mesenchymal progenitor cells derived from human trabecular bone. *Stem Cells* **21**(6): 681–693.

Wognum AW, Eaves AC, Thomas TE. 2003; Identification and isolation of haematopoietic stem cells. *Arch Med Res* **34**(6): 461–475.

Wu Z, Xie Y, Bucher NL, Farmer SR. 1995; Conditional ectopic expression of C/EBPβ in NIH-3T3 cells induces PPARγ and stimulates adipogenesis. *Genes Dev* **9**(19): 2350–2363.

Yamazaki M, Nakajima F, Ogasawara A, *et al*. 1999; Spatial and temporal distribution of CD44 and osteopontin in fracture callus. *J Bone Joint Surg Br* **81**(3): 508–515.

Yeh WC, Cao Z, Classon M, McKnight SL. 1995; Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes Dev* **9**(2): 168–181.

Yen BL, Huang HI, Chien CC, *et al*. 2005; Isolation of multipotent cells from human term placenta. *Stem Cells* **23**(1): 3–9.

Zheng YX, Ringe J, Liang Z, *et al*. 2006; Osteogenic potential of human periosteum-derived progenitor cells in PLGA scaffold using allogeneic serum. *J Zhejiang Univ Sci B* **7**(10): 817–824.

Zuk PA, Zhu M, Mizuno H, *et al*. 2001; Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* **7**(2): 211–228.