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Original article

Isolation and cellular properties of mesenchymal stem cells from human periosteum

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Background: Cell-based therapy has achieved good functional recovery for tissue repair. Mesenchymal stem cells (MSCs) exhibit multilineage potential, long-term viability, and capacity for self-renewal. Periosteum-derived mesenchymal stem cells (PD cells) may be an attractive cell source for tissue engineering because of their easy accessibility and reduced ethical concerns.

Objectives: To isolate and investigate the phenotypic and functional characteristics of mesenchymal stem cells derived from human periosteum. We also examined the differentiation of PD cells with a trilineage differentiation assay to determine whether they were MSCs.

Materials and Methods: Periosteum-derived cells were cultured in osteogenic, chondrogenic or adipogenic media to evaluate their multilineage differentiation potential. Adherent fibroblast-like cells were analyzed by flow cytometry for MSC cell surface markers. Differentiation of PD cells into osteogenic, chondrogenic, and adipogenic lineages was also evaluated by von Kossa, Alizarin red, Alcian blue, and oil red O stains, respectively. Expression of mesenchymal stem cell markers were assessed using reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

Results: We successfully isolated and expanded MSCs from human periosteum. Flow cytometry revealed that PD cells were positive for mesenchymal adhesion cell markers (CD29, CD44, CD90, and CD105) and negative for hematopoietic markers (CD34 and CD45). In osteogenic differentiation, calcium accumulation (positive von Kossa and Alizarin red) and *RUNX2, alkaline phosphatase, collagen type I, osteopontin* genes were detected. In adipogenic differentiation, the cells displayed oil red O positive and expressed *lipoprotein lipase* and *peroxisome proliferator-activated receptor-gamma (PPAR-γ)* associated with adipogenesis. The cells grown in chondrogenic conditions were positively stained for Alcian blue and expressed *SOX-9*.

Conclusion: PD cells presented osteogenic, chondrogenic, and adipogenic differentiation abilities *in vitro* and could provide an alternative cellular source for tissue repair in clinical applications.

Keywords: Adipogenic, chondrogenic, differentiation, mesenchymal stem cells, osteogenic, periosteum

Mesenchymal stem cells (MSCs) are a type of stromal stem cells exhibiting great promise as a cell source for future clinical therapy [1]. MSCs are adult stem cells typically obtained from bone marrow stroma [2]. In general, MSCs are proliferated as plastic-adherent cells with a fibroblast-like morphology and differentiate into cells of the mesodermal lineage, including osteoblasts, chondroblasts, and adipoblasts depending on suitable stimuli and local environment [3]. Human MSCs are attractive candidates for clinical use because they are readily expanded in culture, multipotent, and amenable to genetic manipulation. Different protocols exist for isolation and expansion of MSCs: e.g., umbilical cord MSCs according to Salehinejad and colleagues [4], mesenchymal progenitor cells according to Colter *et al.* [5], and bone marrow stromal stem cells according to Simmons and colleagues [6]. Verfaillie *et al.* [7] described multipotent adult progenitor cells as a subpopulation

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of cells that copurify with MSCs and have unique properties. MSCs have low immunogenicity, can be immunosuppressive [8, 9], and are multipotent [10-13]. MSCs function as precursors of postnatal osteoblasts [14, 15] and can be utilized in bone tissue engineering [16].

Human MSC have been used in clinical trials and without any problems for children with osteogenesis imperfecta [17, 18] or metabolic diseases [19], in autologous bone marrow transplant to support engraftment [20], and to prevent and treat graft-versus-host disease in allogeneic stem cell transplantation [21]. Further applications in cell therapy (stem cell expansion, gene therapy, and tissue engineering) are being developed using bioreactors or animal models, and may soon be available in the clinic [1].

While numerous articles describe isolating MSCs from a variety of human tissues, there are limited reports of isolating MSCs from human periosteum. For bone tissue, it was found that the periosteum displayed a potential MSC source [22, 23]. The periosteum is a membrane that lines the outer surface of all bones, except for the joints of long bones. The periosteum consists of dense irregular connective tissue. The periosteum is divided into an outer "fibrous layer" and inner "cambium layer" (or "osteogenic layer"). The fibrous layer contains fibroblasts, while the cambium layer contains progenitor cells that develop into osteoblasts. These osteoblasts are responsible for increasing the width of a long bone and the overall size of the other bone types. After a bone fracture the progenitor cells develop into osteoblasts and chondroblasts, which are essential to the healing process [24].

In the present study, we examined the cellular properties of periosteum-derived cells (PD cells) as MSCs. The PD cells exhibited a typical fibroblast-like morphology and strongly expressed mesenchymal cell markers. They showed profound potential for differentiation into osteogenic, chondrogenic, and adipogenic cells *in vitro*. These properties of PD cells should provide them with several advantages for clinical use, and suggest that they might be an attractive alternative to allogeneic MSCs for use in regenerative medicine.

Materials and methods

This study was carried out in accordance with the guidelines of the Helsinki Declaration, and approval was granted to use human periosteum. The protocol was accepted by the Ethical Committee of the Faculty of Medicine, Chulalongkorn University.

Cultivation of periosteum-derived cells

Periosteum tissues were collected, with written informed consent, from human donors at King Chulalongkorn Memorial Hospital and stored in sterile saline at 4°C before use. The PD cells were prepared as follows. The tissues were rinsed several times in sterile phosphate-buffered saline (PBS) to remove blood components. The tissues were dissected into small pieces (1 mm) and placed directly into 25 cm^2 flasks (Corning, USA) for culture expansion in alpha-minimum essential medium (α-MEM, Hyclone, USA) containing 10% fetal bovine serum (FBS, Hyclone), 200 U/ml penicillin/streptomycin (Hyclone) at 37°C, and 5% (v/v) CO_2.

PD cells were washed once in Dulbecco's phosphate-buffered saline (PBS; Gibco, USA), incubated with 0.25% trypsin-EDTA (Gibco) for 5 min and brought into suspension by vigorously shaking. The trypsin reaction was blocked by adding the supernatant followed by another washing step. Cells were usually trypsinized at more than 80% confluency.

Flow cytometry analysis

Single-PD cell suspensions were prepared using enzymatic dissociation with trypsin–EDTA. Cell suspensions were washed three times in cold PBS, counted, and adjusted to the appropriate concentrations; 10^6 cells were used per sample. The cells were incubated for 30 min at 4°C with monoclonal antibodies against human anti-CD29, CD34, CD44, CD45, CD90, and CD105 conjugated with PE, FITC, APC, and PE/Cy5 (Bioscience, USA). Stained samples were analyzed using a FACSCalibur cytometer (BD Biosciences).

In vitro differentiation of mesenchymal stem cells

To confirm the multipotent differentiation capacity of MSCs, PD cells at passage 2 (P2) were treated with different induction media for 21 days. Osteogenic induction medium contained 1% FBS, 10 mM β-glycerophosphate, 100 nM dexamethasone, and 100 μg/ml ascorbate in α-MEM. Chondrogenic induction medium was Dulbecco's modified Eagle medium (DMEM, Hyclone) supplemented with 1% FBS, 10 nM dexamethasone, 10 ng/mL transforming growth factor-β3, and 6.25 μg/ml insulin–transferrin–selenium

(ITS supplement). Adipogenic induction medium comprised 3% FBS, 5 µg/ml insulin, 0.5 µM 3-isobutyl-1-methylxantine and 200 µg/ml indomethacin in DME/Ham's F12 medium. Upon completion of the differentiation process, cultured cells were fixed with 10% formaldehyde and stained for osteoblasts with alkaline phosphatase, Alizarin red, and von Kossa, for chondrocytes with Alcian blue, and for adipocytes with oil red O, respectively.

RT-PCR for osteogenic, chondrogenic, and adipogenic cell differentiation

To analyze the relative expression of different mRNAs in differentiated PD cells, total RNA was isolated using the RNeasy Minikit (Qiagen, USA). RT-PCR was performed according to the manufacturer's protocol. The primers were as follows (**Table 1**): *GAPDH* (454 bp) forward, 5′-ACC ACA GTC CAT GCC ATC AC-3′, reverse, 5-TCC ACC ACC CTG TTG CTG TA-3′; *RUNX2* (422 bp) forward, 5′-TGA GCA GGT CCT GGT AC-3′, reverse, 5′-TAT GGC ACT TCG TCA GGA TCC-3′; *ALP* (453 bp) forward, 5′-TGG AGC TTC AGA AGC TCA ACA CCA-3′, reverse, 5′-ATC TCG TTG TCT GAG TAC CAG TCC-3′; *Collagen type I* (461 bp) forward, 5′-TAA CCA CTG CTC CAC TCT GG-3′, reverse, 5′-GGA CAC AAT GGA TTG CAA GC-3′; *Osteopontin* (162 bp) forward, 5′-GAG ATT TCT CTG TAT GGC ACC-3′, reverse, 5′-CTG CAA ATG AGA CAC TTT CTC-3′; *SOX-9* (264 bp) forward, 5′-ATC TGA AGA AGG AGA GCG AG-3′, reverse, 5′-TCA GAA GTC TCC AGA GCT TG-3′; *Lipoprotein lipase* (*LPL*; 277 bp) forward, 5′-GAG ATT TCT CTG TAT GGC ACC-3′, reverse, 5-CTG CAA ATG AGA CAC TTT CTC-3′; *peromisome proliferator–activated receptor gamma(PPAR-γ)*, (351 bp) forward, 5′-GCT GTT ATG GGT GAA ACT CTG-3′, reverse, 5′-ATA AGG TGG AGA TGC CTC-3′. The PCR cycling parameters were 94°C for 5 min; 30 cycles of 30 s at 94°C, 1 min at 60°C, and 1 min at 72°C; and final extension at 72°C for 10 min. After amplification, the PCR products were resolved by agarose gel electrophoresis.

Results

Characterization of multilineage potential of human PD cells

PD cells at P2 appeared as a monolayer of spindle, stellate shaped cells (**Figure 1**). Flow cytometry revealed that isolated PD cells expressed CD29 (92.99%), CD44 (98.04%), CD90 (99.43%), and CD105 (55.47%) although a few CD34- and CD45-positive cells were present (0.02% and 0.29%; **Figure 2**), which indicated the isolated PD cells were not contaminated with cells of hematopoietic origin. To determine the multipotency of PD cells, cells were treated with appropriate lineage-specific induction media. Osteogenic differentiation was verified by Alizarin red staining and confirmed with von Kossa staining in order to detect deposited calcium (**Figure 3A, 3B**). Chondrogenic differentiation was determined by the production of glycosaminoglycan using Alcian blue staining (**Figure 3C**). Adipogenic differentiation was confirmed by oil red O staining (**Figure 3D**).

RT-PCR analysis showed that the expression of *runt-related transcription factor 2 (RUNX2)*, a master regulator of osteogenesis, was upregulated in differentiated PD cells, but remained unchanged in the controls (**Figure 4A**). The levels of osteoblast differentiation-related genes, *alkaline phosphatase (ALP), collagen type I (COL 1)*, and *osteopontin (OPN)*, respectively were also upregulated (**Figure 4B-4D**). The expression of *SRY-box9,* a master regulator of chondrogenesis, was *SOX-9* upregulated in PD cells after 4 weeks of differentiation (**Figure 4E**). The adipocyte differentiation-related gene *lipoprotein lipase (LPL)* increased in differentiated PD cells after 4 weeks of differentiation (**Figure 4F**). The expression of *PPAR-γ,* a master regulator of adipogenesis, was upregulated in the induced PD cells (**Figure 4G**). *GAPDH* was used as internal control for all genes (**Figure 4H**).

Discussion

Human mesenchymal stem cells have been used in research for tissue engineering of bone on scaffolds. Kasten *et al.* used MSCs obtained from adult donors in order to compare three resorbable biomaterials for cell penetration into the matrix, cell proliferation, and osteogenic differentiation [25]. In recent years, periosteum-derived cells have been commonly utilized in basic science and clinical research. The first successful culture of periosteum has been reported by Fell in 1932 [23]. The periosteum can be described as an osteoprogenitor cell containing bone envelope, which can be activated to proliferate by trauma and lymphocyte mitogens [24, 26]. Cells located within the periosteum and bone marrow can differentiate into fibroblasts, osteogenic or reticular cells [23, 27].

Table 1. Mesenchymal differentiation induction and detection of lineage specific markers

Lineage	Differentiation-induction media components	Staining	mRNA expression by RT-PCR		
			Gene name (gene symbol)	GenBank	Primer sequences for PCR amplification*
Osteogenic	□-glycerophosphate (10 mM) Dexamethasone (100 nM) Ascorbic acid 50 (μM)	Alkaline phosphates kit	*Runt-related transcription factor 2* (*RUNX2*)	NM_001015051.3	S: 5′-TGAGCAGGTCCTGGTAC-3′ A: 5′-TATGGCACTTCGTCAGGATCC-3′
			Alkaline phosphatase (*ALP*)	NC_000001.10	S: 5′-TGGAGCTTCAGAAGCTCAACACCA-3′
			Collagen type I, alpha I (*COL1A1*)	NC_000017.10	A: 5′-ATCTCGTTGTCTGAGTACCAGTCC-3′
			Osteopontin	NC_000004.11	S: 5′-TAACCACTGCTCCACTCTGG-3′ A: 5′-GGA CAC AAT GGA TTG CAA GC-3′ S: 5′-GAGATTTCTCTGTATGGCACC-3′ A: 5′-CTGCAAATGAGACACTTTCTC-3′
Chondrogenic	Dexamethasone (10nM) Transforming growth actor-□3 (10 ng/ml) Insulin–transferrin–selenium (ITS, 6.25 μg/ml)	Acidified Alcian blue (1%)	*Sex determining region Y-box 9* (*SOX-9*)	NC_000017.10	S: 5′-ATC TGA AGA AGG AGA GCG AG-3′ A: 5′-TCA GAA GTC TCC AGA GCT TG-3′
Adipogenic	Insulin (5μg/ml) 3-isobutyl-1-methylxantine (0.5 μM) Indomethacin (200 μg/ml)	Oil Red O	*Lipoprotein lipase* (*LPL*)	NM_000316	S: 5′-GAGATTTCTCTGTATGGCACC-3′ A: 5′-CTGCAAATGAGACACTTTCTC-3′
			Peroxisome proliferator- activated receptor gamma (*PPAR-γ*)	NM_005037	S: 5′-GCTGTTATGGGTGAAACTCTG-3′ A: 5′-ATAAGGTGGAGATGCCTC-3′

*S = sense, A = antisense

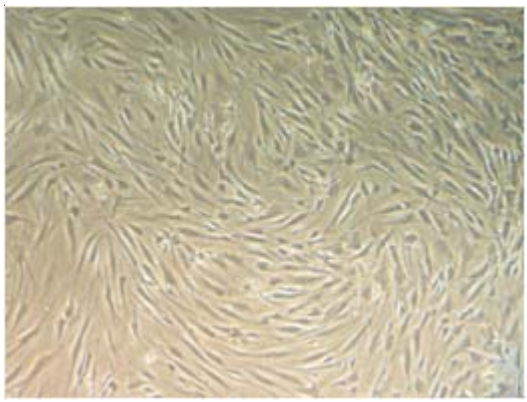

Figure 1. Characterization of human periosteum-derived mesenchymal stem cells (PD cells). PD cells at passage 2 are a monolayer of spindle, stellate shaped cells under phase contrast. Original magnification: x 100

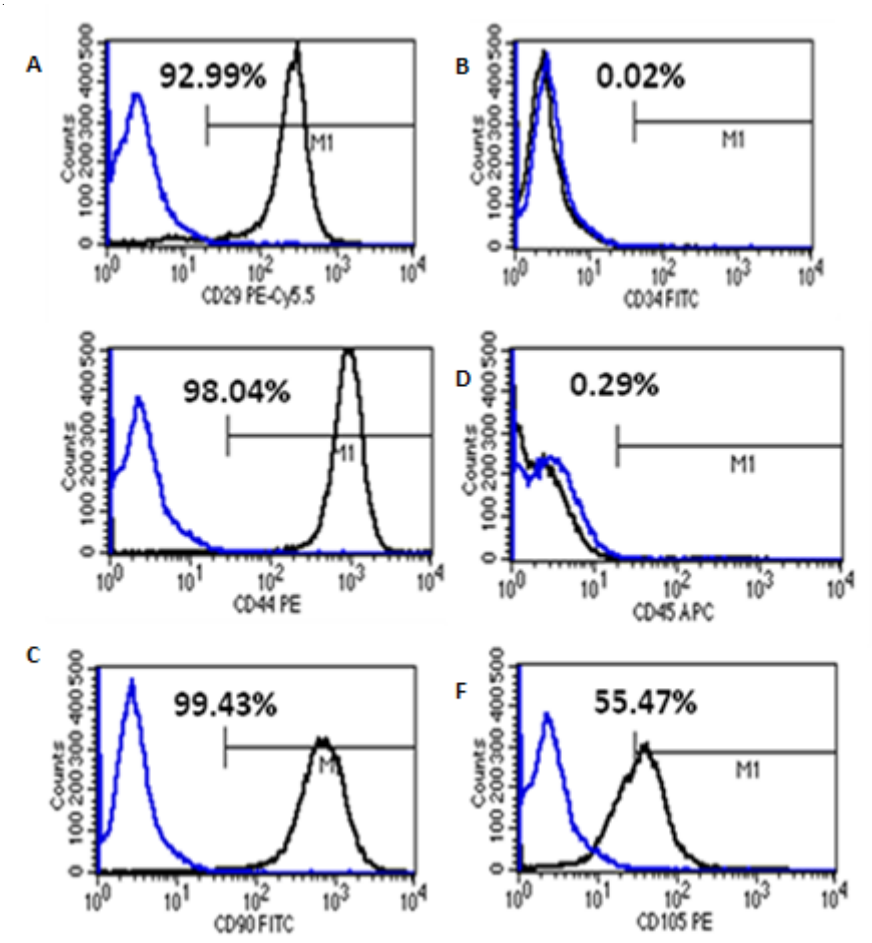

Figure 2. Flow cytometry analysis of human periosteum-derived mesenchymal stem cells (PD cells). FACS analysis shows PD cells to be positive for CD29, CD44, CD90, and CD105 and negative for CD34 and CD45.

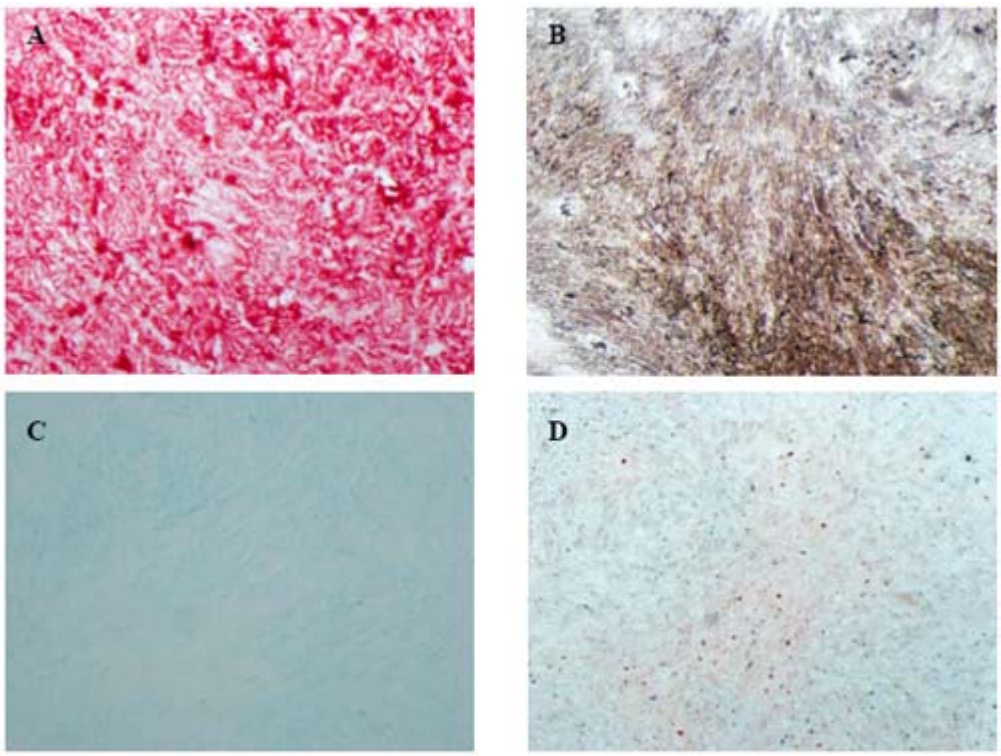

Figure 3. Multilineage potential of PD cells. Cultured PD cells differentiated into osteoblasts (**A**: Alizarin red staining, **B**: von Kossa staining), **C**: chondrocytes (Alcian blue staining), **D**: adipocytes (oil red O staining). Original magnification: x 100

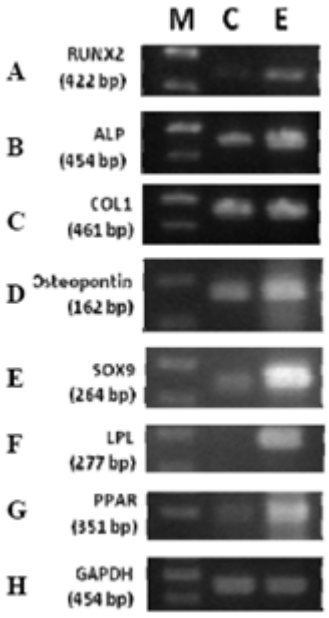

Figure 4. RT-PCR results of MSC-specific genes in control medium and conditioned medium. **A, B, C, D**: osteogenic genes: *RUNX2*, *ALP*, *COL1*, and *Osteopontin*, **E**: chondrogenic gene: *SOX-9*, **F, G**: adipogenic genes: *LPL* and *PPAR-γ*, **H**: *GAPDH*. Lane M = DNA markers, lane C = PD cells cultured in control medium, lane E = PD cells cultured in conditioned medium.

Periosteum-derived mesenchymal precursor cells can generate progenitor cells committed to one or more cell lines with an apparent degree of plasticity. Outgrowth cultures of periosteum pieces favor the coculture of different cell types. Periosteum-derived cells expanded in culture are able to heal a segmental bone defect after implantation and induce osteogenic tissue when seeded into diffusion chambers [28].

To evaluate the phenotype of the expanded periosteum-derived cells, we performed FACS analysis, testing a marker set associated with multipotent MSCs from other tissue sources including bone marrow. CD45, a marker of hematopoietic lineage cells not expressed by MSCs, was not detected in any of the donors tested [10]. A previous study by Roberts *et al.* has reported that human periosteum-derived cells can be cultured with calcium phosphate scaffolds [29]. They have shown that PD cells display classical surface markers used for identification of MSCs including CD73, CD90, and CD105. Additionally, the population showed low expression of CD34 (hematopoietic progenitor) and was negative for CD45, CD20, and CD14 [29]. Moreover, Eyckmans and Luyten detected expression of CD73, CD105, and CD166 in PD cells. Furthermore, CD45 (hematopoietic stem cell (HSC) marker) was not found in PD cells. These data indicate that cells contained in the periosteum display a high self-renewal capacity upon culture expansion and a phenotype suggestive of multipotent MSCs. Thereafter, multipotency towards the mesenchymal lineages was assessed [30].

Recently, the periosteum has received considerable attention as a grafting material for the repair of bone and joint defects. Findings of bone formation induced by cultured human periosteum-derived cells using a rat model have been presented. Schantz and colleagues reported that cultured human periosteum cells harvested from calvarial bone in combination with a three dimensional scaffold expressed osteogenic markers and induced new bone formation in the back muscles of nude mice [28].

In our study, we have successfully isolated a universal cell population with mesenchymal stem cell characteristics from human periosteum. We have studied the morphology, immunophenotype, gene expression of mesenchymal stem cells, and differentiation ability of PD cells. The study has demonstrated adult stem cell activity for three cell types in human periosteum: osteoblasts, chondrocytes, and adipocytes. The PD cells isolated from periosteum tissue showed positive expression of MSC surface markers such as CD29, CD44, CD90, and CD105, but did not express HSC markers such as CD34 and CD45. Moreover, the results revealed that PD cells are superior in terms of osteogenic, chondrogenic, and adipogenic capacity *in vitro* as shown by evidence that PD cells could differentiate into three lineages of osteogenic, chondrogenic, and adipogenic lineages depending on culture conditions. In addition, the expression of osteogenic (*RUNX2, ALP, COLI*), chondrogenic (*SOX-9*), and adipogenic (*LPL, PPAR-γ*) genes were highly upregulated following induction. The data demonstrated that PD cells could be a new cell source that may be more suitable for bone and cartilage repair.

Moreover, our results resemble those of the study by Gargette and coworkers that characterized MSCs from human endometrium [31]. They showed that endometrium-derived cells can differentiate into osteoblasts, chondrocytes, adipocytes, and smooth muscle cells. RT-PCR analyses confirmed this differentiation in that the endometrium derived cells expressed *PTHR1, Collagen type II, LPL*, and *Caldesmon* that represent osteogenic, chondrogenic, adipogenic, and myogenic differentiation. Furthermore, upon flow cytometry, the endometrium-derived cells expressed the MSCs markers CD29, CD44, CD90, CD105, including CD140B, and CD146, but not the hematopoietic markers CD31, CD34, or CD45 [31].

In conclusion, human periosteum-derived cells exhibited a typical fibroblast-like morphology. The expression patterns of CD antigens on the PD cells were almost identical to MSCs, and *in vitro* assays showed that the PD cells could differentiate into osteoblasts, chondrocytes, or adipocytes. These findings demonstrate that the PD cells, which are mesenchymal lineage cells originating from periosteum, have MSC-like properties in periosteum tissue.

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