Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Osteogenic potential of human periosteum-derived progenitor cells in PLGA scaffold using allogeneic serum*

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Received Nov. 15, 2005; revision accepted Mar. 2, 2006

Abstract: The use of periosteum-derived progenitor cells (PCs) combined with bioresorbable materials is an attractive approach for tissue engineering. The aim of this study was to characterize the osteogenic differentiation of PC in 3-dimensional (3D) poly-lactic-*co*-glycolic acid (PLGA) fleeces cultured in medium containing allogeneic human serum. PCs were isolated and expanded in monolayer culture. Expanded cells of passage 3 were seeded into PLGA constructs and cultured in osteogenic medium for a maximum period of 28 d. Morphological, histological and cell viability analyses of three-dimensionally cultured PCs were performed to elucidate osseous synthesis and deposition of a calcified matrix. Furthermore, the mRNA expression of type I collagen, osteocalcin and osteonectin was semi-quantitively evaluated by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). The fibrin gel immobilization technique provided homogeneous PCs distribution in 3D PLGA constructs. Live-dead staining indicated a high viability rate of PCs inside the PLGA scaffolds. Secreted nodules of neo-bone tissue formation and the presence of matrix mineralization were confirmed by positive von Kossa staining. The osteogenic differentiation of PCs was further demonstrated by the detection of type I collagen, osteocalcin and osteonectin gene expression. The results of this study support the concept that this tissue engineering method presents a promising method for creation of new bone in vivo.

Key words: Tissue engineering, Poly-lactic-*co*-glycolic acid polymer, Periosteum-derived progenitor cells, 3-dimensional culture
doi:10.1631/jzus.2006.B0817 **Document code:** A **CLC number:** Q819

INTRODUCTION

Regenerative medicine or tissue engineering offers a promising approach for the regeneration of tissue and organ defects (Sittinger *et al*., 1996; Brittberg *et al*., 1994). In contrast to conventional treatment approaches, tissue engineered transplants are formed here by using harvested and ex vivo expanded cells delivered in a synthetic biocompatible resorbable scaffold. For successful tissue regeneration, it is indispensable to give the cells an environment suitable for regeneration induction and development of neo-tissue. Hereon, the 3-dimensional (3D) structure of biomaterials plays a major role in the physiological switch of cultured cells, the formation of new tissue into an anatomically relevant shape, and in vivo evolvement of engineered tissue (Sittinger *et al*., 2004; Ouyang *et al*., 2002).

The use of pluripotent or multipotent stem cells in place of committed tissue-specific cells represents an exciting new approach for tissue engineering because of their pronounced ex vivo expansion capacity, their remarkable plasticity and therefore, their potential to regenerate complex tissue defects (Ringe *et al*., 2002). Recent evidences suggest that connective tissue progenitors are resident in bone marrow (Pittenger *et al*., 1999), trabecular bone (Nöth *et al*., 2002), periosteum (Ringe *et al*., 2005), adipose tissue

* Project supported by the Investitionsbank Berlin (IBB), Germany (No. 10020666) and the Science and Technology Bureau of Zhejiang Province, China (No. 991110052)

(Zuk *et al*., 2002), muscle (Peng and Huard, 2004) and synovial membrane (de Bari *et al*., 2001). All these progenitors show a multilineage differentiation potential in vitro, and represent very prominent candidates for tissue engineering approaches to connective tissue replacement (Barry and Murphy, 2004). Periosteum-derived progenitor cells (PCs) are derived from the outer cambial layer of the periosteum. It was shown recently that PCs have a similar adipogenic potential as bone marrow-derived mesenchymal stem cell (BMSC) (Ringe *et al*., 2005).

We developed a new scaffold-based 3D cell culture technique in 1996 (Sittinger *et al*., 1996), and applied tissue engineered PCs with bioresorbable fleece scaffolds into animal segmental bone repair experiments (Perka *et al*., 2000; Gröger *et al*., 2003). To our knowledge, the first commercially available bone tissue engineering product for augmentation of the maxilla (sinus lift) was initiated (Schmelzeisen *et al*., 2003). Although the results are exciting, further research on the proliferation and differentiation character of human PCs seeded within 3D poly-lactic-*co*-glycolic acid (PLGA) scaffolds are of special interest.

The aim of this study was to characterize the osteogenic differentiation of PCs in 3D PLGA constructs cultured in medium containing allogeneic human serum. The ethical committee of the Charité-University Medicine Hospital in Berlin, Germany has approved this study.

MATERIALS AND METHODS

Preparation of allogeneic human serum

Venous whole blood (German Red Cross, Germany) without any anticoagulation factors was centrifuged at 3500×g for 10 min (Centrifuge 5804, Eppendorf). Heat inactivation of the sera was performed at 56 °C for 30 min. The sera were and stored at –20 °C until use.

Isolation and cell expansion of human PC

Periosteal tissues (0.5 cm^2) were harvested from human mastoid of three patients (one female and two males, aged 49~64 years, mean age: 54.7 years) undergoing mastoidectomy. PCs were isolated according to the modifications of an enzymatic digestion method described previously (Redlich *et al*., 1999). Briefly, after purification, the biopsies were enzymatically digested with collagenase II (Biochrom, Germany) in Dulbecco's modified Eagle medium (DMEM)/Ham's F12 medium 1:1 (Biochrom, Germany) supplemented with 10% allogeneic human serum and 100 IU/ml penicillin G, 100 g/ml streptomycin sulfate (Biochrom, Germany) (PC medium). The resulting cells were subsequently harvested, resuspended in PC medium, placed into cell culture flasks and cultured at 37 °C with 5% CO_2 and 95% humidified air. After 4~10 d, non-adherent cells were removed by exchange of medium. Adherent growing PCs were cultured under standard cell culture conditions. When reaching 90% confluence, PCs were sub-cultured by treatment with 0.05% trypsin/0.02% ethylene diamine tetra-acetic acid (EDTA) (Biochrom, Germany) for 5 min and subsequently replated at a density of 6000 cells/cm^2.

Preparation and cultivation of the cells/PLGA constructs

Cell-seeded 3D constructs were prepared as described previously (Perka *et al*., 2000). In brief, PCs of the third passage (P3) were trypsinized and resuspended in osteogenic medium consisting of DMEM/Ham's F12 medium 1:1 with 5% allogeneic human serum, 2.5% Hepes, 100 IU/ml penicillin G, 100 g/ml streptomycin sulfate, 0.285 mmol/L ascorbate-2-phosphate, 10 mmol/L β-glycerophosphate, 10^{-7} mol/L dexamethasone, 230 mg/L calciumchloride and mixed with fibrinogen (Tissucol®, Baxter-Immuno GmbH, Germany).

PLGA-fleeces (Ethisorb®, columnar shape, d=8 mm, h=2 mm, Ethicon, Johnson & Johnson, Germany) were soaked in the cell/fibrinogen (5.0×10^6 cells per fleece), and polymerized by dropping thrombin/PBS solution (Tissucol®, Baxter-Immuno GmbH, Germany) onto each side of the fleece. After polymerization of the fibrin gel, constructs were placed in six-well culture dishes (Falcon, Becton Dickinson). The constructs were cultivated with osteogenic medium at 37 °C, with 5% CO_2 and 95% humidified air. Medium was changed every other day. Samples were taken on days 7, 14, and 28. Two pieces of the constructs per time point were subjected to histological examination and fluorescent assessment. On day 7 and day 28, two pieces (approx. 30 mg/piece) per time

point were subjected to real-time RT-PCR analysis.

Histological examination

For histology, one of the cells/PLGA constructs was harvested at each time point, embedded in Tissue-Tek O.C.T Compound (Sakura Finetek Europe, the Netherlands), and cryosectioned (8 μm thickness) using a cryomicrotome (CM1900, Leica, Bannockburn). Sections were stained with haematoxylin and eosin (HE) (Merck, Germany) and for osteogenic differentiation according to the von Kossa method. This stain substitutes calcified matrix Ca^{2+} ions with silver atoms, revealing the calcium minerals as black spots.

Fluorescent assessment of cell viability

To assess cell viability, the cell/PLGA constructs were prepared for fluorescent microscopy by staining the cells with fluorescent dyes. Constructs were placed in 2 mg/ml fluorescein diacetate (FDA, Sigma) and incubated for 15 min at 37 °C, to stain viable cells green. After being rinsed twice in PBS solution, the samples were placed in 1 mg/ml propidium iodide solution (PI, Sigma) for 2 min at room temperature, to stain dead cells red. The samples were then rinsed twice in PBS solution and observed under a fluorescent microscope (AX70, Olympus). The detection principle is that PI dye cannot penetrate viable cells. Nonviable cells, on the other hand, are permeable and can therefore be stained with PI dye.

RNA isolation, real-time RT-PCR

For RNA isolation and cDNA synthesis, the cells on the fleeces were lysed and homogenized with a rotor-stator homogenizer (Ultra-Turrax, Janke & Kunkel IKA Labortechnik). Cells were treated with RLT-buffer (Qiagen, Germany) and 2-mercaptoethanol (100:1) (Sigma), and the total RNA was isolated using Qiagen RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Subsequently, 5 μg total RNA from each sample was reverse-transcribed after annealing with 500 ng oligo-(dT) primer (Gibco, Germany) and 5 units of SuperScript reverse transcriptase (Gibco) in 70 μl reaction mixture. The relative expression level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the marker gene expression in each sample in different concentrations. Real-time PCR using the iCycler PCR system (BioRad, Germany) was performed with 1 μl of the single-stranded cDNA sample, using the SYBR Green PCR Core Kit (Applied Biosystems, Germany). For each sample, the Cycle threshold value (Ct value) was determined as the cycle number at which the fluorescence activity reached the exponential phase. Relative quantitation of osteogenic marker genes was given as a percentage of the GAPDH product. The nucleotide sequences of the oligonucleotides and product size, and annealing temperatures for GAPDH and marker genes are summarized in Table 1. Each sample was analyzed at least in triplicate.

Statistical analysis

Values are given as mean value±standard deviation. Statistical analysis was performed by two-tailed *t*-test with equal variances. Value of $P<0.05$ was considered to be statistically significant.

Table 1 Sequences of forward and reverse oligonucleotides

Gene	Accession number	Oligonucleotides (5′→3′) (up/down)	Product size (bp*)	Annealing temperature (°C)
GAPDH	XM_006959	GGC GAT GCT GGC GCT GAG TAC	149	62
		TGG TCC ACA CCC ATG ACG A		
Collagen Ia1	NM_000088	CGA TGG CTG CAC GAG TCA CAC	180	62
		CAG GTT GGG ATG GAG GGA GTT TAC		
Osteocalcin	X_51699	GAG CCC CAG TTC CCC TAC CC	103	58
		GCC TCC TGA AAG CCG ATG TG		
Osteonectin	Y_00755	AGA AGC TGC GGG TGA AGA A	127	54.8
		TGC CAG TGT ACA GGG AAG ATG		

*bp: Base pairs

RESULTS

Isolation and expansion of PCs in allogeneic human serum

PCs were successfully isolated from periosteum derived from mastoid bone. Within 5 d in culture, PCs attached to the culture plates. Round shaped erythrocytes were removed during the exchange of culture medium. PCs grew in distinct colonies by days 7~10 and displayed a typical fibroblast-like morphology (Fig.1a) which remained stable during prolonged culture and subculture in monolayer. In higher cell passages, PCs appeared slightly larger and more stretched in a homogeneous cell compartment (Fig.1b).

Morphology and cell viability in cells/PLGA constructs

Seeding of the expanded PCs was technically feasible. The fibrin gel immobilization technique provided homogeneous cell distribution without significant cell loss during the seeding process. Initially, the constructs displayed a transparent appearance (A in Fig.2a). Light microscopic investigation showed a switch of the fibroblastic phenotype to cuboid cells, similar to osteoblasts. Round cells were homogeneously distributed in the fibrin gel and spread in the fibrin network within the PLGA scaffolds (Fig.2b). After 14 d in culture, the architecture of constructs showed bony appearance (B, C in Fig.2a).

FDA/PI live-dead staining showed the vital PC inside the PLGA scaffolds. PLGA fibers remained intact by day 7 (Fig.3a). On day 28, the viability rate of PC was nevertheless estimated at approx. 90%, while PLGA fibers came into degradation phase indicated by red fluorescence (Fig.3b).

Histology

HE sections of cell/PLGA constructs confirmed a homogeneous distribution of cells within the scaffolds as well. The pores of the PLGA fleece matrix were completely filled with fibrin gel on day 7. Cell clusters were seen within the deep constructs. Some were attached to the fleece matrix fibers; others still adhered to the fibrin fibrils in the pores of the matrix (Fig.4a). Between day 14 and 28, however, a change in distribution occurred with a decrease of cell density inside the constructs (Fig.4b). FDA/PI staining investigation revealed that degraded PLGA fibers and fibrin are observable on day 28 by HE staining.

Von Kossa staining showed osteogenic structures (matrix mineralization) on the surface of the PLGA constructs and the presence of calcified deposits between the fibers indicated by black staining of the matrix around the cells (Figs.4c and 4d). Bridges between the fibers of PLGA fleeces and osseous nodule formation on the surface of the constructs after 28 d of cultivation were also observed (Fig.4).

Quantitative real-time RT-PCR

Expression of type I collagen, osteocalcin and osteonectin on the mRNA level could be seen over the whole observation period. The expression of type I collagen showed a strong decrease on day 28 (6.2-fold decrease in comparison with day 7; $P<0.05$). The expression of osteocalcin and osteonectin in percent of the expression of the house keeping gene GAPDH showed no significant decrease from day 7 to day 28 of the cultivation period ($P>0.05$) (Table 2).

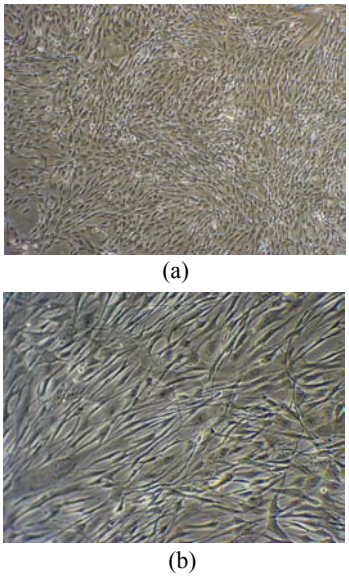
Table 2 Expression of bone-related genes by semi-quantitative real-time RT-PCR

	Day 7	Day 28	*P* value
Type I collagen	176.84±77.82	28.70±11.77	<0.05
Osteocalcin	6.82±1.99	6.09±2.28	>0.05
Osteonectin	18.80±11.01	10.19±6.80	>0.05

Note: Results were normalized to the relative expression level of the house keeping gene GAPDH

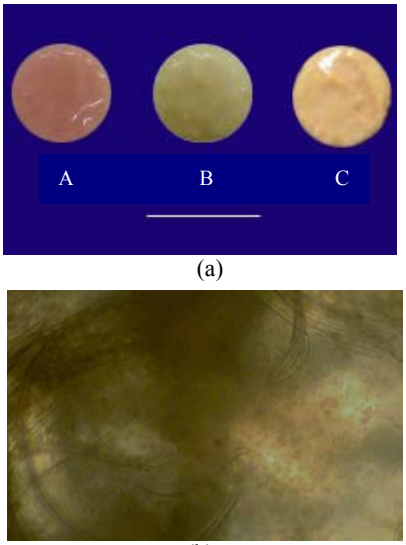
DISCUSSION

Successful harvest and isolation of host cells from a small biopsy, expansion of cells in suitable culture devices, the combination of such cells with bioresorbable biomaterials, and finally the re-implantation into the defect provide the potential for a biological route to restore joint, bone or connective tissue function. In skeletal tissues, cells are distributed within a dense extracellular matrix (ECM) made up of collagens, proteoglycans, a complex mixture of phosphoproteins and other inorganic materials. For regeneration of these tissue types, the spatial distribution and guidance of progenitor cells in all areas of the repair tissue is preferred (Sittinger *et al*., 2004; Chen *et al*., 2005). While cell seeding onto porous membranes



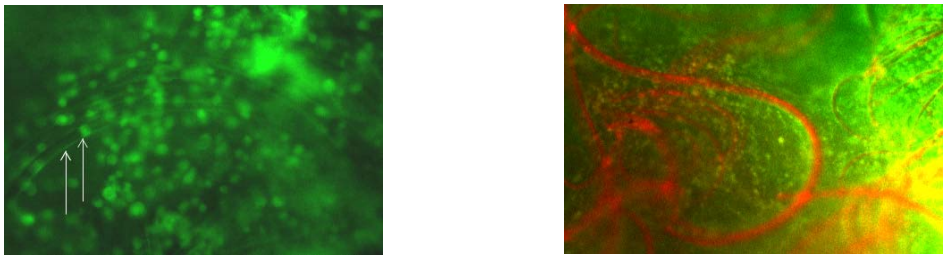
(a) (b)

Fig.1 Morphological appearance and expansion capacity of human PCs in monolayer culture. (a) By day 7, PCs showed a typical fibroblast-like morphology when cultured in medium supplemented with 10% allogeneic human serum (×40); (b) During subculture PCs (passage 3) appeared slightly larger and had a more stretched morphology (×100)



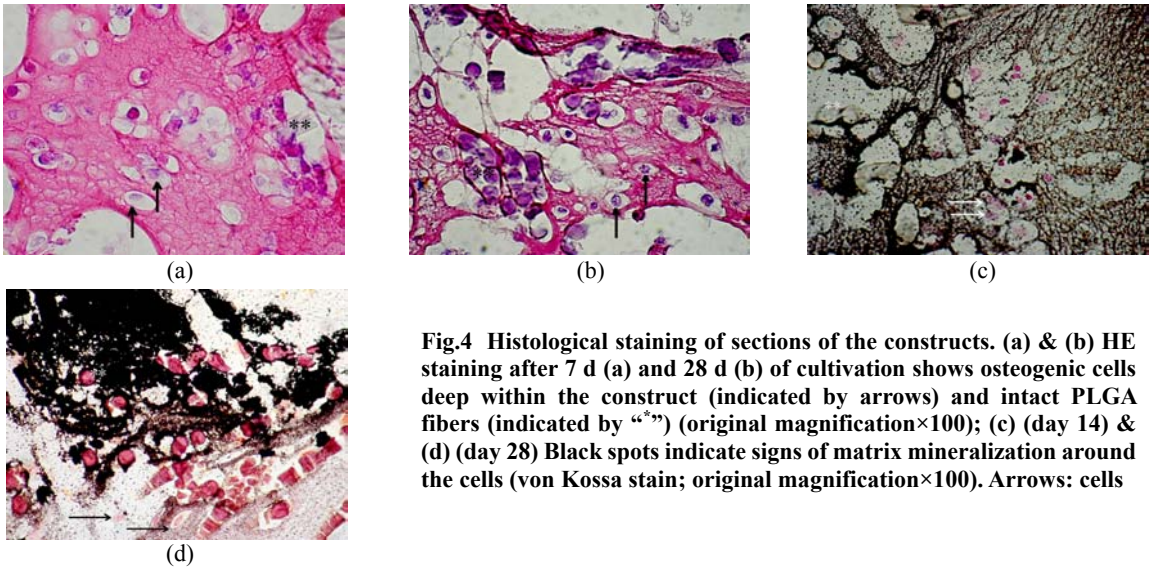
(a) (b)

Fig.2 Morphology in cells/PLGA constructs. (a) The appearance of a tissue-engineered bone constructs. A: day 7, B: day 14, C: day 28; (b) Light microscopic investigation showed round cells homogeneously distributed in fibrin gel and spread in the fibrin network within the PLGA scaffolds



(a) (b)

Fig.3 Live-dead (FDA/PI) assay of PCs in PLGA scaffolds. (a) Day 7. Green: live cells, arrows: PLGA fibers; (b) Day 28. Green: live cells, red: dead cells and PLGA fibers



(a) (b) (c) (d)

Fig.4 Histological staining of sections of the constructs. (a) & (b) HE staining after 7 d (a) and 28 d (b) of cultivation shows osteogenic cells deep within the construct (indicated by arrows) and intact PLGA fibers (indicated by "*") (original magnification×100); (c) (day 14) & (d) (day 28) Black spots indicate signs of matrix mineralization around the cells (von Kossa stain; original magnification×100). Arrows: cells

or solid scaffolds typically does not result in an even cell distribution, various studies have shown that embedding cells in a gel and a specifically designed textile scaffold allows the cells to produce an ECM, which is kept within the scaffold.

Fibrin gel is biodegradable, biocompatible, and not immunogenic. Fibrin supports the migration of cells, allows the diffusion of growth factors and nutrients, and has some osteoinductive properties itself (Karp *et al*., 2004). The initial immobilization of PCs in fibrin gel allowed efficient cell seeding with minimal cell loss and homogeneous cell distribution in PLGA scaffolds. Fibrin-filled scaffolds provide homogeneous 3D cell distribution and can be considered as a means to provide early stabilization, ease of handling and delivery of cells.

We mixed the cells with fibrinogen solution that subsequently formed fibrin as embedding gels for immobilizing the seeded cells. The findings from morphological and histological examination confirmed an even distribution of cells within PLGA scaffolds, and therefore demonstrated that 3D culture of fibrin gel-immobilized PCs in porous PLGA is feasible. Sufficient pore size allows the cells to migrate or adhere on the surface layer of scaffold matrices. Since PLGA scaffolds are bioresorbable and may be sutured or glued into the defect site, this 3D culture system can temporarily simulate the properties of the ECM until the transplanted cells gradually reconstitute a new ECM in vivo.

Since the proliferation capacity of adult organ-specific cells is low during long-term in vitro cultivation, in particular, reduces their functional quality, more and more attention has been drawn to pluripotent embryonic (ES) or multipotent somatic stem cells for tissue engineering application (Ringe *et al*., 2002). In adults, stem or progenitor cell populations are present virtually in all tissues. Connective tissue progenitors are attractive candidates for clinical use for regeneration of connective tissues because they are readily expanded in the culture, multipotent and amenable to genetic manipulation. They also share two important features related to stem cells: the capacity for asymmetric cell division and self-renewal (Muschler and Midura, 2002). Although BMSC has been considered as an ideal candidate for therapeutic applications, many studies showed that ex vivo expansion of BMSC appears a major hurdle for clinical use: cells tend to senesce and lose their multidifferentiation potential with time in culture. Therefore, very limited clinical studies have been performed (Derubeis and Cancedda, 2004).

As a promising alternative, the periosteum is a candidate as it has a relatively simple histological structure. On the basis of gene microarray technology and flow cytometric analysis, we demonstrated the absence of characteristic embryonic stem cell or hematopoietic stem cell markers and the presence of several mesenchymal stem cell (MSC)-related markers in PCs. These findings clearly support, for the first time, our hypothesis that PCs show an MSC-like character and even adipogenesis potential in vitro (Ringe *et al*., 2005).

PCs can be liberated from periosteum by enzymatic digestion, can be easily expanded in culture and are phenotypically stable. Moreover, PCs of passage 3 showed 37-fold increased cell number compared to primary cultures (data not shown). This impressing expansion capacity ex vivo enables tissue-engineered bone and cartilage repair using cultured PCs. In addition, this experiment was performed in medium containing allogeneic human serum as with clinical relevance for tissue engineered constructs. In contrast, optimal conditions for isolation and expansion of BMSC mostly require medium supplemented with fetal calf serum to generate clinically relevant numbers of cells. BMSC adherence and growth strongly depends on proper serum batches (Lennon *et al*., 1996).

Studies in animal models of 3D composites by use of autologous PC combined with PLGA and fibrin gel showed the potential of these composites to build tissue in vitro (Sittinger *et al*., 1996; Perka *et al*., 2000; Arnold *et al*., 2002). More complete understanding of the biological behavior and the gene consequences involved in cell differentiation within PLGA matrices in vitro is required. Although the implantation of cell-loaded constructs directly after seeding is feasible, the induction of differentiation in vitro allows a safe and reproducible "programming" of the cells within the matrix.

The osteogenic differentiation and matrix mineralization of PC in 3D PLGA constructs was investigated in the current study. Analyses showed a switch from cell expansion towards differentiation after osteogenic induction in PLGA constructs. As the most

abundant protein in bone, type I collagen is essential for bone formation and contributes to matrix production (Ignatius *et al*., 2005). Osteocalcin is the most abundant non-collagenous protein in bone and an important marker for terminal osteoblast differentiation. Osteocalcin influences bone mineralization, in part through its ability to bind with high affinity to the mineral component of bone, hydroxyapatite (Chenu *et al*., 1994). Osteonectin is a gene regarded as potential regulator of mineralization in calcified tissues because of its affinity to hyaluronic acid and type I collagen. Bone-related gene expression in osteogenesis is a developmental stage regulated process (Sommer *et al*., 1996). Taken together, type I collagen, osteocalcin and osteonectin have an abundance of functions in physiological processes of bone metabolism.

Our results implicated a relatively constant level of matrix deposition, and clearly confirmed the osteoblastic phenotype of the PCs grown in 3D cell-seeded constructs under osteogenic differentiation conditions. The PCs displayed a complex pattern of differentiation markers on stimulation. Type I collagen, osteocalcin and osteonectin gene expression indicated that the physiological processes of bone formation is mimicked in the PC-seeded bone constructs. On the basis of the observation of physiological PCs differentiation within PLGA matrices in vitro, we assume that this tissue engineering technique is a promising method for creation of bone neotissue in vivo.

However, expression of type I collagen in PLGA constructs was fairly lower on day 28 than on day 7. This may due to insufficient nutrition of the in vitro cultivated cells within PLGA polymers. This result indicates that in PLGA constructs matrix mineralization starts at an early stage, but decreases with expanding culture time. Further research is needed for better understanding of the global gene expression profile of PC grown in these constructs.

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