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Osteogenic Induction of Human Bone Marrow-Derived Mesenchymal Progenitor Cells in Novel Synthetic Polymer–Hydrogel Matrices

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ABSTRACT

The aim of this project was to investigate the *in vitro* osteogenic potential of human mesenchymal progenitor cells in novel matrix architectures built by means of a three-dimensional bioresorbable synthetic framework in combination with a hydrogel. Human mesenchymal progenitor cells (hMPCs) were isolated from a human bone marrow aspirate by gradient centrifugation. Before *in vitro* engineering of scaffold–hMPC constructs, the adipogenic and osteogenic differentiation potential was demonstrated by staining of neutral lipids and induction of bone-specific proteins, respectively. After expansion in monolayer cultures, the cells were enzymatically detached and then seeded in combination with a hydrogel into polycaprolactone (PCL) and polycaprolactone–hydroxyapatite (PCL–HA) frameworks. This scaffold design concept is characterized by novel matrix architecture, good mechanical properties, and slow degradation kinetics of the framework and a biomimetic milieu for cell delivery and proliferation. To induce osteogenic differentiation, the specimens were cultured in an osteogenic cell culture medium and were maintained *in vitro* for 6 weeks. Cellular distribution and viability within three-dimensional hMPC bone grafts were documented by scanning electron microscopy, cell metabolism assays, and confocal laser microscopy. Secretion of the osteogenic marker molecules type I procollagen and osteocalcin was analyzed by semiquantitative immunocytochemistry assays. Alkaline phosphatase activity was visualized by *p*-nitrophenyl phosphate substrate reaction. During osteogenic stimulation, hMPCs proliferated toward and onto the PCL and PCL–HA scaffold surfaces and metabolic activity increased, reaching a plateau by day 15. The temporal pattern of bone-related marker molecules produced by *in vitro* tissue-engineered scaffold–cell constructs revealed that hMPCs differentiated better within the biomimetic matrix architecture along the osteogenic lineage.

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INTRODUCTION

BONE MATRIX is produced by cells defined as osteoblasts, which arise from mesenchymal progenitor cells in a multistep lineage cascade.[1,2] The osteogenic developmental sequence of mesenchymal progenitors *in vitro* is characterized by three defined periods: proliferation, extracellular matrix maturation, and mineralization.[3] *In vivo*, early bone formation is characterized by the deposition of extracellular matrix (ECM) known as osteoid, which is subsequently mineralized. The basic compounds of ECM are collagen type I (95–97%) and collagen type V (3–5%) and specialized noncollagenous matrix molecules, such as proteoglycan and glycosaminoglycan. The major, fibril-forming collagen types I, II, and III are all synthesized as procollagen molecules whose N- and C-propeptides removed outside the cell before or soon after fibril formation. In the formation of bone collagen type I, a fraction of the N-propeptides are retained in the bone matrix after it mineralizes in a form that leaves a single phosphorylated serine residue. Hence, one of the physical properties that sets demineralized bone matrix apart from the soft tissue ECM is a failure to swell in acid and a specific profile for cross-linking amino acids that leads to superior mechanical properties. In the early phase of bone healing or remodeling, only a few areas of mineralization are observed. After prolonged maturation, minerals—especially hydroxyapatite crystals—are embedded in the ECM. *In vivo*, fully mineralized ECM is defined as bone matrix.

At bone defect sites, on a cellular level, the above-described biochemical cascades are initiated to form appropriate bone tissue by osteoblast progenitors, which are recruited from tissues outside the bone (periosteum) and inside the bone (bone marrow). Repair of bone defects may be limited by insufficient self-regeneration potential. However, bone repair may be supported by biocompatible osteoinductive or osteoconductive grafts. Nevertheless, a major challenge for reconstructive surgery is the closure of critical-size defects caused by tumor resection or trauma and to diminish bone loss associated with osteoporosis and osteoarthritis. Current, clinically established therapeutic approaches focus on the implantation of allografts,[4] metal devices,[5] or porous glasses and ceramics[6] to assist repair of bone defect sites.

However, all these techniques have disadvantages that are discussed in detail elsewhere.[7] These constraints have triggered a need for new therapeutic concepts to design and fabricate better functioning bone grafts. It is within this context that the field of tissue engineering has emerged.[8] The primary objective of a variety of tissue engineering strategies is to regenerate structural and functional tissue, using living cells in combination with a scaffold. For example, poly(lactic-co-glycolic acid)/poly-(ethylene glycol) (PLGA/PEG) foams as a three-dimensional model system have been used to study the development of mesenchymal tissues *in vitro*.[9] Porous sponges fabricated from PLGA and PEG has also been applied as scaffold material for mammalian bone marrow-derived cells. In other experiments composites constituted of porous hydroxyapatite (HA) seeded with mesenchymal cells showed *in vivo* osteogenic potential.[10] Cross-linked hyaluran sponges (ACP) as well as benzylated hyaluran (HYAFF-11) have been used in experiments dealing with the chondrogenic induction of mesenchymal stem cells (MSCs) and to treat osteochondral defects.[11]

The use of synthetic or natural polymer matrices with low mechanical properties (high porosity) and fast degradation kinetics results in tissue-engineered grafts with high biological activity, but with poor structural properties, in particular low strength and stiffness. Such tissue-engineered grafts can be used in a manner similar to cancellous bone chips, for example, in non-load-bearing applications or in combination with internal or external fixation devices. In contrast, for the reconstruction of large and/or load-bearing defects, scaffolds able to withstand the forces and stress not only of the wound contraction but also of a highly dynamic biomechanical environment, while the matrix is formed, are required. Scaffolds made of polycaprolactone (PCL) fabricated by the rapid prototyping technology of fused deposition modeling (FDM) exhibit the mechanical properties to be used in a load-bearing implantation site and a matrix architecture that allows for rapid vascularization.[12,13] In addition, use of the highly reproducible and computer-controlled FDM technique for the manufacturing of scaffolds allows the fabrication of tissue-engineered grafts that have been designed on the basis of computed tomography (CT) scans of individual defect sites.[14,15]

Currently, one major research thrust in *in vitro* bone engineering is focused toward the induction of hard tissue formation by seeding and altering progenitor cells in such three-dimensional constructs.[16] These mesenchymal progenitor cells reside in various tissues such as muscle bone marrow and fat.[17] Human mesencymal stem cells (hMSCs) can be isolated from adult bone marrow and grown *in vitro*.[18,19] Important characteristics of hMSCs are their ability to proliferate in culture with an attached fibroblastic morphology, the presence of specific marker proteins on their surface, and their *in vitro* multilineage potential. This potential to differentiate into distinct mesenchymal tissues such as bone, cartilage, fat, tendon, muscle, and stroma[19] offers new opportunities for tissue reconstruction. Although mesenchymal stem cells are present at a low frequency in bone marrow, their capacity to undergo extensive replication without loss of differentiation potential makes them promising candidates for the regeneration of connective tissue and, especially, osteogenic tissue repair applications.[16,20–22]

In the past the osteogenic differentiation of bone marrow-derived mesenchymal progenitors isolated from rabbits and pigs were studied *in vitro* and *in vivo* in designed

and fabricated by FDM scaffolds.[13,14] In the presented *in vitro* study we evaluated the osteogenic potential of human bone marrow-derived mesenchymal progenitor cells when seeded in combination with a biomimetic hydrogel into a novel bioresorbable, fully interconnected, three dimensional construct of caprolactone or polycaprolactone–hydroxyapatite.

MATERIALS AND METHODS

Scaffold design and fabrication

Polycaprolactone (PCL) scaffolds were fabricated by fused deposition modeling (FDM) as previously described by Hutmacher *et al.*,[14] except that the newest FDM techniques (FDM 3000; Stratays, Eden Prairie, MN) were employed. PCL and PCL–hydroxyapatite (90:10%) scaffolds, each with a lay-down pattern of 0/60/120° and a porosity of 65% and measuring 10 × 10 × 4 mm, were used (Fig. 1). One day before cell seeding, the scaffolds were sterilized in 70% ethanol overnight. The ethanol was removed by washing three times in changes of phosphate-buffered saline solution (PBS). The scaffolds were then transferred for 24 h into an incubator at 37°C for drying.

Cell isolation and culture

Mesenchymal progenitor cells were isolated via gradient centrifugation, as described previously,[23] from iliac crest bone marrow aspirates taken from a 2-year-old

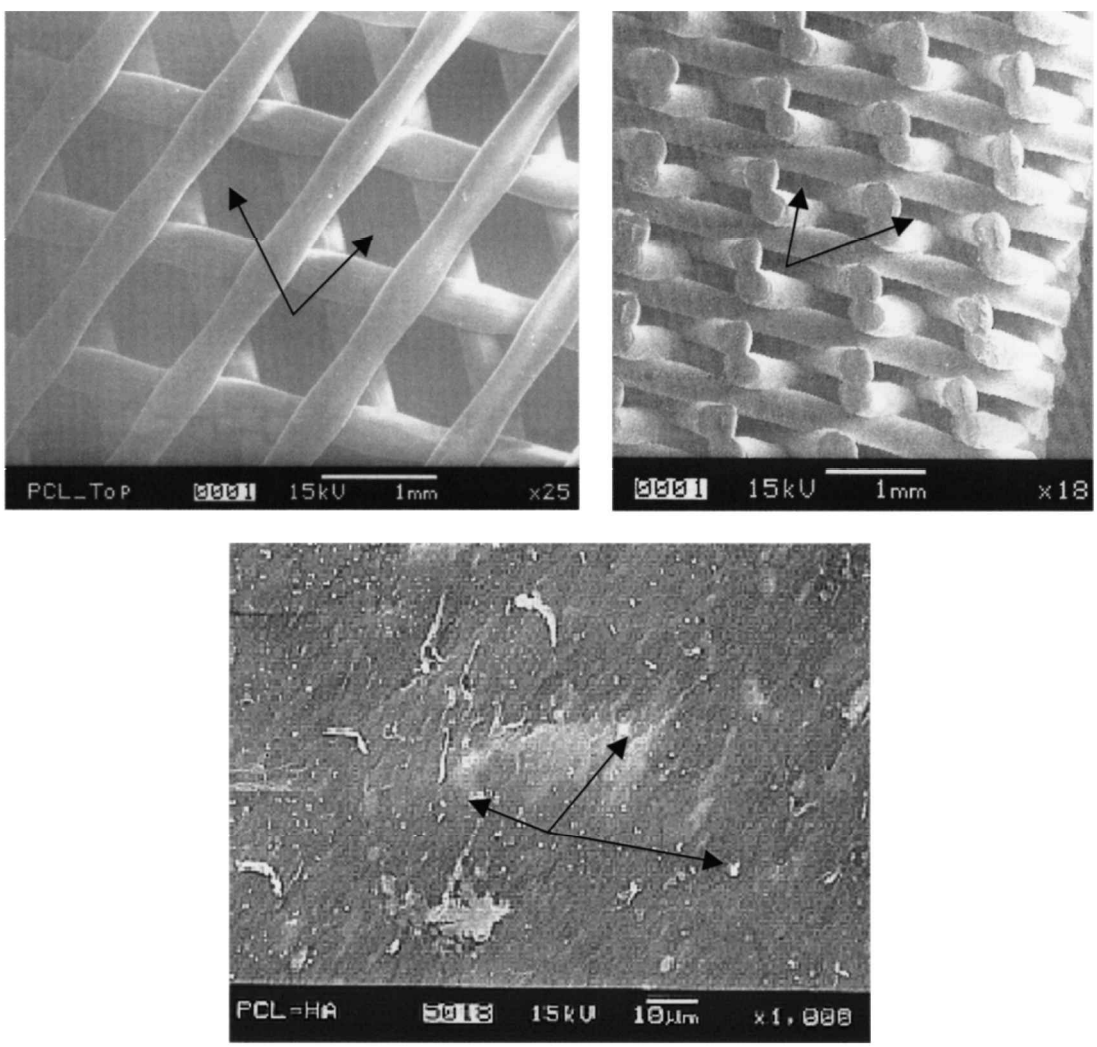

FIG. 1. Representative scanning electron micrographs of a PCL scaffold with a lay-down pattern of 0/60/120° and a porosity of 65%. The honeycomb pore architecture can be viewed in the $-z$ direction of the FDM build process (*top left*, arrows). Computer-controlled alignment of bars and struts in three dimensions results in a fully interconnected channel-like matrix architecture (*top right*, arrows). High-resolution scanning electron micrograph of a PCL–hydroxyapatite (90:10%) scaffold shows that the HA is exposed on the surface of the matrix (*bottom*, arrows).

patient undergoing elective surgery. Cells were seeded at a density of 1.6×10^4 cells/cm^2. The cells were cultivated under standard culture conditions with low-glucose Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Rockville, MD) supplemented with 10% fetal calf serum, penicillin (50 U/mL), and streptomycin (50 μg/mL). Medium was changed after 4 days and then every 3 days thereafter. When cells had reached 70% confluence they were detached by the addition of a solution containing 0.25% trypsin–EDTA and replated at a density of 5.5×10^3 cells/cm^2.

Assessment of differentiation potential in two-dimensional culture system (control)

After passage 5, the cells were differentiated into osteoblasts and adipocytes with the respective induction medium to prove the multilineage potential of the isolated bone marrow-derived mesenchymal progenitor cells. Osteogenic and chondrogenic differentiation was initiated according to the protocol of Owen *et al.*[24] For adipogenesis, a modified protocol was used to induce differentiation of progenitor cells by the use of 0.2 mM indomethacin.[25] Chondrogenic lineage formation was analyzed by histology. Osteogenic differentiation was demonstrated by alkaline phosphatase staining with fast red and immunoassaying with a monoclonal mouse anti-human antibody (100 μg/mL; 1:50 dilution) against osteocalcin (Biodesign International, Kennebunk, ME). For visualization the EnVision system (horseradish peroxidase [HRP], diaminobenzidine [DAB]; Dako, Carpinteria, CA) was used according to the manufacturer's protocol. Adipogenic differentiation was demonstrated by oil red O staining.

Assessment of differentiation potential in three-dimensional culture system

Noninduced progenitor cells of passage 5 were detached by trypsin–EDTA treatment, washed with PBS, and seeded into polycaprolactone (PCL) and PCL–hydroxyapatite (90:10%) scaffolds. The cells were resuspended in fibrinogen at a ratio of 2:1, reaching a concentration of 3×10^6 cells/mL. Both PCL and PLC–HA frameworks were loaded with 40 mL of fibrinogen–cell suspension, resulting in a seeding density of 3×10^6 mesenchymal progenitor cells (MPCs). Twenty milliliters of thrombin (500 U) was used to polymerize the cell–fibrinogen matrix inside the completely interconnected and honeycomb pore architecture. The specimens were cultured under static conditions in standard cell culture medium for 2 days. Thereafter, medium was changed and the specimens were cultured in osteogenic medium I for 7 days and for 5 weeks in osteogenic medium II as described.[24] During the culture period, supernatants were collected after specific time points as indicated before medium exchange and stored at -80°C.The cell culture media protocol for osteogenic differentiation in monolayers was transferred directly to three-dimensional cultivation of progenitor cells within PCL and PCL–HA scaffolds. However, for three-dimensional reassembly of mesenchymal progenitors and optimal seeding efficiency and efficacy, cells were embedded in a biomimetic fibrin matrix to temporarily immobilize them and to obtain a three-dimensional homogeneous distribution of cells within the large size and fully interconnected pore architecture (Figs. 1–3).

Histology

Five-micron-thick sections from the 6-week culture group of scaffold–cell constructs were cut with a cryomicrotome (RM 2165; Leica, Bensheim, Germany) and mounted on poly-L-lysine (Sigma, St. Louis, MO)-coated slides. Slides were stained with von Kossa silver nitrate.

Metabolic assay

Both scaffold–cell construct groups were assayed for procollagen type I, osteocalcin, and alkaline phosphatase as osteogenic markers, The C-terminal propeptide of collagen type I was determined in the supernatants ($n = 2$) of the seeded specimen, using a human-specific enzyme-linked immunosorbent assay (ELISA) (Prolagen-C; Metra Biosystems, Mountain View, CA) according to the manufacturer's protocol. The concentration of bone GIa protein (BGP-osteocalcin) in the supernatants ($n = 2$) of cell-bearing matrices and two-dimensional cell cultures

FIG. 2. (**A**) Morphological appearance of isolated human progenitor cells after 9 days in monolayer culture. (**B**) Phase-contrast microscopy of a confluent monolayer of hMPCs induced down the chondrogenic pathway. (**C**) Immunocytochemistry with an osteocalcin antibody after osteogenic differentiation in monolayer culture. (**D**) Adipogenic differentiation detected via oil red O staining of lipid droplets and lipid vacuoles. Original magnification: (**A** and **B**) ×40; (**C** and **D**) ×100.

FIG. 3. (**A–D**) Representative phase-contrast microscopy images 2 days after seeding undifferentiated human progenitor cells into PCL scaffolds (**A** and **B**) and PCL–HA frameworks (**C** and **D**). Independent of the framework matrix, the cells initially remain round (arrows), when fibrin as a temporary biomimectic cell carrier is used. Seven days postseeding undifferentiated human progenitor cells have established a fibroblast-like morphology (arrows) and migrate and proliferate in three dimensions within the hydrogel (**B** and **D**).

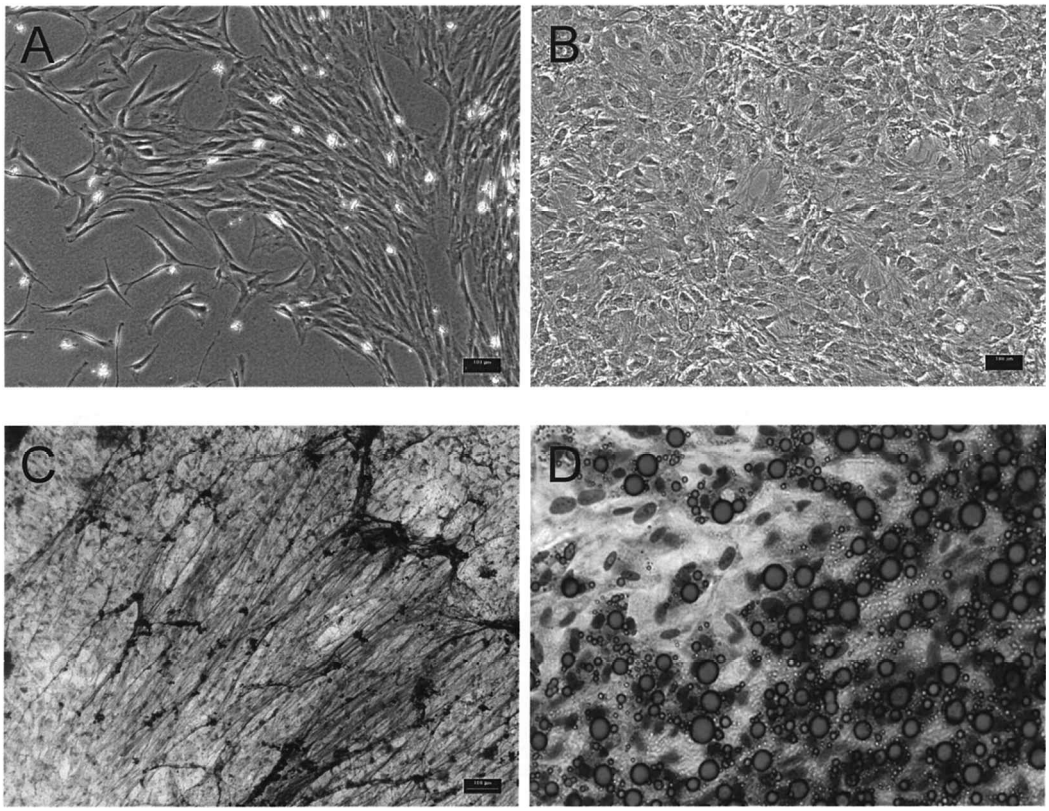

FIG. 2.

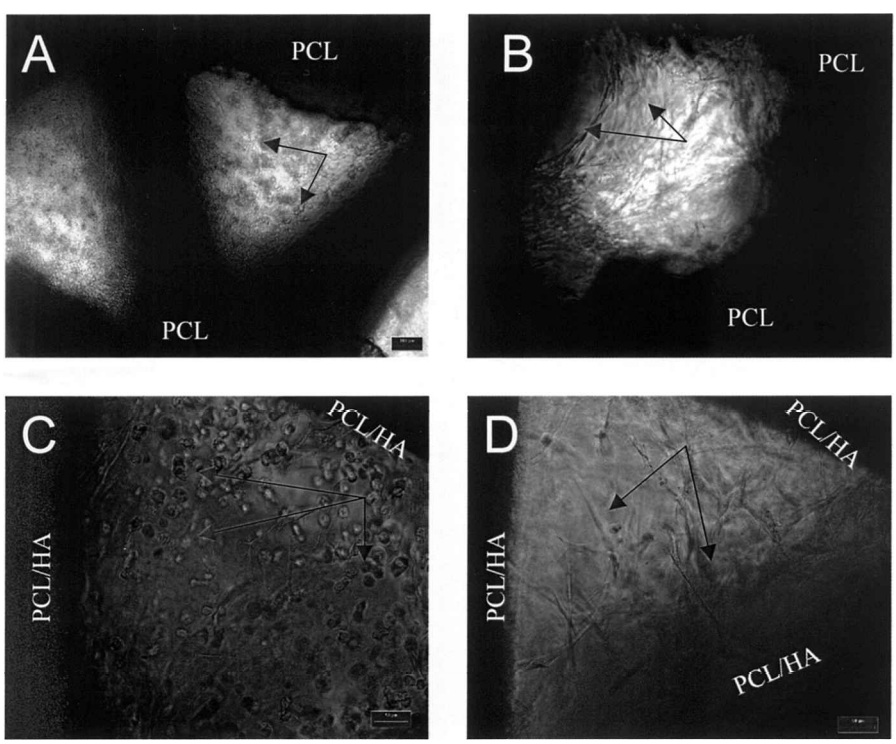

FIG. 3.

was determined by an osteocalcin ELISA (h-Ost EAIA kit; Biosource, Nivelles, Belgium) according to the manufacturer's protocol. Extinction was measured at 405 nm, using an Antos Hill microplate reader. The amount of alkaline phosphatase (ALP) ($n = 1$) was quantified by measuring the conversion to *p*-nitrophenol from *p*-nitrophenyl phosphate according to the manufacturer's protocol (Sigma). Cell metabolism and viability were analyzed via MTS test according to the supplier's protocol (Promega, Madison, WI).

Phase-contrast, scanning electron, and confocal laser microscopy

Adhesion, proliferation, and distribution of the cells were studied by phase-contrast light microscopy (IX70; Olympus, Tokyo, Japan). The establishment of the cell phenotype, intercellular connections, and extracellular matrix production were examined every second day. Routine qualitative analysis of cell attachment and proliferation in the scaffold–cell constructs was performed by phalloidin–propidium iodide (PI) staining whereas cell viability was documented via fluorescein diacetate–propidium iodide (FDA–PI) staining as described previously,[26] using an Olympus IX70-HLSH100 Fluoview confocal laser microscope. A Leica TCS SP2 (Leica Microsystems, Singapore) in combination with a custom-made Leica long lens objective (×10) was used to view the proliferation and ECM formation deep inside the scaffold–cell constructs. Depth projection images were constructed from up to 50 horizontal image sections (20 μm each) through the stained scaffold–cell constructs.

For scanning electron microscopy (SEM), specimens were fixed with 2.5% glutaraldehyde for 4 h at 4°C. Samples were dehydrated in a graded ethanol series of 30, 50, 90, and 100%, dried, gold sputtered, and examined by SEM (JSM-5800LV; UEOL, Tokyo, Japan) at 15 kV, using the low-vacuum mode.

RESULTS

Isolation and differentiation in two-dimensional cultures

Human mesenchymal progenitors were successfully isolated from bone marrow aspirates, via gradient centrifugation, expanded in monolayer culture, and evaluated for osteogenic, chondrogenic and adipogenic differentiation potential (Fig. 2). The progenitor cells grew in distinct colonies and demonstrated a fibroblastic morphology (Fig. 2A) as first described by the groups of Caplan[16] and Haynesworth.[19] In addition, a small number of cells described in the literature as flat cells were also observed.[27,28] The fibroblastic morphology remained stable during prolonged culture and subculture in monolayers (Fig. 1, left). With passaging, routine phase-contrast microscopy did show that flat cells were overgrown by the fibroblast-like cells (micrographs not shown).

Following *in vitro* induction mesenchymal progenitors continued to proliferate and readily formed multilayers showing differentiation along all three lineages. By day 7, the chondrogenic (Fig. 2B) and osteogenic (Fig. 2C) differentiation potential was confirmed via cell morphology as well as visualization of alkaline phosphatase (ALP) activity (data not shown) and via immunostaining of the bone-specific matrix protein osteocalcin. After *in vitro* induction of adipogenic differentiation, staining with oil red O revealed cells filled with neutral lipid droplets, which substantiated the potential of the human bone marrow-derived progenitors to differentiate along the adipogenic lineage (Fig. 2D).

FIG. 4. (**A** and **D**) Representative scanning electron microscopy images of human progenitor cells adhering at the bars of PCL (**A**) and PCL–HA frameworks (**B**) and building cell bridges within the interconnected pore architecture. Cells use the biomimetic hydrogel as a three-dimensional template to migrate and proliferate inside the pore architecture to bridge the bars and struts of the PCL-based framework. The viability of the cells in the scaffold was detected via confocal laser microscopy (**C** and **D**) by using fluorescence markers that allow life/death stain (PI/FDA): dead cells were stained red whereas viable cells were stained green. The qualitative image analysis revealed that a large number of cells stayed viable throughout the entire culture period. Original magnification: (**A**) ×1609; (**B**) ×804; (**C**) ×100; (**D**) ×100.

FIG. 5. (**A** and **B**) Special confocal laser microscopy was performed to view proliferation and ECM formation deep inside scaffold–cell constructs. A custom-made Leica long lens objective (×10) allowed generation of depth projection images of up to 1 mm. Three-dimensional images were constructed from up to 50 horizontal image sections (20 μm) taken within the scaffold–cell constructs. In this phalloidin–propidium iodide-stained specimen, adhesion and proliferation of the MPCs on the PCL surface (*left*) and cell-to-cell contact are shown by studying the actin fiber (green) and cell nucleus (red) arrangement. Intracellular bundles of F-actin filament (stress fibers) indicate the formation of focal adhesions. The cells are forming focal contact patches on the bars and struts of the framework whereas prominent intracellular F-actin filaments can be detected when cells are bridging throughout pores (*left and right*, arrows). Comparing these micrographs obtained by phalloidin–PI staining with the SEM images in Fig. 4A and B further reveals the proliferation and colonization pattern of the hMPCs. Starting at week 2, the cells appear to span three-dimensionally across the pore structure by cell-to-cell and cell-to-ECM contact, preferably by way of the interjunctions of the columns and rods of the honeycomb scaffold architecture (*right*, arrows).

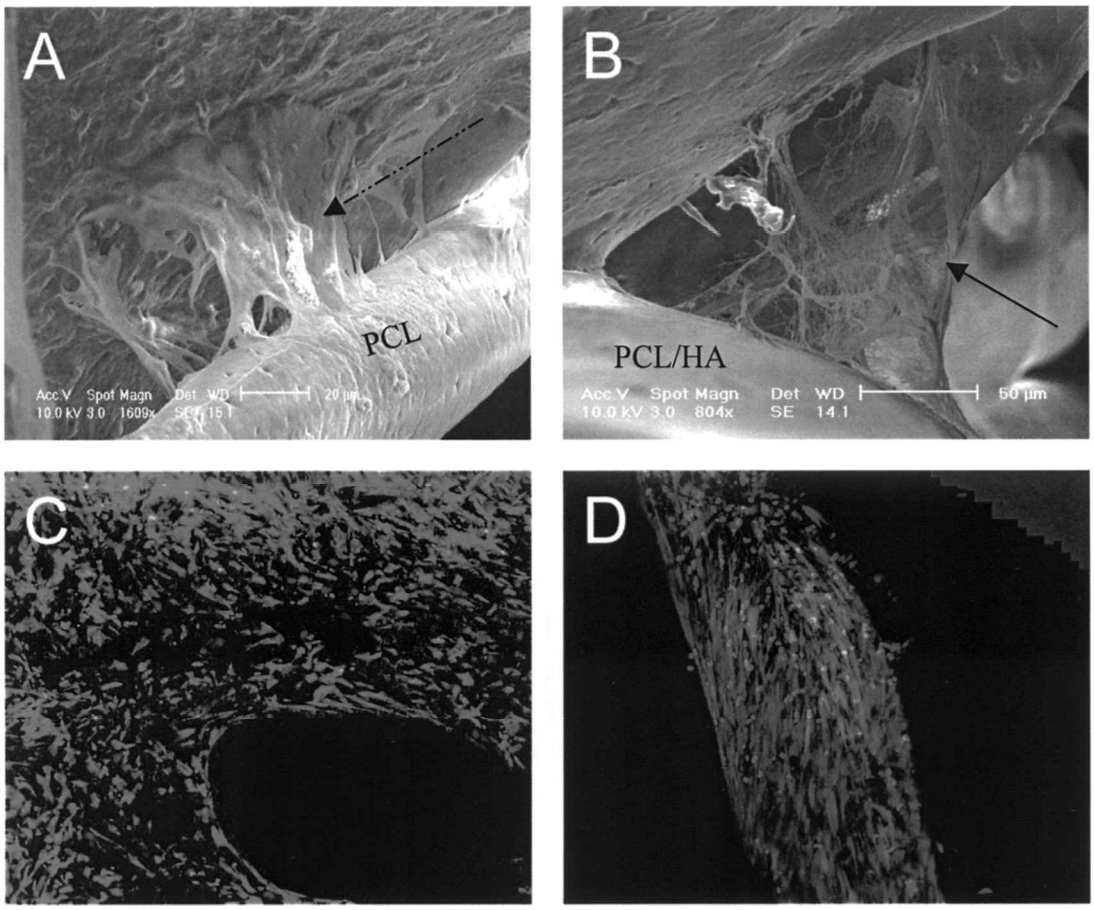
A
PCL
Acc.V Spot Magn Det WD 20 µm
10.0 kV 3.0 1609x SE 15.1
B
PCL/HA
Acc.V Spot Magn Det WD 50 µm
10.0 kV 3.0 804x SE 14.1
C
D

FIG. 4.

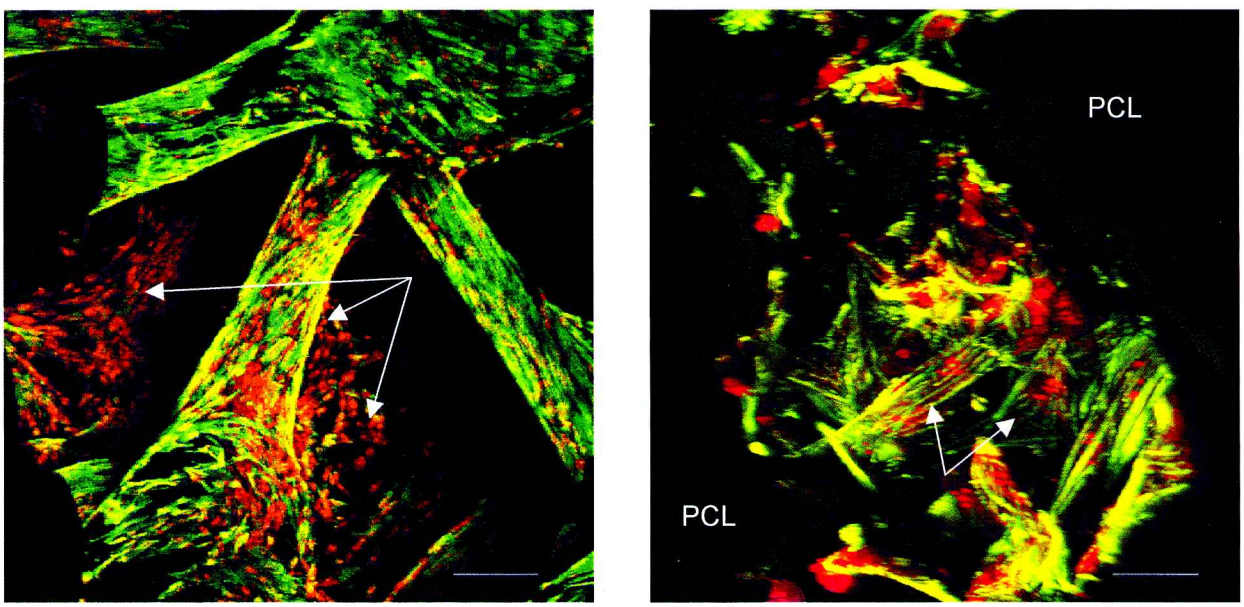
PCL
PCL

FIG. 5.

Cell growth and differentiation in scaffold–cell constructs

During the first 4 days of culture, the cells remained round shaped within the hydrogel and revealed a homogeneous distribution within the PCL (Fig. 3A) and PCL–HA (Fig. 3C) scaffolds. Starting on day 5, the cells began to stretch and proliferated within the biomimetic hydrogel and showed a fibroblast-like phenotype within both matrix materials (Fig. 3B and D). Scanning electron (Fig. 4A and B) and confocal laser (Fig. 4C and D) microscopy revealed the adherence of mesenchymal progenitors onto the PCL and PCL–HA fibers and, after 2 weeks in culture, bridging the pores of both scaffolds via the production of ECM. Henceforth, phase–contrast microscopy and SEM showed that after 3 weeks the entire architecture of both scaffold groups was filled with cells and ECM. This was in accordance with confocal laser microscopy (CLM) images taken deep inside the FDM-fabricated frameworks 3 weeks postseeding (Fig. 5). Qualitative examination via routine CLM of cells did reveal that a large number of cells stayed active for the entire culture period, which was in conformity with the semiquantitative immunoassays (Fig. 6).

On the basis of the imaging data a significant difference between PCL and PCL–HA with respect to the proliferation pattern of the induced hMPCs could not be detected. The MTS test revealed an almost linear increase in cell metabolism for 15 days until a constant metabolic level was reached after 20 days of culture (Fig. 6A). This was in accordance with image analysis, which revealed that after prolonged cultivation of scaffold–cell con-

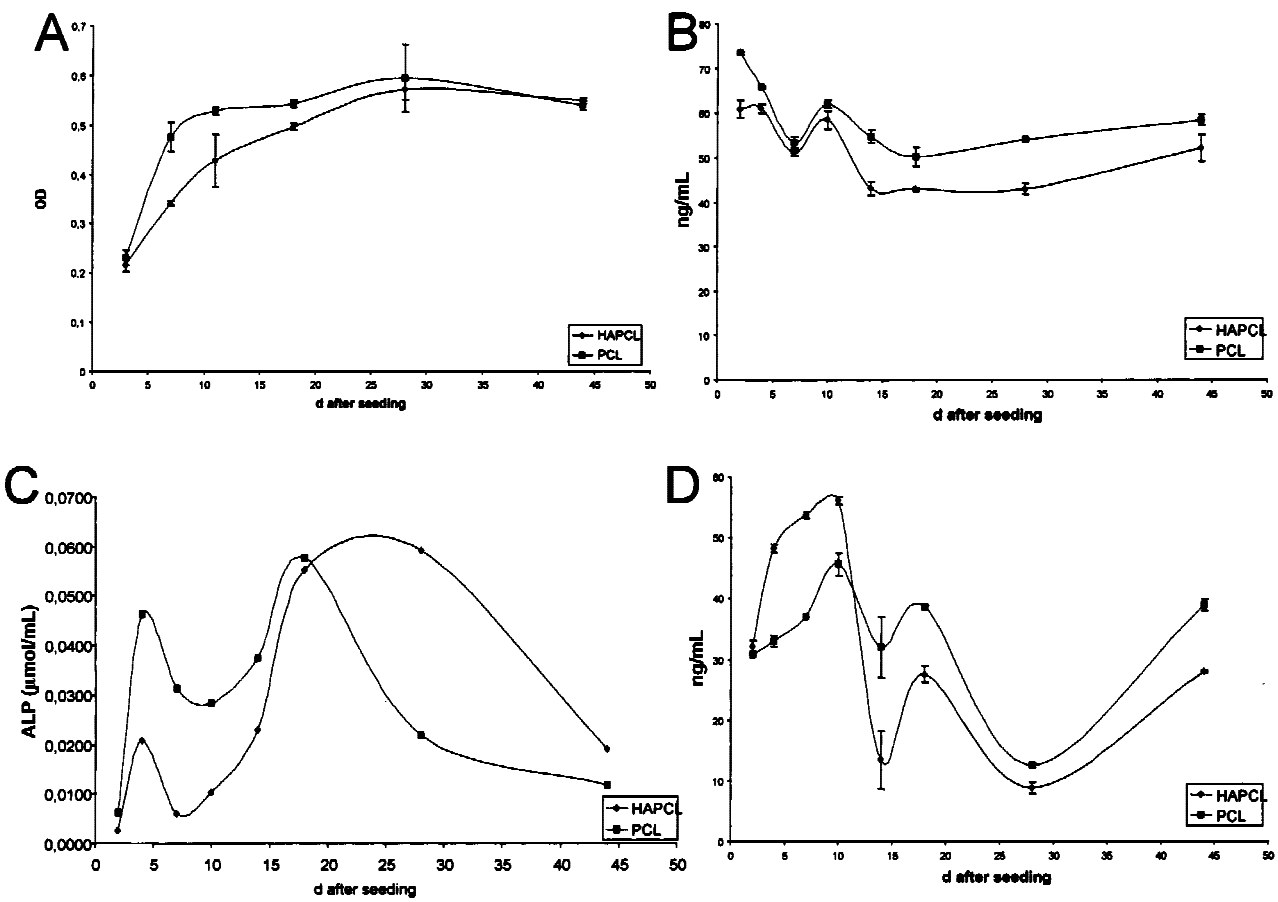

FIG. 6. (**A–D**) Cell metabolism in the scaffold–cell constructs of both groups was detected via MTS test during the entire culture period (**A**). Also assessed was procollagen I (**B**), alkaline phosphatase (**D**), and osteocalcin (**D**) secretion by human progenitor cells in a three-dimensional environment during 6 weeks of culture. The up-and-down regulation of ALP and osteocalcin (OC) activity for both construct groups is related to the fact that the MPC population is inhomogeneous, that is, they are in different stages of their cell cycle. ALP is a marker for actively proliferating cells and it can be argued that most of the MPCs stayed in the proliferative phase until the entire pore architecture of the framework was filled with cells and ECM (week 3 to 4). In contrast, OC is known to be secreted by differentiated cells. It could be detected at the late stage of the culturing period; when the entire construct was filled with cells and mineralized matrix (see also von Kossa staining) OC showed a strong linear increase because most of the induced MPCs were driven down the osteogenic pathway.

structs, by day 28, FDA–PI staining still showed vital fibrocytic cells inside the honeycomb scaffold architecture (data not shown).

During osteogenic differentiation of human mesenchymal progenitors in three-dimensional PCL and PCL–HA scaffolds, viability, physiological activity, as well as osteogenic marker proteins were documented by MTS (Fig. 6A) and also by quantitative ELISAs. The osteogenic marker proteins collagen type I, alkaline phosphatase (ALP), and osteocalcin were also assessed (Fig. 6B–D). The qualitative determination of cell viability and physiological activity via MTS assay revealed a steadily increasing activity of mesenchymal progenitors within the first days of three-dimensional culture in PCL scaffolds. By day 15, the activity reached a plateau and remained constant during further cultivation. Initially cells in the PCL–HA scaffolds showed less metabolic activity reaching the same plateau as the PCL specimen by day 28 (Fig. 6A).

Quantitative analysis of osteogenic marker proteins revealed a decreased secretion of procollagen type I for mesenchymal progenitors seeded onto PCL scaffolds during the culture in osteogenic medium I (OM-I) up to day 7. After stimulation with osteogenic medium II (OM-II) the amount of procollagen type I increased slightly, but decreased again to reach a relatively stabilized secretion level between 48 and 58 ng/mL. The course of PCL–HA is similar to PCL but reached a secretion level between 42 and 40 ng/mL (Fig. 6B). The amount of secreted ALP, instead, showed a more dynamic course during the culture period (Fig. 6C). Directly after stimulation of mesenchymal progenitors with OM-I, the amount of secreted ALP increased and displayed a maximum ALP level of 47 nmol/mL for PCL and 20 nmol/mL for PCL–HA on day 4. Up to day 7, the amount of ALP decreased for both materials and was induced again by stimulation with OM-II, resulting in a maximum secretion of 58 nmol/mL. By day 16, the cultivation of progenitors within the PCL scaffolds with OM-II revealed a decreasing secretion of ALP, which reduced the amount of ALP to 12 nmol/mL on day 45. PCL–HA showed also a decreasing secretion of ALP to a minimum of 20 ng/mL on day 45.

Compared with ALP, secretion of osteocalcin demonstrated an almost inverse course (Fig. 6D). During the early periods of osteogenic stimulation up to day 14, the amount of osteocalcin decreased when ALP was induced by OM-I or OM-II and vice versa. Remarkably, on day 28, the amount of osteocalcin was low (12 ng/mL, PCL; 31 ng/mL, PCL–HA), but increased again during further culture in osteogenic medium, leading to osteocalcin secretion at 38 ng/mL on day 45 in PCL scaffolds and at 26 ng/mL in PCL–HA scaffolds. These results were confirmed by von Kossa staining (Fig. 7), which showed mineralized areas on the scaffold bars and struts as well as in the honeycomb pore architecture after 6 weeks of culturing in an osteogenic medium.

DISCUSSION

Adult stem cells are sparsely distributed in the body and their main functions are 2-fold: first, to produce identical copies of themselves for long periods of time, which is also referred to in the stem cell literature as the capacity for long-term self-renewal; second, to engender transitional cell types before they reach the end of the differentiation cascade. The intermediate cell is defined as a progenitor or precursor cell, a cell that is regarded as committed to differentiate along a particular cellular pathway.[16,17]

In the present study, we have reported the *in vitro* osteogenic differentiation potential of human bone marrow-derived mesenchymal progenitors in novel matrix architectures that were built by using a three-dimensional bioresorbable synthetic framework in combination with a hydrogel. The PCL and PCL–HA scaffolds have honeycomb matrix architecture and mechanical properties suitable for maintenance of the structural integrity of tissue-engineered bone grafts in load-bearing applications. The fibrin glue-based hydrogel presents to the cells a biomimetic environment that supports cell attachment, migration, and production of ECM in three dimensions.

Bone marrow has been considered a source of progenitor cells for mesenchymal and hematopoetic tissues. Previous results indicated that mesenchymal progenitor cells from human bone marrow have the potential to differentiate into distinct mesenchymal lineage cells including osteoblasts,[29] chondrocytes,[30] adipocytes,[31] tenocytes,[32] and marrow stromal cells.[21] The multipotent nature of bone marrow-derived cells might be based on particular mono- or bipotential precursor cells, which are able to differentiate into distinct mesenchymal cell types, or on multipotent stem cells differentiating into various mesenchymal tissues.[16,18] We demonstrated that for this experiment isolated mesenchymal progenitors derived from juvenile bone marrow have the potential to undergo multiple differentiation pathways. Although it has been suggested that osteoblasts and adipocytes share common precursors within the adult stromal system,[31] human bone marrow-derived precursors showed no obvious differentiation into adipocytic cells, when stimulated with osteogenic medium supplemented with dexamethasone (Dex) in monolayers. In other studies, depending on the presence of Dex in primary or secondary cultures of marrow stromal cells, an inverse relationship between the differentiation of adipocytic and osteogenic cells in marrow stromal cells has been reported.[33]

A great number of processing techniques have been developed to design and fabricate three-dimensional scaf-

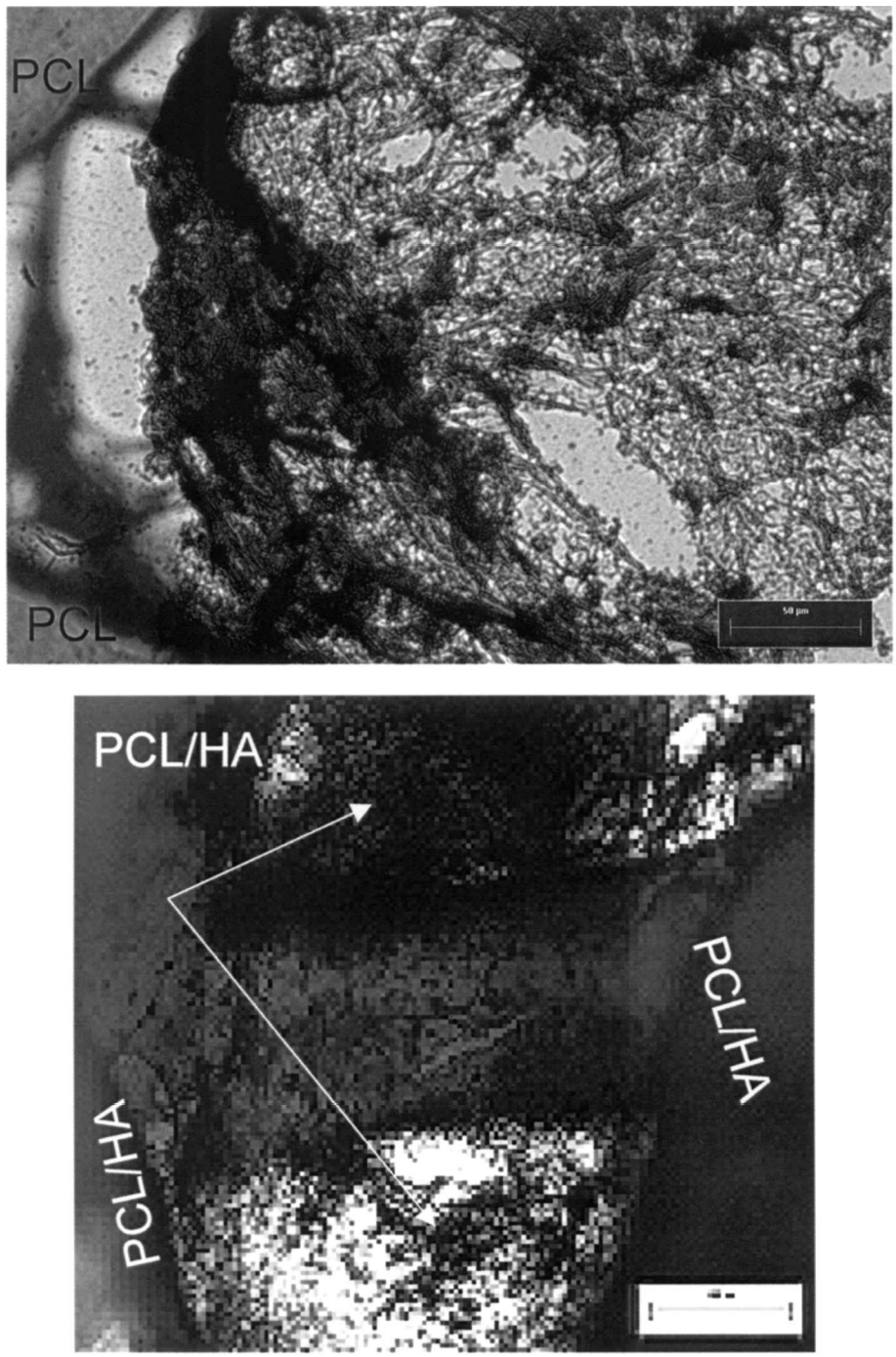

FIG. 7. Representative histological sections of cultured scaffold–cell constructs (*top*, PCL; *bottom*, PCL–HA). Osteogenic induction and long-term culturing (6 weeks) resulted in mineralized ECM formation. Both groups showed, on von Kossa silver nitrate staining, dark brown-black areas (arrows) of calcium phosphate deposits on the bars and struts as well as in the pore architecture. Scale bars: *top* and *bottom*, 50 μm.

folds for bone tissue engineering.[33a] A wide range of scaffold characteristics, such as porosity and pore size, has been reported using such fabrication techniques.

However, no single technique has allowed researchers to design and fabricate scaffolds with a completely interconnected pore network, highly regular and reproducible scaffold morphology, microstructure that varies across the scaffold matrix, and that is solvent-free, using a computer-controlled process. These are essential scaffold features to facilitate cell proliferation and differen-

tiation, extracellular matrix synthesis, and flow transport of nutrients and wastes. Equally important from a clinical point of view is to apply a design and material strategy in which the scaffold should protect proliferating cells and their extracellular matrix, inside the pore network, from being mechanically overloaded for a sufficient period of time and maintain the shape until a mature tissue is formed inside the entire porous scaffold architecture.[15]

Rapid prototyping technologies have emerged that can be used to manufacture scaffolds with more suitable structural and mechanical properties for bone and cartilage regeneration. Hutmacher and co-workers[12–14] designed and fabricated novel polycaprolactone (PCL) scaffolds by fused deposition modeling, which offers the possibility to design and fabricate highly reproducible bioresorbable three-dimensional scaffolds with a fully interconnected pore network and outstanding physical properties. Arranged in a regular manner, interconnected three-dimensional channels are produced and the design of a pore morphology, which varies across the scaffold structure, is allowed. Scaffolds with a porosity of 60 to 65% have mechanical properties in the range of cancellous bone and have been studied in bone tissue engineering by using differentiated osteoblast-like cells.

A great number of the currently applied scaffold designs (foams, textiles, etc.) use fast-degrading polymers combined with a high porosity matrix (>90%). These types of matrices do not possess the structural stability to be applied in the reconstruction of bone defects that are medium to large and/or load bearing.[15] Therefore, we present in this article a scaffold–cell construct design that consists of a mechanically stable structural framework fabricated from a slow-degrading polymer. Beside mechanical stability, another important property of this construct is to support three-dimensional tissue development and progenitor cell differentiation within the honeycomb matrix architecture by using a biomimetic hydrogel as temporary cell carrier.

Surface engineering may potentially be used to create matrices that elicit controlled cellular adhesion and maintain differentiated phenotypic expression. Such modifications generally involve enriching surfaces with extracellular matrix (ECM) components (such as the adhesive protein fibronectin, FN), or their functional domains.[33b] However, a technically much easier and more physiological approach is to use a biomimetic cell carrier, which contains ECM factors, to deliver the cells into a scaffold made of a synthetic polymer. On the basis of this strategy the advantages of synthetic and natural polymers can be straightforwardly combined.

Healing of most mesenchymal tissue in the body depends on the blood clot and consequently fibrin formation (fibrinogen is converted to fibrin in the formation of a blood clot, via the enzymatic action of thrombin), as a matrix for cell migration and a subsequent cascade of biological activities that lead to the eventual formation of reparative tissue in the defect.[40] As reported by a number of tissue-engineering groups and confirmed once again by the results of this study, fibrin provides an excellent biomimetic environment for delivering and culturing mesenchymal cells in a synthetic polymer-based three-dimensional scaffold.

It is known that if cells are directed into the osteogenic lineage, ALP is an early differentiation marker but the cells maintain a strong proliferative capacity. Hence, an increased production of ALP was observed until it peaked on day 20 for PCL and on day 25 for PCL–HA. SEM, phase-contrast light microscopy, and confocal laser microscopy confirmed that at this period of culturing the entire scaffold architecture was filled with cells and ECM.

Several authors have studied the synthesis of ALP and osteocalcin (OC) of osteoblast-like cells *in vitro*.[24,41–43] ALP is an early marker in osteogenesis whereas OC is known as a late marker. In this study, a strong cyclic expression of osteocalcin was observed for the first 3 weeks. This is most likely related to the inhomogeneous cell population, which is common for primary cells from bone marrow aspirates. However, it was during the late stage of the culturing period, when the entire scaffold architecture was filled with cells and mineralized matrix (see von Kossa staining), that osteocalcin showed a strong linear increase.

Fibrillar type I collagen of the ECM of bone differs in physical and chemical properties from polymers of the same molecular type of collagen in nonmineralized tissues. The unique physical properties of bone collagen have not been fully explained at the molecular level, although the distinctive cross-linking chemistry is one suspected factor. Carboxy-terminal propeptide of type I collagen (PICP) and bone Gla-protein-osteocalcin (BGP) are among the most important components of the organic bone matrix and play a key role in bone formation.[44–45] Propeptides of collagen types I, II, III are present in serum and as by-products of metabolic activity. Hence, clinical immunoassays for measuring propeptides in the blood have been developed and routinely applied. Although propeptide I evaluated in serum correlates with serum osteocalcin and alkaline phospatase activity, it is generally less sensitive than these other two bone formation markers.[46]

To our knowledge this is the first time that a procollagen type I ELISA kit was used to study its expression in *in vitro* bone engineering. PICP expression showed a cyclic up- and downregulation for both scaffold types for the first 15 days of culture. One week after the second time of induction PCIP showed a slow linear increase for the rest of the culture period. These semiquantitative data correlate with the microscopy data, which showed that

both PCL- and PCL–HA-based constructs were completely filled with cells and ECM at the end of the culturing period.

At present, aliphatic polyesters, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and PCL, are by far the most applied synthetic polymer materials in the fabrication of scaffolds. In general, polymers of the aliphatic polyester group undergo bulk degradation and their molecular weight, in addition to their mechanical properties, begin to decrease within weeks [PGA, poly(D-lactic acid) (PDLA), etc.] or within a few months to years [poly(L-lactic acid) (PLLA), PCL, etc.] of placement in an aqueous medium. However, the mass loss does not start until the molecular chains are reduced to a size that allows them to freely diffuse out of the polymer matrix. This phenomenon, described and analyzed in detail by a number of research groups,[47–50] results in accelerated degradation and resorption kinetics until the physical integrity of polymer matrix is compromised. The mass loss is accompanied by a release gradient of acidic by-products.

Potential problems of biocompatibility in tissue-engineering bone, using synthetic polymer scaffolds, may also be related to biodegradability and bioresorbability. Rotter *et al.*[51] report that a high amount of resorption by-products of fast-degrading textile-based scaffold made of PGA–PLA (10/90%) can lead to cell death. The incorporation of tricalcium phosphate (TCP),[52] hydroxyapatite (HA),[53] and basic salts[54] into a polymer matrix system produces a hybrid or composite material. These inorganic fillers allow tailoring of the degradation and resorption kinetics of the polymer matrix. A composite material would also improve biocompatibility and hard tissue integration in that ceramic particles, which are embedded into the synthetic polymer matrix, allow for increased initial flash spread of serum proteins compared with the more hydrophobic polymer surface. In addition, the basic resorption products of HA or TCP would buffer the acidic resorption by-products of the aliphatic polyester and may thereby help to avoid the formation of an unfavorable environment for the cells caused by decreased pH.

The data generated in this study did not show a statistically significant difference when human mesenchymal progenitor cells in combination with fibrin as cell carrier were cultured over period of 3 weeks in honeycomb-like scaffold architectures made of PCL or PCL–HA. As a next step toward a clinical application, the cell response as well as the properties of the tissue-engineered bone inside the novel PCL and PCL–HA scaffold must be studied in an accelerated degradation model or in real-time experiments spanning a period of up to 2 years. Such studies are currently being performed in the Tissue Engineering Laboratory at the National University of Singapore.

CONCLUSION

The concept of a mechanically stable framework made of a slow degrading polymer and hydrogel with biomimetic properties has allowed the design of a new generation of scaffold/cell/tissue constructs. Fibrin gel might be the ideal temporary biomimetic cell carrier in such composite constructs because it is a clinically approved material, has been used extensively in the clinical setting as a tissue adhesive, can also be obtained from a patient's own blood, and stimulates *in vivo* vascularization. This *in vitro* study demonstrated that human bone marrow derived osteoprogenitor cells delivered in a fibrin matrix can be induced to the osteogenic lineage in novel PCL and PCL–HA scaffold architectures.

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