CHAPTER 7

A Newly Developed Rotating Bed Bioreactor for Bone Tissue Engineering

C. Kasper*, K. Suck, F. Anton, T. Scheper, S. Kall, M. van Griensven

Summary

The influence of a new 3D macroporous ceramic scaffold (Sponceram®) on the differentiation process of preosteoblastic MC3T3-E1 cells into bone tissue was initially investigated under static conditions in cell culture dishes. Moreover, cultivations of the cell-seeded scaffolds were performed using a newly developed rotating bed bioreactor system in the presence or absence of bone morphogenetic protein 2 (BMP-2). Von Kossa staining showed a significant calcification of the extracellular matrix in the presence of BMP-2 but even in the absence of BMP-2 mineralization was observed. This could also be confirmed by mRNA expression of collagen I, osteocalcin and bone sialoprotein. Furthermore, the capability of this approach was verified by the outcome of a cultivation of human primary osteoblasts on the Sponceram® discs in the rotating bed bioreactor for 26 days yielding in a dense layer of mineralized ECM layer.

Keywords: Rotating bed bioreactor, Sponceram®, bone tissue engineering, differentiation

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Introduction

Tissue engineering is an emerging, interdisciplinary modern field in cell culture applications. It aims at the development of biological tissue substitutes to restore, remain or improve diseased or damaged tissues [1].

The essentials for tissue engineering are shown in figure 1. For the successful generation of functional tissue, the different components (cells, medium and growth factors, scaffold material) must be provided in a suitable environment (formation of cell-cell contacts, mechanical loading, bioreactor).

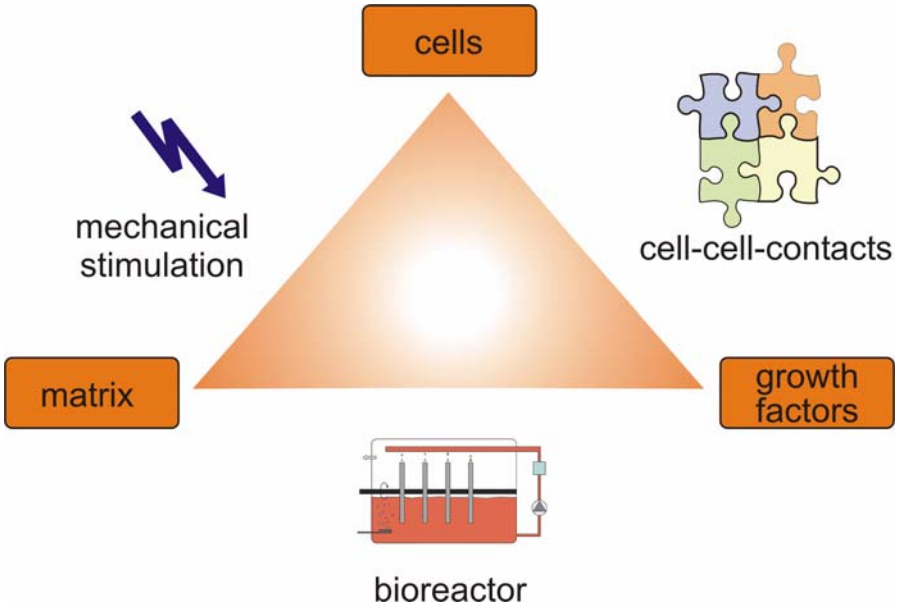


Fig. 1. What is essential in Tissue Engineering?

Depending on the tissue that is supposed to be replaced, substrate materials have to be provided for cell growth, which need to fulfil different requirements with regard to their mechanical stability, biodegradability and porosity. These matrices have to be biocompatible, should support cell attachment, growth and differentiation towards the desired phenotype. Highly porous materials provide space for the bone tissue and allow an optimal cell growth inside the scaffold. Many materials have been reported to fulfil these requirements including natural/synthetic polymers, metals and ceramics [2-6]. Ceramics like hydroxyapatite, calciumphosphates, aluminia, zirconia and composite materials etc. are widely used as scaffold material [7-12]. Moreover, hydroxyapatites, calcium phosphates or composite materials are known to enhance the osteogenic differentiation when seeded with progenitor cells or preosteoblastic MC3T3-E1 cells [13, 14]. Furthermore, a controllable degradation of

scaffolds is often desired, which should not cause any inflammatory reactions *in vivo*. After transplantation, the scaffold is invaded by cells of the surrounding tissue.

Large bone defects caused by tumours, infectious diseases or trauma clearly result in the medical need for bone regeneration. If possible an autologous bone graft (mostly derived from the iliac crest) is carried out. However, the quantity of the obtained material is often not sufficient in case of a large bone defect. Alternatively, allogeneic bone from donor (corpse) patients (e.g. cryopreserved) is transplanted, which is critical with regards to infection risks (AIDS, hepatitis). Therefore, alternative methods have been developed. One innovative approach to bone tissue engineering is to seed osteoblasts or their precursor cells onto an appropriate 3D matrix and to culture this scaffold *in vitro* before implantation into the defect of a patient. During the generation of bone tissue different osteoblastic markers such as alkaline phosphatase (AP), collagen I (COL I), osteocalcin (OC) and bone sialoprotein (BSP) are chronologically expressed [15]. Finally, the cells are embedded in their extracellular matrix and begin to mineralize by depositing mineral along and within the grooves of the collagen fibrils [16].

The differentiation of cells into bone cells are mediated by growth factors. Specific growth factors for bone differentiation are bone morphogenetic proteins (BMPs), members of the TGF-β superfamily [17-19]. BMPs stimulate the differentiation of different cell types to osteoblasts including undifferentiated mesenchymal cells, bone marrow stromal cells and preosteoblasts [20]. For the *in vitro* promotion of the differentiation process into bone tissue mainly BMP-7 and BMP-2 are used [20, 21], whereas BMP-2 increases the AP and OC level in MC3T3-E1 cells [22]. This certifies the eminent influence BMP-2 and of BMPs in general during the process of bone tissue formation and is thus considered as vital for bone tissue engineering.

For an optimal supply of the cells with oxygen and nutrients within the scaffold the cultivation is ideally performed in a bioreactor system, since static cultures are insufficient to mimic the *in vivo* conditions. In a bioreactor oxygen, pH and the transport of nutrients and metabolic waste in the tissue microenvironment can easily be controlled. The most commonly used bioreactors for bone tissue engineering are the spinner flask, perfusion culture system and the Rotating Wall Vessel Reactor (RWVR) (Fig. 2). In spinner flasks the cells can be cultured either on scaffolds fixed on needles or seeded onto microcarriers [23, 24]. Perfusion culture systems provide a continuous exchange of medium which ensures the removal of metabolic waste and supplementing essential nutrients throughout the cultivation.

Perfusion systems are frequently applied for the cultivation of bone tissue [25-29]. Using the RWVR, the scaffolds are cultured in a free fall manner. The RWVR has already been used successfully for the cultivation of bone and cartilage cells [30].

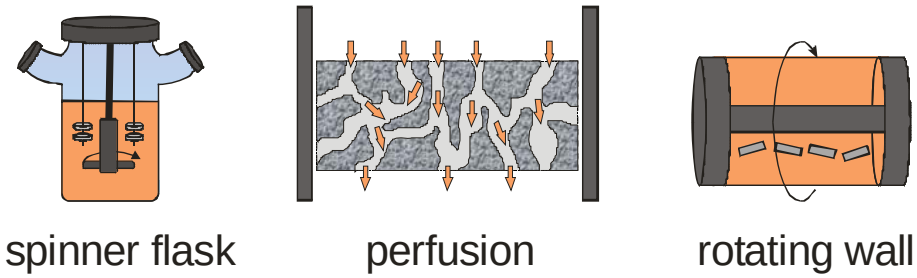
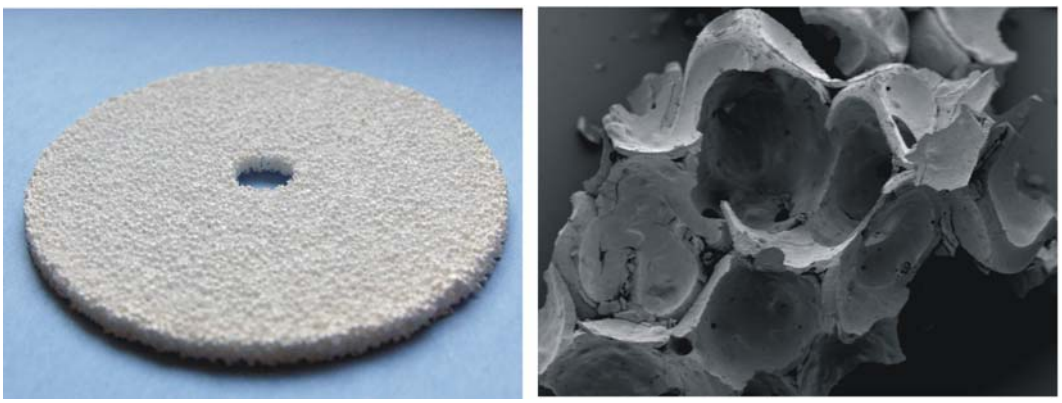
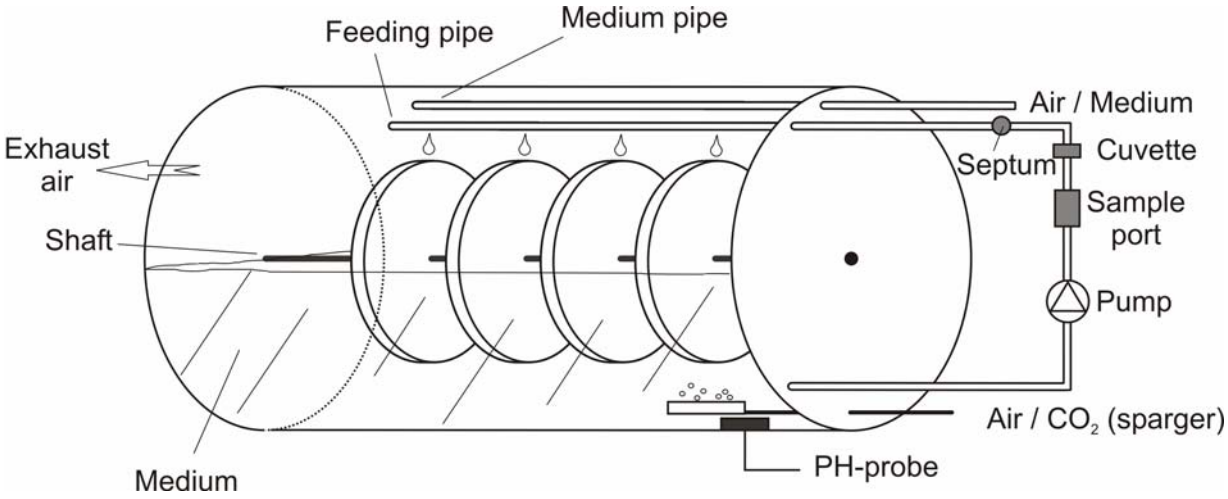


Fig. 2. Bioreactors for tissue engineering.

The approach presented in this study is based on the use of a newly developed bioreactor system BIOSTAT® Bplus RBS (rotating bed system) 500 with special ceramic carrier discs (Sponceram®) (Fig. 3). Sponceram® carrier discs consist of doped zirconium oxide and possess a macroporous structure covered by a microporous layer. The large surface (2 m^2/g) enhances cell adherence and stimulates the formation of extracellular matrix. The discs are fixed on a shaft and rotate during cultivation. The alternating contact of cultured cells with medium and a gas atmosphere above in this system guarantees excellent supply with oxygen and nutrients.



(A)



(B)

Fig. 3. (A). Sponceram® disc and scanning electron microscopic picture of non-seeded macroporous structure of the material. The discs are fixed on a shaft and rotate with a speed of 2 rpm during cultivation (B). Scheme of the BIOSTAT® Bplus RBS 500 system setup.

In initial pre-screening experiments we investigated the applicability of Sponceram® and the influence of the 3D macroporous ceramic on the differentiation process of MC3T3-E1 cells as a potential scaffold for bone tissue engineering under static conditions in 96-well dishes in comparison to a commercially available tricalcium phosphate (TCP) scaffold (Fig. 4).

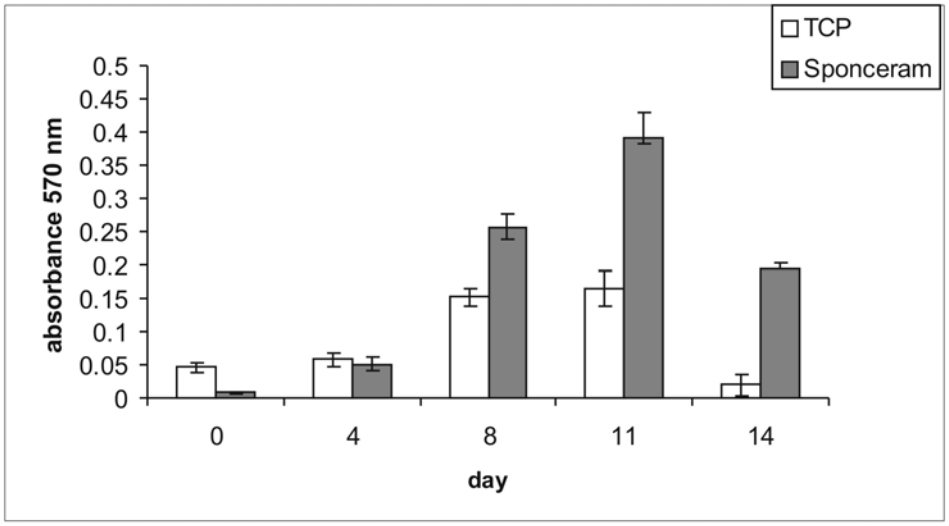


Fig. 4. Cell proliferation of MC3T3-E1 cells cultured on Sponceram® and TCP using standard medium in 96-well dishes for 14 days. Values represent the mean of 5 samples of cultured scaffolds +/- SD.

Furthermore the rotating bioreactor system BIOSTAT® Bplus RBS 500 was used for cultivation of MC3T3-E1 cells during a period of 3 weeks. The differentiation into the osteoblastic phenotype with concomitant mineralization in the presence and absence of BMP-2 under dynamic culture condition was investigated after cultivation (Fig. 5 and Fig. 6).

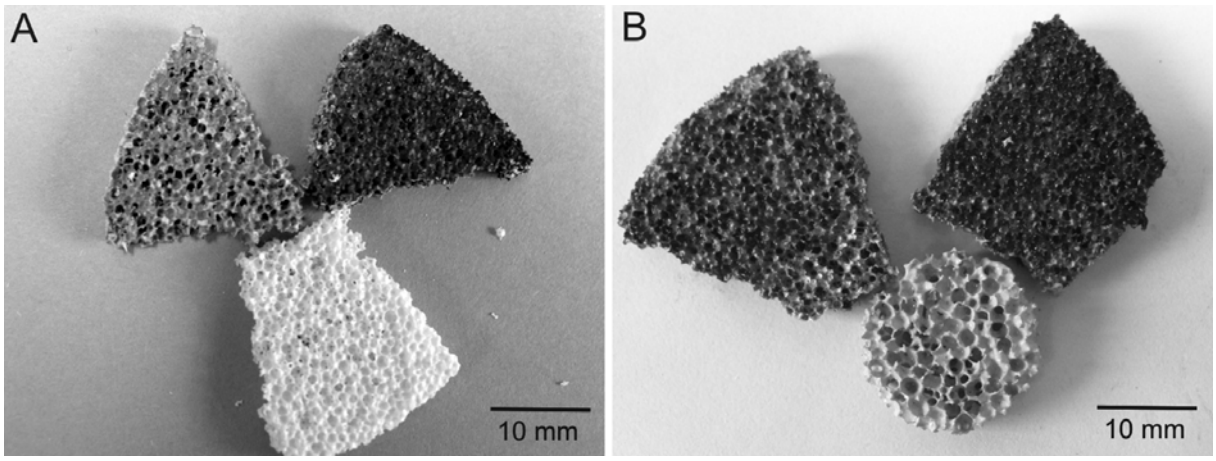


Fig. 5. Specific staining of mineralized ECM after cultivation of MC3T3-E1 cells in the BIOSTAT® Bplus RBS 500. **(A)** Von Kossa staining of non seeded Sponceram® (middle), cultured in standard medium (upper left) and in BMP-2 medium (upper right). **(B)** Alizarin red staining of non seeded Sponceram® (middle), cultured in standard medium (upper left) and in BMP-2 medium (upper right).

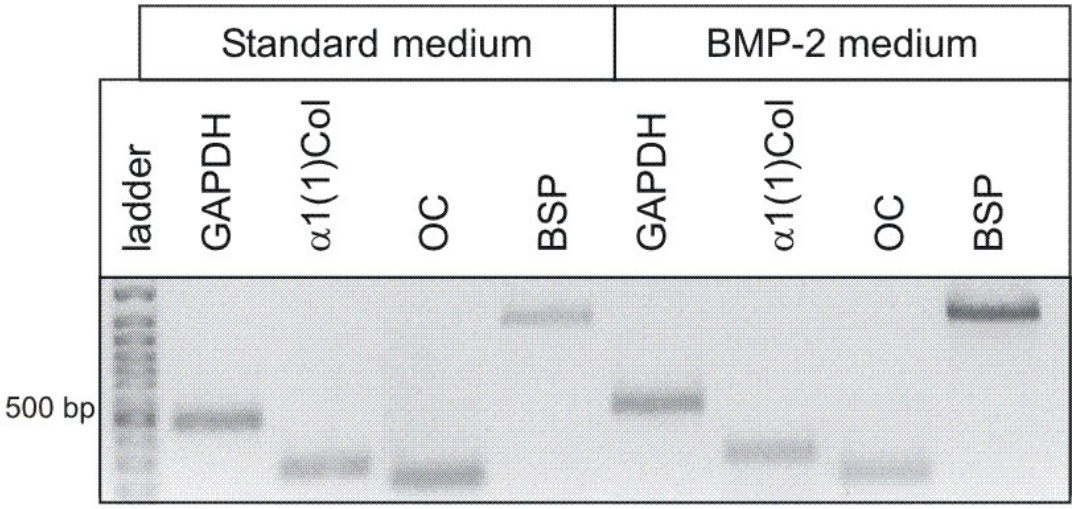


Fig. 6. RT-PCR analysis of alpha 1 (1) collagen, osteocalcin and bone sialoprotein mRNA of MC3T3-E1 cells after cultivation in the BIOSTAT® Bplus RBS 500. Cell culture was performed in standard medium (left) and in BMP-2 medium (right).

Subsequently, human primary osteoblasts were also cultivated under defined conditions in the bioreactor systems (Fig. 7 and Fig. 8). For the generation of functional bone tissue, a physiological environment is desired. Some studies investigated the influence of mechanical load on the differentiation process to osteoblasts [31]. First results of the study of the effect of mechanical strain on the differentiation of bone marrow stromal cells. After 60 minutes of cyclic longitudinal strain, cells showed significant mineralization (von Kossa staining). Static control cells displayed no mineralization at all. After 15 minutes of strain, no mineralization was observed (ebook: Topics in Tissue Engineering, Vol. 2, Chapter 12). The application of mechanical strain to stem cells accelerates the differentiation toward osteoblasts. Thus it is anticipated that this "pre-treatment" of the stem cells prior to the bioreactor cultivation could speed up the cultivation time for generating bone tissue.

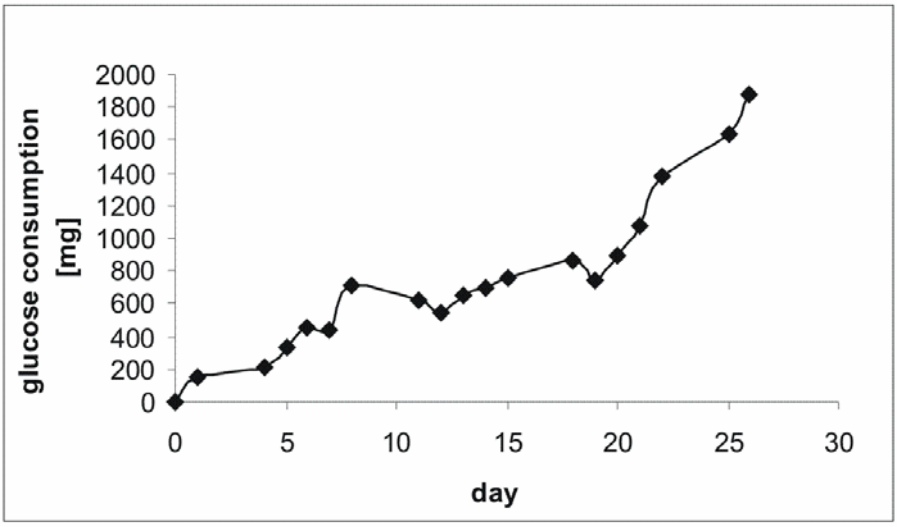


Fig 7. Glucose consumption during bioreactor cultivation of human osteoblasts

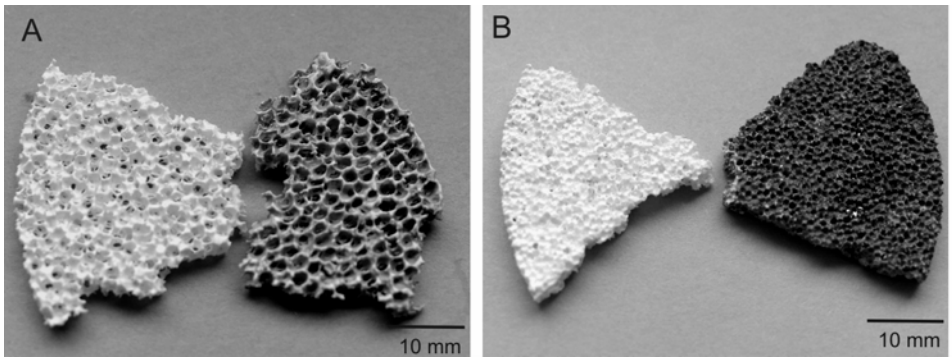


Fig 8: Specific staining of mineralized ECM (van Kossa) after bioreactor cultivation; Sponceram® (right picture) and Sponceram HA (left picture) seeded with primary osteoblasts after 26 days of cultivation (the unseeded material is shown in the left part of each picture).

MATERIALS AND METHODS

All chemicals were purchased from Fluka/Sigma (Taufkirchen, Germany) and were of *pro analysi* quality (if not stated otherwise). Deionized water (ARIUM, Sartorius AG, Goettingen, Germany) was used for the preparation of media and buffers.

Sponceram®

Sponceram® is a ceramic support material consisting of doped zirconium oxide. The structure of the material combines a unique mixture of macro- and micro pores. Technical data about the surface area and pore size are summarized in table 1. For the cell cultivation under static culture conditions in 96-well dishes samples of Sponceram® with a size of approximately 3 mm x 3 mm x 4 mm were used. The cultivation in the bioreactor system was performed with identical Sponceram® (for MC3T3-E1 cells). This means that both Sponceram® (pore size: 900 µm) as well as Sponceram® with hydroxyapatite (HA) coating (pore size 600µm) (for cultivation of primary osteoblasts) were used as carrier discs (65 mm in diameter, 3 mm thickness) for the BIOSTAT® Bplus RBS 500.

Table 1. Surface characteristics of Sponceram

	Sponceram® sample	**Sponceram® disc**
Pore size [µm]	600	600
Surface area (BET) [m^2/g]	2	2
Total surface area per disc [m^2/disc]	-	14
Thickness [mm]	3	3
Diameter [mm]	-	65
Density [g/ml]	0.7	0.7

Cell cultivation under static conditions 96-well dishes

Samples of Sponceram®, Sponceram/HA® (Zellwerk GmbH, Oberkraemer, Germany) and TCP (beta-tricalcium phophate, chronOS™) (Mathys, Bettlach, Switzerland) (approximately 3 mm x 3 mm x 4 mm) were preconditioned in standard medium for 24 h in cell culture medium at 37 °C/5 % CO_2. Subsequently, 1.5 x 10^4 MC3T3-E1 cells in 80 µl medium were seeded on each scaffold in 96-well dishes for 30 min at gentle stirring at 37 °C/5 % CO_2. Non-attached cells were removed and the wells were filled up with 200 µl medium: 1. Standard medium: DMEM (Sigma, Taufkirchen, Germany) containing 10 % FCS (PAA, Coelbe, Germany), penicillin (50 U/ml), streptomycin (50 µg/ml) (PAA, Coelbe, Germany); 2. Differentiation medium: standard medium, 1 µM dexamethasone, 10 mM beta-glycerolphosphate, 50 µg/ml ascorbic acid; 3. BMP-2 medium: differentiation medium + 10 ng/ml BMP-2. Before each analysis the scaffolds were placed into a new 96 well dish. Since it is not possible to estimate the numbers of attached cells on the scaffolds directly the first measurements were performed immediately after cell seeding (day 0). The cultivations were performed in 96-well culture dishes with 200 µl of medium at 37 °C/5 % CO_2. The medium was changed every second to third day.

Cell proliferation assay (static cultivation conditions)

Cell proliferation was assayed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromid (Sigma-Aldrich, Steinheim, Germany). Five replicates for each experimental condition were placed in a 96-well culture dish. 100 µl of fresh medium and 10 µl of MTT solution (5 mg/ml in PBS) was added to each well and incubated for 4 h at 37 °C/5 % CO_2. Subsequently, 100 µl of 10 % (weight/volume) SDS in 0.01 M HCl was added and the samples were incubated for 16 h using the same conditions. The transmission signal was measured at 570/630 nm using a Microplate Reader (Bio-Rad, Muenchen, Germany).

Statistical Analysis (static cultivation conditions)

The experiments were carried out twice with each time n=5 per sample. The data are presented as mean values ± standard deviation.

Cell cultivation in the BIOSTAT® Bplus RBS 500 (dynamic cultivation)

The BIOSTAT® Bplus RBS 500 can be equipped with up to 20 thin Sponceram® discs. In our study, the reactor was equipped with 4 Sponceram® carrier discs (65 mm in diameter, 3 mm thickness) and respective spacers for the cultivation of MC3T3-E1 cells. Cell inoculation was carried out with a total volume of 2 ml cell suspension/disc (seeding cell number: 55 x 10^6/disc). For the cultivation of primary osteoblasts the reactor was equipped with 2 Sponceram® and 2 Sponceram/HA® carrier discs and respective spacers for the cultivation. Osteoblasts of the third passage were used and the inoculation was carried out with a total volume of 2 ml cell suspension/disc (seeding cell number: 1 x 10^7/disc).

The cell suspensions for the cultivations were injected through a feeding pipe onto the carrier discs (Fig. 3). To distribute the cell suspension homogeneously onto the discs a rotation speed of 4 rpm during cell seeding was applied. To allow adhesion onto the Sponceram® surface the reactor was filled with 300 ml of medium 30 min after cell inoculation. The following cultivation was performed at 37 °C, 2 rpm and a pH of 7.3.

One cultivation of the MC3T3-E1 cells was performed for 21 days using standard medium, the second one was cultivated for 10 days using standard medium followed by 11 days cultivation using BMP-2 medium (medium composition: see above). The cultivation of the osteoblasts was performed for 26 days in differentiation medium. After the cultivations in the BIOSTAT® Bplus RBS 500 the disc shaped scaffolds were used for the investigation of matrix mineralization, scanning electron microscopy and RT-PCR analysis.

Glucose assay (dynamic cultivation conditions)

Cell growth during the bioreactor cultivations was determined by the estimation of glucose consumption using the YSI 2700 automated glucose analyzer (Yellow Springs Instruments, USA).

Matrix mineralization (static and dynamic cultivation condition)

The Sponceram® discs were removed from the bioreactor after cultivation, washed with PBS and fixed in ice cold 100 % ethanol for 20 min at room temperature. The Alizarin red staining was performed by washing with PBS followed by staining with 1 % Alizarin red in 2 % of ethanol for 15 min at room temperature. For von Kossa staining fixed cells were washed with deionised water and incubated for 30 min in 5 % $AgNO_3$ in the dark, washed again and

exposed to ultraviolet light for 2 min. Cells were fixed with 5 % sodium thiosulphate for 2 min and washed 3 times with deionised water.

Scanning electron microscopy (static and dynamic cultivation condition)

Cell grown Sponceram® discs were fixed in Karnovsky buffer at 4°C over night prior to scanning electron microscopy. Samples were then dehydrated in solutions containing increasing percentages of acetone (10 %, 30 %, 50 %, 70 %, 90 %, 100 %) and subsequently imaged with a JEOL JSM-6700F scanning electron microscope.

Reverse transcriptase-polymerase chain reaction (RT-PCR) (dynamic cultivation condition)

MC3T3-E1 cells were cultivated on scaffolds in the BIOSTAT® Bplus RBS 500 as described above. Cells were removed from the disc by incubation in the enzyme mix ZW-DT-04 (Zellwerk GmbH, Oberkraemer, Germany) at 37 °C for 2 h and centrifuged at 400 ×g for 5 min. Cells were disrupted with RiboLyse tubes green (Hybaid, Heidelberg, Germany) for 40 s at 6.0 Fast Prep FP 120 (Bio 101® Systems, Qbiogene, Heidelberg, Germany). The RNA was isolated by the SV total RNA Isolation System (Promega, Mannheim, Germany).

RT was carried out with 2 µg RNA using the Superscript II system (Promega, Mannheim, Germany) with oligo dT primer in a total volume of 40 µl. PCR was performed in a PCR-Thermocycler (MWG Biotech, Ebersberg, Germany) using specific primers. The reaction volume was 50 µl with an equivalent RNA concentration of 0.1 µg.

Amplification reactions were performed using the following primers (each 10 pmol) and protocols (30 cycles): **α1 (I) collagen:** *forward*: 5`-TTC TCC TGG TAA AGA TGG TGC-3`, *reverse:* 5`-GGA CCA GCA TCA CCT TTA ACA-3` (annealing 57°C, 255 bp product) (Roth, Karlsruhe, Germany); **Osteocalcin (OC):** *forward:* 5`-ACA AGT CCC ACA CAG CAG CTT-3`, *reverse:* 5`- GCC GGA GTC TGT TCA CTA CCT-3`(annealing 62°C, 187 bp product) (Roth, Karlsruhe, Germany); **Bone sialoprotein (BSP):** *forward:* 5´-CTG TAG CAC CAT TCC ACA CT –3´, *reverse:* 5´-ATG GCC TGT GCT TTC TCG AT-3´(annealing 56°C, 1055 bp product) (MWG Biotech, Ebersberg, Germany); **GAPDH:** *forward:* 5`- GCC ACC CAG AAG ACT GTG GAT-3`, *reverse:* 5`- TGG TCC AGG GTT TCT TAC TCC-3` (annealing 60°C, 455 bp product) (Roth, Karlsruhe, Germany).

Results and Discussion

Results

The results of the static pre-screening experiments for testing the applicability of Sponceram® as a potential scaffold for bone tissue engineering were compared to the data obtained from the employment of TCP after cultivation of the MC3T3-E1 cells in standard medium for 14 days. The results revealed that the proliferation of MC3T3-E1 cells was up to 60 % higher on Sponceram® (Fig 4, day 11). Results of the cultivation of MC3T3-E1 cells cultured in the BIOSTAT® Bplus RBS 500 bioreactor system on Sponceram® in standard medium and BMP-2 medium respectively showed interestingly a clear mineralization by von Kossa and Alizarin red staining when MC3T3-E1 cells were cultured in standard medium (Fig. 5 A and B, upper left).

These finding were confirmed by RT-PCR. The results of mRNA levels showed that collagen I, osteocalcin and bone sialoprotein were expressed independently of the presence of BMP-2 in the medium (Fig. 6) but the intensity of the resulting protein bands was stronger when the cultivation was performed in the presence of BMP-2 in the medium. Thus the differentiation of the MC3T3-E1 cells to osteoblastic lineage [32] was demonstrated.

A cultivation of primary human osteoblasts was also performed in the BIOSTAT® Bplus RBS 500. As an indicator for cell proliferation the glucose consumption was determined during the cultivation in the BIOSTAT® Bplus RBS 500 (Fig. 7). The results revealed that the glucose consumption was 14.05 g total over 26 days of cultivation in differentiation medium. The final specific Alizarin Red and von Kossa stainings for mineralization were clearly positive and the SEM pictures show articulate parallel structures of collagen strands with mineralised ECM (Fig. 9).

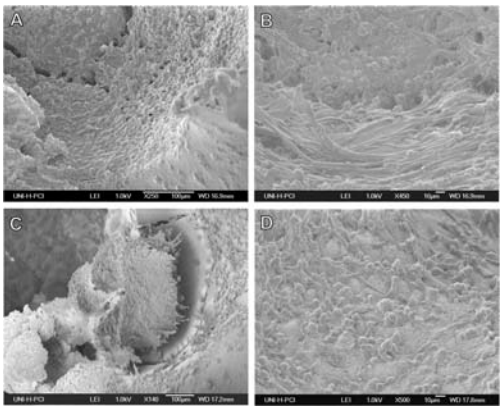


Fig. 9: Scanning electron micrographs after the cultivation of primary osteoblasts for 26 days in the BIOSTAT® Bplus RBS 500 on Sponceram® (A, B) and Sponceram® HA (C, D).

Conclusion

Within this study macroporous Sponceram® biomaterial based on zirconium dioxide was tested towards its suitability as biomaterial for bone tissue engineering. The scaffold used for tissue engineering has to provide all the necessary signals for the cells to grow, differentiate and interact, forming the desired structure and thus mimic extra cellular matrix (ECM) features. The results of the static cultivations revealed a limitation of the cultivation due to confluent cell layer on the biomaterial pieces used. This lead to cell death after 11 days of cultivation because the delivery of nutrients and oxygen as well as the elimination of metabolic waste products is not efficient Thus a newly developed rotating bed bioreactor systems was combined with the Sponceram® ceramic discs and successfully applied for dynamic cultivation of MC3T3-E1 cells and primary human osteoblasts since an appropriate dynamic culture system that mimics the *in vivo* environment (e.g. mechanical stimulation) is needed for the generation of a functional bone tissue substitute.

The results obtained by RT-PCR, Alizarin and von Kossa staining as well as glucose consumption and SEM pictures confirm that the scaffold itself is able to induce differentiation of MC3T3-E1 cells in bone cells with concomitant mineralization when cultured in the bioreactor system. This promotion can be due to the composition of the Sponceram® scaffold and/or is related to its 3D structure. Additionally, the alternate contact of cells to the medium and the oxygen atmosphere within the rotating bioreactor system supported the proliferation and differentiation process of cells within the scaffold.

Outlook

In future experiments, the application of mechanical strain to bone marrow and fat tissue derived stem cells prior to the bioreactor cultivation will be studied in detail. The differentiation process will be the focus of the work since the mechanical load accelerates to differentiation in 2D cultures (ebook: Topics in Tissue Engineering, Vol. 2, Chapter 12), therefore the conditions for the 3D culture will be optimized with regard to generation of functional bone tissue.

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