JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med 2010; **4**: 30–37.
Published online 19 October 2009 in Wiley InterScience (www.interscience.wiley.com) **DOI:** 10.1002/term.213

RESEARCH ARTICLE

A tissue-like construct of human bone marrow MSCs composite scaffold support *in vivo* ectopic bone formation

D. Ben-David[1], T. Kizhner[1], E. Livne[1]* and S. Srouji[1,2]
[1]*Department of Anatomy and Cell Biology, Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel*
[2]*Department of Oral and Maxillofacial Surgery, Carmel Medical Centre, Haifa, Israel*

Abstract

Biocompatible and osteoconductive cell–scaffold constructs comprise the first and most important step towards successful *in vivo* bone repair. This study reports on a new cell–scaffold construct composed of gelatin-based hydrogel and ceramic ($CaCO_3/\beta$-TCP) particles loaded with human MSCs producing a tissue-like construct applied as a transplant for *in vivo* bone formation. Bone marrow-derived human MSCs were cultured in osteogenic induction medium. 5×10^5 (P_2) cells were loaded on a mixture of hydrogel microspheres and ceramic particles, cultured in a rotating dynamic culture for up to 3 weeks. Both hydrogel microspheres and ceramic particles coalesced together to form a tissue-like construct, shown by histology to contain elongated spindle-like cells forming the new tissue between the individual particles. Cell proliferation and cell viability were confirmed by Alamar blue assay and by staining with CFDA, respectively. FACS analysis conducted before loading the cells, and after formation of the construct, revealed that the profile of cell surface markers remained unchanged throughout the dynamic culture. The osteogenic potential of the cells composing the tissue-like construct was further validated by subcutaneous transplants in athymic nude mice. After 8 weeks a substantial amount of new bone formation was observed in the cell-construct transplants, whereas no bone formation was observed in transplants containing no cells. This new cell construct provides a system for *in vivo* bone transplants. It can be tailored for a specific size and shape as needed for various transplant sites and for all aspects of regenerative medicine and biomaterial science.

Received 25 December 2008; Revised 26 July 2009; Accepted 27 July 2009

Keywords **hydrogel scaffold; MSCs; osteoprogenitor cells; *in vivo* transplants; bone repair**

1. Introduction

Bone is the most commonly replaced organ of the body. Due to limitations associated with allografts, xenografts and synthetic bone substitutes, autografts have been the clinical 'gold standard' for bone grafting. However, autograft transplantation suffers from insufficient supply and surgical morbidity of the donor site (Leong *et al*., 2006). Therefore, there is significant interest in tissue-engineered substitutes for use as allografts. The optimal scaffold should be biocompatible, biodegradable and osteoconductive to induce new bone formation (Leong *et al*., 2006). The major challenge is the production of an ideal scaffold or synthetic matrix that will mimic the structure, mechanical aspects and biological functions of the natural extracellular matrix (ECM) (Hutmacher, 2000; Livingston *et al*., 2002; Meinel *et al*., 2004a, 2004b). Scaffolds have been composed of various classes of biomaterials, such as synthetic polymers, ceramics, native polymers (hydrogels) and their composites (Barrilleaux *et al*., 2006; Dubruel *et al*., 2007; Srouji *et al*., 2005a, 2006). The current design and fabrication of organic scaffolds in skeletal tissue engineering involves a range of various materials: protein-based polymers, carbohydrate-based polymers, natural polymers, synthetic polymers and composite materials of hydrogels and inorganic compounds. As a result of this, scaffolds can take on

*Correspondence to: E. Livne, Department of Anatomy and Cell Biology, Faculty of Medicine, Technion-Israel Institute of Technology, PO Box 9649, Haifa 31096, Israel.
E-mail: livne@tx.technion.ac.il

various forms, from porous solid meshes to hydrogel networks. Although solid scaffolds provide a mechanically strong substrate for seeded cells, hydrogel scaffolds that can physically entrap the cells are becoming increasingly popular as tissue-engineered matrices (Fedorovich *et al*., 2007). Ceramics such as calcium phosphates, calcium sulphates, calcium carbonates and bioactive glass have been also used as matrices for bone regeneration (Ducheyne *et al*., 1999; Livingston *et al*., 2002). These substances, especially the calcium phosphates, are ideal candidates for use as matrices in bone tissue engineering because the inorganic component of bone is composed of the ceramic calcium hydroxyapatite.

In cell therapy-based approaches, mesenchymal stem cells (MSCs) are regarded as an excellent source of cells for bone tissue engineering because of their self-replication and osteogenic differentiation capabilities (Bruder *et al*., 1998; Srouji *et al*., 2005a, 2008). Tissue-engineering protocols that include integration of osteo-progenitor cells within custom-shaped porous scaffolds offer a promising strategy for *de novo* bone formation (Hasegawa *et al*., 2007; Ishaug *et al*., 1997; Ohgushi *et al*., 1999).

The purpose of the present study was to design a cell–scaffold composite, retaining the advantages of both hydrogel and ceramic, to create a construct in which osteoprogenitor cells remained viable and to preserve their osteogenic capacity to be able to form new bone *in vivo* for bone tissue engineering.

2. Materials and methods

2.1. Cell source and culture conditions

Human bone marrow-derived MSC progenitor cells were cultured (37 °C, 5% CO_2, 1 week) in control medium [α-MEM; 10% fetal calf serum (FCS), 2 mM L-glutamine and Pen–Strep (penicillin–streptomycin; 100 U/ml, 100 µg/ml; Biological Industries, Beith Haemek, Israel)]. At 70–80% confluence, the cells were trypsinized, counted and passaged (P_1). To induce osteogenic differentiation in culture, P_1 cultures were cultured for 4 additional weeks in α-MEM medium containing 10% FCS, 2 mM L-glutamine, Pen–Strep (both 100 U/ml), 100 µg/ml ascorbic acid and 10^{-8} M dexamethasone (osteogenic induction medium). The resulting osteoprogenitor cells (5×10^5, P_2) were loaded onto the hydrogel–ceramic mixture and cultured for an additional 2 weeks and then the whole tissue-like construct was used for the *in vivo* ectopic transplants.

2.2. Preparation of hydrogel microspheres and microspheres–ceramic mixture

Hydrogel scaffold (95%wt) in the form of microspheres was prepared by chemically crosslinking 10% aqueous acidic gelatin (Nitta Gelatin Co., Osaka, Japan) solution with 12.5 mM glutaraldehyde at 4 °C. The microspheres were prepared by transferring the 10% aqueous acidic gelatin hydrogel solution dropwise at room temperature through an oil layer laid over 12.5 mM glutaraldehyde solution baths subjected to stirring. The resulting hydrogel microspheres were rinsed twice in double-distilled water (DDW), immersed twice in 100% ethanol and twice in autoclaved DDW to obtain sterilized hydrogel. The sterilized microspheres were aseptically freeze-dried (1 h; particle size, 0.5–1 mm) and exposed to UV (1 h) prior to their use.

A mixture of equally measured volumes (20 µl) of microspheres and 20 µl ceramic particles of ProOsteon (Coral/HA particles; calcium carbonate particles covered by hydroxyapatite (HA); particle size, 0.5–1 mm). The mixture was prepared in a 50 ml special sterile culture tubes allowing the exchange of oxygen and CO_2 (TRP; Innovation in Plastic, Switzerland). The hydrogel microspheres and ProOsteon particles were visualized and photographed by binocular (Olympus SZX9, USA).

2.3. Seeding the cells onto the hydrogel microspheres–ceramic mixture

Confluent cultures (P_2) were trypsinized, washed with PBS and 5×10^5 cells (in 500 µl medium) were added carefully to the hybrid construct in the tubes. The tubes were rotated in the incubator (37 °C; 1 h; Pelco R2 rotary mixer, 7.5 rpm) to allow cell adhesion to the construct. After 1 h an additional 9.5 ml medium was added to make a total of 10 ml. The mixture was further rotated for up to 21 days. Half the volume of the medium was changed twice weekly. The cell proliferation curve was determined on days 3, 7, 14 and 21, using the Alamar blue assay. Triplicates were used for the assay ($n = 3$).

2.4. Scanning electron microscopy (SEM)

For SEM studies, samples were fixed in 3% glutaraldehyde in 0.1 cacodylate buffer, pH 7.2 (24 h), followed by 1% OsO4 (1 h) and 2% tannic acid. They were then dehydrated in graded ethanol solutions, immersed in hydroxymethyl xylazine (HMSM), sputter-coated with gold palladium and examined by scanning electron microscope (100 QT, operating at 100 V).

2.5. Fluorescence microscopy

Imaging of viable cells was performed using Vybrant [carboxyfluorescein diacetate succinimidyl ester) CFDA SE) cell Tracer kit (V12 883, Invitrogen, CA, USA)], according to the manufacturer's instructions. Briefly, the tissue-like constructs were washed with prewarmed PBS (37 °C) and incubated in 2 µM CFDA solution (in PBS) for 15 min at 37 °C. The constructs were then washed with PBS and put into growth medium. The microscopic analysis was performed using a Zeiss

DOI: 10.1002/term

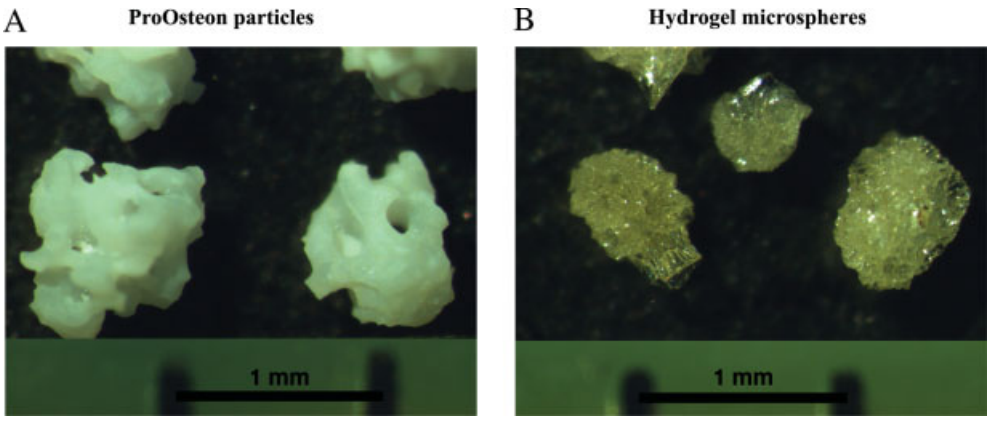

Figure 1. (A) ProOsteon particles. (B) Hydrogel gelatin-based microspheres

Axioscop 2 fluorescent upright microscope (Carl Zeiss MicroImaging, NY, USA).

2.6. Fluorescence-activated cell sorting (FACS) analysis

Cell–scaffold constructs cultured for 2 weeks in the dynamic culture system were carefully removed to static culture plates, allowing the cells to migrate out from the construct; upon confluence (80%) the cells were trypsinized. P_1 MSCs (prior to seeding the cells on the constructs) were similarly trypsinized and both were tested for MSC-specific markers using FACS analysis; using the classical unique set of surface markers, mesenchymal cells are positive for CD90, CD105, CD73 and negative for CD71, CD63 and CD34.

The cells were labelled with the following monoclonal antibodies: CD105 (266; BD PharMingen); CD73 (AD2; BD PharMingen); CD90 (CBL415F; Chemicon International); CD71 (sc-7327; Santa Cruz); CD63 (557 288; BD PharMingen); and CD34 class III (K3; Dako). Acquisition and analysis was performed on a FACS Calibur flow cytometer (Becton-Dickenson, USA). All antibodies were used at a concentration of 0.5 μg/10^6 cells in a volume of 100 μl, unless otherwise recommended by the manufacturer. Isotype-specific negative control antibodies were purchased from Dako.

2.7. *In vivo* transplantation in athymic nude mice

Eight week-old athymic nude mice were used for *in vivo* transplantation. The surgical protocol was approved by the institutional guidelines of the Animal Ethics Committee of the Technion–Israel Institute of Technology, Haifa, Israel

Under anaesthesia (xylazine : ketamine, 1 : 1) a mid-sagittal incision was performed in the animal's back. The tissue-like constructs were subcutaneously transplanted in five animals ($n = 5$). An additional five animals were transplanted with similarly prepared control constructs but without cells ($n = 5$). After surgery, the skin was carefully sutured and topically dressed with antibiotic ointment (3% syntomycin). All animals recovered well from the surgery, were housed separately in plastic cages and were followed for up to 8 weeks. Upon termination of the experiment (after 8 weeks), the animals were sacrificed and the transplant samples were fixed in NBF, decalcified lightly in 10% ethylene diaminetetra-acetic acid (EDTA; 5 days, room temperature), followed by immersion in graded ethanols (70–100%) and embedded in paraffin. Serial sections (6 μm thick) were stained with haematoxylin and eosin (H&E).

2.8. Statistics

Comparisons of the means of histogram analysis were made using the unpaired two-tailed Student's *t*-test, with significant values set at $p < 0.05$. The results of the experiments were expressed as mean ± SEM.

3. Results

3.1. Hydrogel microsphere–ceramic construct

ProOsteon particles and hydrogel microspheres (Figure 1A, B) are shown. Note the porous structure of the surfaces of both particles. MSC-derived progenitor cells were loaded onto the mixture of both particles and cultured (osteogenic induction medium) in a dynamic rotating system. Four days after loading the cells, a semi-solid construct (Figure 2A), composed of cells covering all surfaces (Figure 2B), was shown as viewed by scanning electron microscopy (SEM). Histology sections stained with H&E revealed the appearance of new tissue-like bridges composed of MSC-derived osteoprogenitor cells that were observed around and between the individual particles. These tissue bridges glued and connected the particles together, resulting in a semi-solid construct with a tissue-like structure (Figure 2C). Histological examination of the new tissue between the individual particles showed typical loose connective tissue containing

DOI: 10.1002/term

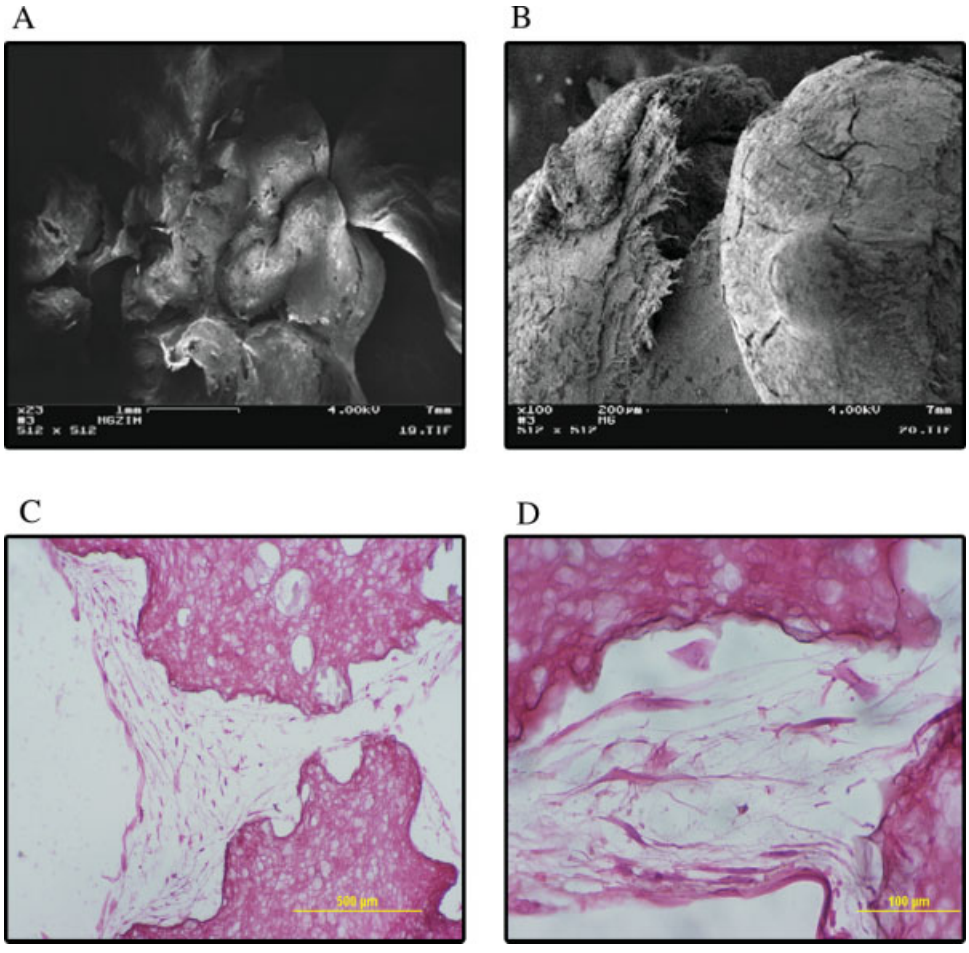

Figure 2. Scanning electron microscope (SEM) and histology sections images of the tissue-like construct after 2 weeks in dynamic culture. (A) Low magnification (×25) showing the overall shape of the construct. (B) Higher magnification (×50) showing the cells adhering to the microsphere particles. (C) Histology section demonstrating the formation of new tissue composed of fibroblast-like elongated cells between and around the scaffold. (D) Higher magnification showing details of spindle shaped cells residing in a loose connective tissue

elongated fibroblast-like cells (Figure 2D). Cell viability was demonstrated by staining the construct with CFDA cell viability fluorescent marker (Figure 3A, B), which showed numerous cells labelled with CFDA, indicative of live cells. A gradual increase in the size of this new tissue was observed throughout the culture period and was also reflected in increased cell proliferation that was monitored by Alamar blue assay (Figure 3C). A gradual increase of cell number was observed up to 2 weeks, followed by a more pronounced increase between the second and third weeks of dynamic culture.

3.2. FACS analysis

Immunotyping of the cells by FACS before loading the cells onto the hydrogel–ceramic construct and 2 weeks after culture in the dynamic system revealed that the MSC surface markers profile did not change throughout the culture period and that the cells retained their MSC marker profile: CD90, CD105 and CD73 remained positive, and CD71, CD63 and CD34 remained negative (Figure 4).

3.3. *In vivo* validation

To determine whether these tissue-like constructs retained their osteogenic potential, tissue-like constructs were transplanted for 8 weeks subcutaneously in athymic nude mice. After 8 weeks, histological analysis of the cell–scaffold constructs clearly showed new bone formation, including typical bone trabeculae in the ectopic site (Figure 5A, C). No bone formation was observed in the control transplants containing no cells (Figure 5B, D). Histomorphometrical measurements showed that the amounts of new bone formed in the tissue-like construct-transplanted animals were approximately 25-fold more than the new bone formed in the control cell-free constructs (Figure 5E).

4. Discussion

The use of *ex vivo* cells cultivated on a scaffold to achieve vital bone tissue constructs has been shown to be an attractive alternative to the use of allogenous or synthetic

DOI: 10.1002/term

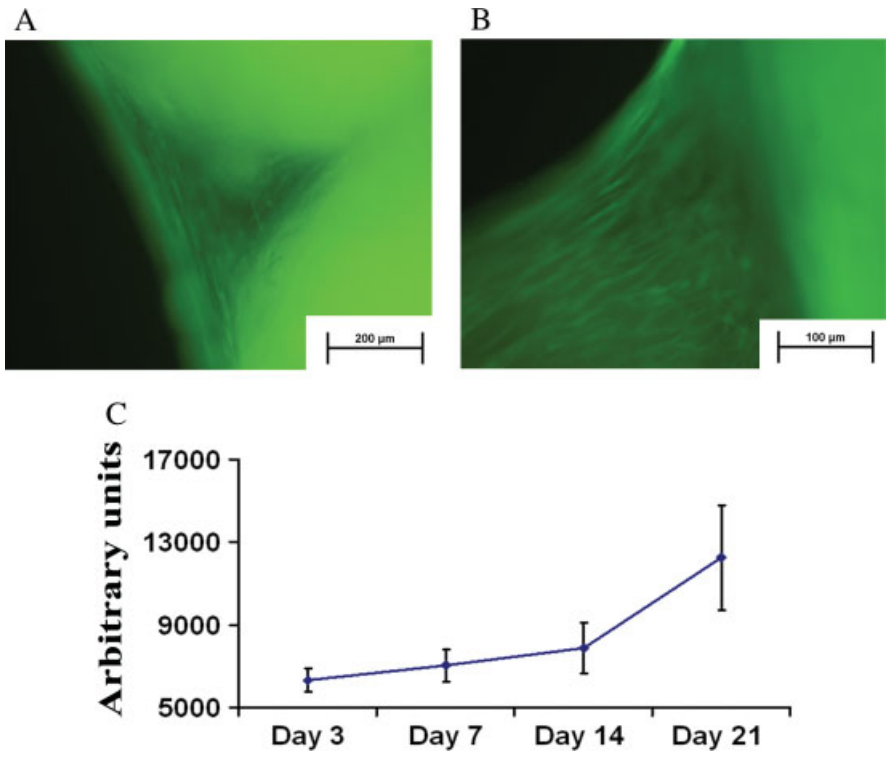

Figure 3. Live cell imaging and proliferation profile of the tissue-like construct after 2 weeks in dynamic culture. (A, B) CFDA fluorescence cell viability marker; fibroblast-like elongated viable cells are seen in the newly formed tissue connecting the particles. (C) Proliferation curve of the tissue-like construct for up to 3 weeks in the dynamic culture system based on the Alamar blue assay. A gradual increase up to 2 weeks is followed by a more pronounced increase of cell proliferation between the second and third weeks

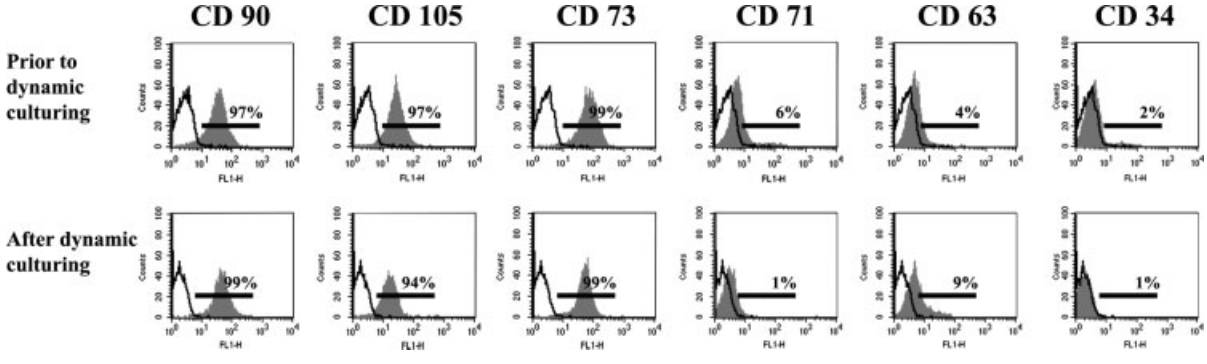

Figure 4. FACS analysis using the classical mesenchymal cell markers before loading the cells on the microsphere–ceramic construct and 2 weeks after rotating in the dynamic culture system revealed that the cell surface markers did not change throughout the culture period

bone substitutes. The aim of the present study was to imitate the structural composition of normal healthy bone, by designing a construct that will contain viable osteoprogenitor cells adhering to a collagenous matrix mixed with ceramic hydroxyapatite (ProOsteon) particles, to finally serve as a potent osteogenic transplant in preclinical animal models.

In the present study, a hydrogel–ceramic composite material was designed. Both polymers and ceramics have their advantages and drawbacks, but it is possible to minimize these drawbacks and maintain the advantages by combining polymers and ceramics into one composite material.

The resulting 3D composites were also tested for their ability to support MSC-derived osteoprogenitors (Fedorovich *et al.*, 2007; Khan *et al.*, 2008; Randle *et al.*, 2007). However, the three-dimensional (3D) culture of MSCs on such porous scaffolds presents several challenges. A major limitation of this technique is the insufficient nutrient and oxygen transport to, and removal of waste products from, the cells at the interior of the scaffold. Consequently, decreased proliferation and differentiation, along with non-homogeneous distribution of cells in the centre of the scaffold, can be observed, restricting the size of scaffolds that can be cultured under conventional static conditions (Ishaug-Riley *et al.*, 1998;

DOI: 10.1002/term

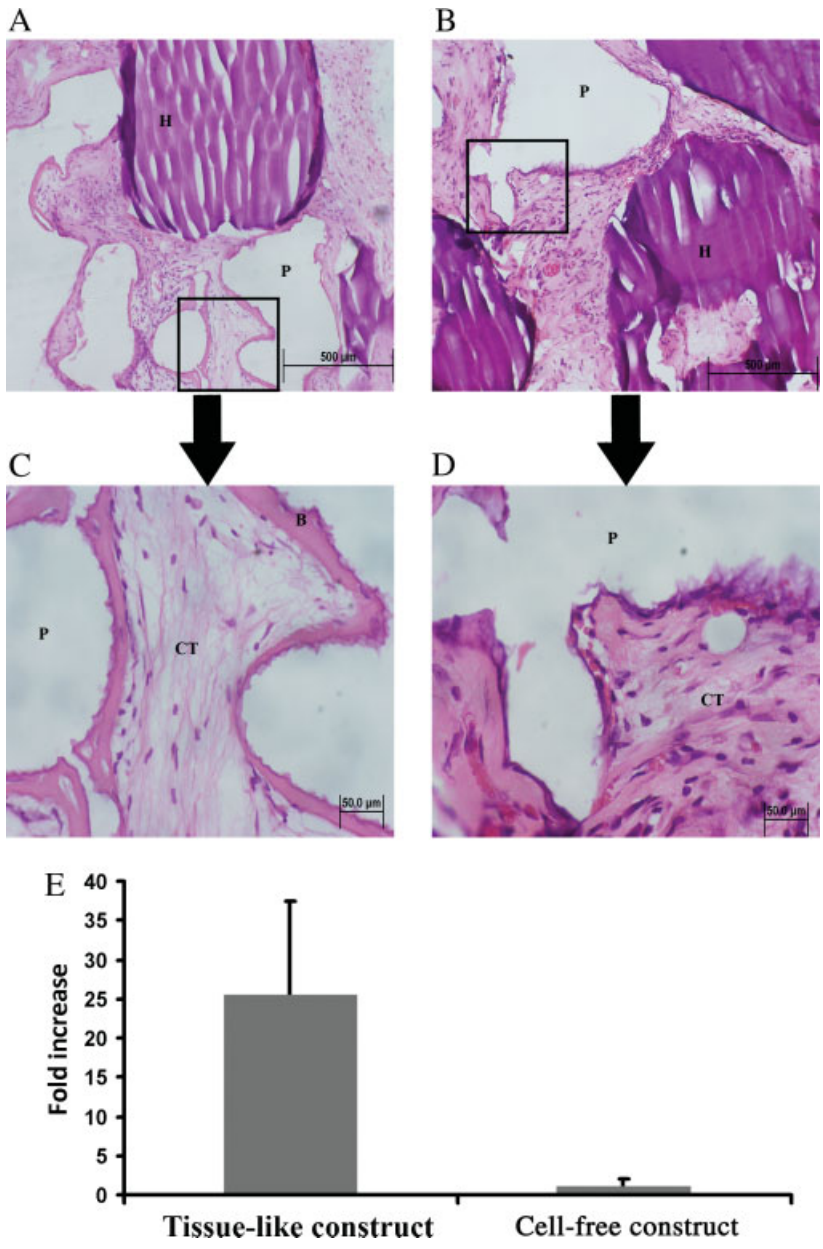

Figure 5. Histology sections of the tissue-like construct 8 weeks after transplantation into athymic nude mice (A) Typical bone tissue formed in the transplanted site along the interface between the new bone and the particles of the construct. (C) A higher magnification of (A), demonstrating new bone formation in the construct. (B) No bone formation is observed in control transplant containing no cells. (D) A higher magnification of (B). No bone formation is observed. (E) Summary of histomorphometrical measurements; tissue-like constructs show ~25-fold increased new bone formation compared to cell-free constructs. H, hydrogel; P, ProOsteon; B, bone; CT, connective tissue

Ishaug *et al*., 1997; Sumanasinghe *et al*., 2008). Usually, in most of the studies made on this subject, the cells are seeded on the scaffold at the same day or several days prior to the transplantation. Although the various scaffolds have different porosity levels, the cells mostly adhere to the surface, with limited deeper penetration. When transplanted they usually present a monolayer of cells that cover the scaffold's surface. To overcome these limitations, a dynamic cell culture technique has been proposed in the present study, since one of the main challenges in cell therapy using 3D scaffolds is to overcome the limited nutrition and cell death inside the scaffold.

Our cell–scaffold construct was designed in order to provide a system in which osteoprogenitor cells will be viable, well nourished, they will preserve their osteogenic capacity and will be able to form new bone *in vivo*. In order to achieve these goals, the following two approaches were combined: the scaffolds were designed in the shape of microspheres; in addition, a dynamic culture system was used. The hydrogel–microspheres design was the most suitable for this situation because this maximizes the surface area for cell adherence. Moreover, cells adhered to the individual microspheres and formed cell–cell and cell–ECM interactions and, as a result, the particles coalesced and gradually formed the composite.

DOI: 10.1002/term

Additionally, applying this dynamic technique enabled the two different types of particles to be combined in a non-chemical method by relying on the cells' ability to adhere and form cell–cell and cell–ECM interactions, to finally create a 3D tissue-like construct around and between the scaffold particles. The cells were thus spread to all depths and on all surfaces of the construct. The dynamic culture system provided a method in which the two types of particles were combined in one composite, exhibiting collectively the unique characteristics of both scaffolds and cells, including the biocompatibility of the hydrogel microspheres, osteoconductivity of the ceramic particles and osteogenicity of the osteoprogenitor cells.

Moreover, besides enhancing cell–cell and cell–ECM interactions, the dynamic culture system supported cell nourishment and proliferation. Indeed, staining with CFDA for live cells enabled us to conclude that within this tissue-like construct the cells adhered to the individual particles and were viable throughout the culture period. In addition, Alamar blue proliferation assay showed that the cell number increased gradually up to 21 days in culture, indicative of a viable growing tissue. Histological analysis showed the formation of a tissue-like structure, indicating the formation of the new tissue composed of MSC-derived osteoprogenitor cells, a feature that was also confirmed by SEM images. The dynamic culture of the cells did not affect the immunotyping profile of the cells and, as shown by FACS analysis, it was shown that the cells retained their typical mesenchymal surface markers after this procedure.

There are many studies that address the issue of bone tissue engineering using biomaterials loaded with bone marrow-derived MSCs; most of the biomaterials tested in these studies were calcium-based ceramics. There are only few studies which combined ceramics with biodegradable polymers and bone marrow-derived MSCs. Na *et al*. (2007) used hydroxyapatite and poly(*N*-isopropylacrylamide-co-acrylic acid) copolymer loaded with rabbit bone marrow-derived MSCs; ectopic *in vivo* bone formation was shown mostly in BMP-2-expressing cells cultured in this hybrid scaffold and not when cultured with non-engineered cells. Trojani *et al*. (2006) used hydroxyapatite/tricalcium phosphate (HA/TCP) particles in suspension in a self-hardening Si–hydroxypropylmethylcellulose (HPMC) hydrogel; the scaffold was loaded with mouse bone marrow-derived MSCs. This study showed that the ceramic–hydrogel construct loaded with MSCs supported bone formation *in vivo*, but no quantitation of the amount of new bone was made.

In the present study, in order to evaluate the osteogenic potential of the cells in the tissue-like hydrogel–ceramic constructs, they were transplanted ectopically in the backs of immunodeficient athymic nude mice. Histological analysis, 8 weeks after transplantation, showed that transplanted tissue-like constructs formed typical bone trabeculae in the construct site with osteocytes residing in typical lacunae, whereas control scaffolds transplanted without cells did not form bone tissue. Histomorphometrical measurements showed that the amount of new bone formed in the tissue-like constructs transplanted animals was approximately 25-fold more than the new bone formed in the control scaffolds transplanted without cells.

Transplantation of live autologous tissue is not an innovative idea in the regenerative medicine field, but generating osteogenic potent tissue-like constructs *in vitro* prior to transplanting for bone tissue regeneration is a relatively new idea and extensive research still needs to be conducted in this area. The present study provides a method for designing an autograft-like construct, capturing the advantages of both components (hydrogel and ceramics), imitating native bone structure and harbouring viable committed cells. Moreover, based on the ability of hydrogel microspheres to be also complexed with growth factors (Srouji *et al*., 2005b), in the future tissue-like structures could also be used as a growth factor delivery system to enhance the new bone formation *in vivo*.

This study provides a new method that can be used to design tissue-like constructs imitating the components of native bone, for use as transplants bearing variable shapes and sizes adaptable for various transplant sites in a broad range of tissue-engineering applications.

Acknowledgements

This study was supported by EU Genostem Grant No. LSHB-CT-3003-503161. The authors wish to acknowledge the excellent technical work of Mrs Pessia Shenzer and Janette Zavin.

References

Barrilleaux B, Phinney DG, Prockop DJ, *et al*. 2006; Review: *ex vivo* engineering of living tissues with adult stem cells. *Tissue Eng* **12**(11): 3007–3019.

Bruder SP, Jaiswal N, Ricalton NS, *et al*. 1998; Mesenchymal stem cells in osteobiology and applied bone regeneration. *Clin Orthop Rel Res* (355 suppl): S247–256.

Dubruel P, Unger R, Vlierberghe SV, *et al*. 2007; Porous gelatin hydrogels: 2. *In vitro* cell interaction study. *Biomacromolecules* **8**(2): 338–344.

Ducheyne P, Qiu Q. 1999; Bioactive ceramics: the effect of surface reactivity on bone formation and bone cell function. *Biomaterials* **20**(23–24): 2287–2303.

Fedorovich NE, Alblas J, de Wijn JR, *et al*. 2007; Hydrogels as extracellular matrices for skeletal tissue engineering: state-of-the-art and novel application in organ printing. *Tissue Eng* **13**(8): 1905–1925.

Hasegawa S, Neo M, Tamura J, *et al*. 2007; *In vivo* evaluation of a porous hydroxyapatite/poly-DL-lactide composite for bone tissue engineering. *J Biomed Mater Res* **81**(4): 930–938.

Hutmacher DW. 2000; Scaffolds in tissue engineering bone and cartilage. *Biomaterials* **21**(24): 2529–2543.

Ishaug-Riley SL, Crane-Kruger GM, Yaszemski MJ, *et al*. 1998; Three-dimensional culture of rat calvarial osteoblasts in porous biodegradable polymers. *Biomaterials* **19**(15): 1405–1412.

Ishaug SL, Crane GM, Miller MJ, *et al*. 1997; Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J Biomed Mater Res* **36**(1): 17–28.

Khan Y, Yaszemski MJ, Mikos AG, *et al*. 2008; Tissue engineering of bone: material and matrix considerations. *J Bone Joint Surg* **90**(suppl 1): 36–42.

Leong NL, Jiang J, Lu HH. 2006; Polymer–ceramic composite scaffold induces osteogenic differentiation of human mesenchymal stem cells. *Conf Proc IEEE Eng Med Biol Soc* **1**: 2651–2654.

Livingston T, Ducheyne P, Garino J. 2002; *In vivo* evaluation of a bioactive scaffold for bone tissue engineering. *J Biomed Mater Res* **62**(1): 1–13.

Meinel L, Karageorgiou V, Fajardo R, *et al*. 2004a; Bone tissue engineering using human mesenchymal stem cells: effects of scaffold material and medium flow. *Ann Biomed Eng* **32**(1): 112–122.

Meinel L, Karageorgiou V, Hofmann S, *et al*. 2004b; Engineering bone-like tissue *in vitro* using human bone marrow stem cells and silk scaffolds. *J Biomed Mater Res* **71**(1): 25–34.

Na K, Kim SW, Sun BK, *et al*. 2007; Osteogenic differentiation of rabbit mesenchymal stem cells in thermo-reversible hydrogel constructs containing hydroxyapatite and bone morphogenic protein-2 (BMP-2). *Biomaterials* **28**(16): 2631–2637.

Ohgushi H, Caplan AI. 1999; Stem cell technology and bioceramics: from cell to gene engineering. *J Biomed Mater Res* **48**(6): 913–927.

Randle WL, Cha JM, Hwang YS, *et al*. 2007; Integrated three-dimensional expansion and osteogenic differentiation of murine embryonic stem cells. *Tissue Eng* **13**(12): 2957–2970.

Srouji S, Kizhner T, Livne E. 2006; 3D scaffolds for bone marrow stem cell support in bone repair. *Regen Med* **1**(4): 519–528.

Srouji S, Kizhner T, Suss-Tobi E, *et al*. 2008; 3D Nanofibrous electrospun multilayered construct is an alternative ECM mimicking scaffold. *J Mater Sci* **19**(3): 1249–1255.

Srouji S, Maurice S, Livne E. 2005a; Microscopy analysis of bone marrow-derived osteoprogenitor cells cultured on hydrogel 3D scaffold. *Microsc Res Techn* **66**(2–3): 132–138.

Srouji S, Rachmiel A, Blumenfeld I, *et al*. 2005b; Mandibular defect repair by TGFβ and IGF-1 released from a biodegradable osteoconductive hydrogel. *J Craniomaxillofac Surg* **33**(2): 79–84.

Sumanasinghe RD, Osborne JA, Loboa EG. 2008; Mesenchymal stem cell-seeded collagen matrices for bone repair: effects of cyclic tensile strain, cell density, and media conditions on matrix contraction *in vitro*. *J Biomed Mater Res* **88**: 778–786.

Trojani C, Boukhechba F, Scimeca JC, *et al*. 2006; Ectopic bone formation using an injectable biphasic calcium phosphate/Si-HPMC hydrogel composite loaded with undifferentiated bone marrow stromal cells. *Biomaterials* **27**(17): 3256–3264.