Human triple cell co-culture for evaluation of bone implant materials†

Cite this: *Integr. Biol.*, 2013, 5, 703

Frederik Wein and Arie Bruinink*

Central to the formation of tissue at implant surfaces are the interactions between multiple cell types including fibroblasts, endothelial cells and, in the case of bone, cells of the osteoblastic lineage. To date the importance of population dynamics and interactions have been largely neglected in the *in vitro* evaluation of biomaterials. To fill this gap we have developed a co-culture system using 3 cell types, primary human bone marrow stromal cells (HBMC), microvascular endothelial cells (HMVEC) and abdominal dermal fibroblasts (HDF). Proliferation of each cell type separately and differentiation of HBMC were determined by flow cytometry analysis. The medium used promoted HBMC differentiation toward osteoblasts without affecting the state of differentiation of HDF and HMVEC. Furthermore, HBMC are strongly affected by HDF and HMVEC, and *vice versa*. When used on a titanium coated substrate the triple cell culture system identified preferential HBMC proliferation relative to HDF if HMVEC was present. This developed culture system represents a new, optimised and potentially predictive approach to evaluate biomaterial biocompatibility early in development.

Received 17th October 2012,
Accepted 27th January 2013

DOI: 10.1039/c3ib20250j

www.rsc.org/ibiology

Insight, innovation, integration

The current *in vitro* set-up to evaluate biocompatibility (non-toxicity and correct tissue response) is limited since only one cell type is used. *In vivo*, however, multiple cells not only influence each other's functionality but also compete for the implant surface space. This may critically determine the kind of tissue which is finally formed at the tissue implant interface. In the present paper we propose a new advanced *in vitro* setup taking the latter aspects into account. It is based on the co-cultivation on the test material of 3 key cell types of the bone implant tissue interface (osteogenic cells, fibroblasts and endothelial cells) determining their population size separately and degree of differentiation in the case of osteogenic cells after 5 days of culture.

Introduction

Each year a broad variety of novel and promising bone implant materials and surface designs are proposed but only a few make their way into the market. The evaluation of biocompatibility (non-toxicity and correct tissue response) is the first crucial hurdle for new implant materials. *In vitro* tests are used as a cost efficient way to help identify biocompatibility of promising materials for subsequent and more costly tests in animals.¹ Current *in vitro* approaches, however, poorly represent the physiological environment as they use cell lines frequently of a single cell type which is often of non-human origin.² As such their prognostic value for the *in vivo* situation in humans is now recognised as being limited.³

The integration of a permanent implant in patients depends on complex interactions between a variety of cell types that come into contact with the implant surface and compete for implant surface space⁴ through both cell–material interactions⁵ and cell–cell interactions.⁶ The cellular niche at the implant–tissue interface is therefore complex with cell–implant and cell–cell interactions influencing cell proliferation and differentiation and tissue formation by direct physical and indirect paracrine signalling.⁷ Increasing the complexity of *in vitro* methods to better replicate the *in vivo* situation is essential to improve the prognostic value of *in vitro* tests and aid design of surfaces and implant materials that favour integration.⁸

Several co-culture systems have been proposed to investigate heterotypic cell–cell interactions *in vitro* that relate to bone regeneration. These have been used to investigate fibroblast–chondrocyte,⁹ osteoblast–chondrocyte,^{7a,10} osteoblast–endothelial cell^{6,11} and marrow stromal cells–endothelial cells¹² interactions. Neoangiogenesis around implant surfaces can be supported by cell types such as activated fibroblasts.¹³ Osteoblast and

Materials-Biology Interactions Lab, EMPA - Materials Science & Technology, Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland.

E-mail: arie.bruinink@empa.ch; Fax: +41 58 7657788; Tel: +41 58 7657695X765

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3ib20250j

endothelial cells affect each other⁶ by production of diffusible factors and direct cell–cell interactions in both two-dimensional and three-dimensional co-culture systems.¹⁴ These interactions are believed to be important in angiogenesis and osteogenesis *in vivo*.^{14b,15} Thus cells of the osteoblastic lineage, endothelial cells and fibroblasts represent key regulators for the regenerative process at the bone implant surface. However, optimal integration may in part depend on which cell type has a relative survival and proliferation advantage at the implant surface. Surface colonization by fibroblasts crowding out osteoblasts on permanent bone implants will probably lead to soft tissue formation and ultimately to implant failure,¹⁶ whereas the direct apposition of bone forming cells on the implant surface would probably lead to new bone formation and implant osseointegration.¹⁷ Conversely, for non-permanent bone implants soft tissue rather than bone formation is preferred. Recent studies have attempted to use co-culture systems to assess biocompatibility of biomaterial surfaces but these have been restricted as they are either only applicable to evaluate 2D biomaterial surfaces or are not able to differentiate effects of hard relative to soft tissue forming cells.^{6,18}

In the present study an *in vitro* system, which takes preferential colonisation of the biomaterial surface by individual cell types into consideration, is proposed for evaluating biomaterials. For this adult human bone marrow stromal cells (HBMC) in the presence or absence of primary human dermal fibroblasts (HDF) and/or microvascular endothelial cells (HMVEC) were cultured as single, double and triple cell co-culture (Fig. 1A) with the population size of each cell type and the state of differentiation of osteoblast lineage cells being used as indices indicating competitive advantages of particular cell types (Fig. 1B). As a conceptual proof we investigated the effect of titanium coating of cell culture dish surfaces on the population size of each cell type as titanium is the current “gold standard” for permanent implants for treating skeletal disorders.¹⁹

Materials and methods

Materials

Cell culture dishes (tissue culture treated polystyrene, TCPS) (35 mm diameter; Becton Dickinson, Franklin Lakes, NJ, USA) were used. Part of the dishes was coated with a nanometer sized layer of titanium (Ti) to ensure that surface structure is only negligibly modified. The Ti coatings were prepared by DC magnetron sputtering (Generator Sparcle V, Advanced Energy) in a home-built pilot-scale web coater, which is described in more detail elsewhere.²⁰ Ti sputtering (99.9% purity ensuring a defined surface chemistry) was carried-out from a 1161 cm² planar target, at a constant power of 2 kW and under an Ar pressure of 0.008 mbar. The resulting Ti coating thickness was approximately 70 nm. The thickness was measured using a profilometer (HRP, KLA – Tencor, USA) with a diamond tip (diameter 2.5 µm).

Cells and cell culture

Human bone marrow specimens were obtained from the femurs of 12 healthy donors (age between 70 and 82) undergoing hip

Fig. 1 Schematization of the current set-up and possible outcome. (A) The cell type composition of the evaluated (co)cultures. (B) A triple cell type co-culture at start and the possible outcome after 5 days of culture regarding advantage for surface colonization by HBMC and HDF.

surgery with informed consent (ethical approval EKSG 08/014). The marrow containing trabecular bone pieces was obtained from the site of hip implant stem insertion. Human bone marrow stromal cells (HBMC) were isolated from bone marrow specimens as described previously.²¹ In brief, samples including pieces of trabecular bone were incubated in isolation medium (25 mM HEPES, 128.5 mM NaCl, 5.4 mM KCl, 5.5 mM D(+)-glucose, 51.8 mM D(+)-saccharose, 0.1% BSA) for at least 12 h at 4 °C before centrifugation at 110g for 15 min. The obtained pellet was resuspended in fresh isolation medium. This procedure was repeated twice. After resuspension it was filtered three times through a 200 µm polyethylene terephthalate (PET) mesh to remove soft tissue and bone pieces. Cells were thereafter centrifuged again at 110g for 15 min. The pelleted cells were resuspended in HB(M)C expansion medium (α -MEM supplemented finally with 10% FBS, 1% PSN, 1 ng ml⁻¹ FGF-2; all from Invitrogen, Luzern, Switzerland) and 1.0×10^7 cells were seeded into T75 culture flasks. After 24 h medium was replaced with fresh medium to remove non-adhering cells. Thereafter, the medium was replaced twice a week. Cells were harvested at 70–80% sub-confluence. Only cells of this passage 1 were used for the present experiments to

minimise cell ageing effects and alteration of potency to differentiate into different cell types.

Human bone cell (HBC) cultures were prepared as described previously.²¹ Briefly, trabecular bone pieces were isolated from the bone marrow specimens. They were repeatedly flushed with isolation medium to remove all blood cells, bone marrow and soft tissue, until the bone pieces were white and clean. The obtained bone fragments were transferred into 75 cm² culture flasks (Greiner Bio-One, Reinach, Switzerland) and incubated in HB(M)C expansion medium. Cells grow out of the bone pieces and colonized the culture flask. Cells were harvested until 70–80% subconfluency is reached (normally after 3 to 4 weeks in culture). As for HBMC only cells of this passage 1 were used for the present experiments.

Human abdominal dermal fibroblasts (HDF) were expanded in the laboratory from an adult human abdominal skin biopsy with informed consent (ethical approval EKSG 06/095). HDF were routinely cultured in HDF expansion medium (DMEM (Invitrogen, Luzern, Switzerland) supplemented with 10% FCS and 1% PSN). Passages 4 to 12 were used in the experiments.

Human microvascular endothelial cells: the Clonetics® HMVEC-dAd-Adult Human Dermal Microvascular Endothelial Cells (HMVEC) were purchased from Lonza (Cryo: CC-2543) and expanded up to 90% confluence in HMVEC expansion medium (EGM-2MV BullerKit medium, Lonza) and incubated at 37 °C, in an atmosphere of 95% O₂, 5% CO₂. Cells from passage 3–6 were used for experiments.

Osteogenic media: the standard osteogenic induction medium (OS) is composed of α -MEM supplemented with 10% FCS, 1% PSN antibiotic mixture, 50 μ M ascorbic acid phosphate, 2 mM β -glycerophosphate and 10 nM 1,25-dihydroxyvitamin D₃. The addition of these osteogenic components to the medium evoked the induction of alkaline phosphatase and osteocalcin expression in HBC and HBMC cultures but not in HDF cultures.¹⁸ For co-culture experiments a triple cell type medium (TCM) was developed consisting of EGM-2MV BullerKit medium (Lonza) supplemented with 1% PSN and the same set of osteogenic inducing factors as used for OS, *i.e.* ascorbic acid phosphate (50 μ M), β -glycerophosphate (2 mM) and 1,25-dihydroxyvitamin D₃ (10 nM).

Co-culture experiments: HBMC were cultured alone (single), with HDF or HMVEC and with the combination of HDF plus HMVEC on TCPS and Ti. The seeding cell density was 180 HDF, 720 HBMC and 900 HMVEC per cm². By that in a triple co-cultural setup an initial ratio of 1:4:5 HDF, HBMC and HMVEC with a total cell density of 1800 cells per cm² was seeded. The ratio was based on published findings of co-cultures using 2 out of these 3 cell types.^{12,18} For each condition the cells were cultured for 5 days (HMVEC were seeded 24 hours earlier) in 2 ml TCM. The cell numbers used ensured that all cells still exhibited exponential growth even if under triple cell conditions all cells would have been of the same cell type. The harvested cell numbers were sufficient for flow-cytometric analyses.

Vital labelling

The vital cell labelling dye (DiD) (Vybrant, Molecular Probes, Luzern, Switzerland) was used for microscopic or flow cytometry

analyses according to Kaiser and Bruinink.²² For flow cytometry analysis HBMC were labelled with the carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE; Molecular Probes, Invitrogen, Karlsruhe, Germany). In brief HBMC were washed in PBS and treated with CFSE at a final concentration of 7.5 μ M for 15 min at 37 °C, washed in PBS and incubated for a further 30 min at 37 °C with HBMC expansion medium followed by three subsequent washes. For evaluation of cell culture morphology HBMC were labelled with the fluorescent membrane-dye PKH2 (SIGMA, Buchs, Switzerland). To analyse the effects of co-culturing different cell types by FACS HBMC were labelled by CFSE and PKH2 to enhance the green signal as stated in the text.

Immunohistochemistry

For fluorescence microscopy, cell cultures were washed twice with PBS, fixed with 2% paraformaldehyde with 0.3% Tween 20 and subsequently kept in PBS. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, CH) at 2 μ g ml⁻¹ after fixation. The samples were analyzed using a fluorescence microscope (Axio Imager.M1, Carl Zeiss, Oberkochen, Germany) and filter sets corresponding to the fluorochromes of interest. Living or fixed cells were stained. Staining against osteogenic (bone specific alkaline phosphatase, bALP) and endothelial markers (CD31; VEGF receptor-2, VEGF-R2) were performed using anti-bALP (1:1000; clone B4-78; Developmental Hybridoma Bank, Iowa City, IA, USA), anti-D31 (1:400; Clone: WM-59; Sigma, Cat. No. P8590) and/or anti-VEGF-R2 (1:400; clone KDR2; Sigma, V9134). For flow cytometry, we preferred a directly conjugated anti-CD31-allophycocyanin (APC; 1:20; Clone: WM-59; NatuTec, eBioscience, Frankfurt, Germany, 17-0319-71). As secondary antibodies we used goat anti-mouse IgG Alexa Fluor 488 (1:400, Molecular Probes, A11029) for HMVEC (fluorescent microscopy) or goat anti-mouse IgG R-Phycoerythrin (1:500; P852; LuBioScience; Lucerne Switzerland, Cat. No. 605 630). In the case of double staining setups for flow cytometry, we labeled bALP-positive cells with the secondary R-Phycoerythrin antibody and added the directly conjugated anti-CD31-APC antibody after multiple washing steps to avoid cross-staining artifacts.

Flow cytometry analyses

Cell types were distinguished and their concentrations quantified by flow cytometry (Partec CyFlow space; Görlitz, D) after day 5 of culture. Prior to seeding only HBMC were labelled with unspecific vital cell dyes emitting light in the green region of the spectrum (CFSE and PKH2; Ex/Em: 492/517 nm and 490/504, resp.) the signal of which was strong enough to identify all cells deriving from the initially seeded HBMC. At the end of the experiment all cultures were treated with antibodies against bALP and CD31. The staining for the osteogenic marker bALP allowed us to distinguish within the HBMC population the cells that are differentiated and those that are not differentiated towards (pre-)osteoblasts (bALP⁺ and bALP⁻ HBMC subpopulations, respectively). HMVEC were positive for the constitutively expressed surface protein CD31. HDF were identified by the absence of a specific and positive signal. In detail, the gating combinations were channels FL1-FITC

to identify the initially labelled HBMC (CFSE and PKH2 signal), the FL2 channel for bALP-PE expression levels (PE signal) and FL4 to identify HMVEC expressing high levels of CD31-APC (APC signal). Single and co-cultured fibroblasts being negative for bALP and CD31 emitted only auto fluorescence signals in FL1 and FL4 (Fig. S1A and B, ESI[†]). Cells stained with an isotype antibody or secondary antibody served as controls. Here the lower limit for cells considered as positive for the marker was defined by the upper limit of the fluorescence signal of the controls with 99% being negative for this marker. HBMC single cell type cultures have been used as a reference to define gateings of bALP[−] and bALP⁺ HBMC. The bALP⁺high comprise the highest 50% of the bALP⁺ HBMC subpopulation as defined by the reference cultures. The definition of the reference culture is mentioned in the text. Identical FACS-gatings to comprise the entire core population of all cells and to define each cell type with a specific region were used for each set of experiments. The cell type ratios were assessed using the Partec Flomax Software. Absolute cell numbers were measured using FACS as a cell counting device. To estimate the probability of differences in subpopulations we used the paired Student's *t*-test.

Results

Medium selection

A necessity for co-culturing is a medium which supports, appropriately, each cell type. In the current study we aimed to stimulate the differentiation of HBMC (or HBC), without affecting the state of differentiation of HDF and HMVEC. This medium termed TCM induced cell growth of all three cell types comparable to cultures containing their expansion media. Furthermore, TCM was able to induce osteogenic differentiation in HBC and HBMC but not HDF and HMVEC cultures (Fig. 2).

HDF remained negative for CD31 and VEGF2, whereas in HMVEC cultures, both media (expansion medium and TCM) maintained CD31 and VEGF2 marker expression. In TCM, HBC and HBMC displayed a well-spread morphology.

Fig. 2 Effect of culture medium on expression on cell differentiation markers. Flow cytometry analysis of osteogenic (bALP) and endothelial marker proteins (CD31, VEGFR2) of single cell type (HDF, HBMC and HMVEC) cultured in the original expansion medium and TCM after 7 days. Bars represent mean over three independent experiments $\pm$ S.E.M.

The proportion of HBMC and HBC that was bALP⁺ was clearly increased in comparison to cultures kept in HB(M)C expansion medium (Fig. 2). Flow cytometry was undertaken in order to evaluate the extent to which TCM is superior to OS in promotion of osteogenesis. We distinguished between the bALP negative (bALP[−]) and all bALP positive (bALP⁺all). Of the bALP⁺all the faintly bALP positive (bALP⁺low) and a bALP positive HBMC population whose fluorescent intensity was the highest (bALP⁺high) were assessed using cells kept in OS. The range of bALP⁺high cells was defined by the lower limit resulting in 50% of the bALP⁺ cells being located in the strongly labelled cell range. The same settings were thereafter used for cultures in TCM. In comparison to culture in OS there was a tendency for more HBC and HBMC cells to be bALP⁺ in TCM (Fig. S2A, ESI[†]). Furthermore, in the case of HBC the percentage of strongly labelled bALP⁺ shifted from $49.8 \pm 0.2\%$ (mean $\pm$ S.E.M over 4 values of 4 patients) in OS to $72.4 \pm 5.9\%$ in TCM and in the case of HBMC from $49.7 \pm 0.4\%$ in OS to $71.5 \pm 8.8\%$ in TCM (Fig S2B, ESI[†]). The effect of TCM on bALP expression was similar in the case of HBC and HBMC. Thus TCM fulfilled all criteria and was used for all subsequent experiments.

Cell type selection

In the bone niche near permanent implants multiple cell types are present. Key-players are certainly fibroblasts and endothelial cells, whereas cells of the osteoblast lineage are important for bone formation. HBMC or HBC lend themselves as a source for cells of the osteoblast lineage. In order to select which osteoblast lineage source is most optimal to be taken for the third component of our triple cell co-culture model we compared specifically the osteogenic differentiation potential of HBC and HBMC in TCM. Differentiation of HBC and HBMC in OS and TCM after the culture period of 7 days was measured taking bALP expression as an index for osteogenic differentiation. In each experiment the proportion of bALP⁺ in HBMC cultures was similar or higher than the one in HBC cultures (Fig. S2A, ESI[†]). This was the case if the cells were cultured in TCM or in OS. The percentages of bALP positive (bALP⁺) HBMC and HBC being strongly bALP⁺ (bALP⁺high) were comparable (Fig. S2B, ESI[†]). Based on these results and the fact that HBMC are a more prominent component of the bone marrow, HBMC were selected as the source for the third cell type in our triple cell type system model.

Cell culture morphology and FACS analysis

The morphology and spatial distribution of the fluorescently labelled three cell types in single and triple cell cultures was analysed after 72 h in TCM using fluorescence microscopy. The morphology of the endothelial cells seems to be altered in the presence of HDF and HBMC (Fig. 3). In single cell type cultures HMVEC displayed a broad and plain morphology (Fig. 3E) whereas in the presence of HDF and HBMC a more clustered and bulky appearance was observed (Fig. 2C). Furthermore, HDF and HBMC were often seen in close proximity to HMVEC (Fig. 3A–D).

Fig. 3 Confocal laser scanning microscopy picture of a triple cell co-culture of HBMC, HDF and HMVEC (A–D) and of a HMVEC single cell type culture (E). Human HBMC, HDF and HMVEC were co-cultured for 72 hours. HDF and HBMC were labelled before seeding with the unspecific cell membrane dyes DiD (orange, A) and PKH26 (red, B) respectively. HMVEC being not susceptible for membrane dyes were stained for CD31 using CD31-Alexa488 (green, C and D) FITC at the day of analyses. A–E: Cell nuclei were stained with DAPI (blue). (A) Merge of orange and blue; (B) merge of red and blue; (C) merge of green and blue; (D) merge of all signals (appears partly yellow); (E) merge of blue and green. Scale bar: 100 μm .

The effects of co-culturing were evaluated using HBMC cultures that have been kept on TCPS and Ti as single culture or as co-culture with HDF or HMVEC, and with the combination of HDF plus HMVEC. As measured after 5 days in culture the presence of HDF or HMVEC or both cell types caused a clear and significant enhancement in the total number of HBMC (Fig. S3A, ESI[†]). In parallel, the percentage of HBMC being bALP⁺ was

Fig. 4 Flow cytometry analysis of single and the 3 types of co-cultures. The number of each cell type was set relative to values of the single cell type cultures (single) kept on TCPS. *: Significant effects relative to single cell type culture controls. #: significantly different from the single cell type in the corresponding triple cell type culture ($P < 0.05$; two-sided t test; $n = 3$).

found to be reduced (Fig. S3B, ESI[†]). This reduction is due to the fact that especially the number of bALP[−] HBMC is enhanced (TCPS: Fig. 4, Ti: Fig. S4, ESI[†]).

In order to evaluate the effect of co-culturing on the extent of bALP expression, additionally a gate setting was used to discriminate faintly and strongly expressing bALP⁺ HBMC. For defining the range settings pure cultures of HBMC kept on either surface were used. The limit of faintly and strongly expressing bALP⁺ cells was chosen in a way that 50% of the bALP⁺ HBMC was located in the range of the faintly and 50% in the strongly bALP⁺ expressing bALP⁺ HBMC. Discriminating subpopulations of preosteoblasts regarding their state of differentiation by taking staining intensity of ALP as an index has been shown before.²³ Based on this we found that the extent of bALP expression was only negligibly affected by co-culturing (except in HBMC plus HMVEC co-cultures), *i.e.* the faintly and strongly bALP expressing HBMC populations in the different co-cultures were similar. Furthermore, it was found that the presence of HBMC negatively affected the HMVEC population (60% reduction). This was more pronounced (significant) in HBMC plus HDF plus HMVEC co-cultures where additionally HDF was present. Also the HDF population was reduced due to the presence of HBMC. However, this decrease was significantly less pronounced in the latter co-culture combination, suggesting that HMVEC was able to counteract the effect of HBMC.

Effects of Ti coating

We found that except in the case of HBMC plus HDF co-culture a titanium coating of TCPS significantly enhanced the bALP[−] HBMC cell number (Fig. 5) as analysed after 5 days of culture. Only marginal effects were seen on the total number of bALP⁺ HBMC. Also here we discriminated between faintly and strongly expressing bALP⁺ HBMC. However, in this case for the definition the same type of culture (single, HBMC plus HDF, HBMC plus HMVEC, HBMC plus HDF plus HMVEC,) kept on TCPS was used to define the 50% range setting. Expression of bALP was reduced by the Ti coating in those cultures where an increase in the bALP[−] HBMC cell number was observed, *i.e.* in single,

Fig. 5 Flow cytometry analysis of single, and the 3 types of co-cultures. The effect of Ti coating on the cell number after 5 days in culture. The number of each cell type on Ti relative to the same cell type and type of cultures kept on TCPS is given. *: significantly different from the same cell type in the same type of culture but kept on TCPS ($P < 0.05$; two-sided t test; $n = 3$). Data represent mean $\pm$ S.E.M. over three independent experiments.

HBMC plus HDF, HBMC plus HMVEC, HBMC plus HDF plus HMVEC (co-)cultures. In single HBMC cultures the number of faintly bALP expressing HBMC (bALP^{low}) was significantly increased, and in the HBMC plus HDF plus HMVEC co-cultures the number of strongly bALP expressing HBMC (bALP^{high}) was significantly decreased due to the Ti coating. A Ti coating resulted in an increase in the HDF cell number if seeded as single culture but in a decrease in the HBMC plus HDF plus HMVEC co-culture situation. A titanium coating significantly reduced the HMVEC cell number if cultured as single culture whose reduction was negligible if HBMC (with or without HDF) was also present (although significant in the case of the HBMC plus HDF plus HMVEC cell type co-culture).

Discussion

Current *in vitro* approaches to screen for potentially optimal implant materials and surface designs are limited.³ The importance of different cell types that mutually affect each other and the fact that *in vivo* these multiple cell types compete for the free space at the implant surface is not taken into account in the evaluation. Cell types, which have an advantage relative to others in colonizing biomaterial surfaces may determine which tissue finally is formed at the implant–tissue interface. To overcome this we have developed a system based on a 3 cell population co-culture on the biomaterial of interest. Crucial premises for such an advanced set-up are: (i) the ability to determine the cell population size of each cell type separately, (ii) proof that the culture medium supports each cell type without inducing a cell type change (trans/dedifferentiation), (iii) the correct selection of the cell type ratio, (iv) evidence that cells are mutually influencing each other, and (v) proof that the nature of substratum differently affect the cell population sizes.

The ability to determine the cell population size of each cell type separately is technically very challenging in a co-culture system. Generally, only mean values over the whole co-culture are studied (e.g. ref. 7b,12,14b). Solutions to solve this problem are to label one cell type and to count the labelled and unlabelled cells using a cell counting chamber according to which the obtained total cell culture data are mathematically normalized (e.g. ref. 24), by sorting different cell types and measuring the effects on the obtained populations separately as described by Guillotin *et al.*,²⁵ Grellier and co-workers,²⁶ or by flow cytometry, as proposed by Zhang and co-workers⁶ and in the present study. Here we could show that flow cytometry is a simple and reproducible one step method to separate and quantify each population separately in a multiple population context based on differences in fluorescent labelling. By that the first premise for an advanced set-up is fulfilled.

A general difficulty inherent to co-cultures is the definition of the culture medium that should support all cell types without inducing aberrant cell type characteristics. Strangely enough, in most co-culture studies this is hardly addressed (e.g. ref. 24,26,27). We selected a medium which supported proliferation of the three cell types similar to their cell type specific expansion medium. The TCM supported HDF and HMVEC without affecting their state of differentiation in

regards to the investigated markers and supported osteogenic differentiation of HBMC and HBC. As a variety of mature cell types including fibroblasts and endothelial cells may develop an osteoblast-like phenotype under the influence of dexamethasone²⁸ it was excluded for TCM. Furthermore, dexamethasone may inhibit osteocalcin mRNA synthesis.²⁹ Enhanced activity of TCM over OS in inducing bALP expression is probably due to the presence of hydrocortisone in EGM 2MV medium, the base of TCM. Ireland and co-workers reported that 0.2 μ M hydrocortisone stimulate the differentiation of primary human osteoblasts taking expression of ALP mRNA after 8 days in culture as an index.³⁰ This concentration does not seem to negatively affect the osteocalcin mRNA synthesis in these cells. Thus TCM proved to be an excellent medium to co-culture the given three cell types, representing the fulfilment of the second premise of an advanced set-up.

The cell ratio used between the various cell types was 1 : 4 : 5 HDF, HBMC and HMVEC. Previously we reported that the ratio of 1 : 4 between fibroblasts and osteoblasts was optimal for investigating fibroblast–osteoblast co-culture effects.¹⁸ The used relative concentration of endothelial cells relative to the other 2 cell types is based on findings of Ma and co-workers. They found that a relative concentration of 50% endothelial cells in endothelial cells and mesenchymal stem cells optimally support the osteogenic and angiogenic potential.¹² As a result the 1 : 4 : 5 ratio as used for the present study was obtained. It must be noted that the exact ratio between fibroblasts, mesenchymal stem cells and endothelial cells in tissues near bone related implants is not determined so far as we know and that this ratio may be expected to greatly vary between locations and patients. Thus it may be assumed that the third premise of an advanced system is also realized.

Analysis of our HBMC plus HDF plus HMVEC co-cultures suggests that morphology of HMVEC was modified in the co-cultures. Only in co-cultures dense HMVEC clusters were seen. HDF and HBMC were often located around them. This is in line with findings of Shah and co-workers who demonstrated that in co-cultures of human primary osteoblasts and HUVEC the endothelial cells and osteoblasts grouped together to form nodule-like structures. Their finding suggests that clustering is based on osteoblast migration, in the direction of the endothelial cells.³¹ Smith and co-workers found evidence that if endothelial cells were co-cultured with fibroblasts HBMC were able to attract fibroblasts. Thus it may be hypothesized that in the triple cell type co-culture HMVEC cluster and attract the HBMC which in turn attract the HDF. It will be an issue of future studies to prove this.

By evaluating cell numbers and cell differentiation we could also show that the three cell types affected each other as evaluated after 5 days in co-culture. On the one hand effects of HDF and HMVEC on HBMC could be detected. HDF and HMVEC were able to increase the number of total HBMC and the number of bALP⁺ HBMC. No indication for an effect on osteoblast cell proliferation was found by Zhang and co-workers if cells were kept on plane titanium surfaces and co-cultured with endothelial cells (being the equivalent to the HBMC plus HMVEC co-culture situation in this study).⁶ One possible reason is that they used an osteoblast cell line and not primary cells as

in the present study. The latter explanation is supported by findings of Saleh and co-workers showing that endothelial cell conditioned medium caused an increase in HBMC colony formation and size, thus promoting HBMC proliferation.³² Under our HBMC plus HMVEC co-culture conditions and especially on TCPS the total number of bALP⁺ HBMC was increased and concomitantly the extent of bALP expression by trend reduced. Regarding effects of endothelial cells on HBMC differentiation contradictory findings were reported. Zhang and co-workers reported that a co-cultivation with endothelial cells increased the ALP enzyme activity per calculated MG63 cell if kept on planar titanium surfaces.⁶ Kaigler, Villars and co-workers²⁴ and Ma and co-workers¹² found positive effects of endothelial cells on HBMC differentiation but only if the culture medium was shared.^{14b,24} In contrast, Meury and co-workers reported that endothelial cells release factors that inhibit osteogenic differentiation of HBMC.^{14c} Differences in culture conditions (medium used, cell ratio, cell density) may be the reason for these reported discrepancies.

HBMC also influence HMVEC and HDF. The presence of HBMC negatively affected HDF and HMVEC cell population sizes. In line with this study we previously reported that HBC were able to inhibit fibroblast proliferation.¹⁸ However, it is in contrast with findings of Smith and co-workers³³ who could show that under paracrine conditions HBMC were able to promote fibroblast cell proliferation. Regarding effects on endothelial cells, Villars and co-workers could show that HBMC conditioned medium was able to stimulate HUVEC proliferation.²⁷ The reason for these differences might be found in the set-up. Our set-up enabled direct contact between HBMC and the other cell types thus simulating a competing environment unlike the *in situ* microenvironment. In both other studies direct contact was absent. Thus, summarising, we could prove that in co-cultures HBMC, HDF and HMVEC were mutually affecting each other representing the fourth premise for the advanced set-up.

In vivo titanium is known to exhibit osteoconductive properties. A premise for osteoconductive materials like titanium is that bone forming cells have a certain advantage over other cell types to colonize the surface. Here we compared current, two proposed and our new set-up composed of three cell types for bone-related biomaterial evaluation by comparing the cell performance on TCPS and Ti. The single cell type set-up represents the currently used set-up to evaluate bone related biomaterials in its most optimal form (comparing the performance of different cell types as single cell type cultures on the various materials). The HBMC plus HDF co-culture represents the set up as we previously have proposed.¹⁸ Our HBMC plus HMVEC set-up is similar to the system proposed by Zhang and co-workers.⁶ The HBMC plus HDF plus HMVEC co-culture set-up is our proposed new set-up and represents a combination of both previously proposed set-up. Using the single cell type set-up we found that both HBMC and HDF cell numbers were increased to a nearly similar extent (no clear predominance in HBMC performance on Ti). With the HBMC plus HDF co-culture set-up no differences in cell performance on TCPS and Ti could be found and by that it failed to make a correct prognosis regarding the osteoconductive effects of

titanium as shown *in vivo*. The HBMC plus HMVEC co-culture set-up provided evidence (like in single cultures) that Ti promoted HBMC performance but this set-up delivered no data regarding fibroblast cells and as a result disabling a prognosis regarding the prevailed tissue that is formed *in vivo*. Only by using the set-up combining HBMC, HDF and HMVEC an indication was obtained that, based on the cell numbers found after 5 days on these surfaces, HBMC has an advantage over HDF to colonize Ti in comparison to TCPS. The latter was expected based on the *in vivo* performance of titanium. These findings suggest that the triple cell type co-culture set-up as presented here have advantages over the single, the HBMC plus HDF and HBMC plus HMVEC co-culture set-up that are currently used, respectively, proposed to evaluate biomaterials.

Conclusions

Here, we introduce a new strategy to evaluate biomaterial surfaces *in vitro* by co-culture and analyses of three different key cell types that are important for bone and soft-tissue formation in relation to implants *in vivo*. We show that these cells mutually influence each other. By comparing cultures on TCPS and Ti we found that only in our triple cell type co-culture system a relative advantage for HBMC over HDF was seen. Thus, the current proposed triple cell type co-culture system may represent a new *in vitro* set-up to evaluate novel bone implant biomaterials and surfaces allowing prediction of tissue that may be expected to be formed at the tissue-implant interface *in vivo*.

Acknowledgements

We would like to thank the Kantonsspital St. Gallen for providing the human tissue samples. We thank Dr G. Sébastien (Empa, CH) for titanium coating of the cell culture dishes and Prof. Dr D. Salter (Uni Edinburgh, UK) for critical reading of the manuscript. This work has been supported by EC FP7 Magister project contract no. NMP3-LA-2008-214685.

Notes and references

- (a) S. R. Winn, J. Mitchell and H. Uludag, in *An Introduction to Biomaterials*, ed. S. A. Guelcher and J. O. Hollinger, CRC Press, Boca Raton, FL, 2006, vol. 15, pp. 63–66; (b) A. Pizzoferrato, G. Ciapetti, S. Stea, E. Cenni, C. R. Arciola, D. Granchi and L. Savarino, Cell culture methods for testing biocompatibility, *Clin. Mater.*, 1994, **15**, 173–190; (c) A. I. Pearce, R. G. Richards, S. Milz, E. Schneider and S. G. Pearce, Animal models for implant biomaterial research in bone: a review, *Eur. Cells Mater.*, 2007, **13**, 1–10.
- (a) L. F. Cooper, T. Masuda, P. K. Yliheikkilä and D. A. Felton, Generalizations regarding the process and phenomenon of osseointegration. Part II. *In vitro* studies, *Int. J. Oral Maxillofac. Implants*, 1998, **13**, 163–174; (b) C. J. Kirkpatrick, S. Fuchs, M. I. Hermanns, K. Peters

- and R. E. Unger, Cell culture models of higher complexity in tissue engineering and regenerative medicine, *Biomaterials*, 2007, **28**, 5193–5198.
- 3 A. Bruinink and R. Luginbuehl, Evaluation of biocompatibility using *in vitro* methods: Interpretation and limitations, *Adv. Biochem. Eng. Biotechnol.*, 2012, **126**, 117–152.
 - 4 J. M. Anderson, in *Bone engineering*, ed. J. E. Davies, Em squared Inc., Toronto, 2000, pp. 81–93.
 - 5 D. A. Puleo and A. Nanci, Understanding and controlling the bone–implant interface, *Biomaterials*, 1999, **20**, 2311–2321.
 - 6 Y. Zhang, O. Andrukhov, S. Berner, M. Matejka, M. Wieland, X. Rausch-Fan and A. Schedle, Osteogenic properties of hydrophilic and hydrophobic titanium surfaces evaluated with osteoblast-like cells (MG63) in coculture with human umbilical vein endothelial cells (HUVEC), *Dent. Mater.*, 2010, **26**, 1043–1051.
 - 7 (a) C. Sanchez, M. A. Deberg, N. Piccardi, P. Msika, J. Y. L. Reginster and Y. E. Henrotin, Subchondral bone osteoblasts induce phenotypic changes in human osteoarthritic chondrocytes, *Osteoarthritis Cartilage*, 2005, **13**, 988–997; (b) I. E. Wang, J. Shan, R. Choi, S. Oh, C. K. Kepler, F. H. Chen and H. H. Lu, Role of osteoblast–fibroblast interactions in the formation of the ligament-to-bone interface, *J. Orthop. Res.*, 2007, **25**, 1609–1620.
 - 8 C. J. Kirkpatrick, S. Fuchs, K. Peters, C. Brochhausen, M. I. Hermanns and R. E. Unger, Visions for regenerative medicine: Interface between scientific fact and science fiction, *Artif. Organs*, 2006, **30**, 822–827.
 - 9 Z. Nevo, J. Silver, Y. Chorev, I. Riklis, D. Robinson and Z. Yosipovitch, Adhesion characteristics of chondrocytes cultured separately and in cocultures with synovial fibroblasts, *Cell Biol. Int.*, 1993, **17**, 255–273.
 - 10 (a) J. P. Spalazzi, K. L. Dionisio, J. Jiang and H. H. Lu, Osteoblast and chondrocyte interactions during coculture on scaffolds, *IEEE Eng. Med. Biol. Mag.*, 2003, **22**, 27–34; (b) J. Jiang, S. B. Nicoll and H. H. Lu, Co-culture of osteoblasts and chondrocytes modulates cellular differentiation *in vitro*, *Biochem. Biophys. Res. Commun.*, 2005, **338**, 762–770.
 - 11 R. E. Unger, A. Sartoris, K. Peters, A. Motta, C. Migliaresi, M. Kunkel, U. Bulnheim, J. Rychly and C. J. Kirkpatrick, Tissue-like self-assembly in cocultures of endothelial cells and osteoblasts and the formation of microcapillary-like structures on three-dimensional porous biomaterials, *Biomaterials*, 2007, **28**, 3965–3976.
 - 12 J. Ma, J. J. J. P. v. d. Beucken, F. Yang, S. K. Both, F.-Z. Cui, J. Pan and J. A. Jansen, Coculture of osteoblasts and endothelial cells: Optimization of culture medium and cell ratio, *Tissue Eng., Part C*, 2011, **17**, 349–357.
 - 13 D. I. T. Lutikhuiizen, M. C. Harmsen and M. J. A. V. Luyn, Cellular and Molecular Dynamics in the Foreign Body Reaction, *Tissue Eng.*, 2006, **12**, 1955–1970.
 - 14 (a) D. S. Wang, M. Miura, H. Demura and K. Sato, Anabolic Effects of 1,25-Dihydroxyvitamin D3 on Osteoblasts Are Enhanced by Vascular Endothelial Growth Factor Produced by Osteoblasts and by Growth Factors Produced by Endothelial Cells, *Endocrinology*, 1997, **138**, 2953–2962; (b) F. Villars, B. Guilloin, T. Amedee, S. Dutoya, L. Bordenave, R. Bareille and J. Amedee, Effect of HUVEC on human osteoprogenitor cell differentiation needs heterotypic gap junction communication, *Am. J. Physiol.: Cell Physiol.*, 2002, **282**, C775–C785; (c) T. Meury, S. Verrier and M. Alini, Human endothelial cells inhibit BMSC differentiation into mature osteoblasts *in vitro* by interfering with osterix expression, *J. Cell. Biochem.*, 2006, **98**, 992–1006.
 - 15 (a) R. Schimming and R. Schmelzeisen, Tissue-engineered bone for maxillary sinus augmentation, *J. Oral Surg.*, 2004, **62**, 724–729; (b) G. Finkenzeller, G. Arabatzis, M. Geyer, A. Wenger, H. Bannasch and G. B. r. Stark, Gene Expression Profiling Reveals Platelet-Derived Growth Factor Receptor Alpha as a Target of Cell Contact-Dependent Gene Regulation in an Endothelial Cell–Osteoblast Co-culture Model, *Tissue Eng.*, 2006, **12**, 2889–2903; (c) B. Mair, G. Fuerst, P. Kubitzky, S. Tangl, H. Bergmeister, U. Losert, G. Watzek and R. Gruber, The anti-angiogenic substance TNP-470 impairs peri-implant bone formation: a pilot study in the rabbit metaphysis model, *Clin. Oral Impl. Res.*, 2007, **18**, 370–375.
 - 16 F. S. Kaplan, W. C. Hayes, T. M. Keaveny, A. Boskey, T. A. Einhorn and J. P. Iannotti, Form and function of bone, in *Orthopaedic Basic Science*, ed. S. R. Simon, American Academy of Orthopaedic Surgeons, Rosemont, IL, 1994, pp. 127–185.
 - 17 P. I. Brånemark, B. O. Hansson, R. Adell, U. Breine, J. Lindström, O. Hallén and A. Ohman, Osseointegrated implants in the treatment of the edentulous jaw. Experience from a 10-year period, *Scand. J. Plast. Reconstr. Surg. Suppl.*, 1977, **16**, 1–132.
 - 18 E. F. Burguera, M. Bitar and A. Bruinink, Novel *in vitro* co-culture methodology to investigate heterotypic cell–cell interactions, *Eur. Cells Mater.*, 2010, **19**, 166–179.
 - 19 C. P. Sharma, *Biointegration of medical implant materials. Science and design*, CRC Press Publishing, Cambridge, 2010, pp. 1–384.
 - 20 (a) D. Hegemann, M. Amberg, A. Ritter and M. Heuberger, Recent developments in Ag metallised textiles using plasma sputtering, *Mater. Technol.*, 2009, **24**, 41–45; (b) M. Amberg, C. Kasdallah, A. Ritter and D. Hegemann, Influence of residual oils on the adhesion of metal coatings to textiles, *J. Adhes. Sci. Technol.*, 2010, **24**, 123–134.
 - 21 R. O. C. Oreffo, S. Bord and J. T. Triffitt, Skeletal progenitor cells and ageing human populations, *Clin. Sci.*, 1998, **94**, 549–555.
 - 22 J. P. Kaiser and A. Bruinink, Investigating cell–material interactions by monitoring and analysing cell migration, *J. Mater. Sci. Mater. Med.*, 2004, **15**, 429–435.
 - 23 (a) N. Kamalia, C. A. McCulloch, H. C. Tenenbaum and H. Limeback, Direct flow cytometric quantification of alkaline phosphatase activity in rat bone marrow stromal cells, *J. Histochem. Cytochem.*, 1992, **40**, 1059–1065; (b) H. Tsangari, D. M. Findlay, A. C. Zannettino, B. Pan, J. S. Kuliwaba and N. L. Fazzalari, Evidence for reduced

- bone formation surface relative to bone resorption surface in female femoral fragility fracture patients, *Bone*, 2006, **39**, 1226–1235.
- 24 D. Kaigler, P. H. Krebsbach, E. R. West, K. Horger, Y. C. Huang and D. J. Mooney, Endothelial cell modulation of bone marrow stromal cell osteogenic potential, *FASAB J.*, 2005, **19**, 665–667.
 - 25 B. Guillotin, R. Bareille, C. Bourget, L. Bordenave and J. Amédée, Interaction between human umbilical vein endothelial cells and human osteoprogenitors triggers pleiotropic effect that may support osteoblastic function, *Bone*, 2008, **42**, 1080–1091.
 - 26 M. Grellier, N. Ferreira-Tojais, C. Bourget, R. Bareille, F. Guillemot and J. Amédée, Role of vascular endothelial growth factor in the communication between human osteoprogenitors and endothelial cells, *J. Cell. Biochem.*, 2009, **106**, 390–398.
 - 27 F. Villars, L. Bordenave, R. Bareille and J. Amédée, Effect of human endothelial cells on human bone marrow stromal cell phenotype: role of VEGF?, *J. Cell. Biochem.*, 2000, **79**, 672–685.
 - 28 (a) R. Aguilar-Vázquez, O. A. Carballo-Molina, O. Collazo-Navarrete, M. Guerrero-Rangel, A. D. Saucedo-Campos, P. Barrera-Lechuga, R. López-Marure and J. R. Cáceres-Cortés, Osteogenesis of human vascular endothelial cells in culture, *Rev. Invest. Clin.*, 2008, **60**, 496–501; (b) C. Shui and A. M. Scutt, Mouse embryo-derived NIH3T3 fibroblast adopt an osteoblast-like phenotype when treated with 1 α , 25-dihydroxyvitamin D3 and dexamethasone *in vitro*, *J. Cell. Physiol.*, 2002, **193**, 164–172; (c) S. Lavenus, P. Pilet, J. Guicheux, P. Weiss, G. Louarn and P. Layrolle, Behaviour of mesenchymal stem cells, fibroblasts and osteoblasts on smooth surfaces, *Acta Biomater.*, 2011, **7**, 1525–1534.
 - 29 N. Leclerc, T. Noh, A. Khokhar, E. Smith and B. Frenkel, Glucocorticoids inhibit osteocalcin transcription in osteoblasts by suppressing Egr2/Krox20-binding enhancer, *Arthritis Rheum.*, 2005, **52**, 929–939.
 - 30 D. C. Ireland, S. Bord, S. R. Beavan and J. E. Compston, Hydrocortisone increases the rate of differentiation of cultured human osteoblasts, *J. Cell. Biochem.*, 2004, **91**, 594–601.
 - 31 A. R. Shah, S. R. Shah, S. Oh, J. L. Ong, C. Wenke and C. M. Agrawal, Migration of co-cultured endothelial cells and osteoblasts in composite hydroxyapatite/polylactic acid scaffolds, *Ann. Biomed. Eng.*, 2011, **39**, 2601–2609.
 - 32 F. A. Saleh, M. Whyte, P. Ashton and P. G. Genever, Regulation of mesenchymal stem cell activity by endothelial cells, *Stem Cells Dev.*, 2011, **20**, 391–403.
 - 33 A. N. Smith, E. Willis, V. T. Chan, L. A. Muffley, F. F. Isik, N. S. Gibran and A. M. Hocking, Mesenchymal stem cells induce dermal fibroblast responses to injury, *Exp. Cell Res.*, 2010, **316**, 48–54.