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EFFECT OF FIBRIN ON OSTEOGENIC DIFFERENTIATION AND VEGF EXPRESSION OF BONE MARROW STROMAL CELLS IN MINERALISED SCAFFOLDS: A THREE-DIMENSIONAL ANALYSIS

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Abstract

The aim of the present study was to test the hypothesis that a fibrin matrix enhances the osteogenic differentiation and expression of vascular endothelial growth factor (VEGF) by human bone marrow stromal cells (hBMSCs) seeded into mineralised scaffolds. Porous calcium carbonate scaffolds were droplet seeded with hBMSCs using a matrix containing 3 % fibrinogen and cultured for 3 weeks. Seeded scaffolds without the fibrin matrix served as controls. The scaffolds were evaluated, using undecalcified thick sections, for fluorescence staining for nuclei, osteocalcin (OC) and VEGF. The sections were systematically scanned using optical sectioning and three dimensional distributions of cells and positive staining indicating expression of OC and VEGF were reconstructed from the z-stacks. The fibrin matrix maintained a significantly higher level of cell numbers after 2 d and 1 week and delayed the onset of osteogenic differentiation while sustaining a significantly higher level of OC and VEGF expression after 2 and 3 weeks, starting from the periphery of the scaffolds. There was a decrease in cell density from the periphery to the centre of the scaffolds in both groups The percentage of cells expressing OC and VEGF was significantly different between the centre and the periphery of the scaffolds in the fibrin(+) group but not in the controls. It is concluded that the fibrin matrix used appears to be a useful adjunct for supporting and sustaining osteogenic and angiogenic activity of hBMSCs in tissue engineered constructs. This could help to improve their performance in a clinical setting.

Keywords: Osteogenic differentiation; vascular endothelial growth factor; tissue engineering; fibrin matrix; human bone marrow stromal cells.

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Introduction

The homogenous distribution and reliable osteogenic differentiation of sufficient numbers of cultured mesenchymal stem cells (MSCs) are considered to be essential factors for the success of cell based techniques of bone tissue engineering *in vitro* (Gomes *et al*., 2003; Meinel *et al*., 2004; Wang *et al*., 2006). The attachment of cells inside the scaffolds after seeding, as well as their proliferation and differentiation during subsequent culture, relies on pathways comparable to the process of MSC homing *in vivo* (Yagi *et al*., 2010). Recently, it has been shown that a fibrin matrix can lead to significantly increased proliferation and survival of human bone marrow stromal cells in porous scaffolds even under static culture conditions (Zhu *et al*., 2010), suggesting that an environment that provides attachment signals similar to the natural stem cell niche is beneficial for the long term survival of bone marrow stromal cells in tissue engineered constructs. It is unclear yet, whether this environment would also promote the ability of the cells inside the scaffolds to support bone formation in the seeded carriers. Besides osteogenic differentiation of seeded cells, an important precondition for bone formation is vascularisation of the implanted carriers. In this respect, it is interesting that bone marrow-derived mesenchymal stem cells have also shown increased production of vascular endothelial growth factor (VEGF) after transplantation facilitating vascularisation and survival of co-transplanted cells (Egana *et al*., 2009; Figliuzzi *et al*., 2009). This suggests that an ideal environment for seeding and culturing of MSCs in carriers for bone tissue engineering would promote not only osteogenic differentiation but also angiogenic activity of the transplanted MSCs for successful application of the seeded constructs *in vivo*.

Unfortunately, assessment of the specific activity of the cells inside the constructs remains difficult. Indirect techniques for the evaluation of cell differentiation by measuring the occurrence of osteogenic markers in the medium surrounding the seeded scaffolds are misleading, because many of these molecules that are produced by the cells will be deposited in the matrix inside the scaffolds (Materna *et al*., 2008). Transcription analysis using PCR can identify osteogenic differentiation (Goessler *et al*., 2006), but homogenisation of the scaffolds makes evaluation of cell numbers and analysis of cell distribution impossible. Therefore, a number of studies have used histologic evaluation to assess the homogeneity of distribution and the differentiation of seeded cells inside the carriers (Solchaga *et al*., 2006; Meretoja *et al*., 2009; Oliveira *et al*., 2006; Gomes *et al*., 2003). Recently, structured illumination of serial

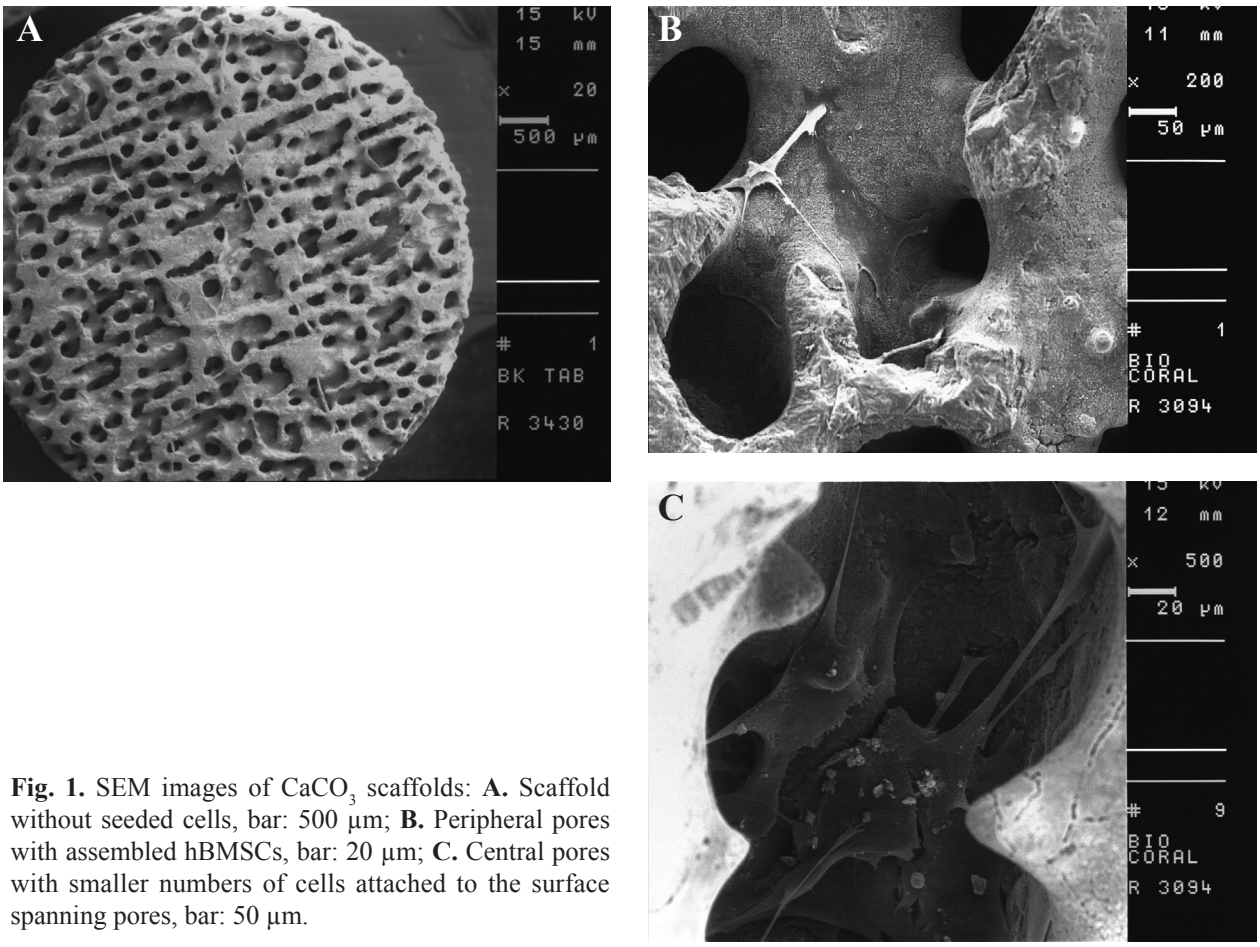

Fig. 1. SEM images of $CaCO_3$ scaffolds: **A.** Scaffold without seeded cells, bar: 500 µm; **B.** Peripheral pores with assembled hBMSCs, bar: 20 µm; **C.** Central pores with smaller numbers of cells attached to the surface spanning pores, bar: 50 µm.

thick section specimens of mineralised scaffolds has been used to identify the three-dimensional position of human bone marrow stromal cells (hBMSCs) inside the seeded scaffolds using immunofluorescence microscopy (Zhu *et al*., 2010). The aim of the present study was, therefore, to use this technique to test the hypothesis that the use of a fibrin matrix enhances not only proliferation but also osteogenic differentiation (expression of osteocalcin (OC)) and angiogenic activity (expression of VEGF) of BMSCs seeded into porous mineralised scaffolds.

Materials and Methods

Human BMSC culture

Human BMSCs were obtained from one healthy donor by bone marrow aspiration during harvesting of bone grafts from the iliac crest. The procedures had been approved by the hospital ethical committee and the donor has given his informed consent. BMSCs were isolated from the collected samples by Lymphoprep® (Axis-Shield Poc AS, Oslo, Norway) density gradient centrifugation and subsequent plating at 2 x 10^6 mononuclear cells/cm^2 in MSC medium. MSC medium was composed of high-glucose DMEM (Gibco, Darmstadt, Germany) and 15 % foetal calf serum (FCS, Biochrom AG, Berlin, Germany), 1 % (5 x 10^{-5} M) β-mercaptoethanol (β-ME, Serva, Heidelberg, Germany) and 1 % non-essential amino acids (NEAA, Gibco), 2 % Gentamycine (PAN biotech GmbH, Aidenbach, Germany) as described by Guan *et al*. (1999). Cell culture plates were incubated at 37 °C in a humidified atmosphere of 5 % CO_2 and medium was replaced every 3-4 d. Adherently growing cells were trypsinised using 0.05 % trypsin, 5 mM EDTA (Biochrom AG) for further subculture as soon as they reached 80 % confluence.

Before seeding, the cells were characterised using FACS analysis (Becton Dickinson GmbH, Heidelberg, Germany) to identify the stem cell markers defined by Dominici and co-workers (CD73, CD90 and CD105 and the lack of expression of CD45) (Dominici *et al*., 2006).

Scaffolds

Calcium carbonate cylinders with a diameter of 5 mm and a thickness of 3 mm (Biocoral®, Biocoral France, La Garenne Colombes, France) were used for seeding. The scaffolds were derived from natural corals and were composed by over 98 % of crystalline calcium carbonate. They had a highly interconnecting porosity with a pore size of 200-300 µm and an overall porosity of ~75 % (Fig. 1A).

Seeding and culture of hBMSC within the scaffolds

Seeding of the scaffolds was performed using cells from the second passage, as described previously (Zhu *et al*., 2010). After pre-wetting of the scaffolds overnight in medium at 5 % CO_2, 37 °C, the cells used for seeding were counted using a CASY cell counter system (Innovatis AG, Bielefeld, Germany) and transferred to the scaffolds by droplet seeding. For scaffolds without a fibrin matrix,

cells were resuspended in medium and 30 µL medium of 5.4 x 10^5 cells/mL (corresponding to a cell number of approximately 16,200 cells) were pipetted into each scaffold. To allow cell adhesion, cells were left for 3 h in an incubator at 5 % CO_2, 37 °C (Fig. 1B and C).

For scaffolds with a fibrin matrix, the cells were resuspended in medium and mixed with fibrinogen solution (Beriplast P, ZLB Behring GmbH, Marburg, Germany) (90 mg/mL) (Tredree *et al*., 2006) at a ratio of 3:1 v/v so that the final cell density was 5.4 x 10^5 cells/mL and the fibrinogen concentration of approximately 3 %. 30 µL of cell/fibrinogen mixture were then pipetted into each scaffold with subsequent addition of 8 µL thrombin solution (Beriplast P, ZLB Behring GmbH, Marburg, Germany) diluted 1:2 with phosphate-buffered saline (PBS) in order to start fibrin polymerisation within the scaffold. Constructs were then placed into an incubator at 5 % CO_2, 37 °C for 30 min to allow the fibrin matrix-cell suspension to polymerise.

After incubation, all scaffolds were transferred into new culture plates with 1 mL of culture medium for subsequent culturing for 3 weeks (37 °C in a humidified atmosphere of 5 % CO_2). Medium was replaced every 3 d. Four seeded scaffolds were collected after 2 d, 1, 2 and 3 weeks for further analysis.

Specimen preparation

The seeded scaffolds were washed in PBS and fixed in 4 % formalin for 24 h, dehydrated and embedded in Technovit 9100 New solutions (Heraeus Kulzer GmbH, Wehrheim, Germany). Longitudinal serial thick sections were prepared parallel to the vertical axis of the undecalcified cylinders at steps of 500 µm using a diamond coated saw (Fa. Exakt, Norderstedt, Germany). The resulting specimens had a thickness of approximately 200 µm and were placed in 2-methoxyethylacetate (MEA, Merck, Darmstadt, Germany) overnight. Subsequently, the embedding material was removed from the sections using xylene 2 x 5 min each and a graded ethanol series for 5 min each. Specimens were re-fixed in 4 % paraformaldehyde for 10 min and permeabilised using 0.1 % Triton for 10 min. Primary antibodies against OC (polyclonal rabbit anti human OC IgG, 1:100; Biotrend Chemikalien, Köln, Germany) and VEGF (monoclonal mouse anti human VEGF IgG, 1:100; Santa Cruz Biotechnology, Heidelberg, Germany) were used according to the instructions of the manufacturers (incubation time 1 h) followed by secondary antibody staining FITC (goat anti-rabbit IgG, 1:100; KPL Inc., Gaithersburg, MD, USA) for OC and Cy3 (goat anti-mouse IgG, 1:100; Jackson ImmunoResearch, West Grove, PA, USA) for VEGF. Subsequently, nuclei were stained with DAPI dilactate 1:1000 (Invitrogen, Darmstadt, Germany) for 15 min and mounted with Prolong Gold Antifade Reagent (Invitrogen). Sections stained without the primary antibodies served as controls to prove specific binding of the respective secondary antibody. Isotype controls for OC (rabbit polyclonal IgG; 1:100; Abcam, Cambridge, UK (ab27472)) and VEGF (Mouse monoclonal IgG2a; 1:100; Abcam, Cambridge, UK (ab18414)) were used to prove specific binding of the respective primary antibodies.

Fluorescence microscopy and image analysis

Each scaffold was completely sectioned resulting in 6 thick section specimens per scaffold corresponding to approximately 25 % of the original scaffold volume. This volume was completely evaluated using fluorescence microscopy with an Apotome equipment that provided structured illumination (Axiovert 200M, Carl Zeiss, Hallbergmoos, Germany) at 100x magnification. Each section was completely scanned using a matrix of 5 x 5 images (units) of 866 µm x 648 µm each (for details see Zhu *et al*., 2010). For evaluation of spatial distribution, the central 9 units (3 x 3) of each section were considered as "centre" whereas the surrounding 16 units were considered as "periphery". Using Axiovision image analysis software in combination with the Apotome equipment, serial optical sections at a distance of 6 µm were performed in z-stacks of 20 sections each. Thus, approximately 3000 images (units) were evaluated for each scaffold comprising 25 % of the original scaffold volume. The number of cells per unit was determined by automatic counting of the DAPI stained nuclei (blue). The blue DAPI stain could be clearly identified by the system at a wavelength of 365 nm and the automated image analysis routines unanimously identified the individual nuclei.

OC staining (green) was detected at an excitation wavelength of 490 nm, VEGF staining (red) was identified at an excitation wavelength of 550 nm. Staining of OC and VEGF within the cells has been specific as shown by the controls using secondary antibodies only. The signals positive for OC and VEGF could be clearly separated by the different wave lengths/filters and were unanimously identified (Fig. 4A-D). The occurrence of positive OC and VEGF signals within the cells was defined as positive expression of OC and VEGF, respectively. Counting of these signals was limited to the immediate vicinity (10 µm) of the nuclei that had been clearly identified. This made sure that false positive signals not associated to cells were not registered or included into the evaluation. Counting was again performed by automated image analysis routines thereby avoiding subjectivity. In this way, effects that could have introduced a subjective bias into the evaluation have been excluded as far as possible on every level of the procedure and it has been ensured that positive counts for OC and VEGF reflected the number of cells actually producing these proteins. No matrix production deposited outside of cells was used for quantitative evaluation of expression of OC/VEGF to avoid bias through occasional autofluorescence at the margins of the scaffold pores.

The data obtained were assigned to the position within the grid and calculated as cells/image and number of positive OC/VEGF stainings/image, respectively. The portion of positively stained cells was expressed as percentage of the total cell count per image.

Statistical analysis

Mean values of counted cell numbers and positive counts for OC and VEGF per unit (image) were averaged for the individual sections of each scaffold. Values from all scaffolds of each group were used to calculate group median values. Longitudinal changes in both groups were

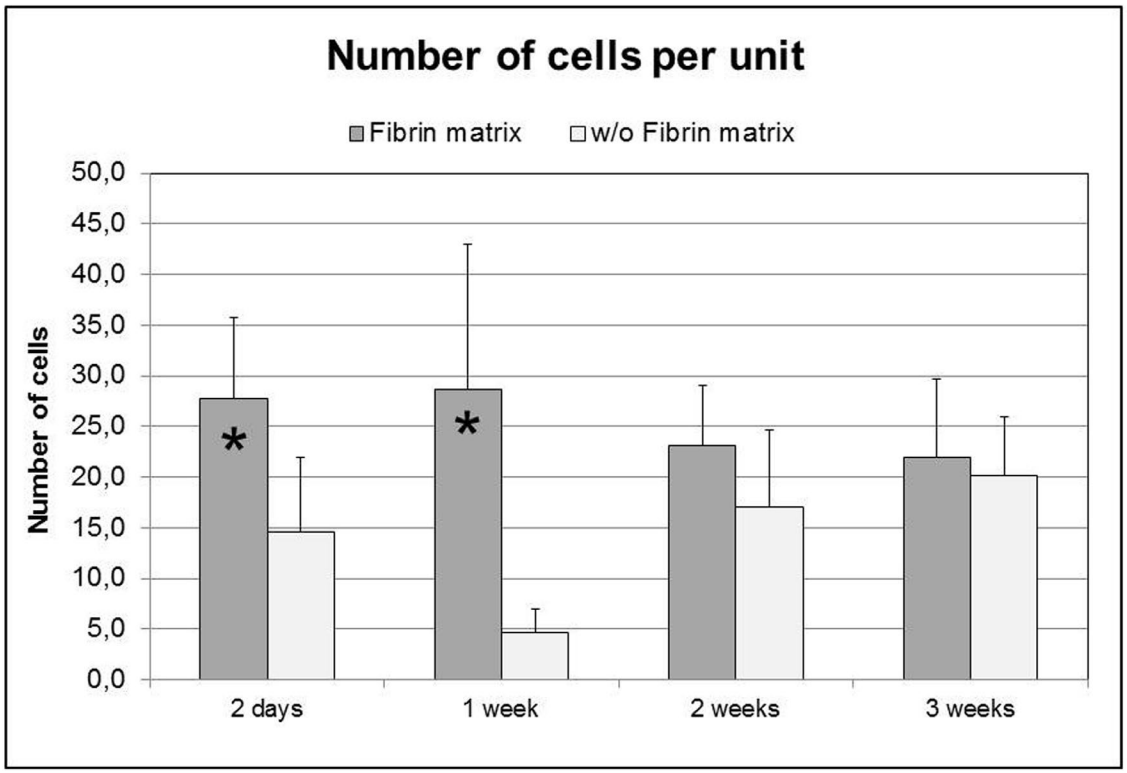

Fig. 2. Evaluation of cell counts/unit (DAPI stain). * Significant with $p < 0.05$.

compared using ANOVA tests with Bonferroni correction. Moreover, for comparison of spatial distribution, the matrix of 5 x 5 units of each section was divided into 9 central units (the central area of 3 x 3 units) and 16 peripheral units. For comparison of the number of both cells and the expression of OC and VEGF in central versus peripheral units, paired *t*-tests were used. For comparison of the two seeding methods, cell counts and OC / VEGF percentages were compared using unpaired *t*-tests. Co-expression of OC and VEGF in each unit was analysed by calculating a Spearman Rho correlation. All tests were performed at a significance level of $p < 0.05$ (SPSS version 19, IBM, Hamburg, Germany).

Results

Cell count and spatial distribution

The mean number of cells per unit in the group of scaffolds with a fibrin matrix was 27.8 (± 7.9) after 2 d of culturing. The number remained rather stable with a slight but insignificant decrease ($p = 0.545$) to 21.9 cells/unit (± 7.8) after 3 weeks. Scaffolds without a fibrin matrix exhibited comparable cell numbers after 2 d of culturing (14.6, ± 7.4) but showed a sharp decrease to 4.6 cells/unit (± 2.4) after 1 week, with a subsequent gradual increase to 20.1 cells/unit (± 5.9) at the end of the observation period ($p < 0.001$) (Fig. 2). The difference in cell counts between the two groups was significant after 2 d and 1 week ($p = 0.014$ and $p = 0.004$, respectively). Analysis of the three dimensional distribution of cells showed a decrease in cell density from the periphery to the centre of the scaffolds in both groups (Figs. 3A-H). This decrease was consistently seen at all intervals during the observation period but was particularly evident in later periods of culture in the group of scaffolds with a fibrin matrix. The difference between cell populations in the units in the periphery and the centre of the scaffolds was significant at all intervals in both groups ($p < 0.05$).

Fluorescence microscopy

The immunofluorescence labelling allowed a clear distinction of positively stained areas and the allocation to cellular contours (Fig. 4). There was frequent co-expression of OC and VEGF in the seeded bone marrow stromal cells. The cells positive for OC and VEGF were seen both spanning across the pore diameters (Figs. 5A and B) and lining the pore walls (Fig. 6A and B). Systematic comparison of the two groups revealed a higher number of cells in many scaffold pores in the group seeded with the fibrin matrix (Fig. 7A-H) than in those seeded without, with the number of cells increasing over time in both groups. Moreover, matrix production in the cells positive for OC and VEGF expression was far more advanced in the scaffolds with fibrin matrix compared to those without.

Expression of OC and VEGF

The mean percentage of cells with positive stains for OC in scaffolds with the fibrin matrix was 15.7 (± 1.8) after 2 d of culturing. This percentage increased to a maximum of 55.4 % (± 6.5) after a period of 2 weeks with a decrease to 45.3 % (± 3.3) 1 week later. The longitudinal changes were significant between each of the intervals ($p < 0.05$). The control scaffolds without fibrin exhibited a slightly

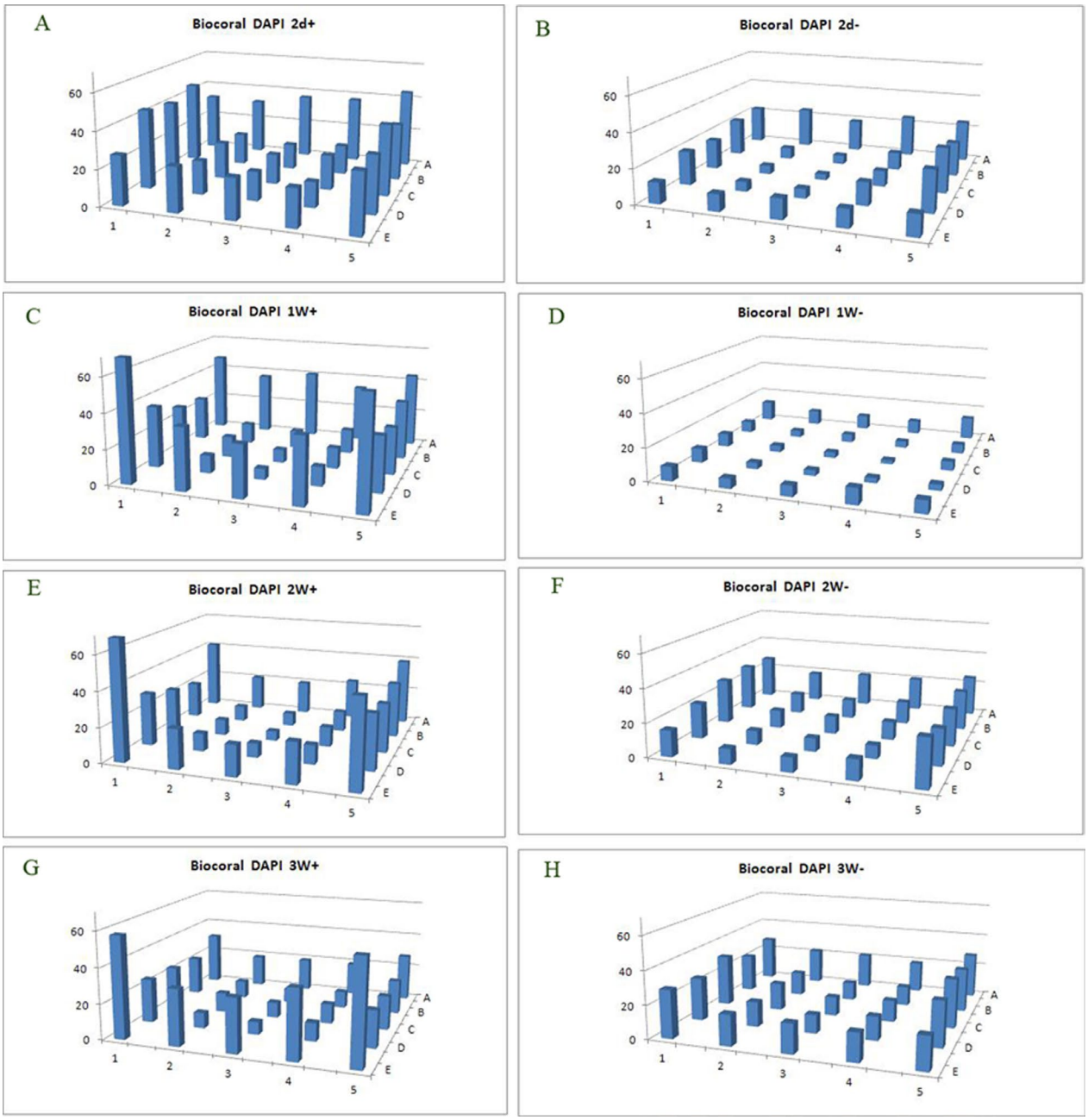

Fig. 3. Spatial cell distribution inside the scaffolds: Vertical axis: cell numbers. Left column **(A,C,E,G)**: Scaffolds with fibrin; **A**: 2 d; **C**: 1 week; **E**: 2 weeks; **G**: 3 weeks. Right column **(B,D,F,H)**: Scaffolds without fibrin; **B**: 2 d; **D**: 1 week; **F**: 2 weeks; **H**: 3 weeks.

higher percentage of positive cells after 2 d (19.9 %; ± 2.3; $p = 0.130$) that increased significantly to 52.4 % (± 4.4) after 1 week ($p < 0.001$) and significantly declined towards the end of the observation period (28.2 % ± 6.1; $p = 0.001$). The differences between the two groups were significant after 1 week ($p = 0.002$), 2 weeks ($p = 0.020$) and 3 weeks ($p < 0.001$), however, with a conversion from a lower to a higher expression of OC in the fibrin(+) group between week 1 and 2. The same held true for the expression of VEGF that showed low expression in both groups (14.5 % / ± 2.2 and 17.6 % / ± 1.5) after 2 d with a subsequent significant increase to 50.7 % (± 5.2) after 2 weeks in the fibrin(+) group and 50.7 % (± 6.3) in the controls already after 1 week ($p < 0.001$ in both groups).

Analysis of expression in the centre and periphery of scaffolds revealed that the conversion started in the periphery of the scaffolds (OC: $p = 0.007$, VEGF: $p = 0.007$) after 2 weeks and affected also the centre after 3 weeks for both OC and VEGF (both: $p = 0.001$) (Fig. 8A and B). Additionally, in the fibrin(+) group there were significant differences in the expression of OC and VEGF between the centre and the periphery after 2 and 3 weeks. This difference did not occur in the control scaffolds without the fibrin matrix. The percentage of positive staining for OC in each individual unit correlated significantly with the level of expression of VEGF ($r = 1.000$, $p < 0.001$).

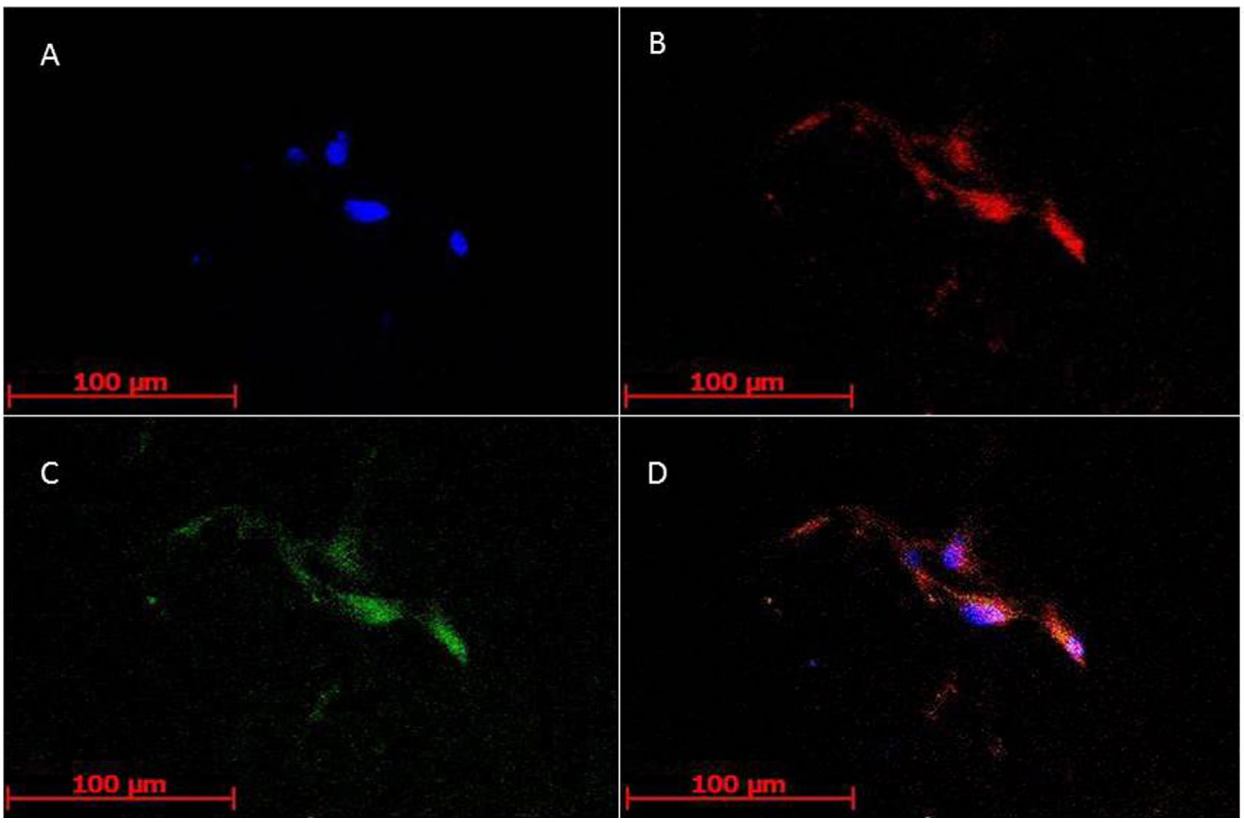

Fig. 4. Immunofluorescence micrographs showing a clear distinction between the three colour components and co-expression of VEGF and OC (**A**: nuclei (DAPI; blue); **B**: OC (FITC; red); **C**: VEGF (Cy3; green); **D** fused image). Incubation time 1 week. Bars: 100 µm.

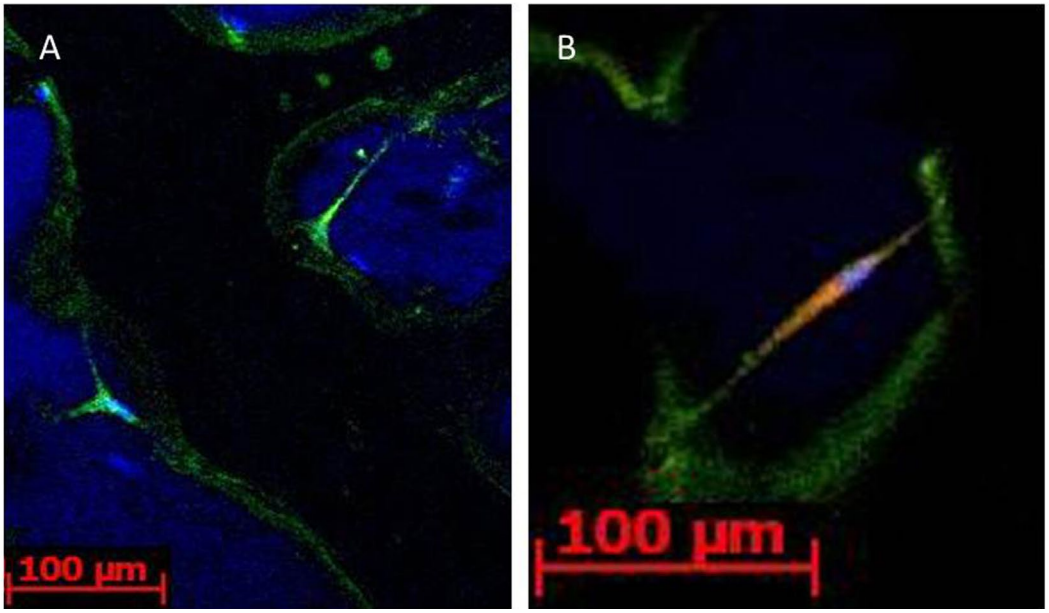

Fig. 5. Immunofluorescence micrographs showing cells spanning scaffold pores in the centre of the scaffold (**A**: 1 week; **B**: 2 weeks). Bars: 100 µm.

Discussion

The present study has shown that a fibrin matrix can enhance the number of bone marrow stromal cells seeded into porous mineralised scaffolds. This was particularly evident 2 d and 1 week after seeding and is in line with previous reports showing that fibrin is supportive of proliferation of mesenchymal stem cells in tissue engineering applications (Galler *et al*., 2011; Nair *et al*., 2009; Trombi *et al*., 2008). The concentration of fibrinogen appears to be influential for the degree of proliferation as it affects the microstructure of the resulting fibrin matrix. Trombi *et al*. (2008) have shown the architecture of a fibrin matrix containing 3 % fibrinogen has shown the most favourable results. The concentration used in the present study is well within the range of 3 % and therefore suitable

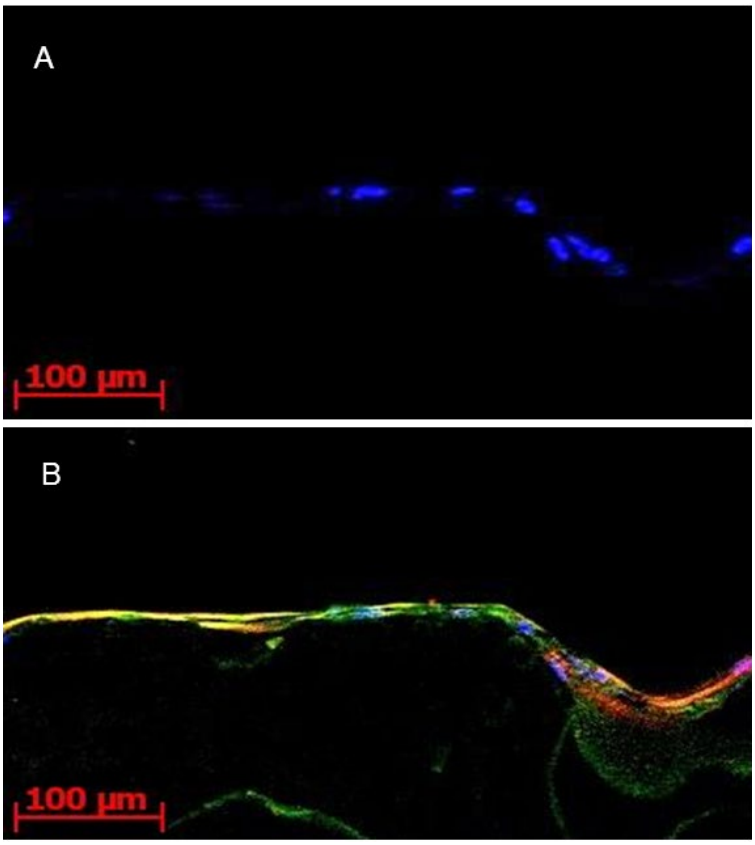

Fig. 6. Immunofluorescence micrographs showing cells lining pore walls (**A**: DAPI stain (blue/nuclei); **B**: Co-expression of OC (red) and VEGF (green)) in the periphery of the scaffold. Incubation time 2 d. Bars: 100 µm.

to achieve a high seeding efficacy. Other than in a previous study, where the use of a fibrin matrix had shown increased proliferation in the centre of the scaffolds, the main effect in the present study appears to be based on a higher maintenance of cell numbers particularly in the periphery of the scaffolds rather than on increased proliferation of seeded cells. The difference in results between the two studies may raise questions about the validity of the method of evaluation. Previous work has shown that the initial number of seeded cells could be identified quite reliably using this approach (Zhu *et al*., 2010). In the present study the cell numbers 2 d after seeding in both groups were 27.8 and 14.6 (+/- fibrin) per image (unit), corresponding to 695 and 365 cells per section, respectively. The evaluated volume would then contain some 4,200 cells (fibrin(+) group) and 2,200 cells (fibrin(-) group), which is well within the order of magnitude previously reported for the same technique and number of seeded cells. The original scaffold volume would then have contained about 16,000 cells and 9,000 cells, respectively, which corresponds nicely to the initially seeded number of cells (16,200) and the difference in seeding efficacy of approximately 90 % and 50-60 % between the two methods of seeding (Materna *et al*., 2008; Zhu *et al*., 2010). This shows that the method of assessment can be considered as reproducible and reliable.

The pattern of cell distribution across the scaffolds with a higher number of cells in the periphery is in line with previously reported results of droplet seeding with subsequent static culturing. Wang *et al*. (2006) have described an accumulation of cells at the scaffold periphery that may have occurred due to air entrapment in the centre of the porous scaffolds (Solchaga *et al*., 2006), so that an uneven penetration of the scaffold would be the result of gravity and flow. More even distribution of cells can be achieved by using dynamic seeding approaches (Solchaga *et al*., 2006; Wendt *et al*., 2003). Depending on the macrostructure of the scaffolds, the technique of seeding may also affect the viability of the seeded cells (Wendt *et al*., 2003). It has also been shown that flow perfusion culturing enhances the osteogenic differentiation of marrow stromal cells (Gomes *et al*., 2003). Nevertheless, strategies have to be considered that can enhance the survival and differentiation under static culturing conditions in order to improve the performance of tissue engineered constructs after transplantation when flow perfusion through a bioreactor is no longer possible. In this way, it would be interesting to combine dynamic seeding and culturing techniques with the addition of fibrin matrices or other extracellular matrix proteins.

In contrast to the rather uniform results with respect to proliferation of mesenchymal cells, the reports on the effect of fibrin on osteogenic differentiation are contradictive. While some studies have reported a clearly enhancing effect on the expression of osteogenic markers that occurs in fibrin clots (Nair *et al*., 2009; Trombi *et al*., 2008; Chen *et al*., 2009) or fibrin-modified scaffolds (Schantz *et al*., 2005), other authors question the value of fibrin (Demol *et al*., 2011). The composition of the fibrin-containing matrix tested appears to play an important role for the expression of bone specific markers. Nair *et al*. (2009) have shown that coating of ceramics with platelet rich plasma has

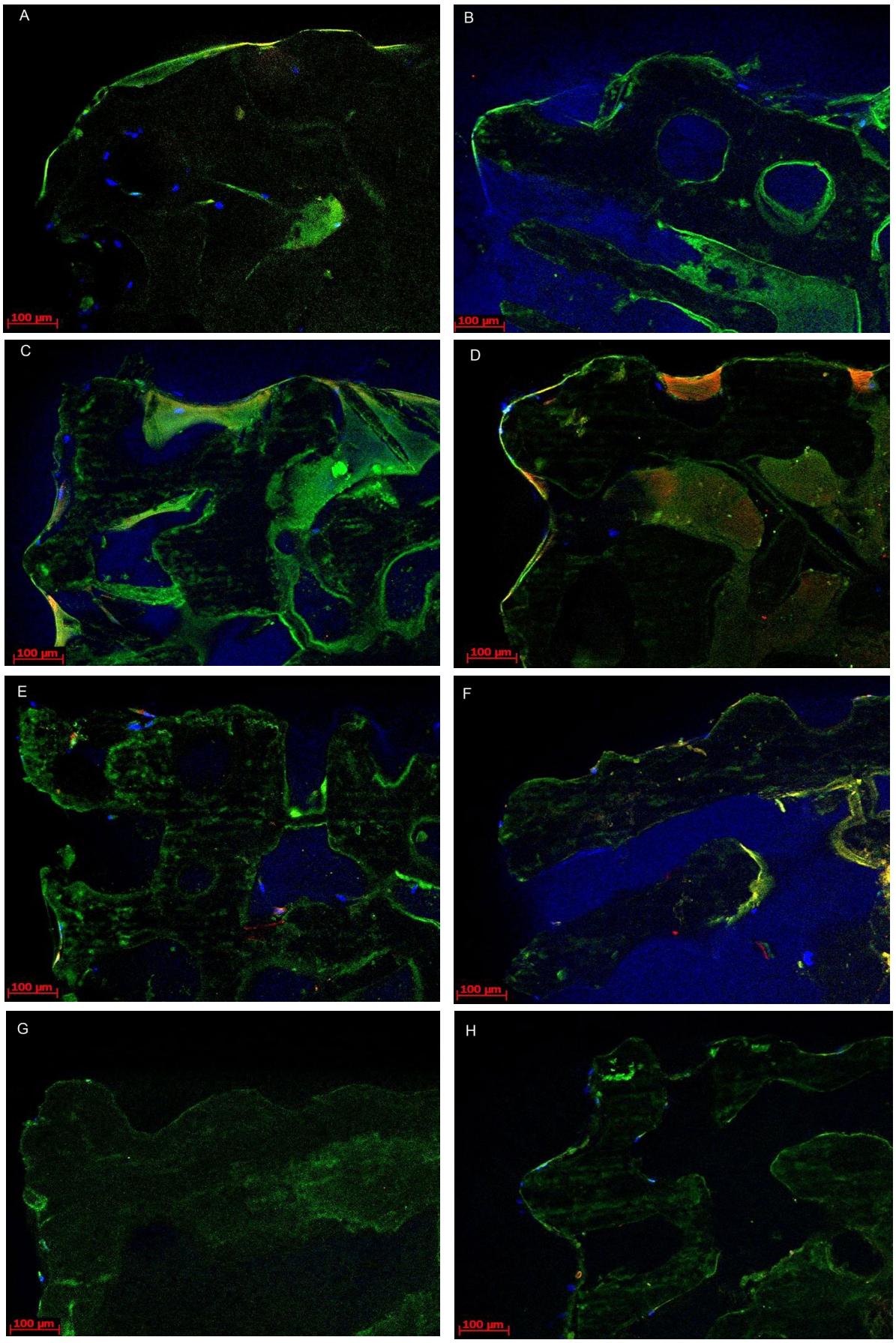

Fig. 7. Immunofluorescence micrographs showing cells and matrix production (green /red) in the scaffolds (dark brown) partially or completely filling pore lumina (dark blue). Top row **(A-D)**: scaffolds with fibrin; **A**: 2 d; **B**: 1 week; **C**: 2 weeks; **D**: 3 weeks. Bottom row **(E-H)**: scaffolds without fibrin; **E**: 2 d; **F**: 1 week; **G**: 2 weeks; **H**: 3 weeks. The clear difference in the production of OC and VEGF-containing matrix can be seen. Matrix production has not been quantified to avoid bias through autofluorescence effects of the scaffolds. Bars: 100 µm.

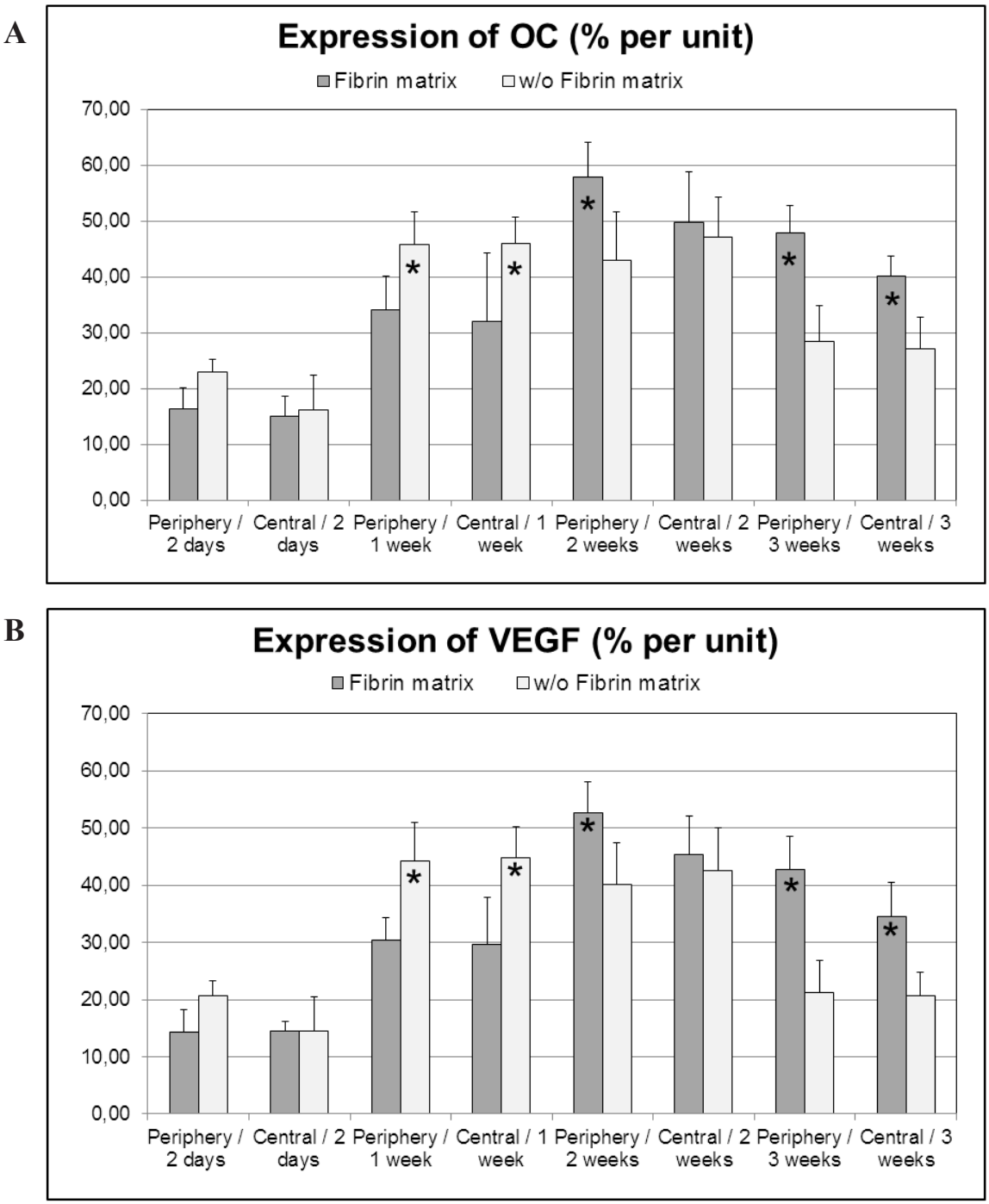

Fig. 8. **A.** Comparison of expression of OC (percentage of positively stained cells/unit) in the central and peripheral units of the scaffolds (*significant with $p < 0.05$). **B.** Comparison of expression of VEGF (percentage of positively stained cells/unit) in the central and peripheral units of the scaffolds (*significant with $p < 0.05$).

not increased the expression nor the activity of alkaline phosphatase in contrast to fibrin glue-coated ceramics, whereas the addition of peripheral blood mononuclear cells did have an enhancing effect on the activity of alkaline phosphatase (Seybold *et al*., 2010).

An unusual finding is the early expression of OC at day 2 despite the fact that OC is considered to be a late marker of osteogenesis, which again touches on the validity of the immunohistochemical staining and evaluation. The majority of reports have used PCR as a reliable method to assess transcription of specific genes in cells seeded into porous scaffolds. Unfortunately, PCR cannot indicate the final expression of the protein, nor can it detect spatial/topical differences inside a scaffold/tissue. For this purpose, immunohistochemistry/immunofluorescence is an accepted and reliable method of analysis that has also been used for the *in vitro* evaluation of bone cells on biomaterials in recent reports (Gomes *et al*., 2006; Saino *et al*., 2010; Yang *et al*., 2012). The fact that the immunofluorescence pictures in the present study correspond well to the morphology and arrangement of the cells displayed in SEM pictures supports the use of this method for three-

dimensional evaluation of protein expression in tissue engineered constructs.

The early expression of OC may be explained by the fact that the occurrence of osteogenic markers *in vitro* may differ from the natural course of osteogenic differentiation because mesenchymal cells tend to show early expression of OC on biomaterials in *in vitro* environments. The results of the present study have shown a rather low expression of OC after 2 d with a significant increase after 7 and 14 d. This parallels recent findings of Xia *et al*. (2011), who reported elevated OC transcription in human periodontal ligament cells already after 4 d with a subsequent increase at days 7 and 10.

The present study showed that the maximum level of expression of OC in bone marrow stromal cells was not increased by the use of a fibrin matrix, but the temporal sequence of expression was significantly altered. While BMSCs seeded without a fibrin matrix have exhibited the highest signal level after 1 week of culture in mineralised scaffolds with a decrease thereafter, the maximum expression of OC in the scaffolds with a fibrin matrix has been retarded to 2 weeks of culture but was sustained at a significantly higher level to the end of the observation period. Thus, it appears that a fibrin matrix used for seeding of BMSCs in mineralised scaffolds delays the onset of OC and VEGF expression but sustains them at a higher level thereafter. The significant increase appears to be based on an enhancement of bone marrow stromal cells in the periphery of the scaffolds and a better maintenance of the level of expression in the centre later on. To the knowledge of the authors, this is the first report that analyses and reconstructs the spatial distribution of expression of OC and VEGF in osteogenic cells inside mineralised scaffolds.

The supportive effect of ECM matrix components such as fibrin and fibronectin on osteogenic differentiation appears to be associated with the type of integrin-specific protein binding motifs. Martino and co-workers have shown that fibronectin fragments specific for $\alpha_5\beta_1$ integrins enhanced osteogenic differentiation of mesenchymal stem cells in a 3D fibrin matrix system over $\alpha_v\beta_3$ integrins, whereas the full length fibronectin motif was only supportive of attachment and proliferation (Martino *et al*., 2009). As fibrin has also shown to present binding sites to both $\alpha_v\beta_3$ (Yokoyama *et al*., 1999) and $\alpha_5\beta_1$ integrin receptors (Caiado *et al*., 2011), it can be hypothesised that the sustaining effect on osteogenic differentiation is associated with the interaction between the fibrin matrix and the respective integrin receptors.

An interesting observation is the co-expression of VEGF and OC in the majority of bone marrow stromal cells seeded into the scaffolds. This is in line with previous *in vitro* observations describing increased VEGF secretion during early stages of osteoblastogenesis (Furumatsu *et al*., 2003). Recent results report even an enhancing effect of VEGF A on osteogenic differentiation and bone formation of human adipose-derived stem cells (Behr *et al*., 2011). When fibrin clots were used as dermal substrates for skin substitutes composed of keratinocytes and dermal fibroblasts, they have shown to induce VEGF production during culturing and a better take after transplantation (Hojo *et al*., 2003).

Conclusions

The present data have shown that the use of a fibrin matrix composed of 3 % fibrinogen used for seeding and culturing of human bone marrow stromal cells in mineralised scaffolds positively affects the osteogenic differentiation of the seeded cells by sustaining the expression of OC and VEGF. It thereby resembles the features described for two dimensional culture systems and appears to be a useful adjunct for fostering osteogenic and angiogenic activity of tissue-engineered constructs.

Acknowledgements

The authors declare that they have no conflicts of interest.

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Discussion with Reviewers

Reviewer II: Do the authors have any data on the degradation rate of the fibrin matrix, and can they speculate on any effects that the degradation of the fibrin over time may have had on their results? Do the authors think that the retardation of the osteogenic differentiation of the cells by fibrin seeding is a biochemical effect or a physical effect, such as one of substrate stiffness?

Authors: Unfortunately we do not have data on the degradation rate of the fibrin matrix within the seeded scaffolds. Recent experimental research has shown that changes of fibrin degradation in carriers using tranexamic acid had only minor effects on osteogenic differentiation across a cultivation period of 21 d (Demol *et al*., 2011). Thus, we would speculate that degradation of fibrin would have only little impact on osteogenic differentiation during the cultivation period. Substrate stiffness would have been affected negligibly by fibrin degradation because the underlying $CaCO_3$ scaffolds are very stiff and brittle and would rather become disintegrated than increase in flexibility.