

Functionalisation of PLLA nanofiber scaffolds using a possible cooperative effect between collagen type I and BMP-2: impact on growth and osteogenic differentiation of human mesenchymal stem cells

Markus D. Schofer · Anne Veltum ·
Christina Theisen · Fei Chen · Seema Agarwal ·
Susanne Fuchs-Winkelmann · Jürgen R. J. Paletta

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Abstract Mesenchymal stem cell differentiation of osteoblasts is triggered by a series of signaling processes including integrin and bone morphogenetic protein (BMP), which therefore act in a cooperative manner. The aim of this study was to analyze whether these processes can be remodeled in an artificial poly-(L)-lactide acid (PLLA) based nanofiber scaffold. Matrices composed of PLLA-collagen type I or BMP-2 incorporated PLLA-collagen type I were seeded with human mesenchymal stem cells (hMSC) and cultivated over a period of 22 days, either under growth or osteoinductive conditions. During the course of culture, gene expression of alkaline phosphatase (ALP), osteocalcin (OC) and collagen I (COL-I) as well as Smad5 and focal adhesion kinase (FAK), two signal transduction molecules involved in BMP-2 or integrin signaling were analyzed. Furthermore, calcium and collagen I deposition, as well as cell densities and proliferation, were determined using fluorescence microscopy. The incorporation of BMP-2 into PLLA-collagen type I nanofibers resulted in a decrease in diameter as well as pore sizes of the scaffold. Mesenchymal stem cells showed better adherence and a reduced proliferation on BMP-containing scaffolds. This was accompanied by an increase in gene expression of ALP, OC and COL-I. Furthermore the presence of BMP-2 resulted in an upregulation of FAK, while collagen had an impact on the gene expression of

Smad5. Therefore these different strategies can be combined in order to enhance the osteoblast differentiation of hMSC on PLLA based nanofiber scaffold. By doing this, different signal transduction pathways seem to be up regulated.

1 Introduction

The reconstruction of large bony defects after injury or tumor resection often requires the use of bone substitution. In Europe 500,000 surgeries/year require bone graft material [1]. Therefore artificial scaffolds based on synthetic biomaterials such as metals, polymers, porous ceramics, hydroxyl apatite, collagen sponge or hydrogels, and composites have been developed [2–8].

Among them, scaffolds based on nanofibers or nano-scaffolds offer great advantages [9, 10]. They are thought to mimic the extracellular matrix (ECM) [11–14] and allow the differentiation of human mesenchymal stem cells (hMSC) towards osteoblasts. However, this process depends on the chemical composition of the nanofibers.

In comparison to other α -(hydroxyester)-based nanofibrous scaffolds, poly-(L)-lactide acid (PLLA) scaffolds supported the highest rate of proliferation of mesenchymal stem cells [15] with a tendency to higher cell densities [16, 17]. However, an initial down regulation of genes associated with the osteoblast lineage [16, 18] can be observed on PLLA nanofibers. This effect can be eliminated by functionalisation of the nanofibers either by blending with collagen type I [19] or the incorporation by recombinant bone morphogenetic protein 2 (BMP-2) [16]. Both strategies enhanced the differentiation of mesenchymal stem cells towards osteoblasts. In terms of collagen it is known that the adhesion to collagen I (COL-I) via the

M. D. Schofer · A. Veltum · C. Theisen · S. Fuchs-Winkelmann ·
J. R. J. Paletta (✉)
Department of Orthopedics, University Hospital of Marburg,
Baldingerstraße, 35043 Marburg, Germany
e-mail: paletta@med.uni-marburg.de

F. Chen · S. Agarwal
Department of Chemistry, University of Marburg,
Hans-Meerwein-Straße, 35032 Marburg, Germany

$\alpha 2\beta 1$ integrin is sufficient to induce osteogenic differentiation of hMSC's, even in the absence of exogenous stimuli [20, 21]. BMP-2 induces different factors involved in the differentiation of hMSC towards osteoblasts [22–31] and is necessary to induce bone formation [32–34]. Furthermore BMP-2 induces the formation of $\alpha v\beta$ -Integrin on osteoblasts [35, 36]. These, in interaction with the ECM, influences the BMP-function [36] resulting in migration, activation of focal adherence kinase and formation of focal adhesion [37, 38]. Suzawa et al. [39] found evidence, that the downstream pathway of integrin is involved in Smad1 signals activated by BMP-2 and provide a possible mechanism for cooperation between intracellular signals activated by integrin and BMP receptors in osteoblastic cells.

In order to analyze whether this cooperative effect can be utilized in PLLA nanofiber based bone replacement material we constructed BMP-2 containing PLLA-collagen type I blended nanofiber scaffolds and analyzed their ability to induce osteoblast differentiation of hMSC.

2 Materials and methods

2.1 Construction of nanofibers and characterization

The preparation of PLLA and PLLA-collagen type I blend nanofibers as well as the incorporation of BMP-2 into PLLA nanofibers by electrospinning has been reported in detail earlier [16, 19, 40]. Briefly, a 4% (w/w) PLLA (Resomer L210, Boeringer Ingelheim Germany) solution in dichloromethane (DCM) was prepared at room temperature by stirring overnight until a homogenous solution was obtained. Spinning process was performed at a flow rate of 14 $\mu\text{l}/\text{min}$ with an applied voltage of 20–30 kV and a distance of 15 cm. The spinning solution had a surface tension of 28.34 ± 0.03 mN/m, a viscosity of 1.561 PaS and a conductivity of ~ 0 $\mu\text{S}/\text{cm}$. Samples of non woven PLLA nanofibers were fixed on 18 mm cover slips for cell culture experiments.

To incorporate collagen type I into the nanofibers, PLLA and collagen were dissolved in hexafluoroisopropanol (HFIP) in a 4:1 ratio resulting in a 4.5% (w/v) polymer solution. Here the spinning solution had a surface tension of 18.33 ± 0.03 mN/m, a viscosity of 1.529 PaS and a conductivity of ~ 4.7 $\mu\text{S}/\text{cm}$. Spinning process was performed at a flow rate of 14 $\mu\text{l}/\text{min}$ with an applied voltage of 10–18 kV and a distance of 15 cm.

In order to incorporate BMP-2 into the nanofibers, 25 μg lyophilized BMP-2 (Reliatech, Braunschweig Germany) was dissolved in 125 μl 50 mM acetic acid and stabilized by the addition of 25 μl fetal calf serum (FCS). This mixture was emulsified in 2.5 ml of a 4.5% PLLA-collagen type I solution (4:1) over a period of 1 min using a vortex mixer (MS 2 Minishaker IKA[®]; 2,500 rpm). This solution was sufficient for

the spinning of 50 cover slips with BMP-2 depending on the spinning and fiber collection process. Spinning process was performed at a flow rate of 14 $\mu\text{l}/\text{min}$ with an applied voltage of 10–18 kV and a distance of 15 cm. In some experiments BMP was replaced by a fluoresceinisothiocyanat (FITC)-labeled protein, to analyze the distribution within the nanofibers and release of growth factors from the PLLA Collagen fibers.

2.2 Pore size distribution

The pore size distribution of the three electrospun scaffold was determined using a capillary flow porometer (type: CFP-1200-AEYL, Porous Materials Inc., USA). A wetting liquid, Galwick[™] (Porous Materials Inc., USA) was applied to spontaneously fill the pores in the scaffold and differential pressure of nitrogen gas was slowly increased on the sample to remove the liquid within the pores and permit gas flow. The differential pressure and flow rates through dry and wet scaffold were measured. All the required pore structure characteristics such as pore size at bubble point mean flow pore and pore size distribution were computed from the measured differential pressures and gas flow rates [41].

2.3 Scanning electron microscopy

For scanning electron microscopy (SEM), samples were sputter-coated with gold in an AUTO-306 (BOC Edwards, Crawley, Sussex, U.K.) high-vacuum coating system and examined in a SEM (S-4100, Hitachi Ltd., Tokyo, Japan) at an accelerating voltage of 5 kV in the SE mode.

2.4 Human mesenchymal stem cell isolation, characterization and culture

HMSC cells were obtained from consenting patients with the approval of the institutional review board. The indication for surgery was primary osteoarthritis of the hip. The patients had no evidence of other bone or auto immune diseases. The routinely removed bone was obtained from the proximal femur, while preparing the implant bed. HMSC were isolated and cultured according to the preparation of Pittenger et al. [42], with minor modification as described by Brendel et al. [43].

Within the experiments, hMSCs preparations negative for CD34 and CD40 and positive for the stem cell markers CD90 and CD105 were used. In order to ensure the osteoinductive potential of the obtained cells, gene expression of alkaline phosphatase (ALP), in response to dexamethasone, was determined prior to the experiments.

For experiments, hMSC were seeded at a density of 3×10^4 cells/cm² on cover slips or cover slips coated with nanofibers in growth medium (DMEM), with low glucose and glutamine (PAA, Linz, Austria) supplemented with

10% fetal calf serum (FCS) from selected lots (Biochrom, Berlin, Germany) and 1% penicillin/streptomycin. Osteogenic differentiation was induced according to Jaiswal et al. [44] after an initial proliferation phase of 3 days. The medium was replaced every second day of culture.

2.5 Gene expression analysis

RNA was extracted from cell layers at days 2, 4, 10 and 22 (after introduction of osteoinductive conditions) using Peq Gold total RNA Kit (PeqLab, Erlangen, Germany) according to the manufacturer and quantified spectrometrically. Starting from 1 µg RNA, 20 µl cDNA were synthesized using Verso cDNA Kit (Thermo Fisher Scientific, Lafayette, USA) with oligo-dT primer in the presence of dNTP. Quantitative RT-PCR reactions were performed and monitored using a Mastercycler[®] ep realplex Detection System (Eppendorf, Hamburg, Germany) and Absolute Blue QPCR Sybr Green Mix (Thermo Fisher Scientific, Lafayette, USA). Genes of interest were analyzed in cDNA samples (5 µl for a total volume of 50 µl/reaction) using DeltaDeltaCt method and CyberGreen. Primers (purchased at TIB Biomol, Berlin, Germany), cycle temperatures and incubation times for human alkaline phosphatase (ALP), collagen I (COL-I), osteocalcin (OC), and 18 s rRNA were previously described [45–47]. The primer sequence for focal adhesion kinase (FAK) was 5'ACC TCA GCT AGT GAC GTA TGG -3' and 5'CGG AGT CCC AGG ACA CTG TG 3' (gen bank L0518666), PCR Primers for Smad5 were obtained ready to use form www.realtimeprimers.com.

2.6 Immunofluorescence microscopy

Samples were fixed in acetone/methanol, washed with PBS (3×), and exposed to blocking buffer (1% donkey serum albumin PBS) over a period of 30 min at room temperature to minimize non-specific absorption of the antibodies. After another wash in PBS (3×), the cells were incubated with primary antibodies against COL-I (Abcam, Ab6308, Cambridge, United Kingdom), OC (Acris, BP710, Hiddenhausen, Germany), Ki-67 (Darko, Hamburg, Germany) FAK (BD Biosciences, San Jose, California, USA) and Smad5 (Santa Cruz Biotechnology, Inc. Heidelberg, Germany). Visualization was done, after washing in PBS (3×) using cy-2- or cy-3-conjugated secondary antibody (Dianova, Hamburg, Germany) at room temperature (1 h). The slices were stained with DAPI and embedded in mounting medium. Fluorescence microscopy was done using a Leica DM5000. Microphotographs of at least three different areas were made and intensity of fluorescence was determined using Quips analysis software. Total cell count of DAPI stained nuclei were obtained. Patient series were analyzed by independent observers.

2.7 Detection of calcium deposition

Von Kossa staining was performed using 1% aqueous silver nitrate solution for 1 h followed by several washing steps in distilled water and incubation in 5% sodium thiosulfate for 5 min. Alizarin red staining was performed using 2 g/100 ml at pH 4.1–4.3.

2.8 Statistics

All values were expressed as mean ± standard error of at least three different patients and compared using student's *t*-test or ANOVA with Bonferroni as a post hoc test. Values of *P* < 0.05 were considered to be significant. Significant values were marked with *.

3 Results

3.1 Characterization of PLLA collagen blend nanofibers with or without BMP-2 incorporation

Scanning electron microscopy of electrospun PLLA nanofibers revealed a 3-D non-woven network with a diameter of 0.775 ± 0.294 µm. Fibers were porous in structure. The PLLA-collagen type I blend nanofibers revealed a 3-D non-woven network with a diameter of 0.421 ± 0.023 µm for the blend (Fig. 1c). The PLLA-collagen type I blend nanofibers presented themselves with a relatively smooth surface (Fig. 1a). The pore size of the resulting scaffolds was about 2 µm as demonstrated in Fig. 1d.

The incorporation of BMP-2 into these fibers reduced the diameter to 0.217 ± 0.009 µm and resulted in a smaller pore size (approximately 0.6 µm) as shown in Fig. 1b and c. When FITC labelled bovine serum albumin (BSA) was used as a model for the incorporation of proteins by electrospinning, fluorescence microscopy showed evidence that the dye was distributed uniformly within the fibers (Fig. 2a). When these fibers were incubated in phosphate buffer at 37°C, the fluorescence remained within the fiber over a period of 14 days (Fig. 2b). Nevertheless the sharpness of the fibers becomes indistinct. Correspondingly using UV-visible-spectroscopy, no time dependent release could be detected (data not shown).

3.2 Adhesion of mesenchymal stem cells on nanofiber scaffolds

In order to describe the biological effects of blending PLLA with collagen as well as the incorporation of BMP-2 into PLLA-collagen type I blend nanofibers, we first analyzed the adhesion of hMSC on the desired scaffolds. Cells were seeded on the scaffolds and allowed to adhere over a period of 12 h. As shown in Fig. 3, during this time,

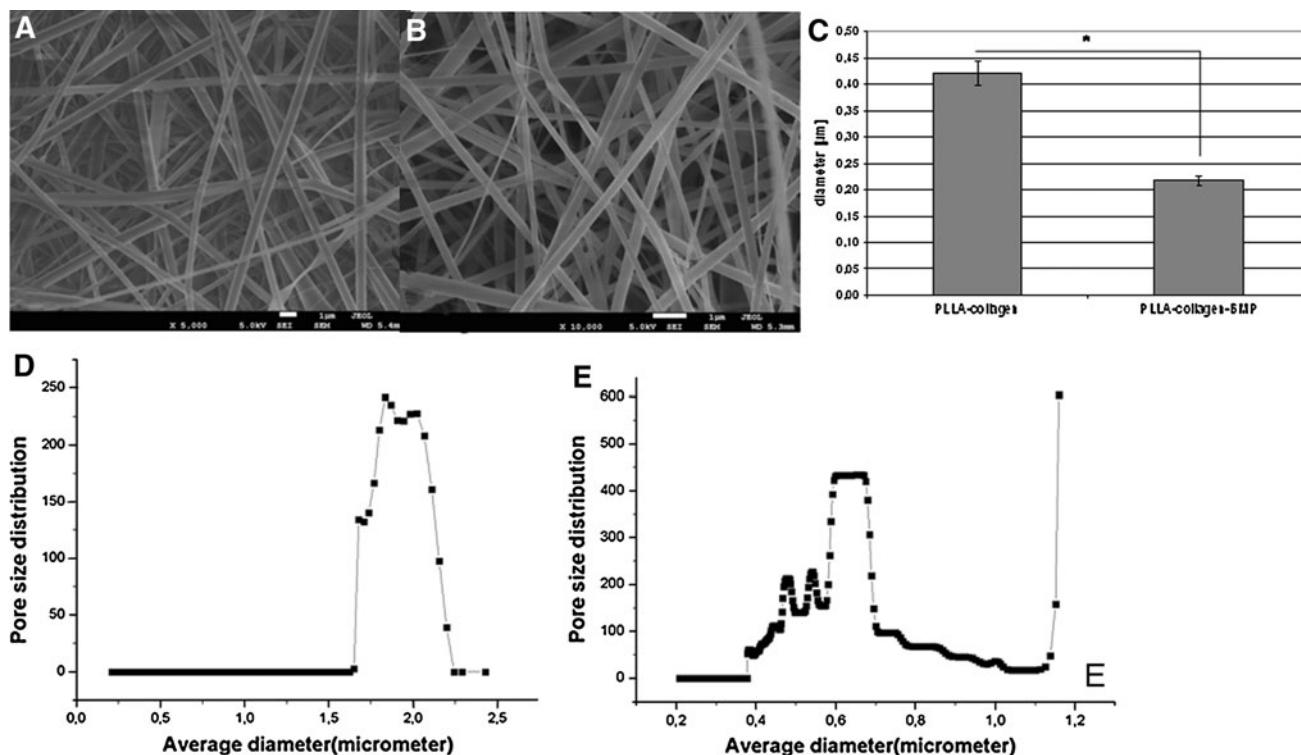


Fig. 1 Fiber characterization. SEM analysis of PLLA-collagen blend (a) and BMP-2 enwrapped PLLA-collagen blend nanofibers (b) and the resulting fiber diameters (c) and pore sizes of the PLLA-collagen blend (d) and BMP-2 enwrapped PLLA-collagen blend nanofibers (e)

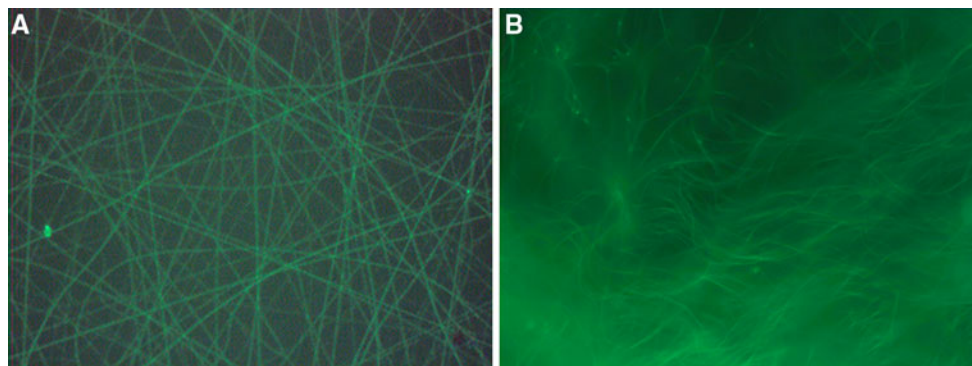


Fig. 2 Release characteristics of BMP-2 enwrapped PLLA-collagen blend nanofibers. Fluorescence microscopy analysis of BMP-2 enwrapped PLLA-collagen blend nanofibers after incubation in aqueous solutions over a period of 1 (a) and 14 days (b)

approximately three times more cells adhered on PLLA-collagen type I blend nanofibers. Nevertheless, this effect was not significant at all. The incorporation of BMP-2 into the PLLA-collagen type I blend scaffolds resulted in a four to five fold increase ($p = 0.044$).

3.3 Growth and proliferation of human hMSC cultured on nanofibers

Focusing on growth of hMSC, we next cultured the cells over a period of 22 days on PLLA, PLLA-collagen type I,

and PLLA-collagen type I nanofiber scaffolds containing BMP-2. Under growth conditions significant differences between cell densities between PLLA and PLLA-collagen type I ($p = 0.000$) as well as PLLA-collagen type I containing BMP-2 ($p = 0.015$) were found after 22 days (Fig. 4a).

Under osteoinductive conditions after 4 days of culture significance was reached (PLLA/PLLA-collagen nanofibers $P = 0.001$; PLLA-collagen nanofibers containing BMP-2 ($p = 0.000$)). In case of PLLA-collagen nanofibers significant higher cell densities were found over the time

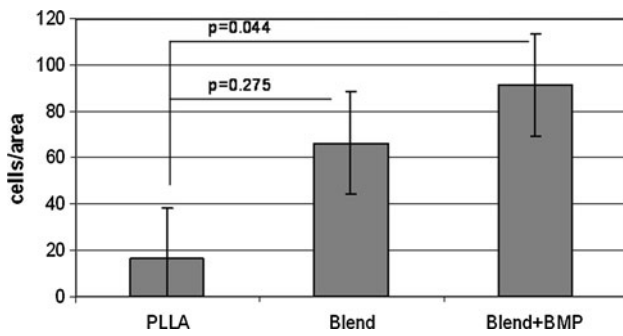


Fig. 3 Influence of PLLA, PLLA-collagen blend and BMP-2 containing PLLA-collagen blend nanofibers on adhesion

course of the experiment as compared to PLLA nanofibers (day 10, $P = 0.004$; day 22, $P = 0.003$). At these time points no significant differences between PLLA and PLLA-collagen type I nanofibers containing BMP-2 were detected (Fig. 4b). This growth pattern was reflected in the gene expression of Cyclin 1D (CCND) normalized to 18 s. Here the gene expression was increased when cells were cultured on PLLA-collagen type I blend nanofibers as compared to PLLA nanofibers. Furthermore the presence of BMP-2 resulted in a reduced CCND gene expression (Fig. 4f). Consequently this pattern could be detected using Ki67 staining (Fig. 4c, d, e).

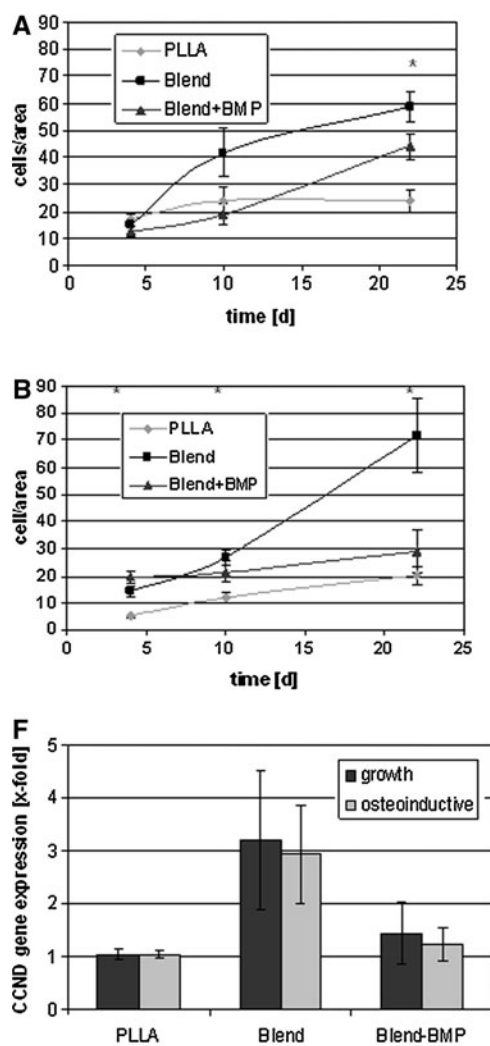
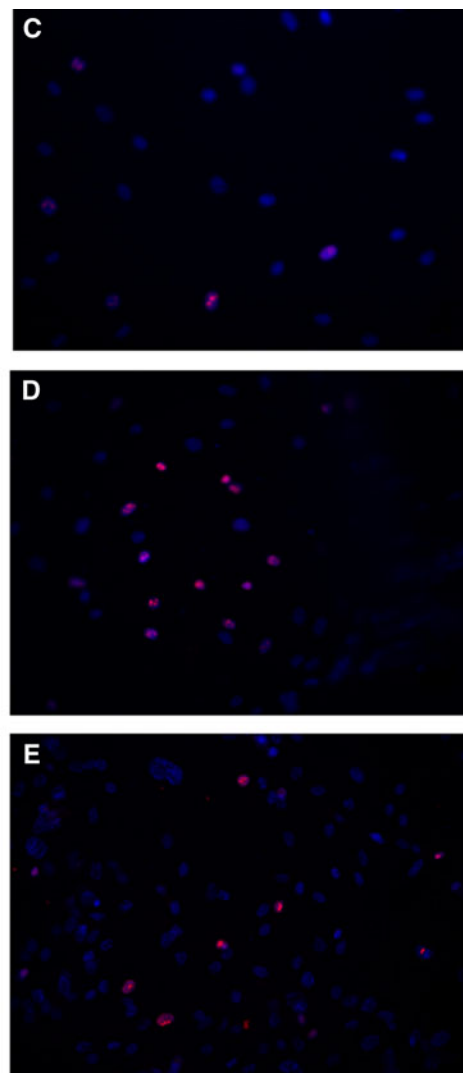


Fig. 4 Influence of PLLA, PLLA-collagen blend and BMP-2 containing PLLA-collagen nanofibers on growth and proliferation of hMSC cultured under growth and osteoinductive conditions. Time course of cell densities cultured under growth (a) and osteoinductive (b) conditions. Immunofluorescence microphotographs of



proliferation as determined by Ki67 staining of hMSC cultured under osteoinductive conditions on cover PLLA nanofibers (c), PLLA-collagen blend nanofibers (d) and BMP-2 envolved PLLA-collagen blend nanofibers (e). Gene expression of CCND after 4 days of cell culture as compared to PLLA (f)

3.4 Influence of PLLA-nanofibers on the differentiation and matrix formation of hMSC

To examine the influence of incorporation of BMP-2 into PLLA-collagen type I on hMSC differentiation into osteoblasts we compared the gene expression of alkaline phosphatase, collagen type-I and osteocalcin of cells, grown on PLLA-collagen type I blend nanofibers with or without BMP-2. Although there was a broad inter-assay variability (mainly responsible for the lack of significance) the presence of BMP-2 resulted in an increase of alkaline phosphatase and osteocalcin (Fig. 5a, b). The reason why osteocalcin and alkaline phosphatase expression was increased in the early stage and decreased during the time course is due to the ddCT method used to compare expression between the scaffolds at each time point and not in a time dependent manner. Here von Kossa as well as alizarin staining gave evidence that

osteogenesis was more dominant when cells were cultured under osteoinductive conditions. Furthermore when BMP was incorporated this effect seems to be enhanced (Fig. 5c).

This was accompanied by a significant increase in the gene expression of collagen type I (Fig. 6a) when cells were cultured on BMP-2 containing nanofiber scaffolds as compared to PLLA-collagen nanofibers alone. This effect was independent from culture conditions and resulted in an enhanced deposition of the corresponding proteins in the nanofiber scaffolds over a period of 22 days as demonstrated by immunofluorescence (Fig. 6b, c, d, e).

3.5 Influence of nanofiber composition signal transduction

Because collagen blended PLLA nanofibers [19] as well as BMP-2 containing PLLA nanofibers [16] enhance the differentiation of mesenchymal stem cells towards osteoblasts

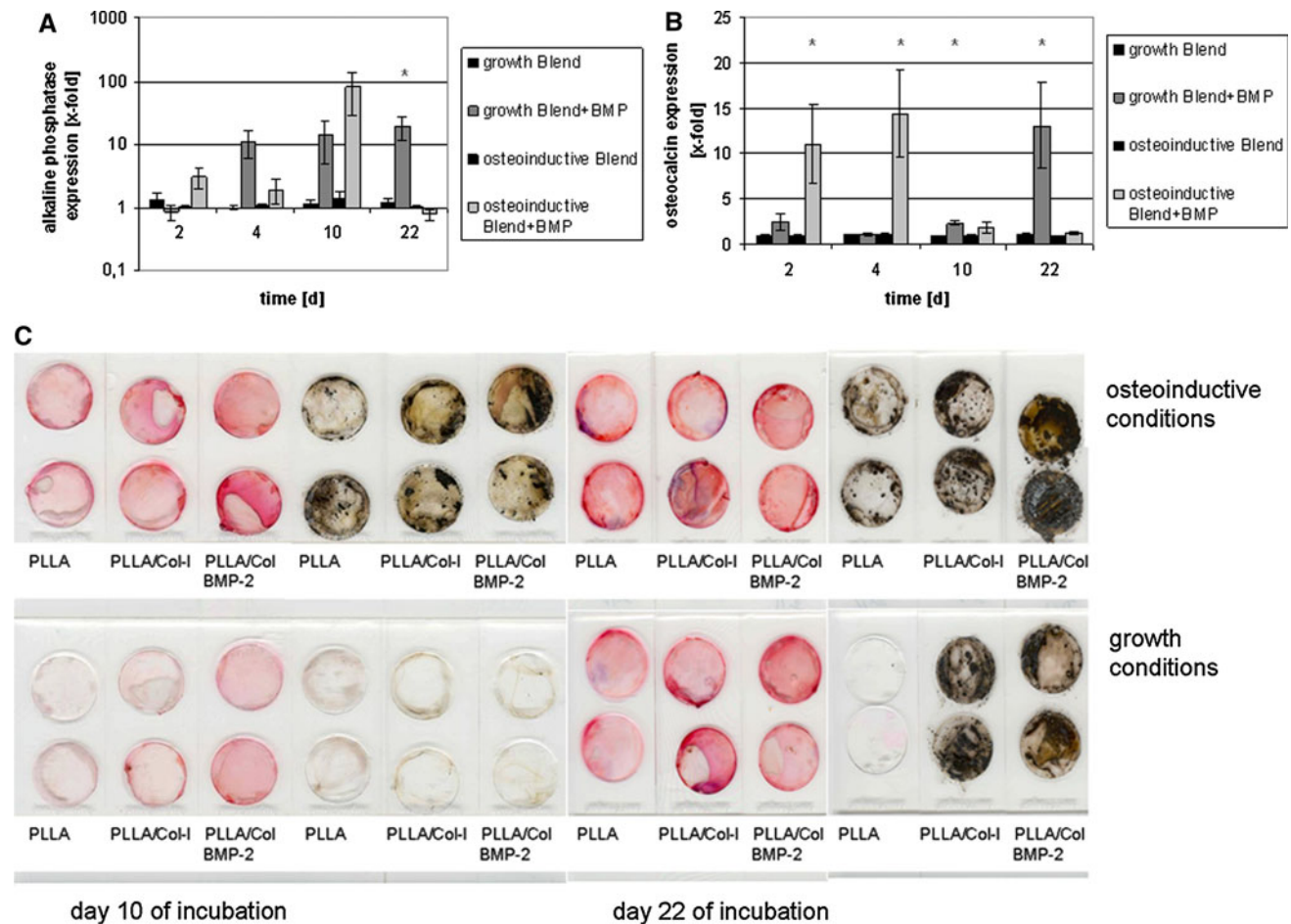


Fig. 5 Influence of BMP-2 containing PLLA-collagen blend nanofibers on differentiation of hMSC towards osteoblasts cultured under growth and osteoinductive conditions. Time course of alkaline phosphatase (a) and osteocalcin (b) gene expression of hMSC cultured under growth and osteoinductive conditions on BMP-2

containing PLLA-collagen blend nanofibers compared to PLLA-collagen blend nanofibers without BMP. c Alizarin (red) and von Kossa staining (black) of hMSC cultured under growth and osteoinductive conditions on different nanofiber scaffolds (Color figure online)

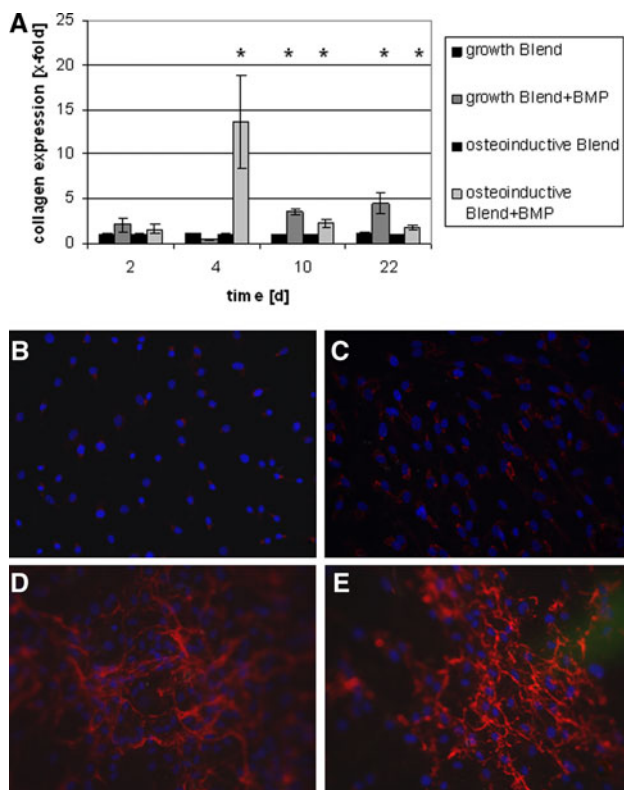


Fig. 6 Influence of PLLA-collagen blend nanofibers and BMP-2 containing PLLA-collagen blend nanofibers on collagen type-I gene expression and matrix deposition of hMSC cultured under growth and osteoinductive conditions. Time course of gene expression of hMSC cultured under growth and osteoinductive conditions (a). Immunofluorescence microphotographs of COL-I (red) deposition after 22 days of culture on PLLA-collagen blend nanofibers (b) BMP-2 containing PLLA-collagen blend nanofibers under growth conditions (c) or osteoinductive conditions (d, e)

and additionally integrin activation by collagen was involved in BMP signals in osteoblastic MC3T3-E1 cells [39] we next analyzed the occurrence of FAK and Smad5 (which are key regulator molecules in the corresponding pathways) using real time PCR and fluorescence microscopy. As compared to PLLA nanofibers alone, the PLLA collagen type I nanofibers showed an increased expression gene of the FAK gene (Fig. 7) which resulted in an increase of FAK within the cells. This effect was boosted when BMP-2 was incorporated into the PLLA collagen type I nanofibers. Similar findings were made with respect to Smad5 (Fig. 8), although the elicited effects by BMP-2 were two times higher as compared to FAK gene expression.

4 Discussion

Tissue-engineering scaffolds should be analogous to native ECM in terms of both chemical composition and physical

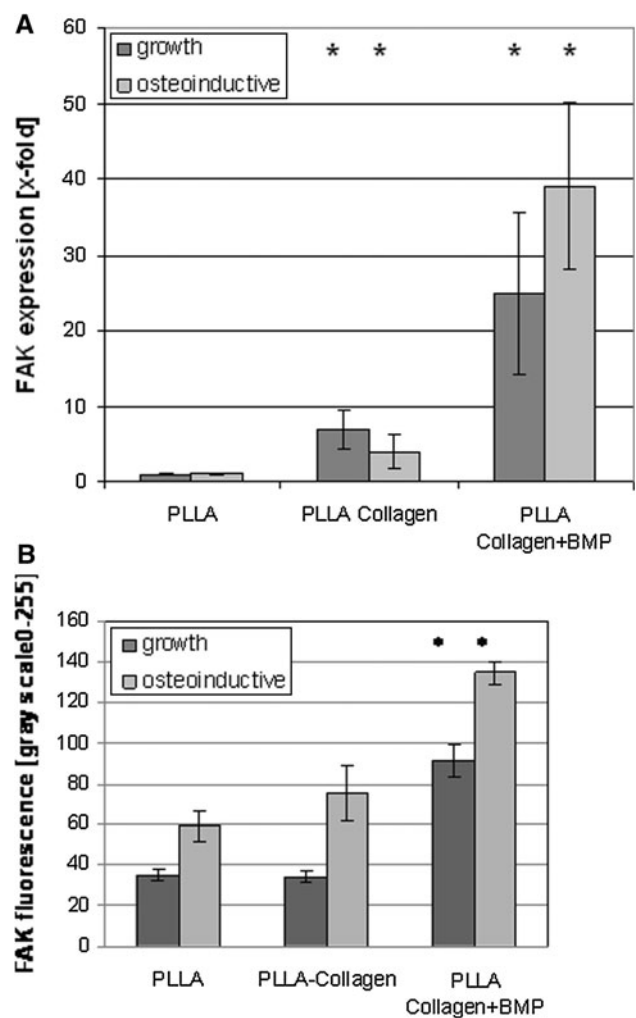


Fig. 7 Influence of PLLA-collagen blend nanofibers and BMP-2 containing PLLA-collagen blend nanofibers on FAK gene expression and immunofluorescence detection of FAK in hMSC cultured under growth and osteoinductive conditions

structure. Polymeric nanofiber matrix, with its nanoscaled nonwoven fibrous structure is a candidate for ECM-mimetic material [48]. Besides the structural effects (elicited by the nanofibers) signals mediated by growth factors and/or collagen sequences like RGD-integrin system are important to develop artificial tissue. In case of bone regeneration, members of the TGF- β superfamily, the bone morphogenetic proteins are necessary [28–31]. We demonstrated earlier that the incorporation of BMP-2 into PLLA nanofiber scaffolds by electrospinning is useful to enhance the osteoblastic differentiation of hMSC as determined by an increased gene expression of genes associated with the osteoblast [16]. Furthermore it was shown, that BMP-2 induces the formation of $\alpha v \beta$ -integrins on osteoblasts [35, 36]. These—in interaction with the ECM—influence the BMP-2 function [36]. Beyond that,

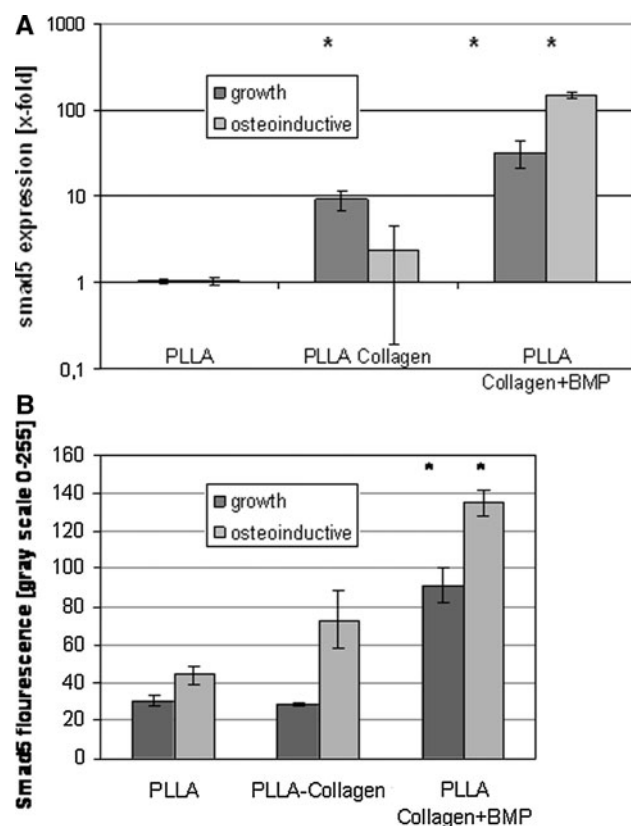


Fig. 8 Influence of PLLA-collagen blend nanofibers and BMP-2 containing PLLA-collagen blend nanofibers on Smad5 gene expression and immunofluorescence detection of smad5 in hMSC cultured under growth and osteoinductive conditions

the adhesion to collagen via the $\alpha 2\beta 1$ integrin is sufficient to induce osteogenic differentiation of hMSC's, even in the absence of exogenous stimuli [20, 21]. Indeed, this property is carried to collagen nanofiber scaffolds [18, 49] as well as nanofiber scaffolds from other polymers, blended with collagen [19, 50].

Within this paper we analyzed whether the combination of both stimuli (BMP-2 and collagen) have a cooperative effect when incorporated into PLLA nanofibers.

With respect to adhesion, the BMP incorporation into PLLA-collagen type I nanofibers resulted in an increased adhesion of hMSC on the nanofibers. Although we cannot exclude that the differences in pore size or fiber diameter were involved in this phenomenon, we speculate, that upregulation of integrins by BMP-2 [35, 36] could be responsible for this effect. This speculation is supported by the finding that cells cultured on this fiber type possess higher gene expression levels as well as protein levels of focal adhesion kinase, a key regulator of the integrin signaling pathway. However additional studies are necessary to support this speculation.

As reported earlier, the collagen nanofiber scaffold increased cell densities [19] as well as proliferation.

The additional incorporation of BMP-2 resulted in a reduced proliferation and lower cell densities. This can be explained by the osteoinductive stimulus elicited by BMP-2. In fact we found an increased gene expression of alkaline phosphatase and osteocalcin, marker of osteoblast differentiation. Furthermore this speculation is supported by an upregulation of Smad5 which is involved in the BMP-2 signal transduction pathway. In addition, this effect was enhanced when osteoinductive conditions were applied. The latter finding is in accordance with cooperative effects between dexamethason and BMP-2 on hMSC differentiation [51]. Taken together this indicates that BMP-2 and not the altered fiber size is responsible for the biological effects on hMSC. Therefore our study indicates that the nanofiber scaffolds can be used to combine the activation of different signal transduction pathways. This facilitates the use of cooperative effects—at least in the differentiation of hMSC towards osteoblasts.

Whether this is sufficient for a bone replacement material, depends although on physical prosperities of the scaffold. The design of scaffolds for bone tissue engineering is based on the physical properties of bone tissue such as mechanical strength, pore size, porosity, hardness, overall 3D architecture and—when used as a drug carrier system—the presentation of the growth factor. Although PLLA is known to possess poor protein release properties [52, 53], during electro-spinning process hydrophilic compounds were accumulated on the fiber surface [54]. The large surface and the porous structure of PLLA nanofibers, might facilitate contact to the BMP-2 receptor of the growing cell and elicit differentiation [16]. In case blend nanofibers similar behavior can be expected. The latter showed that the growth factor was detectable by fluorescence microscopy over a period of 14 days. Although the blend fibers formed a gel-like structure over time, which was observed earlier in unfixed collagen blended nanofibers [19], this effect had no impact on the release kinetics of the incorporated BMP-2. This might be explained by the collagen binding sequence, presented in BMP-2 [55, 56], retarding the growth factor without detectable effect on the BMP-bioavailability. Maybe this form of BMP-2 presentation is similar to the extra cellular matrix, facilitating the BMP-2 contact to its receptors.

More problematic might be the pore size of the nanofiber scaffolds. For bone tissue engineering, scaffolds with a pore size in the range of 100–350 μm are preferred for better cell/tissue in-growth and hence enhanced bone regeneration [57, 58]. Here the PLLA-collagen type I as well as the BMP-2 enovved PLLA-collagen I nanofiber scaffolds show some disadvantages concerning their pore size. Whether this might be compensated by the formation of the gel like structure, or the degradation of collagen after implantation has to be analyzed in vivo.

5 Conclusion

It has been shown earlier that PLLA nanofiber scaffolds can be functionalized by either growth factors or components from the ECM. Here we demonstrated that these different strategies can be combined in order to enhance the osteoblast differentiation of hMSC. By doing this, different signal transduction pathways seemed to be up regulated.

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