J Mater Sci: Mater Med (2009) 20:767–774
DOI 10.1007/s10856-008-3634-8

Influence of nanofibers on the growth and osteogenic differentiation of stem cells: a comparison of biological collagen nanofibers and synthetic PLLA fibers

Markus Dietmar Schofer · Ulrich Boudriot · Christina Wack · Irini Leifeld · Christian Gräbedünkel · Roland Dersch · Markus Rudisile · Joachim Heinz Wendorff · Andreas Greiner · Jürgen Rudolf Josef Paletta · Susanne Fuchs-Winkelmann

Received: 10 August 2008 / Accepted: 16 October 2008 / Published online: 6 November 2008

Abstract The aim of this study was to compare biological collagen I (ColI) and synthetic poly-(L-lactide) (PLLA) nanofibers concerning their stability and ability to promote growth and osteogenic differentiation of human mesenchymal stem cells in vitro. Matrices were seeded with human stem cells and cultivated over a period of 28 days under growth and osteoinductive conditions and analyzed during the course. During this time the PLLA nanofibers remained stable while the presence of cells resulted in an attenuation of the ColI nanofiber mesh. Although there was a tendency for better growth and osteoprotegerin production of stem cells when cultured on collagen nanofibers, there was no significant difference compared to PLLA nanofibers or controls. The gene expression of alkaline phosphate, osteocalcin and collagen I diminished in the initial phase of cultivation independent of the polymer used. In the case of PLLA fibers, this gene expression normalized during the course of cultivation, whereas the presence of collagen nanofibers resulted in an increased gene expression of osteocalcin and collagen during the course of the experiment. Taken together the PLLA fibers were easier to produce, more stable and did not compromise growth and differentiation of stem cells over the course of experiment. On the other hand, collagen nanofibers supported the differentiation process to some extent. Nevertheless, the need for fixation as well as the missing stability during cell culture requires further work.

M. D. Schofer (✉) · C. Wack · I. Leifeld · C. Gräbedünkel · J. R. J. Paletta · S. Fuchs-Winkelmann
Department of Orthopedics, University of Marburg, Baldingerstraße, 35043 Marburg, Germany
e-mail: schofer@med.uni-marburg.de

U. Boudriot
Department of Orthopedics, Sankt-Elisabeth-Hospital Gütersloh, Kattenstroth 103, 33332 Gütersloh, Germany

R. Dersch · M. Rudisile · J. H. Wendorff · A. Greiner
Department of Chemistry, University of Marburg, Hans-Meerwein-Straße, 35032 Marburg, Germany

1 Introduction

Tissue engineering involves the in vitro seeding of cells onto scaffolds supporting cell adhesion, migration, proliferation, and differentiation, and defines the three-dimensional shape of the tissue to be engineered. Among the various types of scaffold architecture available, scaffolds based on nanofibers offer great advantages [1, 2]. These nanofibers mimic the extra cellular matrix [3, 4] and serve as a three dimensional matrix for growing cells and allow the differentiation of human mesenchymal stem cells (hMSC) towards osteoblasts [5–10] or chondrocytes [11].

Beside this, one of the most important factors, when tailoring the artificial graft for the specific tissue, is the material used. The spectrum of materials used in preparing nanofiber-based scaffolds for tissue engineering is extremely broad and includes biocompatible and biodegradable polymers of natural and synthetic origin such as poly (glycolic acid) (PGA), poly(L-lactic acid) (PLLA), poly (ε caprolactone) (PCL) as well as copolymers from the corresponding monomers in various compositions, segmented polyurethanes, polyphosphazenes, collagen, gelatin, and chitosan [4, 12]. This allows the production of a broad spectrum of nanofiber-based scaffolds with different mechanical and biophysical properties. It also offers the chance to incorporate growth factors in order to use the nanofibers as a drug carrier system.

Although most nanofibers made of these polymers were tested concerning their suitability in tissue engineering, only few studies focus on the differentiation of human mesenchymal stem cells [5–10] or rat mesenchymal stem cells [13, 14] towards osteoblasts. With respect to PLLA, we reported earlier that nanofibers made of this polymer, did not interfere with growth and differentiation of hMSC as demonstrated by van Kossa staining as well as immunofluorescence staining towards osteocalcin [15]. Furthermore PLLA supports proliferation of hMSC's more than other chemosysnthetic polymers [10].

Nevertheless, the influence of PLLA nanofibers on the course of differentiation (in terms of the expression of genes related to the osteoblast phenotype) is unclear. Furthermore, there are no data available comparing the PLLA nanofibers with nanofibers made from collagen I (ColI), which is one of the main components of bone with respect to osteoblastic stem cell differentiation and growth.

Therefore, the aim of this study was to characterize the course of growth and differentiation of stem cells on synthetic PLLA nanofibers and to compare it with that of stem cells grown on biological ColI nanofibers in vitro.

2 Materials and methods

2.1 Construction of nanofibers and characterization

The preparation of PLLA nanofibers by electrospinning has been reported in detail earlier [16]. Briefly, a 4% (w/w) PLLA (Resomer L210, Boeringer Ingelheim Germany) solution in dichloromethane was prepared by stirring at room temperature over night until a homogenous solution was obtained. Spinning process was performed at a flow rate of 14 µl/min with an applied voltage of 20–30 kV and a distance of 15 cm. Samples of nonwoven poly-L-lactide nanofibers were fixed on 19 mm cover slips for cell culture experiments. In order to prepare ColI fibers, a 5% (w/v) collagen I (Sigma Aldrich, Taufkirchen, Germany) solution in 1,1,1,3,3,3 hexafluoro-2-propanol (HFP) was prepared by steering at room temperature over night until a homogenous solution was obtained. Spinning process was performed at a flow rate of 5 µl/min with an applied voltage of 10 kV and a distance of 15 cm according to Matthews et al. [17]. Due to the fact that native electrospun nanofibers lose their integrity in aqueous solutions, fibers were thermal cross linked according to Weadock et al. (110°C, applying a vacuum of approximately 1 mbar) [18].

Static contact angles of water were measured using the sessile drop method with a G10 Drop Shape Analysis System (Krüss, Hamburg, Germany) and calculated using Data Physics SCA20 Contact Angle Analyzer Software.

2.2 Scanning electron microscopy

For scanning electron microscopy (SEM), samples were fixed in 2% cold buffered OsO_4 containing 0.22 M sucrose for 2–3 h. After the fixation, the specimens were washed with PBS, dehydrated through a graded propanol series, and critical-point dried using CO_2 (CPD 030, Bal-Tec, Schalksmühle, Germany). Specimens 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.

2.3 Human mesenchymal stem cell isolation, characterization and culture

Human mesenchymal stem cells were obtained from male patients with the approval of the institutional review board. The indication for surgery was primary osteoarthritis of the hip with increasing pain, decreased range of motion, and signs of progressive osteoarthritis in radiographs. 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. Mesenchymal stem cells were isolated and cultured according to the preparation of Pittenger et al. [19], with minor modification as described by Brendel et al. [20]. To ensure the purity of hMSC's, flow cytometric antigen expression analysis was performed at passage 3. For staining the surface molecules, 10^5 cells were incubated in 100 µl PBS containing flurochrome labeled monoclonal antibody for 20 min at 4°C. After washing the cells in order to remove dispensable dye, cells were suspended in 300 µl PBS and immediately submitted to fluorescence-activated cell sorting (FACS) analysis (FACScan, Becton Dickinson, San Jose, CA, USA). The following antibodies were employed: CD45-FITC (PharMingen, San Jose, CA, USA), CD90-FITC (PharMingen), CD34-PE (PharMingen), CD14-PE (Becton Dickinson), CD31-PE (Beckman Coulter, Krefeld, Germany), IgG-FITC and IgG-PE (Becton Dickinson). For the CD105 antibody (PharMingen, San Jose, CA, USA), indirect staining was performed using goat anti-mouse PE secondary antibody (PharMingen, San Jose, CA, USA).

Within the experiments, hMSC's preparations were used, showing up negative for haematopoietic cells, macrophages (CD14, CD45), blood progenitor cells (CD34, CD45) and endothelial precursors (CD34, CD31) and positive for stem cell markers (CD90 and CD105). In order to ensure the osteoinductive potential of the obtained cells, gene expression of alkaline phosphatase (AP) in response to dexametasone was determined prior to the experiments.

For experiments, stem cells were seeded at a density of 3×10^4 cells/cm^2 on cover slips or cover slips coated with

either PLLA or ColI nanofibers in growth medium (DMED), with low glucose and glutamine (PAA, Linz, Austria) supplemented with 10% fetal calf serum (FCS) from selected lots (Stem Cell Technologies, Vancouver, Canada) and 1% penicillin/streptomycin. In some experiments osteogenic differentiation was induced by a modified method according to Jaiswal et al. [21]. Here standard medium was replaced by medium containing additionally 0.1 µM dexamethason, 0.05 mM ascorbic acid-2-phosphate and 10 mM ß-glycerolphosphate (all obtained from Sigma, Taufkirchen, Germany) after an initial proliferation phase of 3 days. Medium was replaced every second day of culture.

2.4 Osteoprotegerin determination

Osteoprotegerin (OPG) was determined in culture supernatants at days 4, 10, 16, 22 and 28 using OPG Elisa kit (Immundiagnostik AG, KB 1011, Bensheim, Germany) according to the instructions of the manufacturer. Each sample was diluted 1:20 using PBS and analyzed in triplicate.

Cells were cultured in FCS-containing medium which interferes with the determination of secreted total protein. OPG content was normalized to the culture volume. Therefore, OPG increase might partially be associated with an increase in cell density.

2.5 Gene expression analysis

RNA was extracted from cell layers at days 4, 10 and 22 using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer and quantified spectrometrically. Starting from 1 µg RNA, 20 µl cDNA were synthesized using Omniscript reverse transcriptase and oligo-dT primer in the presence of dNTP (Qiagen GmbH, Hilden, Germany). Quantitative RT-PCR reactions were performed and monitored using a Mastercycler® ep realplex Detection System (Eppendorf, Hamburg, Germany) and RealMaster Mix CyberGreen (Eppendorf, Hamburg, Germany). Genes of interest were analyzed in cDNA samples (5 µl for a total volume of 50 µl/reaction) using $^{\Delta\Delta}$CT method and CyberGreen. Primers, cycle temperatures and incubation times for human AP, ColI, osteocalcin (OC), and 18 s rRNA were previously described [22, 23] and purchased from TIB Biomol (Berlin, Germany). Purity of the single PCR products was verified by melting point analysis.

2.6 Immunofluorescence microscopy

Samples obtained at day 22 were fixed in aceton/methanol, washed with PBS (3×), and exposed to blocking buffer (1% donkey serum albumin PBS) for a further 30 min at room temperature in order to minimize non-specific absorption of the antibodies. After another wash in PBS (3×), the cells were incubated with primary antibodies against ColI (Abcam, Ab6308, Cambridge, United Kingdom). 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).

After DAPI staining for 30 s, slices were washed three times with PBS and embedded in mounting medium. Fluorescence microscopy was done using a Leica DM5000. Microphotographs of at least three different areas were made at a primary magnification of 20-fold.

2.7 Statistics

All values were expressed as mean ± standard deviation of three patients done in triplicate and compared using ANOVA with Bonferroni as a post hoc test. Values of $P < 0.05$ were considered to be significant. Significances in all series were marked with * whereas significances in either of the patient series were marked with +.

3 Results

3.1 Characterization of fibers

SEM of electrospun PLLA nanofibers revealed a 3-D nonwoven network with a diameter of 775 ± 294 nm. Fibers were porous in structure, and had a contact angle of 124.6 ± 5.7° (Fig. 1). In aqueous solutions the PLLA fibers were stable over a period of 30 days.

In contrast, ColI fibers obtained by electrospinning had an apparent diameter of 458 ± 143 nm. The contact angle was 64.8 ± 3.0° (Fig. 1). The contact angles show the increased hydrophobicity of PLLA compared with ColI nanofibers. Thermal cross linked ColI nanofibers presented themselves with a smooth surface and showed no changes in aqueous solutions (Fig. 1b, e–g) over a period of 12 days. However, the stability of ColI fibers was lost in the presence of cells, resulting in a formation of macroscopic visible cracks after 22 days of culture.

3.2 Cell densities

First we analyzed the effect of ColI and PLLA nanofibers on the cell densities of stem cells compared to cover slips without fibers. As shown in Fig. 2a, under osteoinductive conditions there was a significant time dependent increase in cell density within 22 days, independent of whether cells were cultured on glass, PLLA or ColI nanofibers, accompanied by a trend to higher cell densities (ColI > PLLA or glass), after 22 days.

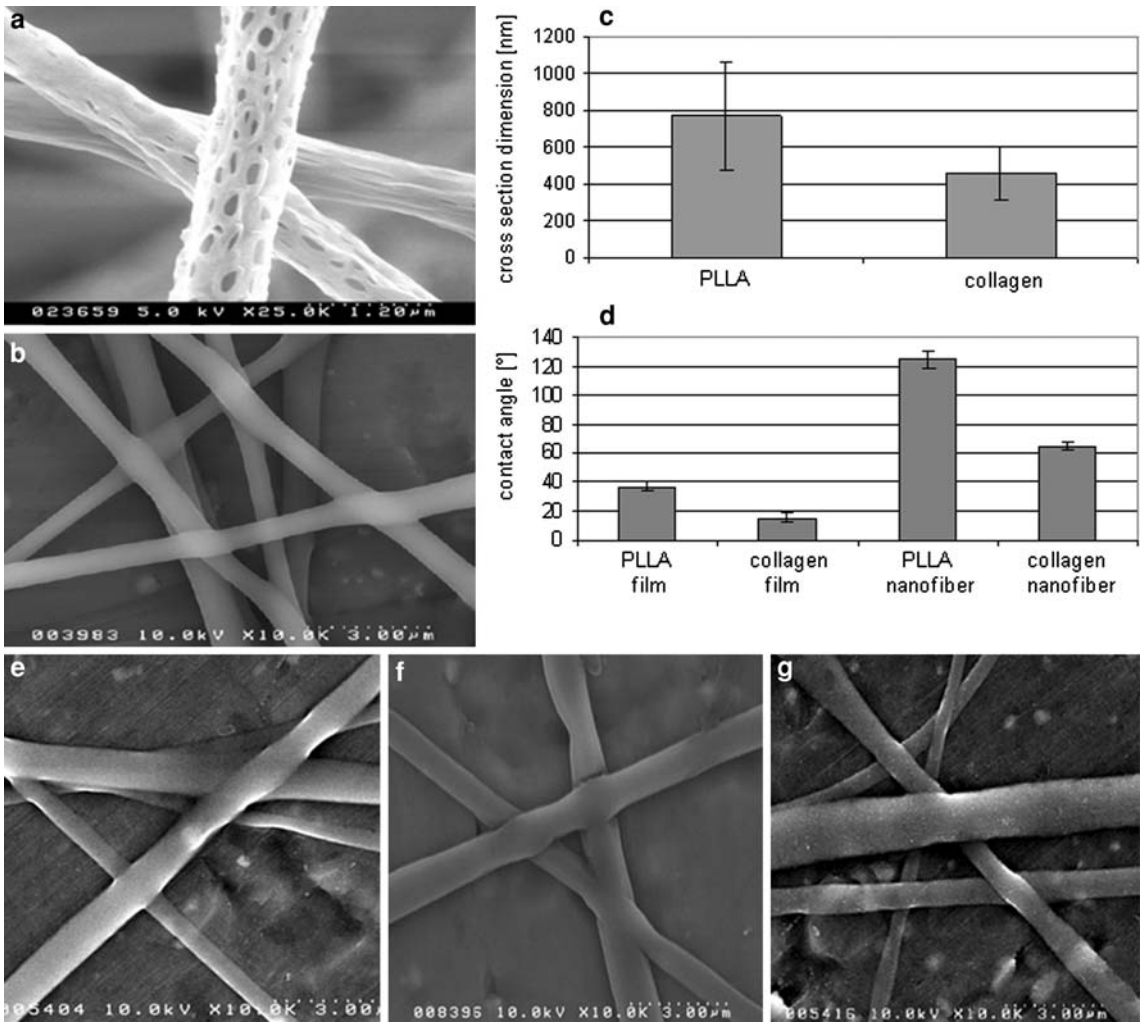

Fig. 1 Characterisation of PLLA and ColI nanofibers. SEM of nanofibers electrospun from PLLA (**a**) and ColI (**b**). Mean and standard deviation of cross section dimension (**c**) and contact angle of water (**d**). Long term stability of thermal cross linked ColI nanofibers in aqueous solutions. SEM was done before (**e**), after 24 h (**f**) and after 12 days in water at 37°C (**g**)

When cultured under growth conditions (Fig. 2b), cell densities only increased until day 10 and remained constant until the end of culture. Here cell densities on PLLA fibers did not differ from control cells in a significant manner. In contrast, ColI fibers showed increased cell densities, especially at day 4 and day 10.

With respect to the stability, PLLA fibers remained robust during cultivation. In contrast, ColI nanofiber meshes lost their integrity, resulting in a scaffold rupture. This phenomenon was obvious in the late stage of culture.

3.3 Osteoprotegerin release

In order to elucidate the impact of PLLA or ColI nanofibers on the course of stem cell differentiation towards osteoblasts we compared the release of OPG of cells cultured on PLLA nonofibers and on ColI nanofibers. As shown in Fig. 2c, d, the addition of dexamethason resulted in a time dependent increase of OPG production. This increase was significant in the case of cells cultured on glass (day 22, day 28) and cells cultured on PLLA fibers (day 16, day 22, day 28). Nevertheless, there was no significant difference in OPG production between cells cultured on PLLA nanofiber or cover slips.

In the case of ColI nanofibers, stem cells showed an increased OPG production when cultured under growth conditions as compared to PLLA nanofiber surface or cover slips.

3.4 Influence of nanofibers or gene expression and deposition of matrix proteins

Osteoblast lineage gene expression was detected using real time PCR analysis for OC, ColI and AP (Fig. 3). In response to dexamethasone, we found a time dependent up-regulation in gene expression (data not shown). This was

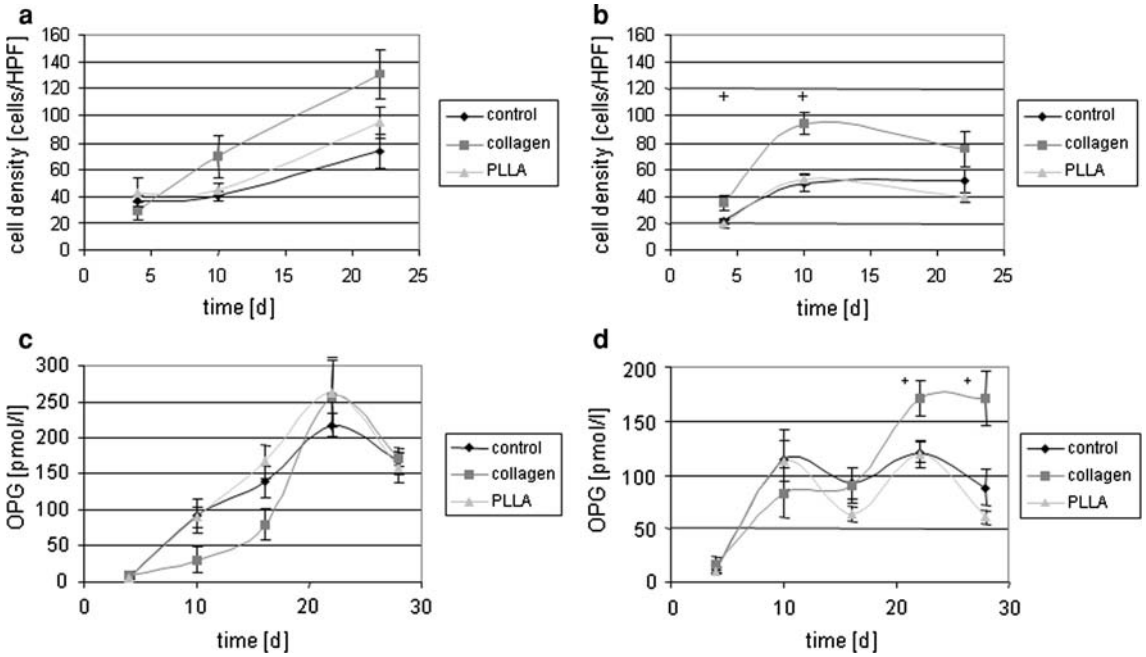

Fig. 2 Time course growth and OPG production during cultivation of hMSC's on nanofibres under growth and osteoinductive conditions. Cells were seeded on ColI and PLLA nanofibers or cover slips and cultured either under osteoinductive (**a**) or growth conditions (**b**). At desired time points, cell densities and OPG were determined as indicated in the text (**c** - osteoinductive and **d** - growth conditions)

accompanied by an increased staining intensity towards ColI and OC using immunofluorescence analysis after 22 days of culture (Fig. 4a, b).

This increase in gene expression was altered by the presence of nanofibers (Fig. 3). Independent of the polymer (PLLA or ColI) or the culture conditions (growth or osteoinductive), gene expression of AP, OC and ColI was reduced significantly during the initial culture compared to cells cultured on cover slips. In the case of PLLA nanofibers, the gene expression conditioned during the time course of cultivation. Here immunfluorescence analysis resulted in comparable staining intensities compared to cover slips (Fig. 4c, d).

In contrast, when cells were cultured on ColI nanofibers, we found an increase of gene expression for AP and OC as well as ColI (Fig. 3), detectable in the late stage of cultivation (day 10 in the case of growth conditions, day 22 in the case of osteoinductive conditions). This increase was accompanied by deposition of ColI and OC as indicated by immunfluorescence (Fig. 4e, f). Here it is remarkable that under growth conditions, the presence of ColI nanofibers resulted in both an increase in gene expression as well as protein deposition of both ColI and OC.

4 Discussion

This study was designed in order to compare nanofibers, based on polymers of natural and synthetic origin, with respect to their suitability in bone tissue engineering and their influence on growth and differentiation of hMSC's on the osteoblast linkage. ColI was chosen due to the fact that it is the main component of bone, whereas PLLA is a FDA approved polymer and maintained a robust scaffold structure upon incubation in physiological solutions [10]. As reported earlier, PLLA [24] as well as ColI [17] could be easily electrospun and reproducible to a 3-D non-woven network. Nevertheless, the thickness of the ColI mesh obtained by electrospinning was limited. This was due to the fact that ColI nanofibers react with air moisture. Therefore an immediate fixation is necessary. The thermal fixation chosen in this study resulted in stable nanofiber mesh in aqueous solutions. However, the presence of cells during the time course of cell culture resulted in an attenuation of the ColI nanofiber mesh. Due to the fact that this phenomenon occurred in the presence of cells, we speculate that it is caused by enzymatic degradation rather than by fragmentation as described for thermal fixation [18].

Besides these material properties, the influence of the polymer on the growth and differentiation of stem cells is of interest. We reported earlier the ability of osteoblast cell line (MG63) to grow on poly-L-lactide nanofibers [16] as well as the osteogenic differentiation of hMSC's on a three-dimensional matrix of these fibers in principle [15]. In this study, we found a significant time dependent increase in cell densities of stem cells on PLLA fibers, indicating that there were no inhibitory effects of the polymer itself, which might be expected with respect to

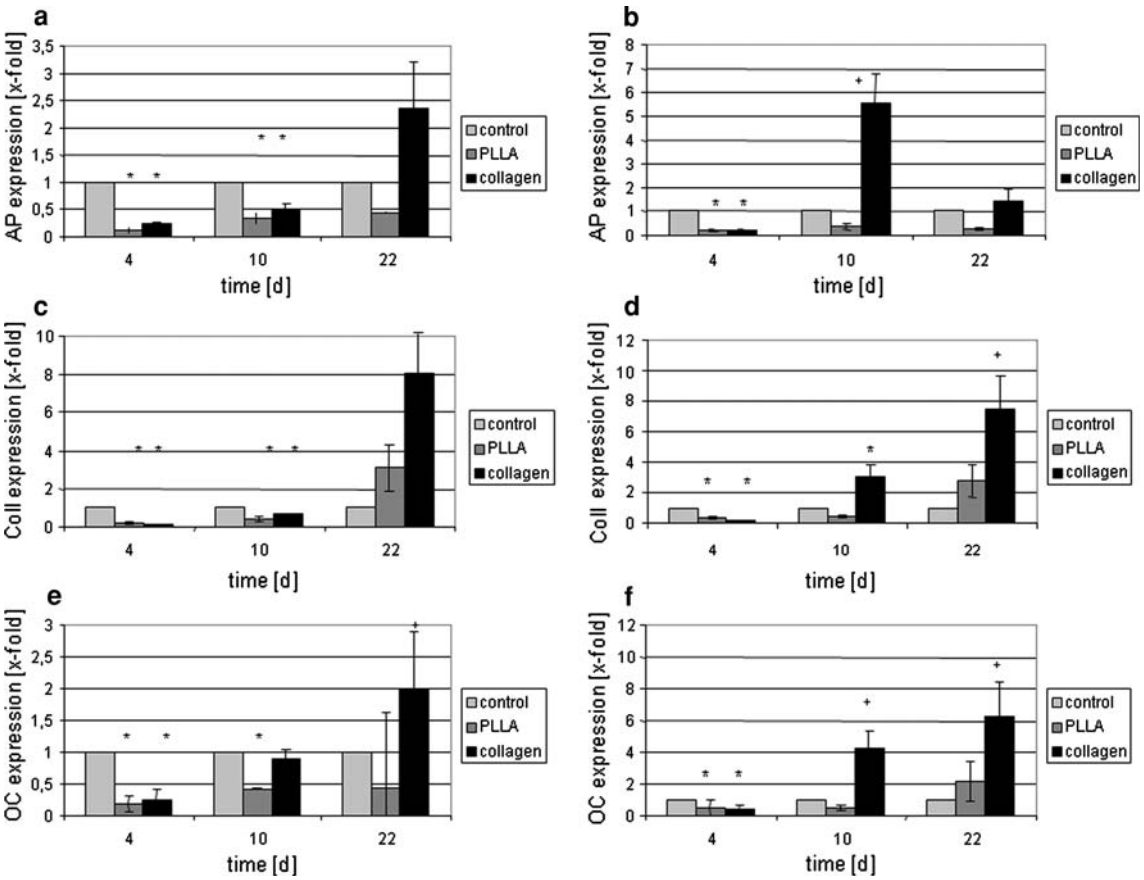

Fig. 3 Gene expression of markers of the osteoblastic linkage and matrix formation during cultivation of stem cells on nanofibers under growth and osteoinductive conditions. Cells were seeded on ColI and PLLA nanofibers or cover slips (control) and cultured either under osteoinductive (**a**, **c**, **e**) or growth (**b**, **d**, **f**) conditions. At desired time points, AP (**a**, **b**), ColI (**c**, **d**) and OC (**e**, **f**) gene expression were analyzed and compared to the gene expression of cells grown on cover slips under comparable conditions

osteolysis due to PLLA implants. Similar findings were made in experiments with thermal cross linked collagen nanofibers. Here, cell densities tend to be higher compared to those of cover slips or PLLA. Although the effect was not significant, it is in accordance with findings of Shih et al. [8], who reported higher cell viability of stem cells on type I collagen nanofibers compared to smooth surfaces. Moreover, this effect was also observed when smooth muscle cells or dermal fibroblast were cultured on collagen nanofibers [25, 26].

More obvious, was the effect of nanofibers on the osteogenic differentiation of stem cells. The initial down regulation of genes, related to osteoblast formation and the finding that this was independent of the polymer used to produce the fibers. One interpretation might be, that during this phase of cultivation the structure of the surface, rather than the biochemical properties influence the differentiation of stem cells. Nevertheless, this effect was not stable during the course of experiments. In the case of PLLA nanofibers, the down regulation of osteoblast marker genes diminished after 22 days of culture. As a consequence, OC as well as ColI deposition, comparable to cells cultured on cover slips could be demonstrated by fluorescence microscopy.

More obvious, was the effect of collagen nanofiber on the differentiation of stem cells. Here the gene expression of AP, OC and ColI increased during the late stages of cultivation compared to cells cultured on glass surface or PLLA nanofibers. Although there was a great variance which we attribute to the instability of the collagen nanofiber constructs, the results are consistent with Shih et al. [8], who reported similar or higher levels of osteoblast-lineage RNA transcript production between the nanofibers and tissue culture polystyrene, indicating that the nanofibers support intrinsic properties of hMSC's differentiation after osteogenic induction.

When cultured under growth conditions, the effect of ColI nanofibers was more obvious. We interpreted that, in thermal fixed collagen nanofibers, the RGD-sequences responsible for integrin interactions, are presented comparably to native collagen. It is known from other studies that the adhesion to ColI via the $\alpha 2\beta 1$ integrin is sufficient

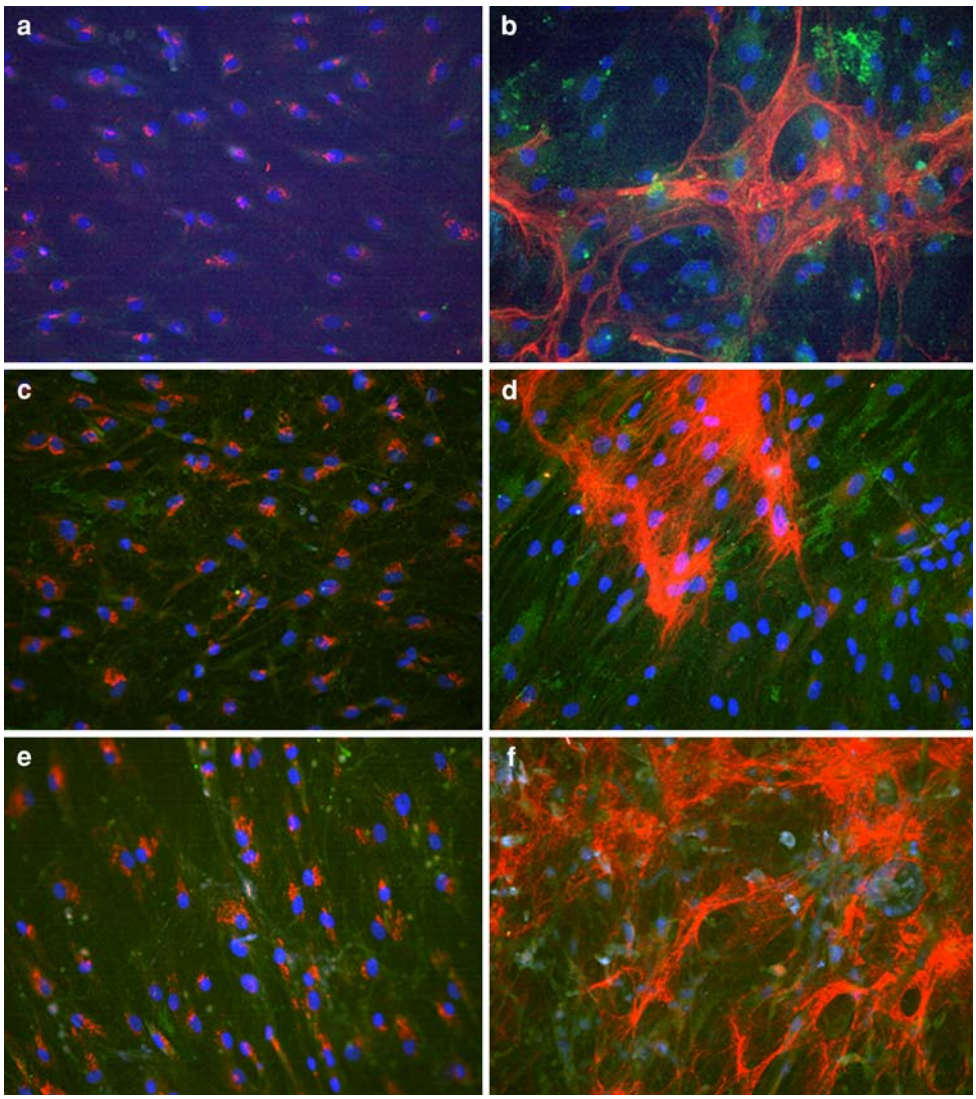

Fig. 4 Immunfluorescence analysis of OC (green) and ColI (red) deposition after cultivation of hMSC's on cover slips (**a**, **b**), PLLA nanofibers (**c**, **d**) and ColI nanofibers (**e**, **f**) under growth (**a**, **c**, **e**) and osteoinductive (**b**, **d**, **f**) conditions

to induce osteogenic differentiation of hMSC's, even in the absence of exogenous soluble stimuli [27, 28].

5 Conclusion

Taken together, this study shows the advantages and disadvantages of PLLA and ColI electrospun nanofibers, with respect to osteoblastic differentiation of stem cells and bone tissue engineering. PLLA fibers were easier to produce, more stable and did not compromise growth and differentiation of stem cells over the course of experiment. On the other hand, collagen nanofibers supported the differentiation process to some extent. Nevertheless, the need for fixation as well as the missing stability during cell culture requires further work.

Acknowledgments This work was supported by the Deutsche Forschungsgemeinschaft (German Research Foundation, Grant No. BO 3065/1-1).

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