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Human platelet-rich plasma induces chondrogenic differentiation of subchondral progenitor cells in polyglycolic acid–hyaluronan scaffolds

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Abstract: Cartilage repair approaches may be improved by addition of human platelet-rich plasma (PRP) that increases chondrogenic differentiation of mesenchymal stem and progenitor cells. The aim of our study was to evaluate the effect of human PRP on the differentiation of multipotent human subchondral progenitor cells in resorbable polyglycolic acid–hyaluronan (PGA-HA) scaffolds. PGA-HA scaffolds were loaded with subchondral progenitor cells and stimulated with transforming growth factor-beta3 (TGFB3) or 5% PRP, whereas nonstimulated cultures served as controls. Chondrogenic differentiation was evaluated by real-time gene expression analysis of typical chondrogenic marker genes and by immunohistochemical staining of extracellular cartilage matrix molecules such as proteoglycans and collagen type II. TGFB3 and PRP induced the expression of chondrogenic marker genes collagen type II and IX, aggrecan, and cartilage oligomeric matrix protein in subchondral progenitor cells cultured in PGA-HA scaffolds compared with nonstimulated controls. Progenitor cells in PGA-HA scaffolds formed an extracellular matrix rich in proteoglycans and collagen type II on treatment with PRP, but to a lesser extent, than in cultures stimulated with TGFB3. The results suggest that PRP induces chondrogenic differentiation of progenitor cells in PGA-HA scaffolds and may be therefore beneficial in scaffold-assisted cartilage repair approaches involving stem and progenitor cells. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater, 102B: 681–692, 2014.

Key Words: platelet-rich plasma, cartilage regeneration, stem and progenitor cells, scaffold, transforming growth factor-beta

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INTRODUCTION

The microfracture technique is the most commonly used first-line treatment option for articular cartilage defects in clinical routine today in which small holes of 3–4 mm in depth are created into the subchondral bone with an awl 3–4 mm apart from each other.[1] In this bone marrow stimulating technique, progenitor cells from the subchondral bone are stimulated to migrate to the site of the defect and develop hyaline-like repair tissue.[2] The subchondral mesenchymal progenitor cells are characterized by their high proliferation capacity and the ability to differentiate into bone, cartilage, and fat.[3] In addition, subchondral progenitor cells show the typical cell surface markers known from mesenchymal stem and progenitor cells, such as CD 73, CD 90, CD 105, and CD 166.[4] However, several studies showed that the cartilage repair tissue obtained after microfracturing is fibrocartilage[5,6] characterized by its high quantity of collagen type I and loss of hyaline cartilage matrix-specific molecules such as glucosaminoglycans, collagen type II, and aggrecan.[7,8] This could be due to an insufficient local stimulation with chondrogenic stimuli that may be necessary to induce or support chondrogenic differentiation of progenitor cells toward formation of hyaline cartilage. Among other techniques, cell-free implants made of resorbable polyglycolic acid–hyaluronic acid (PGA-HA) have been developed[9] for the use in combination with autologous serum or

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platelet-rich plasma (PRP). These scaffolds consist of PGA with a nonwoven, textile structure and an initial tensile strength of 3.6 N/mm^2, which decreases continuously on prolonged incubation in aqueous solution to 1.7 N/mm^2 after 10 days. The PGA scaffolds showed a stiffness of 0.13–0.16 N/mm^2. The diameter of the single scaffold fiber is approximately 17 μm.[10] Hyaluronic acid, with a molecular weight of approximately 1.5×10^6 Da, is incorporated by a freeze-drying process.

Defect cover by the PGA-HA implant after microfracture shall improve quality of repair tissue, assuming that subchondral mesenchymal progenitor cells enter the implant that supports hyaline-like cartilage tissue formation.[11–13] Histochemical analysis of repair tissue obtained after 6 months of implantation of the PGA-HA implant in microfractured ovine cartilage defects showed the formation of cartilaginous repair tissue that was superior to the repair tissue after microfracture alone.[9] The migration and recruitment of such mesenchymal stem and progenitor cells may be mediated by human serum.[14] A recent study showed that PRP stimulates *in vitro* progenitor cell migration and extracellular matrix biosynthesis and enhances chondrogenic differentiation of subchondral progenitor cells.[15] This beneficial effect may be due to the high amount of cytokines and growth factors in PRP, which may stimulate chondrogenic differentiation of progenitor cells and improve chondrocyte proliferation and metabolism. PRP is defined as a volume of the plasma fraction of autologous blood having a platelet concentration above baseline.[16] This may lead consequently to a high content of platelet-derived growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGFB), and others.[17] TGFB is known to induce chondrogenesis of mesenchymal progenitor cells, whereas PDGF supports maintenance of the hyaline-like chondrogenic phenotype and induces proliferation and proteoglycan synthesis of chondrocytes.[18] Because of these properties, PRP is currently used in clinical application especially in combination with biological cartilage repair treatments.[19] Although clinical outcome of such applications can be determined by questionnaires to reveal actual pain situation or quality of life, the biological effect of PRP on cartilage tissue regeneration is still unclear. Therefore, the influence of PRP on cartilage matrix development will be analyzed in PGA-HA scaffolds loaded with human subchondral mesenchymal progenitor cells *in vitro*. In this study, we focus on the differentiation potential of subchondral progenitor cells known to be involved in repair tissue formation during microfracture treatment without consideration of other cell types, which might be involved in articular repair tissue formation *in vivo*.

MATERIAL AND METHODS

Isolation and cultivation of human subchondral progenitor cells

Human subchondral progenitor cells were obtained from femur heads of three donors postmortem (one female, two males, mean age 63 years) and processed separately. The ethics committee of the Charité-Universitätsmedizin Berlin approved the study. The spongious bones of the femur heads were cut into small fragments. These fragments were partially digested for 4 h at 37°C using 256 U/mL collagenase XI (Sigma-Aldrich, St. Louis, MO). After digestion, the remaining bone fragments were placed in Primaria™ cell culture flasks (Becton and Dickinson, Franklin Lakes, NJ) and cultured in DME-medium (Biochrom, Berlin, Germany) containing 10% human serum (German Red Cross, Berlin, Germany), 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL gentamicin, 0.1 μg/mL amphotericin B, 2 m*M* *N*-acetyl-*L*-alanyl-L-glutamine (all Biochrom) and 2 ng/mL human fibroblast growth factor-2 (PeproTech, Hamburg, Germany). Cells that reached 80–90% confluency were subcultivated using trypsin in phosphate-buffered saline (PBS; 0.05% v/v, Biochrom) and re-plated at a density of 8000 cells/cm^2. Medium exchange was performed every 2 or 3 days.

Flow cytometric analysis

Human subchondral progenitor cells (250,000 cells, passage 3, $n = 3$ donors) were washed in PBS/0.5% bovine serum albumin (BSA) and incubated with phycoerythrine (PE) or fluorescein isothiocyanate (FITC)-labeled monoclonal mouse anti-human antibodies CD 34-PE, CD 73-PE, CD 166-PE, CD 45-FITC, CD 90-FITC, and CD 105-FITC (all Becton and Dickinson) for 15 min. Staining of cell surface antigens was analyzed using FACS Calibur (Becton and Dickinson). Apoptotic cells were excluded from analysis using propidium iodide (PI). CD 34-PE/FITC–stained cells served as negative control.

Preparation of human PRP

PRP ($n = 5$) from normal, healthy blood donors was extracted by aphaeresis at the Department of Transfusion Medicine, Charité-Universitätsmedizin Berlin, using an automated blood collection system (Trima Accel®, CaridianBCT, Lakewood, CO) with anticoagulant citrate dextrose-A. Platelet count was defined as $0.6–1.3 \times 10^{10}$ platelets per milliliter and proportion of leukocytes was less than 0.3×10^4 per milliliter. PRP activation was performed according to Weibrich et al.[20] as follows. Platelet concentrates (approximately 300 mL per sample) were frozen overnight at −20°C, thawed, and centrifuged. The supernatant was taken and used for further analysis. Total protein content of PRP was determined using the bicinchoninic acid assay (Sigma-Aldrich) according to the manufacture's recommendations. BSA was used to prepare a standard curve. Samples were measured at 562 nm in a micro-plate reader (Synergy HT, BioTek, Bad Friedrichshall, Germany), and the protein content was calculated using a BSA standard and adjusted with PBS to 110 mg/mL. PRP was stored at −20°C. Before use, PRP was thawed at 4°C, followed by centrifugation at 4°C at 1600*g* for 10 min. The supernatant was used immediately.

Scaffold preparation and cell differentiation studies in three-dimensional culture

Nonwoven polyglycolic acid scaffolds (Alpha Research, Switzerland) were cut into pieces of 10 × 10 × 1.1 mm. Scaffolds were loaded with 110 μL hyaluronic acid (Ostenil, TRB Chemedica, Haar, Germany), freeze-dried for 16 h using

TABLE I. Oligonucleotide Sequences

Gene Name	Acession No.	Oligonucleotide (5′→3′)(up/down)	Base Pairs
Cartilage oligomeric matrix protein	NM_000095	CCG GAG GGT GAC GCG CAG ATT GA/ TGC CCT CGA AGT CCA CGC CAT TGA A	133
Aggrecan	NM_001135	GGC TGC TGT CCC CGT AGA AGA/ GGG AGG CCA AGT AGG AAG GAT	163
Collagen type I	NM_000088.2	CGA TGG CTG CAC GAG TCA CAC/ CAG GTT GGG ATG GAG GGA GTT TAC	180
Collagen type II	NM_001844	CCG GGC AGA GGG CAA TAG CAG GTT/ CAA TGA TGG GGA GGC GTG AG AAT CAG	128
Collagen type IX	NM_001853.2	GCT CTC GAA GCT CAT AAA A/ CCT GCC ACA CCC CCG CTC CTT CAT	100
Collagen type X	NM_000493.2	GAA CTC CCA GCA CGC AGA ATC C/ GTG TTG GGT AGT GGG CCT TTT ATG	145
Osteocalcin	X51699	GAG CCC CAG TTC CCC TAC CC/ GCC TCC TGA AAG CCG ATG TG	103
Fatty acid binding protein 4	NM_001442.1	CCT TAG ATG GGG GTG TCC TGG TA/ AAC GTC CCT TGG CTT ATG CTC TC	156
Glyceraldehyde-3-phospate dehydrogenase	NM_002046.3	GGC GAT GCT GGC GCT GAG TAC/ TGG TCC ACA CCC ATG ACG A	149

a lyophilisator (Leybold-Heraeus, Hanau, Germany), and stored in a dessicator at room temperature (RT). Subchondral progenitor cells (passage 3, $n = 3$ donors) were trypsinized, counted, and seeded into scaffolds with a density of 2.2 million viable cells per scaffold by resuspending in 74 μL cell culture medium and 37 μL fibrinogen (Tissucol, Baxter, Deerfield, IL). The cell-seeded scaffolds were placed on a drop of thrombin (Tissucol, Baxter) and another drop of thrombin solution was added onto the scaffold to start polymerization. After incubation at 37°C and 5% CO_2 for 15 min, each scaffold was transferred into a well of a 6-well plate with 3 mL DME-medium containing 1% ITS+1 (insulin-transferrin-selenium), 1 m*M* sodium pyruvate, 0.35 m*M* L-proline, 0.17 m*M* L-ascorbic acid-2-phosphate, and 0.1 μ*M* dexamethasone (all Sigma-Aldrich). Chondrogenesis was induced by adding 10 ng/mL TGFB3 (PeproTech) or 5% PRP (pool of $n = 5$ PRP preparations). Scaffolds cultured in DME medium containing 1% ITS+1 without TGFB3 or PRP served as controls (noninduced samples). Medium changes were performed every 2–3 days, and the cell-loaded PGA-HA scaffolds were turned upside down at every medium change. Samples for gene expression analysis were taken at day 7 and 14. Samples for PI/fluorescein diacetate (PI/FDA) staining, histology, and immunohistochemistry were taken at day 1, 14, and 21.

PI/FDA staining

For life/death staining, samples ($n = 3$) were covered with 3 μg/mL FDA (Sigma-Aldrich) and incubated for 15 min at 37°C in the dark. Samples were covered with 0.1 mg/mL PI (Sigma-Aldrich) for 2 min at RT in the dark. The scaffolds were analyzed directly under the fluorescence microscope (Olympus CKX41).

Histology and immunohistochemistry

Proteoglycans, a major component of cartilage tissue, were visualized by Alcian blue and safranin O/fast green staining. At each point in time, cryosections (8 μm; $n = 9$) were prepared, stained using Alcian blue 8GX; pH 2.5 (Roth, Karlsruhe, Germany), and counterstained with nuclear fast red (Sigma-Aldrich). Cryosections ($n = 9$) were stained using 0.7% safranin O staining solution (Sigma-Aldrich) and counterstained with 0.2% fast green (Sigma-Aldrich). To detect collagen type II, sections ($n = 9$) were digested for 30 min with 50 U/mL hyaluronidase (Sigma-Aldrich) at RT, followed by staining with primary rabbit anti-human collagen type II antibody (Acris, Hiddenhausen, Germany) for 40 min, followed by colorimetric detection with 3-amino-9-ethylcarbazole (EnVision™, Dako, Glostrup, Denmark)[21] and counterstaining with hematoxylin (Merk, Darmstadt, Germany).

Osteogenic differentiation of subchondral progenitor cells in PGA/HA scaffolds characterized by deposition of mineralized matrix was excluded by von Kossa staining. Therefore, sections ($n = 9$) were incubated in the dark at RT with 5% silver nitrate (Sigma-Aldrich) for 30 min after incubation with 1.7*M* sodium carbonate/10% formalin (v/v) (Sigma-Aldrich) for 5 min. After washing with tap water for 10 min, counterstaining was performed with nuclear fast red for 4 min. Adipogenic differentiation ($n = 9$ sections) with formation of lipid droplets was excluded using oil red O (Sigma-Aldrich) and hematoxylin for counterstaining.

Reverse transcriptase real-time PCR

RNA was isolated from monolayer (passage 3, designated day 0) and scaffold cultures ($n = 3$) as described previously,[22] and 1 μg of RNA was reverse transcribed with the iScript cDNA Synthesis Kit according to the manufacturer's recommendations (BioRad, Munich, Germany). The relative expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the samples. Real-time RT-PCR was performed in triplicates using the i-Cycler PCR System (BioRad) with 1 μL

TABLE II. Data of Gene Expression Were Given as Mean (n = 3) Percentage of the GAPDH Product and the Lower and Upper Limit of the 95% Confidence Interval

Group	n	Rel. Expression in %GAPDH		
		Mean	CI (95%) Lower Limit	CI (95%) Upper Limit
Aggrecan				
d0	3	0.82	0.66	0.98
Noninduced d7	3	1.05	1.15	0.95
Noninduced d14	3	0.71	0.68	0.74
5% PRP d7	3	1.63	1.55	1.71
5% PRP d14	3	1.13	0.93	1.33
TGFB3 d7	3	3.68	3.35	4.01
TGFB3 d14	3	13.66	12.92	14.40
Cartilage oligomeric matrix protein				
d0	3	2.72	2.59	2.85
Noninduced d7	3	1.06	1.07	1.05
Noninduced d14	3	3.67	3.51	3.83
5% PRP d7	3	2.60	2.45	2.75
5% PRP d14	3	5.36	4.36	6.36
TGFB3 d7	3	48.21	43.94	52.48
TGFB3 d14	3	44.51	41.41	47.61
Collagen type II				
d0	3	0.03	0.02	0.04
Noninduced d7	3	0.07	0.05	0.09
Noninduced d14	3	1.30	1.14	1.46
5% PRP d7	3	1.23	1.17	1.29
5% PRP d14	3	7.01	6.48	7.54
TGFB3 d7	3	22.71	20.96	24.46
TGFB3 d14	3	192.05	177.35	206.75
Collagen type IX				
d0	3	0.11	0.02	0.20
Noninduced d7	3	0.31	0.09	0.53
Noninduced d14	3	1.09	0.78	1.40
5% PRP d7	3	3.10	2.68	3.52
5% PRP d14	3	3.09	2.87	3.31
TGFB3 d7	3	35.89	33.57	38.21
TGFB3 d14	3	330.88	310.67	351.09
Collagen type I				
d0	3	279.97	257.10	302.84
Noninduced d7	3	940.18	853.77	1 026.59
Noninduced d14	3	2 875.59	2 728.43	3 022.76
5% PRP d7	3	2 516.35	2 386.47	2 646.23
5% PRP d14	3	1 161.56	963.91	1 359.21
TGFB3 d7	3	1 610.30	1 519.38	1 701.22
TGFB3 d14	3	1 286.62	1 223.57	1 349.67
Collagen type X				
d0	3	0.03	0.01	0.05
Noninduced d7	3	0.40	0.30	0.50
Noninduced d14	3	4.61	3.36	5.86
5% PRP d7	3	8.81	7.36	10.26
5% PRP d14	3	2.42	2.01	2.83
TGFB3 d7	3	100.10	94.95	105.25
TGFB3 d14	3	286.56	259.17	313.95
Osteocalcin				
d0	3	4.04	3.88	4.20
Noninduced d7	3	2.00	1.91	2.09
Noninduced d14	3	2.86	2.66	3.06
5% PRP d7	3	4.98	4.78	5.18
5% PRP d14	3	5.55	5.53	5.57
TGFB3 d7	3	1.28	1.08	1.48
TGFB3 d14	3	1.60	1.47	1.73
Fatty acid binding protein				
d0	3	2.34	1.17	3.50
Noninduced d7	3	36.59	34.61	38.56
Noninduced d14	3	90.12	85.46	94.78

TABLE II. *Continued*

Group	*n*	Rel. Expression in %GAPDH		
		Mean	CI (95%) Lower Limit	CI (95%) Upper Limit
5% PRP d7	3	49.44	45.84	53.03
5% PRP d14	3	60.85	52.71	68.98
TGFB3 d7	3	0.99	0.82	1.16
TGFB3 d14	3	0.25	0.03	0.48

cDNA/sample and the SYBR Green PCR Core Kit (Applied Biosystems, Forster City, CA). Relative quantification of expression levels of marker genes (Table I) was performed ($n = 3$ per sample) and data were given as percentage of the GAPDH product. The mean was calculated; error bars represent the lower and upper limit of the 95% confidence interval (Table II).

Statistical analysis

Statistical analysis was performed with SigmaStat 3.5 (Systat Software, Germany). Normal distribution and equal variance of gene expression values were analyzed using the Kolmogorov-Smirnov test. Subsequently, the nonparametric Mann-Whitney rank sum test was used. Differences were considered significant at $p < 0.05$.

RESULTS

Cell surface antigen pattern of subchondral progenitor cells

Subchondral progenitor cells (Figure 1) were homogenously positive for CD 73 (99.9–100%), CD 90 (99.8–99.9%), CD 105 (94.1–99.6%), and CD 166 (98.6–99.8%). Subchondral progenitor cells did not present the haematopoietic surface antigen CD 34 (0–0.1%) and the leukocyte common antigen CD 45 (0–0.1%).

Cell distribution of subchondral progenitor cells in PGA-HA scaffolds

One day after seeding of subchondral progenitor cells into PGA-HA scaffolds, cells showed homogeneous distribution between the scaffold fibers [Figure 2(A–D); arrows]. Cells are viable [Figure 2(A)]. Neither staining of scaffold fibers nor staining of fibrin nor hyaluronic acid could be detected after Alcian blue staining [Figure 2(B)]. Safranin O/fast green revealed a slightly green staining of the temporary fibrin matrix [Figure 2(C); encircled]. Antibody staining of collagen type II in day 1 samples showed that there is no collagen type II evident.

Viability of subchondral mesenchymal progenitor cells in PGA-HA scaffolds

PI/FDA staining was used to verify viability of subchondral progenitor cells (green staining) in PGA-HA scaffolds (Figure

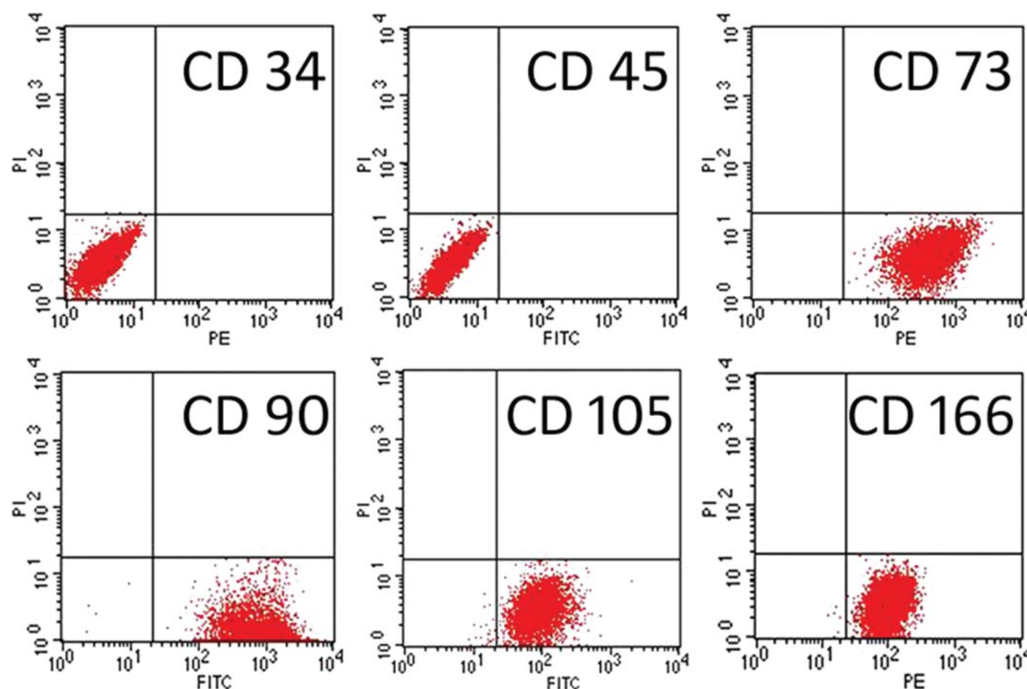

FIGURE 1. Flow cytometric analysis of cultured subchondral progenitor cells. Dot plots obtained from flow cytometric analysis of culture expanded subchondral progenitor cells. Cells were positive for the surface antigens CD 73, CD 90, CD 105, and CD 166 and negative for CD 34 and CD 45. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

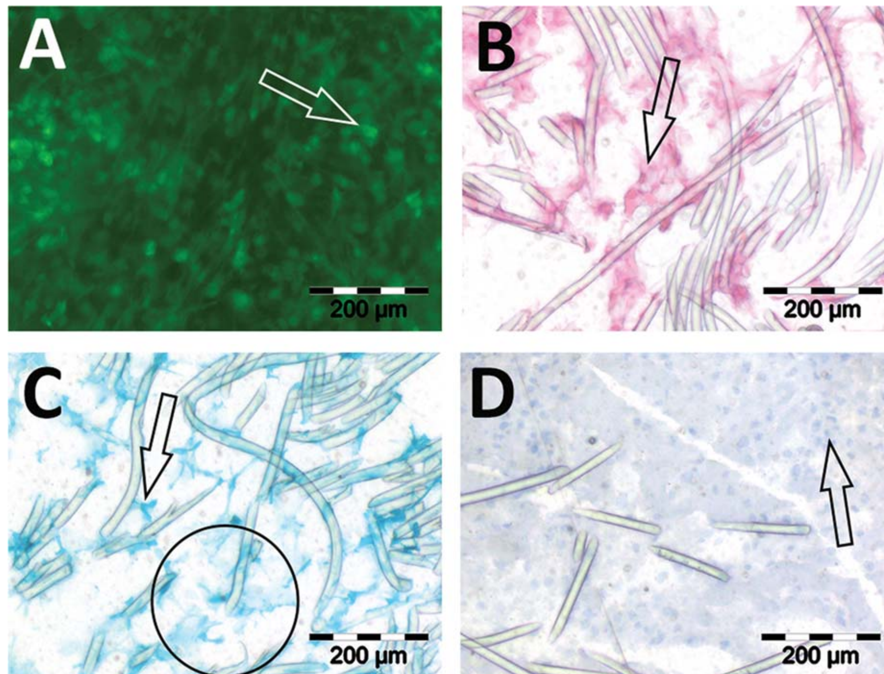

FIGURE 2. Verification of cell distribution in PGA-HA scaffolds 1 day after seeding. PI/FDA staining (A), Alcian blue staining (B), safranin O/fast green staining, and collagen type II antibody staining (C). Arrows show cells within the scaffolds. Staining of fibrin or hyaluronic acid by fast green encircled. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

3). At day 7 and 21, the cells were distributed homogenously and dead cells were not evident (red staining). Cultures, which were stained after 21 days of cultivation, showed red stained scaffold fibers (white arrows) indicating degradation of fibers.

Histological staining of proteoglycans in PGA-HA scaffolds loaded with subchondral progenitor cells

To evaluate formation of extracellular proteoglycans on protein level, PGA-HA scaffolds loaded with subchondral progenitor cells were stained with Alcian blue and safranin O/fast green. In noninduced PGA-HA scaffolds, cells [Figure 4(A), white arrow; Figure 4(C), black arrow] and PGA fibers were evident surrounded by extracellular matrix showing no deposition of proteoglycan as assessed by Alcian blue staining at day 14 [Figure 4(A)] and day 21 [Figure 4(B)]. The matrix showed marginal staining of collagenous components (faint green staining) as assessed by safranin O/fast green staining at day 14 and 21

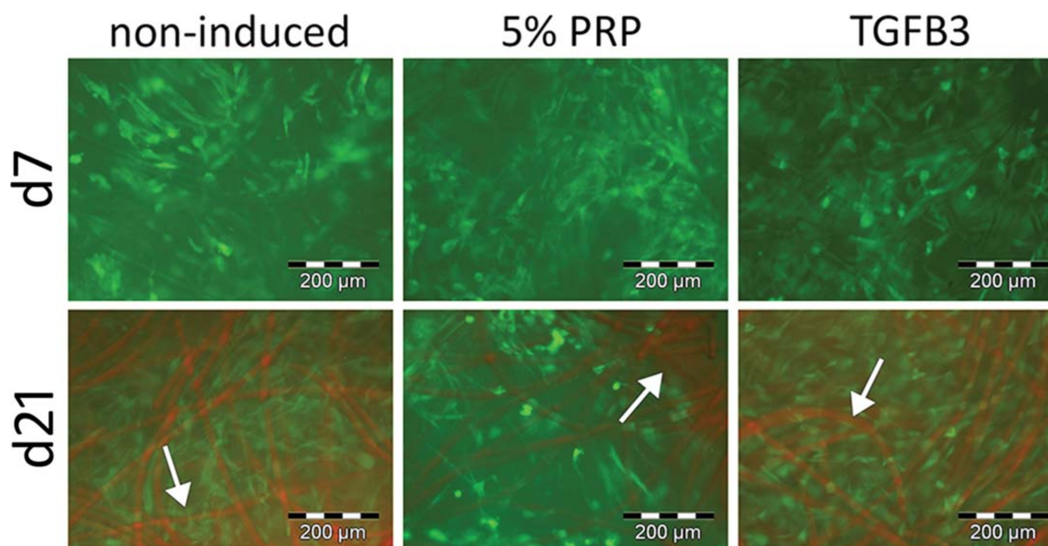

FIGURE 3. PI/FDA staining was used to verify cell viability (green staining) in PGA-HA scaffolds in noninduced, PRP-, or TGFB3-treated groups. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

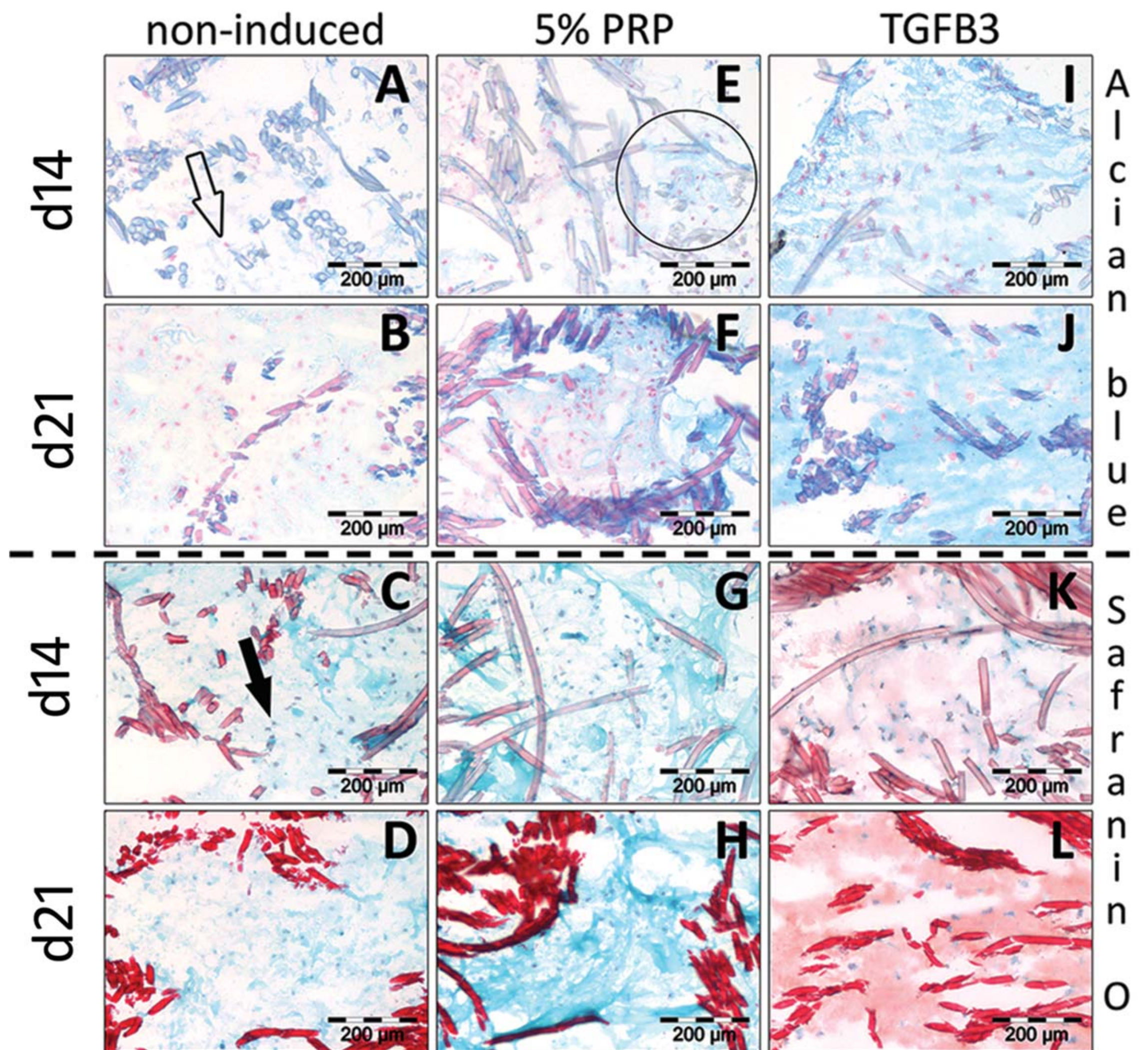

FIGURE 4. Documentation of chondrogenic differentiation of subchondral progenitor cells in three-dimensional PGA-HA scaffolds. The formation of proteoglycan-rich tissue was determined by Alcian blue or safranin O/fast green staining for cell-seeded PGA-HA scaffolds in noninduced, PRP-treated, or TGFB3-treated groups. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

[Figure 4(A,C)]. In the presence of PRP, progenitor cells developed a matrix rich in proteoglycans as indicated by the faint blue alcian blue staining [Figure 4(E,F)]. However, safranin O/fast green staining indicated the development of a collagenous matrix on PRP treatment [Figure 4(G,H)]. The formation of proteoglycans was more pronounced in PGA-HA scaffolds augmented with progenitor cells and stimulated with TGFB3 as assessed by Alcian blue [Figure 4(I,J)] and safranin O/fast green staining [Figure 4(K,L)].

Immunohistochemical staining of collagen type II in PGA-HA scaffolds loaded with subchondral progenitor cells

PGA-HA scaffolds loaded with subchondral progenitors were analyzed for the presence of collagen type II. Cells showed a dark purple color due to hematoxylin counterstaining [Figure 5(A); black arrow]. Collagen type II staining of noninduced PGA-HA scaffolds at day 14 [Figure 5(A)] and day 21 [Figure 5(B)] of cultivation was negative. PGA-HA scaffolds loaded with progenitor cells showed marginal deposition of collagen type II after 14 days of cultivation in the presence of PRP [Figure 5(C); black cycle]. At day 21, collagen type II staining was more pronounced, indicating formation of a cartilaginous extracellular matrix rich in collagen type II on treatment with PRP [Figure 5(D); white arrows]. TGFB3-treated samples showed collagen type II formation at day 14 and 21 [Figure 5(E,F)].

Gene expression analysis of PGA-HA scaffolds loaded with subchondral progenitor cells

To assess early effects of PRP treatment on chondrogenic differentiation of progenitor cells, gene expression analysis for cartilage-related genes was performed at day 0, 7, and 14 (Figure 6).

Human subchondral progenitor cells cultured in PGA-HA scaffolds in the absence of PRP and TGFB3 showed a

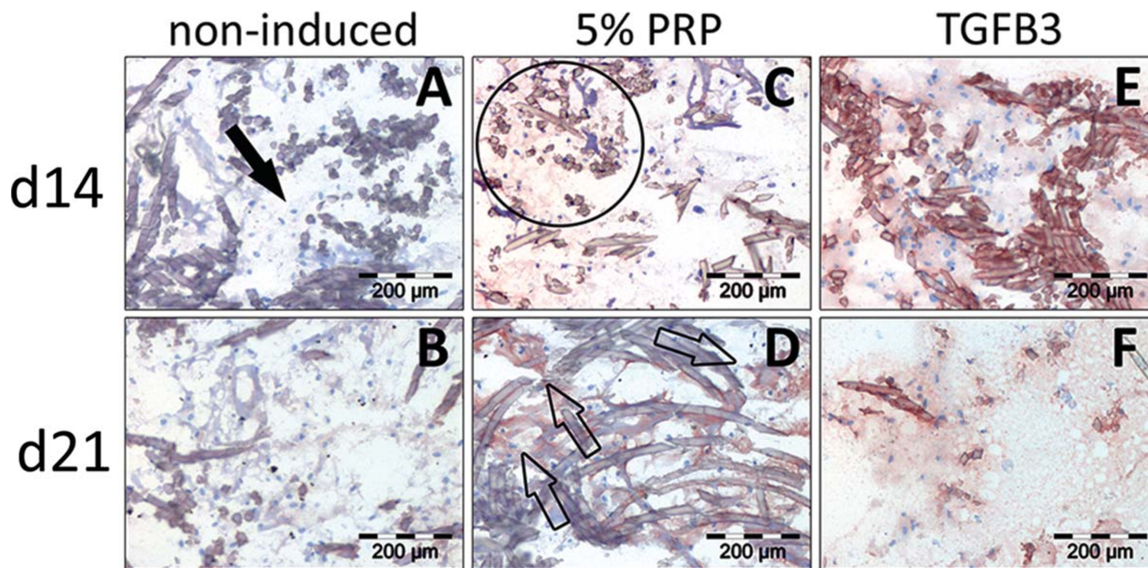

FIGURE 5. Documentation of chondrogenic differentiation of subchondral progenitor cells in three-dimensional PGA-HA scaffolds. The formation of collagen type II was determined by immunohistochemical staining in cell-loaded scaffolds treated without any induction, induced with PRP or TGFB3 over 21 days. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

continuous increase in the expression level of collagen type I from 279% (day 0) in relation to the expression level found for the housekeeping gene GAPDH to 940% (day 7) and 2875% at day 14. The expression of collagen type X was generally low and increased from 0.03% at day up to 4.6% at day 14. Noninduced scaffolds loaded with progenitor cells showed marginal expression of chondrogenic marker genes. The expression level of collagens type II (0.03% at day 0 up to 1.3% at day 14) and type IX (0.11% at day 0 to 1.1% at day 14) showed an increase, whereas the markers aggrecan (0.82% at day 0 to 0.71% at day 14) and cartilage oligomeric matrix protein (COMP; 2.7% at day 0 to 3.7% at day 14) remained stable or were repressed on prolonged culture of nonstimulated subchondral progenitor cells in PGA-HA scaffolds. Treatment of subchondral progenitor cell-augmented PGA-HA scaffolds stimulated with PRP showed a stable expression of aggrecan with an expression level of 1.1% at day 14. The expression of COMP was slightly induced by the addition of PRP from 2.7% at day 0 to a level of 5.4% at day 14. In addition, PRP induced gene expression of collagen type II (from 0.03% at day 0 to 7.1% at day 14) and IX (from 0.1% at day 0 to 3.1% at day 7 and 14). Interestingly, the expression level of collagen type I was high in PRP-treated cultures, but the expression level was induced up to day 7 (2516%) and repressed again at day 14 (1161%). A similar profile was found for collagen type X with an induction of the expression level (0.3–8.8%) up to day 7 and a repression to 2.4% at day 14. Progenitor cells in PGA-HA scaffolds showed induction of the chondrogenic marker genes aggrecan (from 0.8% at day 0 to 13.7% at day 14), COMP (from 2.7% up to 44.5%), and collagen type II (from 0.03% up to 192%) and IX (from 0.1% up to 330.9%) on treatment with TGFB3. The expression level of collagen type I remained on a high level at day 7 (1610%) and day 14 (1287%), whereas the expression level of collagen type X was steadily induced from 0.4% at day 0 to 100% at day 7 and 287% at day 14.

PRP significantly ($p < 0.05$) induced the gene expression levels of the chondrogenic marker genes collagen type II, collagen type IX, aggrecan, and COMP in PGA-HA scaffolds loaded with subchondral progenitor cells at day 7 and day 14, compared with noninduced scaffolds. However, TGFB3-induced PGA-HA scaffolds loaded with subchondral progenitor cells showed a significant ($p < 0.05$) induction of the marker genes collagen type II, collagen type IX, aggrecan, and COMP compared with gene expression levels of PRP-treated and noninduced scaffolds loaded with subchondral progenitor cells at day 7 and 14.

Exclusion of osteogenic and adipogenic differentiation of PGA-HA scaffolds loaded subchondral progenitor cells

For evaluating possible osteogenic or adipogenic differentiation of subchondral progenitor cells on PRP treatment, histological and gene expression analysis of typical adipogenic and osteogenic markers were performed (Figure 7). At day 21, PGA-HA scaffolds loaded with subchondral progenitor cells nonstimulated or stimulated with PRP or TGFB3 showed no calcified matrix as assessed by von Kossa staining [Figure 7(A–C)]. The gene expression level of osteocalcin was low in PGA-HA scaffolds treated with TGFB3 and PRP. Compared to day 0, the expression levels of osteocalcin in nonstimulated or TGFB3-induced subchondral progenitor cells cultured in PGA-HA scaffolds decreased slightly, whereas the expression level remained stable in cultures treated with PRP [Figure 7(G)]. At day 21, subchondral progenitor cell PGA-HA scaffolds nonstimulated or stimulated with PRP or TGFB3 showed no lipid droplets [Figure 7(D–F)]. Treatment of PGA-HA scaffolds seeded with

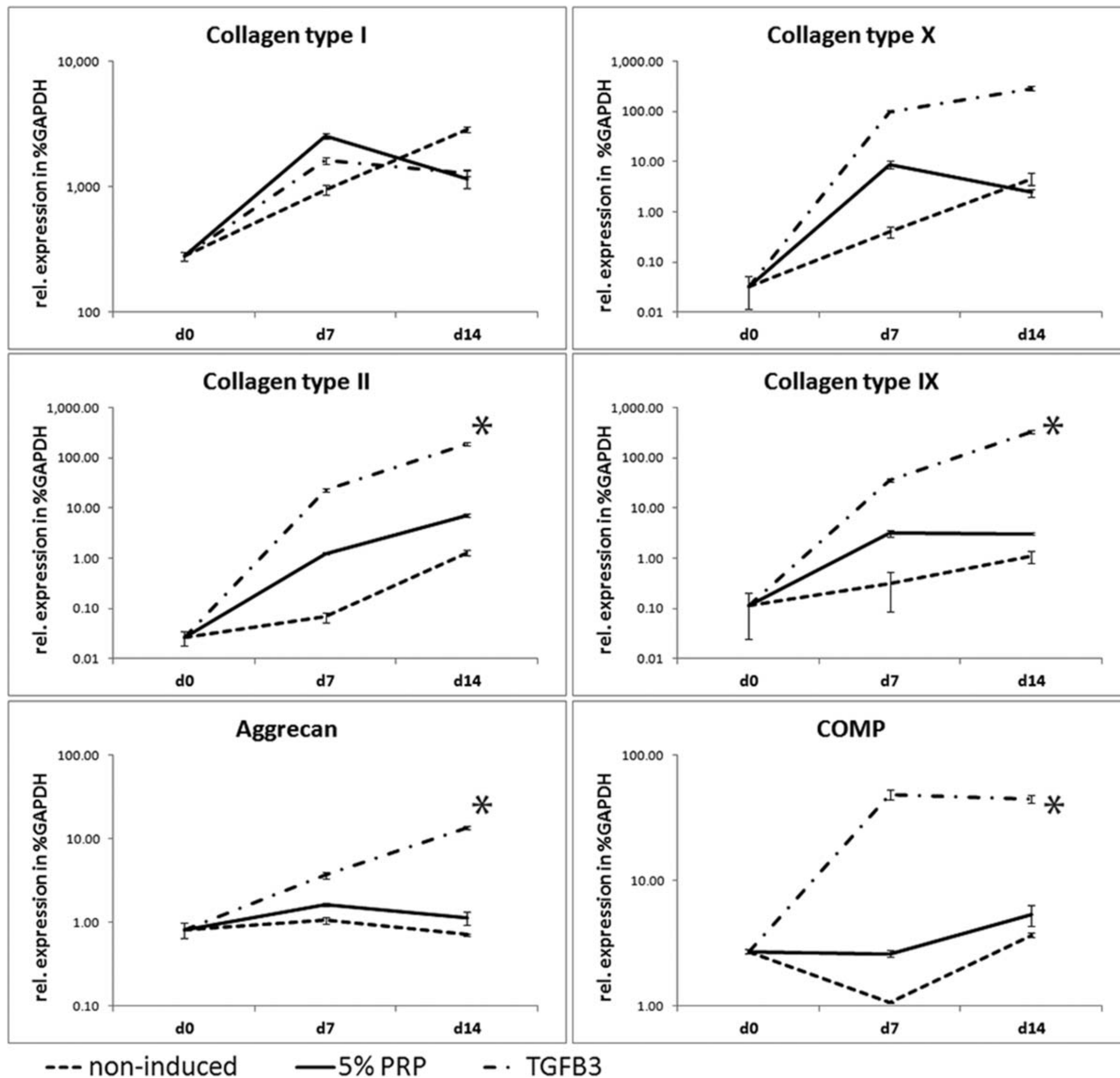

FIGURE 6. Semiquantitative real-time gene expression analysis of subchondral progenitor cells in PGA-HA scaffolds treated with TGFB3 or PRP. The expression level of typical chondrogenic marker genes such as aggrecan, cartilage oligomeric matrix protein (COMP), and collagens type II and type IX was calculated as percentage of the expression level of the housekeeping gene GAPDH. For chondrocyte hypertrophy, the expression level of collagen type X and for fibrous tissue formation, collagen type I were analyzed. Gene expression levels were significantly (*$p < 0.05$) increased in PGA-HA scaffolds loaded with progenitors and treated with TGFB3 compared with PGA-HA if stimulated with PRP or noninduced at day 7 and 14. PRP-treated scaffolds loaded with subchondral progenitor cells showed significantly increased (*$p < 0.05$) gene expression levels compared with noninduced scaffolds at day 7 and 14. The mean ($n = 3$) is represented and error bars represent the lower and upper limit of the 95% confidence interval.

progenitor cells with TGFB3 resulted in a decrease of the expression level of fatty acid binding protein-4 (FABP4), whereas stimulation with PRP had no effect on the expression profile of FABP4 [Figure 7(H)].

DISCUSSION

In the current study, we have shown that human PRP induced the chondrogenic differentiation of human subchondral progenitor cells in three-dimensional PGA-HA scaffolds. In contrast to TGFB3-mediated chondrogenic differentiation that showed a "continuous" increase in chondrogenic marker gene levels and the induction of chondrocyte hypertrophy markers, PRP may induce the chondrogenic developmental sequence to a lesser extent, predominantly in the early phase of stimulation. However, continuous induction of hypertrophic markers like collagen type X is prevented. PRP effectively induced the formation of proteoglycan and collagen-rich cartilage-like matrix. This might depend on that PRP contains a variety of growth factors, cytokines, and chemokines, including TGFB, PDGF, insulin-like growth factors, and interleukins, which are released on activation and clotting of the platelets,[23–25] which are known to affect chondrogenic differentiation in mesenchymal progenitor cells.[26,27] Several *in vitro* studies have demonstrated that human subchondral progenitor cells[3,4] and human trabecular bone-derived cells[28,29] were positive for the antigens CD73, CD90, CD105, and CD166 and have a high

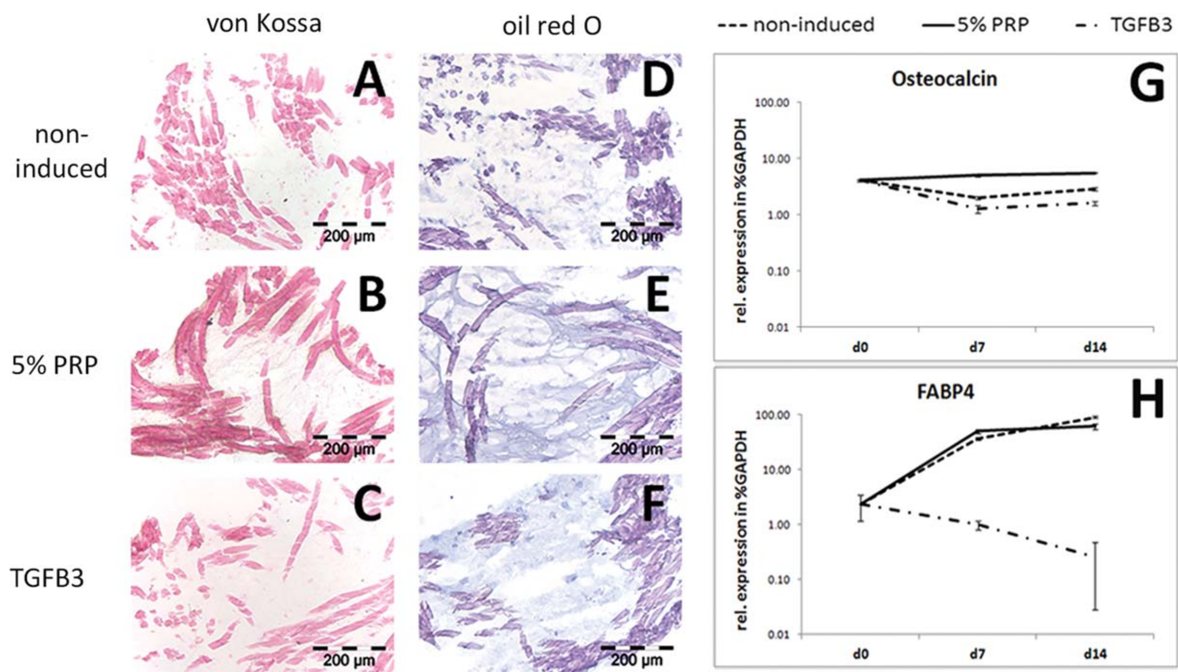

FIGURE 7. Histochemical analysis of matrix mineralization of subchondral progenitor cell-loaded PGA-HA scaffolds. Nonstimulated (A) or stimulated with PRP (B) or TGFB3 (C) showed no matrix mineralization at day 21. The real-time gene expression analysis showed a stable expression of osteocalcin in progenitor cell PGA-HA scaffolds induced with PRP, whereas TGFB3-induced and noninduced samples showed a reduction of osteocalcin expression. Histological analysis of lipid droplets of subchondral progenitor cell-seeded PGA-HA scaffolds noninduced (D) or induced with PRP (E) or TGFB3 (F) showed no evidence for lipid droplets. The real-time gene expression analysis showed an increase in the expression of fatty acid binding protein-4 (FABP4) in PGA-HA scaffolds loaded with progenitor cells comparable to the noninduced group. Induction of subchondral progenitor cell loaded PGA-HA scaffolds with TGFB3 showed a decrease in FABP expression. The mean ($n = 3$) is represented and error bars represent the lower and upper limit of the 95% confidence interval. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

chondrogenic differentiation potential in high-density pellet culture systems. Another study demonstrated that human mesenchymal progenitor cells cultured under serum-free conditions in the presence of hyaluronic acid differentiate along the chondrogenic lineage as shown by the induction of typical chondrogenic marker genes, including aggrecan, COMP, and collagen type II.[30] In this study, PGA-HA scaffolds cultured with PRP and TGFB3 induced the expression of typical chondrogenic marker genes like aggrecan, COMP, and collagen type II, whereas a more fibrocartilage-related markers such as collagen type I remained lower and were repressed. Thereby, the formation of cartilage repair tissue is more pronounced in scaffolds cultured with TGFB3 compared with PRP-treated samples. These findings are in line with a recent study, which showed that PRP exerts chondrogenic differentiation of subchondral progenitor cells in high-density pellet culture with formation of proteoglycan and collagen type II.[15] Because PRP contains high amounts of TGFB isoforms[31] and all isoforms are able to induce the chondrogenesis of mesenchymal stem and progenitor cells,[32,33] it is likely that the high amounts of TGFB may contribute to the PRP-induced chondrogenic differentiation of progenitors in PGA-HA scaffolds. As shown previously, TGFB1 content in PRP has been reported to be between 169.4 ng/mL[17] and 3100 ng/mL.[34] Although PRP is well characterized, no standardized protocol for PRP production is available. Currently, PRP is isolated by centrifugation, plasmapheresis, or commercial PRP isolation Kits that differ in PRP yield and factor concentrations. Therefore, it might be useful to determine the TGFB concentration for each PRP preparation.

In this study, we have shown that PRP may induce a higher expression of chondrogenic marker genes and a more pronounced matrix formation compared to untreated progenitor cell PGA-HA scaffolds. However, the chondrogenic induction seemed to be less in PGA-HA scaffolds seeded with subchondral progenitor cells and treated with PRP compared to TGFB3-treated cultures. Remarkably, progenitor cells cultured in PGA-HA scaffolds in the presence of PRP showed a repression of the gene expression level of the hypertrophic marker gene collagen type X compared with TGFB3-treated cultures. However, it is known that in standard *in vitro* culture systems of mesenchymal stem and progenitor cell differentiation have been shown that the expression of the hypertrophic marker collagen type X is up-regulated, in the presence of TGFB.[32,35] In contrast, it was demonstrated that the expression of collagen type X was inhibited if TGFB3 when combined with growth factors like bone morphogenetic protein-2 (BMP-2), BMP-4, BMP-6, or with hormones like parathyroid hormone-related protein.[36,37] Different bioactive factors in PRP[17,38] may have synergistic or antagonistic effects on TGFB-mediated differentiation and so it may be possible that the suppression of collagen type X is a response of such an effect. Our data suggest that PRP may be beneficial for repair tissue

formation in cartilage repair, in particular in bone-marrow stimulating techniques such as microfracture or drilling by, at least initially, inducing the chondrogenic developmental sequence of subchondral mesenchymal progenitor cells. However, controlled clinical trials have to prove whether the addition of PRP in microfracture-mediated cartilage repair may accelerated or improve cartilage repair compared with microfracture alone or scaffold-assisted microfracture approaches.

In conclusion, PRP as an autologous source of a variety of growth factors supports and/or induces the chondrogenic differentiation of human subchondral mesenchymal progenitor cells in three-dimensional PGA-HA scaffolds used in microfracture-mediated cartilage repair. In addition, the expression level of collagen type X was inhibited in progenitor cell PGA-HA scaffolds treated with PRP compared with cells cultured with TGFB3.

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