### Platelet-Rich Plasma Preparation Types Show Impact on Chondrogenic Differentiation, Migration, and Proliferation of Human Subchondral Mesenchymal Progenitor Cells

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Purpose: To evaluate the chondrogenic potential of platelet concentrates on human subchondral mesenchymal progenitor cells (MPCs) as assessed by histomorphometric analysis of proteoglycans and type II collagen. Furthermore, the migratory and proliferative effect of platelet concentrates were assessed. Methods: Platelet-rich plasma (PRP) was prepared using preparation kits (Autologous Conditioned Plasma [ACP] Kit [Arthrex, Naples, FL]; Regen ACR-C Kit [Regen Lab, Le Mont-Sur-Lausanne, Switzerland]; and Dr.PRP Kit [Rmedica, Seoul, Republic of Korea]) by apheresis (PRP-A) and by centrifugation (PRP-C). In contrast to clinical application, freeze-and-thaw cycles were subsequently performed to activate platelets and to prevent medium coagulation by residual fibrinogen in vitro. MPCs were harvested from the cortico-spongious bone of femoral heads. Chondrogenic differentiation of MPCs was induced in high-density pellet cultures and evaluated by histochemical staining of typical cartilage matrix components. Migration of MPCs was assessed using a chemotaxis assay, and proliferation activity was measured by DNA content. Results: MPCs cultured in the presence of 5% ACP, Regen, or Dr.PRP formed fibrous tissue, whereas MPCs stimulated with 5% PRP-A or PRP-C developed compact and dense cartilaginous tissue rich in type II collagen and proteoglycans. All platelet concentrates significantly (ACP, *P* = .00041; Regen, *P* = .00029; Dr.PRP, *P* = .00051; PRP-A, *P* < .0001; and PRP-C, *P* < .0001) stimulated migration of MPCs. All platelet concentrates but one (Dr.PRP, P = .63) showed a proliferative effect on MPCs, as shown by significant increases (ACP, P = .027; Regen, P = .0029; PRP-A, P = .00021; and PRP-C, P = .00069) in DNA content. Conclusions: Platelet concentrates obtained by different preparation methods exhibit different potentials to stimulate chondrogenic differentiation, migration, and proliferation of MPCs. Platelet concentrates obtained by commercially available preparation kits failed to induce chondrogenic differentiation of MPCs, whereas highly standardized PRP preparations did induce such differentiation. These findings suggest differing outcomes with PRP treatment in stem cell-based cartilage repair. Clinical Relevance: Our findings may help to explain the variability of results in studies examining the use of PRP clinically.

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Datelet-rich plasma (PRP) has been shown to promote chondrogenic differentiation, migration, and proliferation of mesenchymal cell types including multipotent mesenchymal stem and progenitor cells.<sup>1,2</sup> In recent years, PRP has become popular as an easy-toobtain and cost-effective source of autologous bioactive factors for the treatment of a variety of symptoms in sports medicine and orthopaedics.<sup>3</sup> Especially in the management of knee injuries and cartilage repair, PRP is used for intra-articular injection therapy or in combination with bone marrow stimulation techniques to reduce pain and enhance repair. For instance, a recent study reported that injection of PRP in the knee joints of patients with chronic degenerative symptoms showed better results in terms of reducing pain and symptoms, as well as improving articular function, than hyaluronic acid viscosupplementation.<sup>4</sup> Other authors reported significantly reduced pain and improvement in quality of life on repeated injection of PRP in symptomatic knees of patients with tibiofemoral cartilage degeneration, whereas there was no effect on cartilage condition as assessed by magnetic resonance imaging.<sup>5</sup> In a case series of patients with traumatic and degenerative cartilage defects, a resorbable polymer-based cartilage implant immersed with autologous PRP and used to cover tibial and femoral cartilage defects after drilling was shown to effectively improve the patients' situation as assessed by the Knee Injury and Osteoarthritis Outcome Score and to form hyaline-like to hyaline cartilage repair tissue.<sup>6</sup> However, in autologous matrixinduced chondrogenesis, filling of microfractured cartilage defects with PRP gel and subsequent covering with a type I/III collagen membrane resulted in clinical improvement but imperfect defect filling and osteophyte formation in 3 of 5 patients.<sup>7</sup>

Although the results of cartilage repair using PRP are encouraging, there is still a lack of studies that address effectiveness, as well as the underlying biological mechanisms, and that take the great variability in PRP composition, application, and preparation types into account.<sup>8</sup> In addition, variability of PRP may depend on platelet activation methods, as well as donor age and sex.<sup>9</sup> In the past few years, various manual, semiautomatic, and fully automated systems for PRP preparation have been developed and have been made commercially available. The PRP separation systems are based on different preparation methods and result in PRP with different platelet and growth factor compositions and contents.<sup>10,11</sup> Consequently, we used different PRP preparation methods to cover a broad range of platelet concentrates used in clinical routine today.<sup>12-14</sup> Because effective cartilage repair and PRPaugmented stem cell-based cartilage regeneration depend on or may be improved by progenitor cell chondrogenesis, recruitment, and growth, our primary

objective was to evaluate the chondrogenic potential of platelet concentrates on human subchondral mesenchymal progenitor cells (MPCs) as assessed by quantitative histomorphometric analysis of the developed matrix. Furthermore, the migratory and proliferative effect of platelet concentrates were assessed.

### Methods

### **Preparation of Platelet Concentrates**

PRP was prepared using the Autologous Conditioned Plasma (ACP) Kit (double-syringe system; Arthrex, Naples, FL), the Regen ACR-C Kit (gel separator system; Regen Lab, Le Mont-Sur-Lausanne, Switzerland), and the Dr.PRP Kit (1-kit system; Rmedica, Seoul, Republic of Korea) according to the manufacturers' instructions. PRP obtained by apheresis (PRP-A) was provided by the Department of Transfusion Medicine, Charité-Universitätsmedizin Berlin, Germany, using an automated blood collection system (Trima Accel; CaridianBCT, Lakewood, CO). PRP prepared from buffy coats by centrifugation (PRP-C) was provided by the German Red Cross (Berlin, Germany). PRP characteristics are given in Table 1. In contrast to clinical practice, all platelet concentrates were pooled (n = 3) in equal amounts after preparation and stored at  $-20^{\circ}$ C. To activate platelets and to prevent medium coagulation by residual fibrinogen, 3 freeze-and-thaw cycles were performed as described previously.<sup>15</sup> In brief, all frozen platelet concentrates were thawed slowly at 4°C and centrifuged at 1,600g for 10 minutes, and the resulting supernatant was stored at  $-20^{\circ}$ C overnight. After a second freeze-andthaw cycle, the supernatant was again harvested and stored at  $-20^{\circ}$ C. Before use, a third freeze-and-thaw cycle was performed, and the supernatant was used immediately for further experiments. The total protein content of each PRP pool was determined using a bicinchoninic acid assay (Sigma-Aldrich, St Louis, MO) according to the manufacturer's recommendations. The local ethics committee approved the study.

### Isolation and Culture of Human Subchondral MPCs

Human subchondral MPCs were isolated from cortico-spongious bone of human femoral heads post mortem (3 donors [1 female and 2 male donors], aged 53 to 57 years) as described previously.<sup>2</sup> In brief, cortico-spongious bone fragments were digested using 256 U/mL of collagenase XI (Sigma-Aldrich) for 4 hours at 37°C; placed in Primaria cell culture flasks (BD Biosciences, San Jose, CA); and cultured in Dulbecco modified Eagle (DME) medium (Biochrom, Berlin, Germany) containing 10% human serum (German Red Cross), 100 µg/mL of streptomycin (Biochrom), 100 U/mL of penicillin (Biochrom), 100 µg/mL of gentamicin (Biochrom), 0.1 µg/mL of

	ACP	Regen	Dr.PRP	PRP-A	PRP-C
No. of donors	3	3	3	3	12-18 (3 batches)*
Age, yr	27-35	34-39	34-39	Anonymous donors	Anonymous donors
Sex	2 male and 1 female	2 male and 1 female	2 male and 1 female	Anonymous donors	Anonymous donors
Blood withdrawal per donor, mL	15	8	18	Unknown <sup>†</sup>	Unknown <sup>†</sup>
PRP volume per donor, mL	3-4	3-4	3-4	60-90	40-90
Platelet counts <sup>‡</sup>	2- to 3-fold increased platelet concentration	Platelet yield >80%	Platelet concentration of 94%	$0.6-1.3 \times 10^{10}/mL$	$0.7-1.8 \times 10^9/mL$
Leukocyte counts <sup>‡</sup>	Not measured <sup>§</sup>	Not measured <sup>§</sup>	Not measured <sup>§</sup>	$<\!0.3\times10^4/mL$	${<}0.5\times10^4/mL$
ACP, Autologous Con	ditioned Plasma; PRP, plat	elet-rich plasma; PRP-A,	, platelet-rich plasma obta	ined by apheresis; PRP-0	C, platelet-rich plasma

#### Table 1. Characteristics of PRP Pools

obtained by centrifugation.

\*Each batch was prepared by the German Red Cross from 4 to 6 anonymous blood donors with the same blood type.

<sup>†</sup>Blood withdrawal was performed by Transfusion Medicine Berlin Charité or the German Red Cross.

<sup>‡</sup>Manufacturers' information.

<sup>§</sup>Usually not measured in clinical routine.

amphotericin B (Biochrom), and 2 ng/mL of human fibroblast growth factor 2 (FGF-2) (PeproTech, Hamburg, Germany). At 80% to 90% confluence, cells were subcultivated using trypsin (0.05% vol/vol in phosphate-buffered saline solution; Biochrom) and replated at a density of 6,000 cells/cm<sup>2</sup>. Medium was exchanged every 2 to 3 days.

### PRP-Mediated Chondrogenic Differentiation of Human Subchondral MPCs

Chondrogenic differentiation of MPCs (passage 3) was performed under serum-free conditions in highdensity pellet cultures (pool of 3 donors; 250,000 cells per pellet) as described previously.<sup>16</sup> In brief, MPC pellets (n = 3 per experimental group) were cultured in complete DME medium containing 1% ITS+1 (insulintransferrin-selenium), 1-mmol/L sodium pyruvate, 0.35-mmol/L L-proline, 0.17-mmol/L L-ascorbic acid-2-phosphate, and 0.1-µmol/L dexamethasone (all Sigma-Aldrich), as well as 5% (vol/vol) PRP. MPC pellets cultured in complete DME medium without PRP served as controls (n = 3). Four-fifths of the medium was exchanged every second day, and pellet cultures were maintained for 28 days. For histochemical and immunohistochemical staining of typical cartilage components, pellets were embedded in OCT compound (Sakura, Alphen aan den Rijn, Netherlands) and frozen, and cryo-slides (6  $\mu$ m) were prepared and stained with H&E. Proteoglycans were stained with Alcian Blue 8GX (Roth, Karlsruhe, Germany) at pH 2.5, followed by counterstaining with nuclear fast red (Sigma-Aldrich), as well as staining with 0.7% safranin O solution and counterstaining with 0.2% fast green (both Sigma-Aldrich). For type II collagen staining, cryo-slides were incubated for 40 minutes with mouse antihuman type II collagen antibodies (Acris, Herford, Germany).

Mouse IgG served as control (DAKO, Hamburg, Germany). Detection was performed according to the manufacturer's instructions using the EnVision++ System HRP Kit (DAKO), followed by counterstaining with hematoxylin. For each staining, 3 slides per pellet and experimental condition were used and 3 pellets per experimental group were analyzed. As an outcome measure for platelet concentrate-mediated chondrogenic differentiation, formation of cartilaginous matrix was visualized by alcian blue, safranin O, and type II collagen staining and quantified by histomorphometric analysis. Quantitative histomorphometric analysis was performed using Adobe Photoshop software (Adobe Systems, San Jose, CA) as described previously.<sup>17</sup> In brief, a standard color was defined that represents the particular color of the specific staining. The tools "magic wand" and "select similar" were used to select areas of that particular color. The amount of stained pixels in relation to the total amount of pixels of the section gives the percentage of the area positively stained for proteoglycans or type II collagen.

## PRP-Mediated Migration of Human Subchondral MPCs

Migration of MPCs (pool of 3 donors, passage 3) on stimulation with PRP was analyzed in 96-multiwell ChemoTX plates (8- $\mu$ m pore size of polycarbonate membranes; Neuro Probe, Gaithersburg, MD) in triplicate as described previously.<sup>2</sup> In brief, 3 × 10<sup>4</sup> MPCs in DME medium containing 0.1% human serum (German Red Cross), 100  $\mu$ g/mL of streptomycin, and 100 U/mL of penicillin were seeded in the upper wells. The lower wells were filled with 0% to 25% PRP in DME medium containing 0.1% human serum and antibiotics. After 20 hours' incubation at 37°C, cells that migrated through the polycarbonate membrane were fixed with acetone/methanol (1:1, vol/vol). Nonmigrating cells on top of the membrane were removed. Migrated cells were stained for 3 minutes with Hemacolor Rapid Stain (Merck, Darmstadt, Germany) and counted microscopically. Three representative photographs (left, right, and central) of each well were taken, migrated cells per picture were counted using ImageJ (National Institutes of Health, Bethesda, MD), and the total number of migrated cells was extrapolated to the total well.

# PRP-Mediated Proliferation of Human Subchondral MPCs

MPCs (pool of 3 donors, passage 3) were seeded in T25 cell culture flasks (4,000 cells/cm<sup>2</sup>) in DME medium containing 1% ITS+1 for 24 hours. The medium was replaced by DME medium containing ITS+1 and 5% (vol/vol) of individual PRP preparations (n = 3 per point in time and experimental group). Cells cultured in DME medium containing 1% ITS+1 without PRP served as controls (n = 3 per point in time). Medium was exchanged every 3 days, and cells were maintained for up to 9 days. For DNA analysis, cells were harvested using trypsin. The resulting cell pellet was incubated with 1 mL of papain-cysteine hydrochloride (0.125 mg/mL in distilled water) for 16 hours at 60°C. The supernatant was stored at  $-20^{\circ}$ C. Samples were diluted (1:2 to 1:8, vol/vol) in 2-mol/L sodium chloride and 0.05-mmol/L sodium hydrogen phosphate (Sigma-Aldrich). Serial dilutions of calf thymus DNA (Life Technologies, Grand Island, NY) were used as a standard. One hundred microliters of standard or sample was mixed with 100 µL of  $0.67 \mu g/mL$  of bisbenzimide (Invitrogen, Carlsbad, CA) and measured at 360 nm with light emission at 460 nm. The calf thymus DNA values were used to calculate the total DNA content of samples.

### Measurement of Candidate Chondrogenic Growth Factor Content in PRP by Enzyme-Linked Immunosorbent Assay

Growth factor content of PRP was measured in triplicate using commercially available sandwich enzymelinked immunosorbent assay (ELISA) systems. To quantify the content of bone morphogenetic protein 2 (BMP-2; PeproTech), FGF-2 (PeproTech), connective tissue growth factor (CTGF; PeproTech), and transforming growth factor  $\beta_3$  (TGF- $\beta_3$ ; R&D Systems, Minneapolis, MN), ELISAs were performed according to the manufacturers' recommendations and as described in detail previously.<sup>18</sup>

### **Statistical Analysis**

Statistical analysis was performed with SigmaStat, version 3.5 (Systat Software, Erkrath, Germany). Because we obtained only a small number of random

samples (e.g., 3 values of migrated cells), the central limit theorem was used for data interpretation of the population. This theorem implies that 1 random variable X (e.g., 1 value of migrated cells) is the sum of a large number of independent random variables (e.g., blood donor characteristics and temperature), which follow a stable distribution. On the basis of this assumption, it is assumed that variable X (e.g., 1 value of migrated cells) is nearly normally distributed.<sup>19</sup> Regarding our measured values (3 per experiment), we supposed that our values were a product of probability and repeated measurements would lead to normal distribution of our data. Therefore all values were considered normally distributed, and parametric significance tests were performed.

For analysis of cell migration data, 1-way analysis of variance was used, followed by the all-pairwise multiple comparisons procedure (Holm-Sidák method). For analysis of cell proliferation (DNA content) and extracellular matrix content (histomorphometry), 1way analysis of variance was performed, followed by the Student-Newman-Keuls post hoc test. Data are presented as mean values, 95% confidence intervals are plotted, and exact *P* values are given in Tables 2-4. Histomorphometric and migration data of each single PRP preparation group were analyzed independently and compared with a nonstimulated control but not among each other. Data obtained by proliferation assays were analyzed using independent within-group comparisons by comparing DNA content on days 3, 6, and 9 with corresponding day 0 data (before simulation). Significant differences were considered at P < .05.

#### Results

#### Determination of Total Protein Content of PRP Concentrates

The highest protein content was found in the pool of ACP (89.7 mg/mL), followed by PRP-A (84.3 mg/mL), Dr.PRP (78.8 mg/mL), Regen (58.8 mg/mL), and PRP-C (38.1 mg/mL).

### Tissue-Forming Effects of PRP on Human Subchondral MPCs

MPCs stimulated with PRP and nonstimulated controls formed tissues with 28 days of pellet culture (Fig 1). Macroscopically, stimulation with PRP enlarged pellets compared with nonstimulated controls. The largest pellets were found on stimulation of MPCs with ACP, followed by Regen, PRP-A, PRP-C, and Dr.PRP. H&E staining showed that controls and MPCs stimulated with PRP-A or PRP-C evolved a compact and dense pellet rich in cells with negligible amounts of fibrous tissue in the outer layers of the pellets. In

#### Table 2. Histomorphometric Quantification Data

Comparison	% St	tained Area	Student-Newman-Keul
v Control	Mean	95% CI	P Value <sup>*</sup>
Alcian blue			
(proteoglycan)			
Control	0.21	0.13-0.29	—
ACP	0.01	0.00-0.02	.99
Dr.PRP	0.02	0.01-0.03	.98
Regen	0.44	0.34-0.54	.0038
PRP-A	25.00	20.05-29.95	.00012
PRP-C	7.08	6.08-8.08	.00012
Safranin O			
(proteoglycan)			
Control	0.01	0.00-0.02	—
ACP	0.01	0.00-0.02	.99
Dr.PRP	0.02	0.03-0.01	.99
Regen	0.04	0.00-0.08	.99
PRP-A	19.63	9.43-29.82	.00013
PRP-C	1.43	0.15-2.71	.97
Type II collagen			
Control	0.16	0.05-0.27	_
ACP	0.36	0.08-0.64	.99
Dr.PRP	0.16	0.00-0.45	.99
Regen	0.10	22.87-52.41	.98
PRP-A	37.64	1.56-3.82	.00013
PRP-C	2.69	0.66-4.72	.012

NOTE. The proportion of areas stained for cartilage matrix components in mesenchymal progenitor cell pellet cultures on platelet-rich plasma treatment, as well as 95% confidence intervals and *P* values according to 1-way analysis of variance, followed by multiple comparisons procedures (Student-Newman-Keuls method), is given.

ACP, Autologous Conditioned Plasma; CI, confidence interval; PRP-A, platelet-rich plasma obtained by apheresis; PRP-C, platelet-rich plasma obtained by centrifugation.

\*Significance was defined as P < .05.

contrast, MPCs stimulated with ACP or Regen developed a small core of dense tissue surrounded by a distinct amount of loose fibrous matrix (Fig 1). MPCs stimulated with Dr.PRP developed fibrous tissue with some small cell clusters (Fig 1).

## PRP-Mediated Chondrogenic Differentiation of Human Subchondral MPCs

To assess PRP-mediated chondrogenic differentiation of MPCs, cartilaginous matrix components were stained and quantified. The amount of stained matrix stimulated by each individual PRP was compared with a nonstimulated control. MPCs cultured in high-density pellets for 28 days showed chondrogenic differentiation on stimulation with PRP-A and PRP-C, whereas nonstimulated controls and MPCs stimulated with ACP, Regen, or Dr.PRP showed no signs of chondrogenesis (Fig 2). Proteoglycans as assessed by alcian blue staining were evident in pellets stimulated with PRP-A and PRP-C and weakly evident in MPC pellets treated with Regen (Fig 2). Pellets stimulated with ACP or Dr.PRP and nonstimulated controls showed no proteoglycans.

<b>Table 5.</b> Migration Date	Table	e 3.	Migration	Data
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	No. of	Migrated Cells		
Comparison v 0%	Mean	95% CI	Holm-Šidák P Value	
0%	4,567	4,181-4,953	_	
ACP				
2.5%	11,585	10,680-12,490	.00041	
5.0%	12,653	11,546-13,760	< .0001	
10.0%	11,418	9,583-13,253	.00036	
25.0%	8,610	7,719-9,501	.027	
Regen				
2.5%	11,537	9,010-14,064	.00029	
5.0%	14,936	13,641-16,231	< .0001	
10.0%	16,540	14,913-18,167	< .0001	
25.0%	13,857	8,062-19,652	< .0001	
Dr.PRP				
2.5%	11,995	8,953-15,037	.00051	
5.0%	4,833	3,421-6,245	.88	
10.0%	4,397	1,634-7,160	.92	
25.0%	3,074	2,775-3,373	.40	
PRP-A				
2.5%	13,798	11,138-16,458	< .0001	
5.0%	16,281	13,897-18,665	< .0001	
10.0%	16,643	15,305-17,981	< .0001	
25.0%	5,964	3,088-8,840	.43	
PRP-C				
2.5%	17,035	13,654-20,416	< .0001	
5.0%	15,653	12,214-19,092	< .0001	
10.0%	19,370	18,399-20,341	< .0001	
25.0%	17,345	13,861-20,829	< .0001	

NOTE. The number of migrated mesenchymal progenitor cells on platelet-rich plasma treatment, as well as 95% confidence intervals and *P* values according to 1-way analysis of variance, followed by multiple comparisons procedures (Holm-Šidák method), is given.

ACP, Autologous Conditioned Plasma; CI, confidence interval; PRP-A, platelet-rich plasma obtained by apheresis; PRP-C, platelet-rich plasma obtained by centrifugation.

\*Significance was defined as P < .05.

Safranin O staining of sulfated proteoglycans was evident in MPC pellets treated with PRP-A, whereas all other samples were negative. Type II collagen was found in pellets cultured with PRP-A and weakly on stimulation with PRP-C (Fig 2). All other groups showed no type II collagen.

Histomorphometric quantification (Table 2) showed a significant increase in proteoglycans visualized by alcian blue staining in pellets cultured with PRP-A (mean, 25.00%), PRP-C (mean, 7.08%), and Regen (mean, 0.44%) compared with controls (mean, 0.21%). MPC pellets treated with ACP (mean, 0.01%) or Dr.PRP (mean, 0.02%) showed no significant increase in proteoglycans compared with controls. Quantification of sulfated proteoglycans (safranin O staining) showed a significant increase in proteoglycan-rich areas in pellets stimulated with PRP-A (mean, 19.63%), whereas MPC pellets treated with ACP (mean, 0.01%), Regen (mean, 0.04%), Dr.PRP (mean, 0.02%), or PRP-C (mean, 1.43%) showed no significant increase compared with controls (mean, 0.01%). Type II collagen was present and significantly increased in MPC pellets treated with

Та	ble	4.	Prol	ifera	tion	Data	
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Comparison <i>v</i>	Tot Conte	al DNA nt, μg/mL	Student-Newman-Keu	
Day 0 of Group	Mean	95% CI	P Value*	
Control				
Day 0	1.98	1.57-2.39	_	
Day 3	1.76	1.56-1.96	.39	
Day 6	1.78	1.18-2.38	.62	
Day 9	1.50	1.30-1.70	.11	
ACP				
Day 0	1.94	1.85-2.03	—	
Day 3	2.48	2.07-2.89	.0024	
Day 6	2.59	2.48-2.70	.016	
Day 9	2.37	2.26-2.48	.027	
Regen				
Day 0	2.04	1.85-2.23	—	
Day 3	2.15	2.00-2.30	.79	
Day 6	3.65	2.97-4.33	.0098	
Day 9	4.22	3.37-5.07	.0029	
Dr.PRP				
Day 0	1.93	1.82-2.04	—	
Day 3	1.70	1.44-1.96	.16	
Day 6	1.70	1.55-1.85	.065	
Day 9	2.00	1.77-2.23	.63	
PRP-A				
Day 0	2.17	1.98-2.36	—	
Day 3	2.70	2.46-2.94	.017	
Day 6	3.83	3.46-4.20	.00021	
Day 9	4.48	4.39-4.57	.00021	
PRP-C				
Day 0	2.15	1.72-2.58	—	
Day 3	2.57	2.38-2.76	.043	
Day 6	3.26	2.75-3.77	.017	
Day 9	4.31	4.05-4.57	.00069	

NOTE. The DNA content of mesenchymal progenitor cells on platelet-rich plasma treatment, as well as 95% confidence intervals and P values according to 1-way analysis of variance, followed by multiple comparisons procedures (Student-Newman-Keuls method), is given.

ACP, Autologous Conditioned Plasma; CI, confidence interval; PRP-A, platelet-rich plasma obtained by apheresis; PRP-C, platelet-rich plasma obtained by centrifugation.

\*Significance was defined as P < .05.

PRP-A (mean, 37.64%) and PRP-C (mean, 2.69%) compared with controls (mean, 0.16%). ACP (mean, 0.36%), Dr.PRP (mean, 0.16%), and Regen (mean, 0.10%) showed no significant increase compared with controls (mean, 0.16%).

### PRP-Mediated Migration of Human Subchondral MPCs

To assess PRP-mediated migration of MPCs, the number of cells that migrated on treatment with each single PRP was compared with a nonstimulated control (0% PRP). All PRP preparations showed migratory effects to different extents on MPCs (Fig 3, Table 3). Stimulation of MPCs with 2.5% to 25.0% ACP, Regen, or PRP-C significantly increased the number of

migrated cells (ranging from a mean of 8,610 to 19,370) compared with nonstimulated controls (mean, 4,567 spontaneously migrating cells). MPCs treated with 2.5% to 10.0% PRP-A showed significantly increased numbers of migrated cells (ranging from a mean of 13,798 to 16,643), whereas only low amounts of Dr.PRP (2.5%) resulted in significantly increased cell migration (mean, 11,995 cells) compared with the nonstimulated control.

## PRP-Mediated Proliferation of Human Subchondral MPCs

To determine PRP-mediated growth of MPCs, DNA content was measured. In a within-group comparison, DNA content measured on days 3, 6, and 9 was compared with the content on day 0. Stimulation of MPC growth was evident after stimulation with ACP, Regen, PRP-A, and PRP-C, whereas Dr.PRP had no effect (Fig 4, Table 4). Nonproliferating controls showed relatively stable DNA content (mean, 1.98 µg/mL on day 0; mean, 1.50 µg/mL on day 9). MPCs stimulated with ACP (mean, 2.48  $\mu$ g/mL on day 3; mean, 2.37  $\mu$ g/ mL on day 9), PRP-A (mean, 2.70 µg/mL on day 3; mean, 4.48  $\mu g/mL$  on day 9), and PRP-C (mean, 2.57  $\mu$ g/mL on day 3; mean, 4.31  $\mu$ g/mL on day 9) showed significantly elevated DNA content on day 3 to day 9, as compared with the content found on day 0. Stimulation of MPCs with Regen resulted in significantly increased amounts of DNA on day 6 (mean, 3.65 µg/mL) and day 9 (mean, 4.22  $\mu$ g/mL) compared with day 0 (mean, 2.04 µg/mL). The DNA content in MPCs treated with Dr.PRP remained unchanged during the 9 days of cell culture (mean, 1.93 µg/mL on day 0; mean, 2.00 µg/mL on day 9).

# Measurement of Candidate Chondrogenic Growth Factor Content in PRP by ELISA

The content of bioactive factors (BMP-2, CTGF, FGF-2, and TGF- $\beta_3$ ) in PRP preparation pools varied (Fig 5). ACP (mean, 1.10 ng/mL) and PRP-C (mean, 1.11 ng/mL) showed CTGF, whereas BMP-2, FGF-2, and TGF- $\beta_3$  could not be detected in both concentrates. CTGF was highest in Regen (mean, 4.01 µg/mL), followed by Dr.PRP (mean, 3.75 ng/mL) and PRP-A (mean, 1.80 ng/mL). BMP-2 could be found in Regen (mean, 2.32 ng/mL), Dr.PRP (mean, 1.58 ng/mL), and PRP-A (0.51 ng/mL). The mean FGF-2 content was 0.71 ng/mL in Regen, 0.34 ng/mL in Dr.PRP, and 0.19 ng/mL in PRP-A. TGF- $\beta_3$  was highest in Regen (mean, 1.15 ng/mL), followed by Dr.PRP (mean, 0.71 ng/mL) and PRP-A (0.55 ng/mL).

#### Discussion

In this study it has been shown that PRP preparation types have an impact on the chondrogenic

Fig 1. Tissue-forming effects of platelet concentrates on human subchondral mesenchymal progenitor cells (MPCs) in pellet culture: H&E staining and macroscopic view of highdensity pellet cultures of MPCs stimulated for 28 days with platelet concentrates. MPC pellet cultures show increase in pellet size and formation of dense, compact tissue (arrowheads): small cell clusters (double arrowhead); and/or fibrous tissue (asterisks), depending on the type of platelet concentrate. (ACP, Autologous Conditioned Plasma; PRP-A, platelet-rich plasma obtained by apheresis; PRP-C, platelet-rich plasma obtained by centrifugation.)



differentiation, migration, and proliferation of human subchondral MPCs. PRP prepared with commercially available kits failed to induce chondrogenesis of subchondral MPCs in high-density pellet cultures, whereas stimulation of cells with standardized preparations of PRP provided by the Department of Transfusion Medicine, Charité-Universitätsmedizin Berlin, and the German Red Cross initiated the chondrogenic differentiation sequence. All tested platelet concentrates stimulated migration of MPCs, and all but 1 preparation enhanced proliferation of MPCs. The findings suggest that PRP-mediated chondrogenesis may rely on which PRP is used and how it is prepared. Clinically, PRP made by apheresis or centrifugation might have positive effects on cartilage formation, whereas PRP prepared by kit systems might have no chondrogenic effect but may support MPC migration and proliferation. Key biological effects of PRP on the pathologic process of cartilage and thus on cartilage repair have been reviewed recently,<sup>20</sup> suggesting that PRP may exert its beneficial effect on cartilage repair by stimulating stem cell migration, proliferation, and chondrogenic differentiation. In a case series of patients with traumatic and degenerative cartilage defects, a resorbable polymer-based cartilage implant immersed with autologous PRP and used to cover tibial and femoral cartilage defects after drilling has been shown to effectively improve the patients' situation as assessed by the Knee Injury and Osteoarthritis Outcome Score and to form hyaline-like to hyaline cartilage repair tissue.<sup>6</sup> However, in autologous matrix-induced chondrogenesis, filling of microfractured cartilage defects with PRP gel and subsequent covering with a type I/III collagen membrane resulted in clinical improvement but imperfect defect filling and osteophyte formation in 3 of 5 patients.<sup>7</sup> The chondrogenic potential of PRP on mesenchymal progenitors is variable. The migratory effect of PRP on human subchondral MPCs is in line with reports showing recruitment of bone marrow-derived mesenchymal stem cells with PRP treatment.<sup>21,22</sup> In contrast, another group showed that the migratory effect of PRP on human bone marrow mesenchymal stem cells is reduced compared with fetal calf serum,<sup>23</sup> whereas it has been shown that PRP made by apheresis and used in a broad range of concentrations leads to higher numbers of migrating progenitors than human serum.<sup>2</sup>

The mitogenic activity of PRP on stem and progenitor cells is well documented<sup>24,25</sup> and has been confirmed

Alcian blue Safranin O **Collagen Type II** Control ACP REGEN Dr.PRP **PRP-A** PRP-C

Fig 2. Chondrogenic effects of platelet concentrates on human subchondral mesenchymal progenitor cells (MPCs) in pellet culture. Chondrogenic effects have been evaluated by histochemical analysis of extracelcartilaginous lular matrix formation by MPCs on stimulation with platelet concentrates for 28 days in pellet culture. Alcian blue, safranin O, and type II collagen staining showed that MPCs stimulated with plateletrich plasma obtained by apheresis (PRP-A) and with platelet-rich plasma obtained by centrifugation (PRP-C; circle) formed a cartilaginous matrix with proteoglycans and type II collagen. Only a weak staining with alcian blue was evident in MPC pellets treated with Regen (arrowhead). Nonstimulated controls and MPCs stimulated with Autologous Conditioned Plasma (ACP) and Dr.PRP showed no chondrogenic differentiation and no formation of cartilage matrix.

by our study, showing that PRP prepared by different methods leads to growth of human subchondral progenitor cells. Interestingly, PRP prepared using the Dr.PRP Kit failed to induce growth of progenitors. Because Dr.PRP showed no stimulation of progenitor cell migration when higher doses (5% to 25%) were used, the lack of growth-promoting activity on stimulation with 5% Dr.PRP may be due to inhibiting agents or insufficient growth factor content at a particular dose. However, compared with other PRP preparations, the growth factor content of Dr.PRP appeared not to be abnormal.

Because various PRP preparation methods are used in clinical routine and the resulting platelet concentrates



**Fig 3.** Migratory effects of platelet concentrates on human subchondral mesenchymal progenitor cells. Migratory effects have been quantified by enumerating the number of migrating mesenchymal progenitor cells. Stimulation with Autologous Conditioned Plasma (ACP), Regen, and Dr.PRP, as well as platelet-rich plasma obtained by apheresis (PRP-A) or by centrifugation (PRP-C), increases—to different extents—the number of migrating human subchondral mesenchymal progenitor cells. Asterisks indicate a significant (P < .05) increase compared with controls (0%). The bars show the mean (n = 3) and 95% confidence interval.

are obviously not comparable regarding biological activity, the outcomes after PRP treatments are not necessarily predictable. This may explain the variability of results in clinical trials examining the effect of PRP treatment in osteoarthritic knee joints.<sup>26</sup>

In contrast to clinically used PRP, in which platelets are activated by hemostasis and bioactive factors are kept on site by the resulting fibrin clot,<sup>27</sup> in vitro, activation of platelets is induced by repeated freezeand-thaw cycles whereas growth factor containment is achieved by conventional cell culture conditions such as the use of flasks and pellet cultures. PRP activation resulted in a platelet lysate, which made bioactive factors available.<sup>15</sup> During freeze-and-thaw cycles, fibrinogen was precipitated and removed to prevent medium coagulation in cell culture and thus optimization of oxygen and nutrition supply was achieved. However, bioactive factors were still detectable after fibrinogen precipitation<sup>18</sup> and kept on site during cell culture by the in vitro culture system. We suppose that the modification of platelet activation and fibrinogen precipitation does not influence the potential of the PRP used and that the potential may be comparable with PRP prepared for clinical application.

The findings obtained in this pilot study suggest that PRP does have a differentiation-, migration-, and proliferation-stimulating effect on mesenchymal stem and progenitor cells, albeit the "biological" efficacy may depend on factors such as the preparation method and donor-related variability.<sup>10</sup> Although a relation of the growth factor content and biological activity of PRP is not obvious in our study, variability of components and its effects on dosage have been suggested to be one of the key issues in autologous PRP variability.<sup>28</sup> Further explanations for the variability of PRP efficacy may be that different preparation methods lead to biological variations in PRP, such as the content of leukocytes or blood cells with, for example, leukocyte-rich PRP obtained by Regen and leukocyte-poor PRP obtained by ACP. However, the impact of leukocyte content on chondrogenesis appears to be not obvious, with leukocyte-poor PRP enhancing expression of type II collagen and leukocyte-rich PRP inducing hyaluronic acid synthase,<sup>29</sup> both genes being important for chondrogenesis. Therefore, in addition to inherent, patientrelated variability in autologous blood-derived products, there are a variety of differences, such as preparation methods, leading to different concentrations of platelets, leukocyte and growth factor content, or other bioactive compounds that may cause variability and unpredictability of PRP efficacy and effects in cartilage repair. In our study PRP obtained by various preparation methods showed different potentials in terms of stimulating chondrogenic differentiation, migration, and proliferation of human MPCs, which are needed for cartilage repair. This finding may also explain the variability of PRP benefits in clinical applications. With this knowledge, we suggest that characterization and standardization of PRP are needed. However, further animal studies and clinical trials with well-characterized and -standardized PRP are needed before a particular preparation method can be recommended unrestrictedly for cartilage repair.

#### Limitations

In vitro studies do not resemble the clinical situation, especially the strict autologous use of PRP in stem cell-mediated cartilage repair. In contrast to clinical use, PRP activation was performed in vitro by freezeand-thaw cycles. In this context fibrinogen was removed to prevent medium coagulation in cell cultures. However, these modifications may have no influence on the biological potential of the platelet concentrates used. No sample size calculation was performed. The sample size is relatively low, and the study would have been strengthened by using PRP prepared by different methods with blood drawn from the same donors. Such an approach and the use of individual donors would underline variations and probably different biological activities of PRP due to different preparation methods. There are no studies on dosage, and the different PRP preparations have not been normalized. Because only a single dose of 5% was used



**Fig 4.** Proliferative effects of platelet concentrates on human subchondral mesenchymal progenitor cells (MPCs). Proliferative effects have been evaluated by measuring the content of genomic DNA of MPCs on stimulation with platelet-rich plasma for up to 9 days. DNA content and thus the number of MPCs increased after stimulation with Autologous Conditioned Plasma (ACP), Regen, platelet-rich plasma obtained by apheresis (PRP-A), and platelet-rich plasma obtained by centrifugation (PRP-C). Non-stimulated controls and MPCs stimulated with Dr.PRP showed no increase in DNA content. Asterisks indicate a significant (P < .05) increase compared with baseline (day 0). The bars show the mean (n = 3) and 95% confidence interval.

to analyze the effect of platelet concentrates on proliferation and matrix formation, we cannot draw any conclusion concerning the effect of higher or lower doses, which is another limitation of the study.



**Fig 5.** Quantification of candidate chondrogenic growth factors in platelet concentrates. The content of the candidate chondrogenic growth factors bone morphogenetic protein 2 (BMP2), connective tissue growth factor (CTGF), fibroblast growth factor 2 (FGF2), and transforming growth factor  $\beta_3$  (TGFB3) was measured using sandwich enzyme-linked immunosorbent assay systems. The bars show the mean (n = 3) and 95% confidence interval. (ACP, Autologous Conditioned Plasma; PRP-A, platelet-rich plasma obtained by apheresis; PRP-C, platelet-rich plasma obtained by centrifugation.)

Therefore our results may not allow extension to a broader range of doses.

### Conclusions

Platelet concentrates obtained by different preparation methods exhibit different potentials to stimulate chondrogenic differentiation, migration, and proliferation of MPCs. Platelet concentrates obtained by commercially available preparation kits failed to induce chondrogenic differentiation of MPCs, whereas highly standardized PRP preparations did induce such differentiation. These findings suggest differing outcomes with PRP treatment in stem cell—based cartilage repair.

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