

Osteoarthritis and Cartilage



The effect of platelet-rich plasma on the regenerative therapy of muscle derived stem cells for articular cartilage repair

Y. Mifune †‡, T. Matsumoto †‡, K. Takayama †‡, S. Ota †‡, H. Li †‡, L.B. Meszaros †, A. Usas †, K. Nagamune §, B. Gharaibeh †‡, F.H. Fu †, J. Huard †‡||*

† Stem Cell Research Center, University of Pittsburgh, Pittsburgh, PA 15219, USA

‡ Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA 15213, USA

§ Human and Artificial Intelligent Systems, University of Fukui Graduate School of Engineering, Fukui, Japan

|| Departments of Molecular Genetics and Biochemistry, and Bioengineering, University of Pittsburgh, Pittsburgh, PA 15213, USA

ARTICLE INFO

Article history:

Received 29 November 2011

Accepted 24 September 2012

Keywords:

Platelet-rich plasma

Muscle derived stem cells

Osteoarthritis

SUMMARY

Objective: Platelet-rich plasma (PRP) is reported to promote collagen synthesis and cell proliferation as well as enhance cartilage repair. Our previous study revealed that the intracapsular injection of muscle derived stem cells (MDSCs) expressing bone morphogenetic protein 4 (BMP-4) combined with soluble Flt-1 (sFlt1) was effective for repairing articular cartilage (AC) after osteoarthritis (OA) induction. The current study was undertaken to investigate whether PRP could further enhance the therapeutic effect of MDSC therapy for the OA treatment.

Methods: MDSCs expressing BMP-4 and sFlt1 were mixed with PRP and injected into the knees of immunodeficient rats with chemically induced OA. Histological assessments were performed 4 and 12 weeks after cell transplantation. Moreover, to elucidate the repair mechanisms, we performed *in vitro* assays to assess cell proliferation, adhesion, migration and mixed pellet co-culture of MDSCs and OA chondrocytes.

Results: The addition of PRP to MDSCs expressing BMP-4 and sFlt1 significantly improved AC repair histologically at week 4 compared to MDSCs expressing BMP-4 and sFlt1 alone. Higher numbers of cells producing type II collagen and lower levels of chondrocyte apoptosis were observed by MDSCs expressing BMP-4 and sFlt1 and mixed with PRP. In the *in vitro* experiments, the addition of PRP promoted proliferation, adhesion and migration of the MDSCs. During chondrogenic pellet culture, PRP tended to increase the number of type II collagen producing cells and in contrast to the *in vivo* data, it increased cell apoptosis.

Conclusions: Our findings indicate that PRP can promote the therapeutic potential of MDSCs expressing BMP-4 and sFlt1 for AC repair (4 weeks post-treatment) by promoting collagen synthesis, suppressing chondrocyte apoptosis and finally by enhancing the integration of the transplanted cells in the repair process.

© 2012 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.

Introduction

Platelet-rich plasma (PRP) is defined as an autologous concentration of platelets in a small volume of plasma which is considered to be a rich source of autologous growth factors¹. Recently, there has been a great interest in the use of PRP for the treatment of orthopedics-related injuries². PRP contains numerous growth factors and

bioactive proteins in the alpha-granules of blood platelets^{1,3–6}. It has been reported that PRP can promote angiogenesis, collagen synthesis and cell proliferation, and is capable of positively influencing the healing of tendon, ligament, skeletal muscle, and bone^{7,8}. In regard to articular cartilage (AC), it has been reported that PRP stimulates porcine chondrocyte proliferation and matrix biosynthesis⁹, as well as having the ability to enhance the chondrogenic differentiation capacity of mesenchymal stem cells (MSCs) *in vitro*¹⁰. Furthermore, some *in vivo* studies also showed that chondrocyte/PRP composites could enhance the regeneration of AC defects¹¹ and that PRP, in a poly-lactic-glycolic acid scaffold, could improve osteochondral healing in a rabbit model¹².

* Address correspondence and reprint requests to: J. Huard, Stem Cell Research Center, University of Pittsburgh, 450 Technology Drive, 2 Bridgeside Point, Suite 206, Pittsburgh, PA 15219, USA. Tel: 1-412-648-2798; Fax: 1-412-648-4066.

E-mail address: jhuard@pitt.edu (J. Huard).

Our group has investigated the therapeutic potential of muscle derived stem cells (MDSCs) for the treatment of various diseases and conditions of the musculoskeletal system because of their ability to undergo multilineage differentiation^{13–19}. More importantly for the current investigation, we have reported that MDSCs can undergo chondrogenesis *in vivo* and *in vitro*^{20,21}, and that bone morphogenetic protein 4 (BMP-4)-transduced MDSCs could improve AC regeneration after injury²². Moreover, we have shown that soluble Flt-1 (sFlt1; a vascular endothelial growth factor (VEGF) antagonist)/BMP-4 transduced MDSCs could enhance cartilage regeneration in rats with induced osteoarthritis (OA) when transplanted intra-articularly by blocking the intrinsic VEGF catabolic pathway and extrinsic VEGF-induced vascular invasion which prevents chondrocyte apoptosis²³. These results taken together showed that blocking angiogenesis, combined with BMP-4 expressing MDSCs was effective for OA repair. Despite the fact that PRP contains many growth factors and cytokines that promote angiogenesis, PRP has been reported to have a positive effect on cartilage repair^{9–12}. In the current study, we performed a set of experiments in a rat model of OA designed to investigate whether PRP could enhance the therapeutic effect that MDSCs have on OA injured AC, and also determined whether PRP could further increase the beneficial effects that retroviral constructs expressing sFlt1 and BMP-4 have on MDSC mediated AC repair during OA.

Materials and methods

Isolation of MDSCs and PRP preparation

MDSCs were isolated from the hind-limb skeletal muscle of C57-BL10J mice, (Jackson Laboratory, Bar Harbor, ME) via a modified preplate technique, and MDSCs were transduced separately with retroviral vectors encoding for green fluorescent protein (GFP), BMP-4 and GFP (B4-GFP) or sFlt1 and LacZ (sFlt1-LacZ) as previously described²³. PRP was isolated from the whole blood of nude rats (NIH-Whn NIHRNU-M; Taconic, NY, USA) via a double centrifugation technique as previously described²⁴.

Repair of MIA-induced arthritis

The animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee at University of Pittsburgh. Thirty-six female nude rats (NIH-Whn NIHRNU-M; Taconic) were used in this study. The animals were anesthetized with 3% isoflurane in O₂ gas (1.5 L/min). OA-like arthritis was induced by a single intra-articular knee injection of monosodium iodoacetate (MIA) (Aldrich Chemical, Milwaukee, WI) (0.3 mg/150 mg body weight) into the bilateral knee joints of the rats. Two weeks after MIA injection, each group of rats had their knee joints treated as described in Table I. Rats were sacrificed 4 and 12 weeks after cell transplantation ($n = 6$ OA knees from three rats in each treatment group for each time point) as previously described²³.

Table I
Treatment groups in OA model

Group	Cell type	Cell number (cells)	Solution
M + PRP	MDSC	5.0×10^5	PRP 30 μ l + PBS 20 μ l
M	MDSC	5.0×10^5	PBS 50 μ l
PRP	No cells	0	PRP 30 μ l + PBS 20 μ l
PBS	No cells	0	PBS 50 μ l
M-B4/sFlt1 + PRP	sFlt1-MDSC/BMP-4-MDSC	$2.5 \times 10^5/2.5 \times 10^5$	PRP 30 μ l + PBS 20 μ l
M-B4/sFlt1	sFlt1-MDSC/BMP-4-MDSC	$2.5 \times 10^5/2.5 \times 10^5$	PBS 50 μ l

We set these six treatment groups in this study.

Histological evaluation of cartilage repair

Serial sagittal sections, 5 μ m in thickness, were cut and stained with Safranin O-fast green. We evaluated OA repair semi-quantitatively using a grading and staging system²⁵. In this system, there are six histological grades and four histological stages. The total score (score = grade \times stage) ranging from 1 point (normal AC) to 24 points (no repair) was evaluated by three researchers blinded to the treatment groups.

Contribution of transduced MDSCs in the cartilage healing process

To detect transplanted mouse cells in the femoral condyle, immunohistochemistry was performed at week 4 with the following antibodies; rabbit anti-rat type 2 collagen (Col2) (Sigma) to detect rat and mouse chondrocytes²¹, rabbit anti-GFP Alexa Fluor 488 conjugated (Molecular Probe, Eugene, OR) for detection of B4-GFP-MDSCs and GFP-MDSCs, and biotin-conjugated anti- β -galactosidase (β -gal) for detection of sFlt1-LacZ-MDSCs. The numbers of double-positive and Col2-positive cells were morphometrically counted in five randomly selected soft tissue fields in the femoral condyle by using Northern Eclipse software (Empix Imaging Inc., Cheektowaga, NY). The following secondary antibodies were used for each immunostaining: Alexa Fluor 594-conjugated donkey anti-rabbit IgG (Molecular Probes) was used for Col2 staining and Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes) was used for GFP and β -gal staining.

Chondrocyte apoptosis analysis

TUNEL assay was performed on the 4-week time point groups using an Apop Tag Plus Peroxidase In Situ Apoptosis Detection Kit according to the manufacturer's instructions (CHEMICON, Temecula, CA). After the staining, the positive signals were examined by light microscopy ($n = 6$ knees in each group).

Cell functional assays and mixed pellet culture

To assess the effects of PRP, BMP-4 and sFlt1 on cell function of MDSCs including proliferation, adhesion and migration, each assay was performed and the detailed methods and the treatment groups in these assays are shown in [Supplementary Information](#). Pellet cultures were also performed with the mixed-cell components. Mixed pellet cultures consisted of 1×10^5 OA chondrocytes plus: (1) 1×10^5 BMP-4 transduced MDSCs, (2) 1×10^5 sFlt1 transduced MDSCs, or (3) 1×10^5 non-transduced MDSCs, and each of the mixed-cell pellets was cultured in chondrogenic medium with or without PRP for 14 days. Pellets were assessed via Alcian blue and TUNEL staining ($n = 3$). The detailed methods of mixed pellet culture are outlined in the [Supplementary Information](#).

Statistical analysis

All values were expressed as 95% confidence intervals (95% CI). Comparisons among multiple groups were made using analysis of

variance (ANOVA) test followed by the Tukey's test. *Post hoc* analysis was performed by Fisher's Protected Least Significant Difference (PLSD) test. The comparisons of histological scores were analyzed using mixed effects ANOVA using the Statistical Package for Social Sciences (SPSS) (version 15.0; SPSS Inc., IL, USA). A value of $P < 0.05$ was considered statistically significant.

Results

Platelet count

The platelets found in the PRP used in these studies exhibited normal morphology. Platelet counts revealed that the PRP preparation technique produced highly concentrated platelet containing plasma. PRP smears showed higher concentrations of platelets than whole blood ones. The average whole blood platelet count was 42.0×10^4 platelets/mL (95% CI: 34.2×10^4 , 50.7×10^4), whereas the average PRP platelet count was 230.0×10^4 platelets/mL (95% CI: 171.1×10^4 , 275.4×10^4) which is a 5.5-fold increase in platelets.

Effect of PRP on MDSC mediated AC repair (macroscopic and histological evaluation of the femoral condyles)

There was no gross evidence of any side effects from the retroviral transduction such as a reduction in proliferation, changes in the cell differentiation behavior, nor in their neoplastic transformation behavior throughout the observation period. Four weeks after MDSC (M) transplantation into the OA model, macroscopic evaluation of the M + PRP group showed well-repaired articular surfaces, though osteophyte formations were detected in some portions of the experimental animal's joints [Fig. 1(A)]; however, the M, PRP and PBS groups showed marked arthritis including synovial hypertrophy and osteophyte formation. Histologic assessment demonstrated that Safranin O-positive hyaline-like cartilage was present in the M + PRP group [Fig. 1(B)], and was less prevalent in the M group and was completely absent in both the PRP and PBS groups.

Twelve weeks after transplantation in the M + PRP group, the AC surfaces appeared to be smooth though osteophyte formation was more advanced than at the 4-week time point. On the other hand, the M, PRP, and PBS groups all showed a marked progression of arthritis. Histologic assessment also demonstrated destructive events including pannus invasion, osteolysis, cyst formation within the subchondral bone area, and reduction in Safranin O-positive staining in the PRP and PBS groups. A histological grading scale²⁵ was used to evaluate the quality of the repaired tissues. At 4 weeks following transplantation, the total score of the M + PRP group was significantly better than the PRP and PBS groups [$P = 0.0470$ for M + PRP vs PRP group; $P = 0.0330$ for M + PRP vs PBS group (Note that the lower the score the better the cartilage repair)] [Fig. 1(C)]. Twelve weeks following transplantation, the total score of the M + PRP group was still significantly better than the PRP and PBS groups ($P = 0.0340$ for M + PRP vs PRP group; $P = 0.0060$ for M + PRP vs PBS group) [Fig. 1(C)]. There were no significant differences between the M + PRP and M groups at 4 and 12 weeks after transplantation (week 4, $P = 0.1860$; week 12, $P = 0.2520$).

MDSCs contribution to the AC repair process

Double immunohistochemical staining for Col2 and GFP or β -gal was performed using tissue samples obtained 4 weeks post-transplantation which would allow the determination of whether the new chondrocytes were differentiated transplanted MDSCs. All MDSCs used for M + PRP and M groups were transduced to express GFP. GFP-positive cells expressing Col2 were found in the femoral

condyles in all the groups except the PRP and PBS groups [Fig. 2(A)]. Quantification of the double-positive stained cells demonstrated that there was no significant difference between the M + PRP and the M groups ($P = 0.5160$) [Fig. 2(B)]; however, the total number of Col2-positive cells derived from the donor and host cells was found to be significantly higher in the M + PRP group when compared to the other groups ($P = 0.0060$ for M + PRP vs M; $P = 0.0030$ for M + PRP vs PRP; $P = 0.0020$ for M + PRP vs PBS) [Fig. 2(C)].

Chondrocyte apoptosis analyses

Chondrocyte apoptosis was less abundant in the superficial/mid zone of the femoral condyles in the M + PRP group when compared with the PRP and PBS groups [Fig. 2(D)]. Quantification of the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) stained cells showed that the M + PRP group showed significantly fewer apoptotic chondrocytes compared to the PRP and PBS groups ($P = 0.0050$ for M + PRP vs PRP; $P = 0.0020$ for M + PRP vs PBS), however, there was no significant difference between the M + PRP and M groups ($P = 0.0790$) [Fig. 2(E)].

Beneficial effect of PRP on BMP-4 and sFlt1 expressing MDSC therapy

In the current study, PRP provided only a limited effect on the regenerative potential of MDSCs toward cartilage repair after OA (Figs. 1 and 2), when compared with our previous paper which used transduced MDSCs²³. As PRP contains angiogenic factors that can have deleterious effects on the AC repair process, and BMP-4 has been found to be important for MDSC mediated AC repair, we performed additional experiments using PRP and MDSCs that expressed both BMP-4 and sFlt1.

MDSCs (M) were retrovirally transduced with sFlt1–LacZ (sFlt1) or BMP-4–GFP (B4) and then combined at a 1:1 ratio prior to transplantation (M–sFlt1/B4) (Table I). Four weeks after MDSC transplantation, macroscopic evaluation of the groups revealed smooth joint surfaces of AC and no osteophyte formation [Fig. 3(A)]. Histologic assessment demonstrated that more Safranin O-positive hyaline-like cartilage was present in the M–sFlt1/B4 + PRP and M–sFlt1/B4 groups when compared with the M + PRP group [Fig. 3(B)]. Twelve weeks after transplantation, rat knees treated with M–sFlt1/B4 + PRP or M–sFlt1/B4 still showed smooth joint surfaces in most regions of the articular condyles [Fig. 3(A)]. Histologic assessment also demonstrated that more Safranin O-positive tissue and fewer clusters of chondrocytes present within necrotic-like-tissue were found in the M–sFlt1/B4 + PRP and M–sFlt1/B4 groups when compared with the other groups shown in Fig. 1 [Fig. 3(B)].

In the histological grading scale at week 4, the score of the M–sFlt1/B4 + PRP group was significantly better than the M–sFlt1/B4 group ($P = 0.0020$) [Fig. 3(C)]. Twelve weeks following transplantation, there was no significant difference between the M–sFlt1/B4 + PRP and the M–sFlt1/B4 groups ($P = 0.2640$) [Fig. 3(C)]. Of note, the M–sFlt1/B4 + PRP and M–sFlt1/B4 groups maintained scores similar to the scores given at the 4-week time point.

Contributions of BMP-4 and sFlt1 expressing MDSCs to the AC repair process

Differentiated chondrocytes derived from transduced MDSCs were determined at week 4 by detecting cells double stained for Col2 and either GFP (M–B4 cells) or β -gal (M–sFlt1 cells). Within the knee, cells were identified that co-expressed Col2 and GFP or β -gal [Fig. 4(A and B)]. Quantification of the number of double-positive cells of Col2 and GFP or β -gal demonstrated that the M–sFlt1/B4 + PRP group showed a significantly higher number of double-

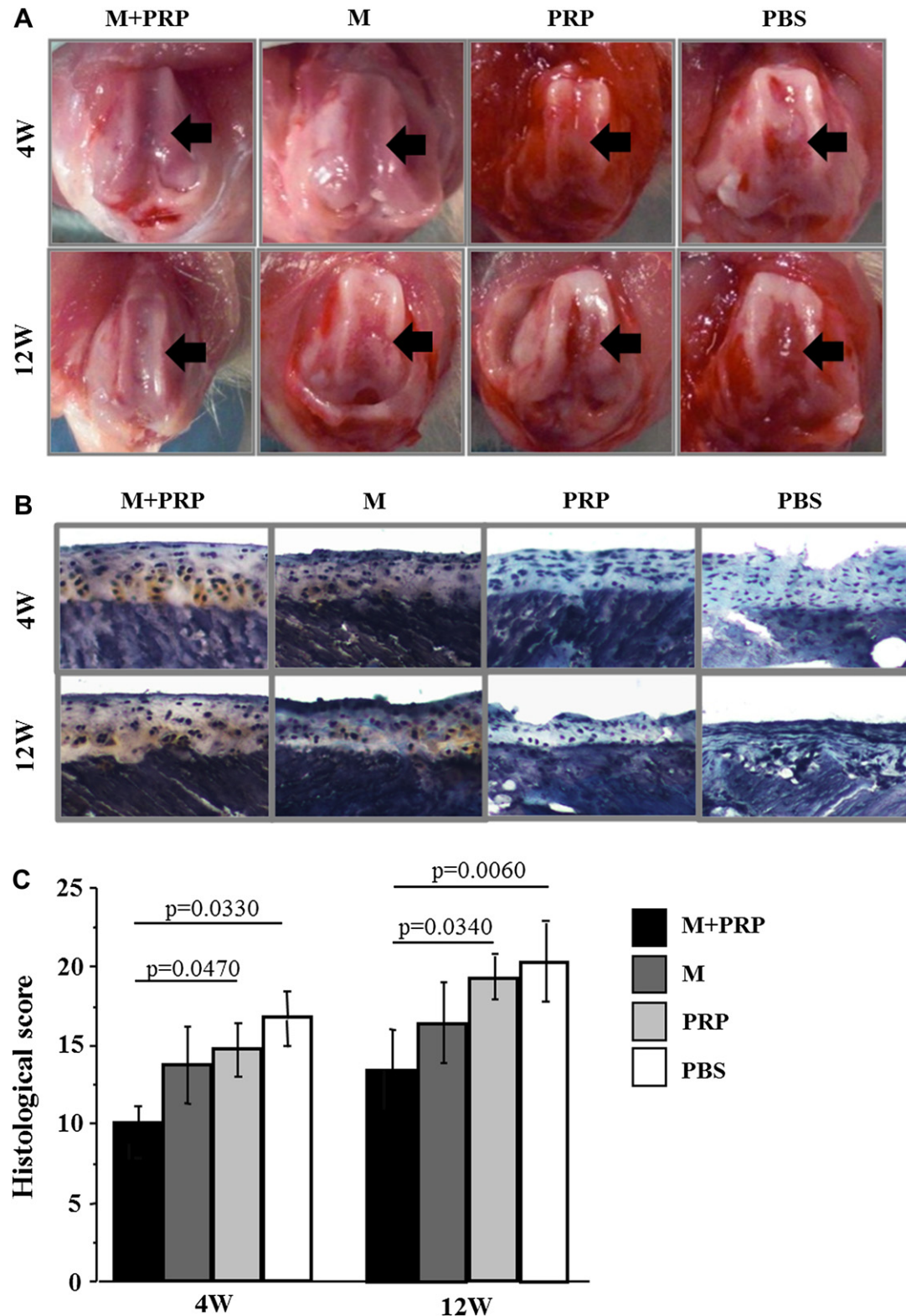


Fig. 1. Macroscopic and histologic evaluation of representative rat joints. (A) Four weeks after MDSC transplantation, macroscopic evaluation of the MDSC (M) + PRP group showed well-repaired articular surfaces, but some parts of the joints included osteophyte formation. The M, PRP and PBS groups showed marked arthritis including synovial hypertrophy and osteophyte formation. Twelve weeks after transplantation, in the M + PRP groups, the AC surfaces tended to be smooth, but osteophyte formation was more advanced than that at 4 weeks (arrows). The M, PRP, and PBS groups showed marked progression of arthritis, synovial hypertrophy, and osteophyte formation (arrows). (B) Histologic assessment at week 4 and 12 demonstrated that Safranin O-positive hyaline-like cartilage (red staining) was present in the M + PRP group, but less prominent in the M group. Safranin O-positive hyaline-like cartilage was completely absent in both the PRP and PBS groups. Original magnification 100 \times . (C) Semiquantitative histologic scores for all groups, 4 and 12 weeks following transplantation. The total score of the M + PRP group was significantly lower than the PRP and the PBS groups at week 4 and 12 ($n = 6$ knees in each group).

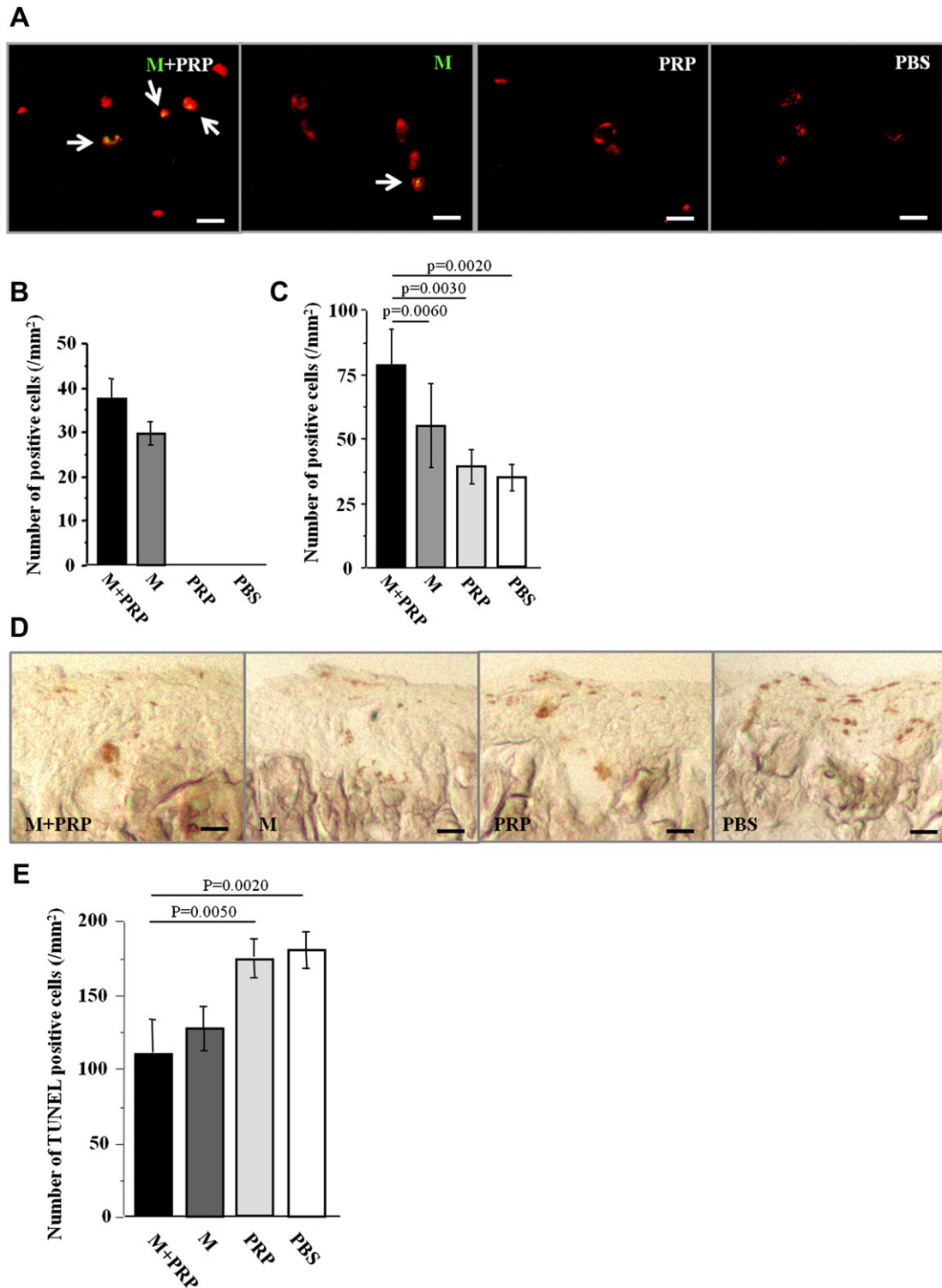


Fig. 2. Cell contribution of MDSCs and chondrocyte apoptosis analysis. (A) Double immunohistochemical staining for type II collagen (Col2) and green fluorescent protein (GFP). GFP-positive cells expressing Col2 were found in the femoral condyles in the M + PRP and M groups (arrow). Bars = 20 μ m. (B) Quantification of the number of double-positive cells staining for both type II collagen and GFP demonstrated that the M + PRP group showed a higher number of GFP labeled cells differentiated into Col2 expressing cells (chondrocytes) compared to the M group (not significant, $n = 6$ knees in each group). (C) The total number of Col2-positive cells was significantly higher in the M + PRP group compared to the other groups ($n = 6$ knees in each group). (D) Chondrocyte apoptosis was less abundant in the superficial/mid zone of the femoral condyle in the M + PRP group when compared with the other groups. Bars = 40 μ m. (E) Quantification of the TUNEL stained cells showed that the M + PRP group showed significantly fewer apoptotic chondrocytes compared to the PRP and the PBS groups ($n = 6$ knees in each group).

positive cells than the M-sFlt1/B4 group ($P = 0.0200$) [Fig. 4(C)], and the number of GFP/ β -gal positive cells was significantly greater in the M-sFlt1/B4 + PRP group than the M-sFlt1/B4 group

($P = 0.0220$) [Fig. 4(D)]. The total number of Col2-positive cells was significantly higher in the M-sFlt1/B4 + PRP group compared to the M-sFlt1/B4 group ($P = 0.0180$) [Fig. 4(E)].

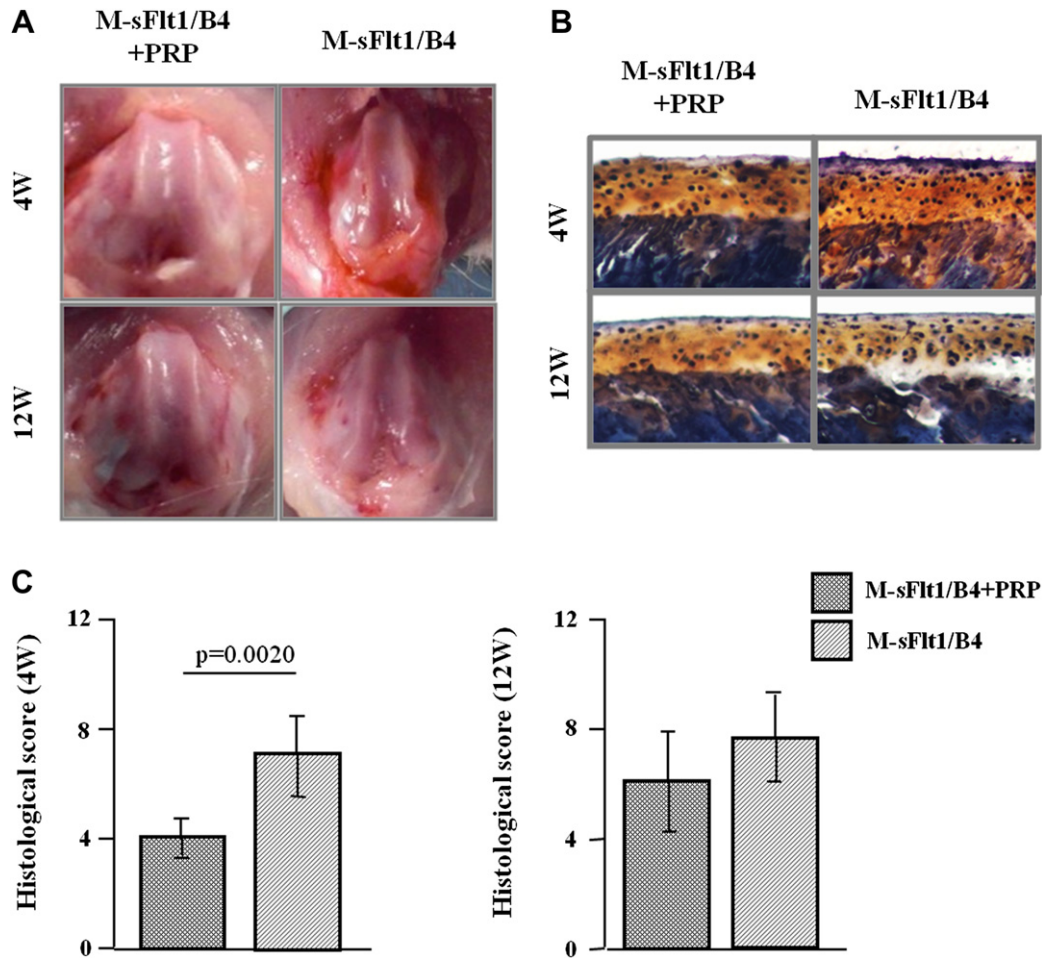


Fig. 3. Macroscopic and histologic evaluation of gene-transduced MDSC therapy. (A) Four weeks after MDSC transplantation, macroscopic evaluation of the M-sFlt1/B4 + PRP and M-sFlt1/B4 groups revealed smooth joint surfaces of AC and no osteophyte formation, and 12 weeks after transplantation, rat knees treated with M-sFlt1/B4 + PRP or M-sFlt1/B4 still showed smooth joint surfaces. (B) Histologic assessment at week 4 demonstrated that Safranin O-positive hyaline-like cartilage was present in the M-sFlt1/B4 + PRP and M-sFlt1/B4 groups, and histologic assessment at week 12 also demonstrated that more Safranin O-positive tissue and few clusters of chondrocytes near necrotic tissue were found in the M-sFlt1/B4 + PRP and M-sFlt1/B4 groups. Original magnification 100 \times . (C) Four weeks following transplantation, the score of the M-sFlt1/B4 + PRP group was also significantly better than the M-sFlt1/B4 group. Twelve weeks following transplantation, there was no significant difference between the M-sFlt1/B4 + PRP and the M-sFlt1/B4 groups ($n = 6$ knees in each group).

Chondrocyte apoptosis analyses in OA knees injected with BMP-4 and sFlt1 transduced MDSCs

Chondrocyte apoptosis was less abundant in the M-sFlt1/B4 + PRP group [Fig. 4(F)]. Quantification of the TUNEL stained cells showed that there were significantly fewer apoptotic chondrocytes in the M-sFlt1/B4 + PRP group than in the M-sFlt1/B4 group ($P = 0.0120$) [Fig. 4(G)].

Mixed pellet culture experiments

To assess the reciprocal effects between MDSCs and OA chondrocytes (OA-C) with/without PRP, we performed mixed-cell pellet culture experiments. As a control group, we set OA-C group which included OA chondrocytes alone, not mixed with MDSCs. All cell groups tested formed pellets. The pellets in the M-B4 + PRP, M-B4 and M-sFlt1 + PRP groups were significantly larger than the M-sFlt1, M + PRP, M and OA-C groups ($P = 0.0026$ for M-B4 + PRP vs M + PRP; $P < 0.001$ for M-B4 + PRP vs M-sFlt1, M and OA-C; $P = 0.0108$ for M-B4 vs M + PRP; $P = 0.0027$ for M-B4 vs M-sFlt1; $P < 0.001$ for M-B4 vs M and OA-C; $P = 0.0140$ for M-sFlt1 + PRP vs M + PRP; $P = 0.0036$ for M-sFlt1 + PRP vs M-sFlt1; $P < 0.001$ for M-sFlt1 + PRP vs M and OA-C). The pellets in the M-sFlt1 group were significantly larger than the M and OA-C groups. ($P = 0.0285$ for

M-sFlt1 vs M; $P < 0.001$ for M-sFlt1 vs OA-C) [Fig. 5(A)]. The pellets from every group showed hyaline cartilage-like extracellular matrix (ECM) that stained positively for Alcian blue and contained well-differentiated, round chondrocyte-like cells [Fig. 5(B)].

To analyze the effect of PRP on chondrocyte apoptosis in cultured pellets, TUNEL staining was performed using pellet samples obtained 3 weeks after cultivation. Chondrocyte apoptosis was less abundant in the M-sFlt1 + PRP and M-sFlt1 groups [Fig. 5(B)]. Quantification of the TUNEL stained cells showed that the M-sFlt1 + PRP and M-sFlt1 groups showed fewer apoptotic cells compared with the other treatment groups ($P < 0.001$ for M-sFlt1 + PRP vs M-B4 + PRP; $P = 0.0018$ for M-sFlt1 + PRP vs M-B4; $P = 0.0021$ for M-sFlt1 + PRP vs M + PRP; $P = 0.0452$ for M-sFlt1 + PRP vs M; $P = 0.0110$ for M-sFlt1 + PRP vs OA-C; $P < 0.001$ for M-sFlt1 vs M-B4 + PRP, M-B4, M + PRP and OA-C; $P = 0.0170$ for M-sFlt1 vs M). The number of apoptotic cells was significantly higher in the M-B4 + PRP group than the M-B4 group, as well as in the M + PRP group than the M groups ($P = 0.0072$ for M-B4 + PRP vs M-B4; $P = 0.0448$ for M + PRP vs M); however, there was no significant difference between the M-sFlt1 + PRP and M-sFlt1 groups ($P = 0.3874$ for M-sFlt1 + PRP vs M-sFlt1) [Fig. 5(C)].

Immunohistochemical staining for Col2 showed that collagen synthesis within the pellets was enhanced in the M-B4 + PRP and M-B4 groups [Fig. 5(B)]. Quantitative analysis revealed that there

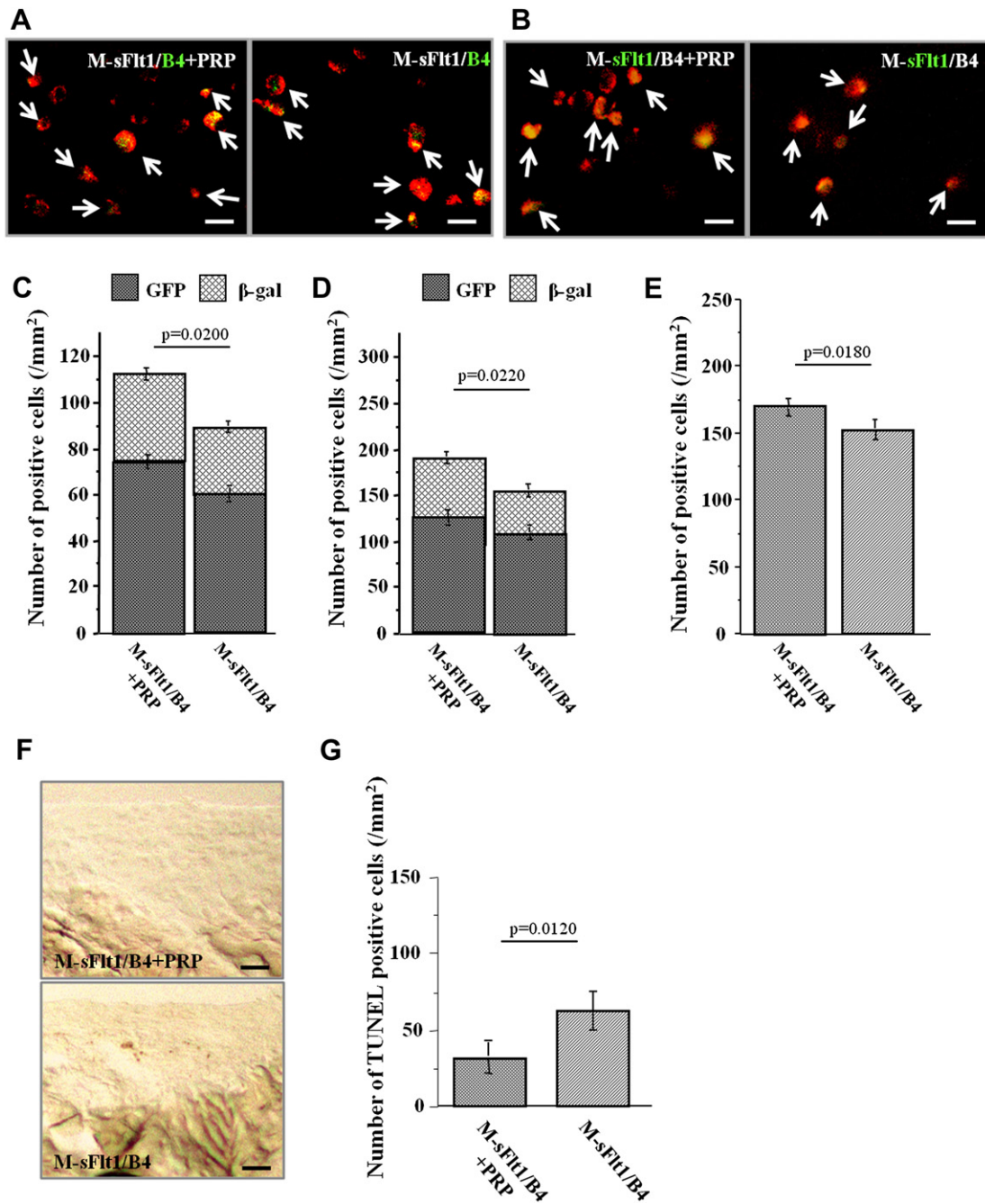


Fig. 4. Cell contribution of gene-transduced MDSCs and chondrocyte apoptosis analysis. (A) Double immunohistochemical staining for Col2 and GFP. The BMP-4 transduced MDSCs and the control MDSCs were also transduced to express GFP. GFP-positive cells expressing Col2 were found in the femoral condyles in the M-sFlt1/B4 + PRP and M-sFlt1/B4 groups (arrow). Bars = 20 μ m. (B) The sFlt1 transduced MDSCs were also transduced to express β -gal. β -gal positive cells expressing Col2 were found in the femoral condyles in the M-sFlt1/B4 + PRP and M-sFlt1/B4 groups (arrow). Bars = 20 μ m. (C) Quantification of the number of double-positive cells of Col2 and GFP/ β -gal demonstrated that the M-sFlt1/B4 + PRP and the M-sFlt1/B4 groups showed a significantly higher number of GFP/ β -gal labeled cells differentiated into Col2 expressing cells (chondrocytes) compared to the other groups. The number of double-positive cells in the M-sFlt1/B4 + PRP group was also significantly higher than the M-sFlt1/B4 group ($n = 6$ knees in each group). (D) The number of GFP/ β -gal positive cells in the M-sFlt1/B4 + PRP group was significantly higher than the M-sFlt1/B4 group ($n = 6$ knees in each group). (E) The M-sFlt1/B4 + PRP group showed a significantly higher number of Col2-positive cells than the M-sFlt1/B4 group ($n = 6$ knees in each group). (F) Chondrocyte apoptosis was less abundant in the superficial/mid zone of the femoral condyle, especially in the M-sFlt1/B4 + PRP group. Bars = 40 μ m. (G) Quantification of the TUNEL stained cells showed that the M-sFlt1/B4 + PRP and the M-sFlt1/B4 groups showed significantly fewer apoptotic chondrocytes compared to the other treatment groups. The number of apoptotic chondrocytes was significantly fewer in the M-sFlt1/B4 + PRP group than the M-sFlt1/B4 group ($n = 6$ knees in each group).

was no significant difference between the M-B4 + PRP and M-B4 groups, as well as between the M + PRP and M groups ($P = 0.2246$ for M-B4 + PRP vs M-B4; $P = 0.1538$ for M + PRP vs M); however, the number of Col2-positive cells was significantly higher in the M-sFlt1 + PRP group than the M-sFlt1 group ($P = 0.0315$ for M-sFlt1 + PRP vs M-sFlt1) [Fig. 5(D)].

Discussion

In the present study, we initially determined whether PRP can improve the beneficial effect imparted by MDSCs for AC repair after OA induction by comparing the regenerative potential of MDSCs + PRP treatment with the MDSCs alone. Although

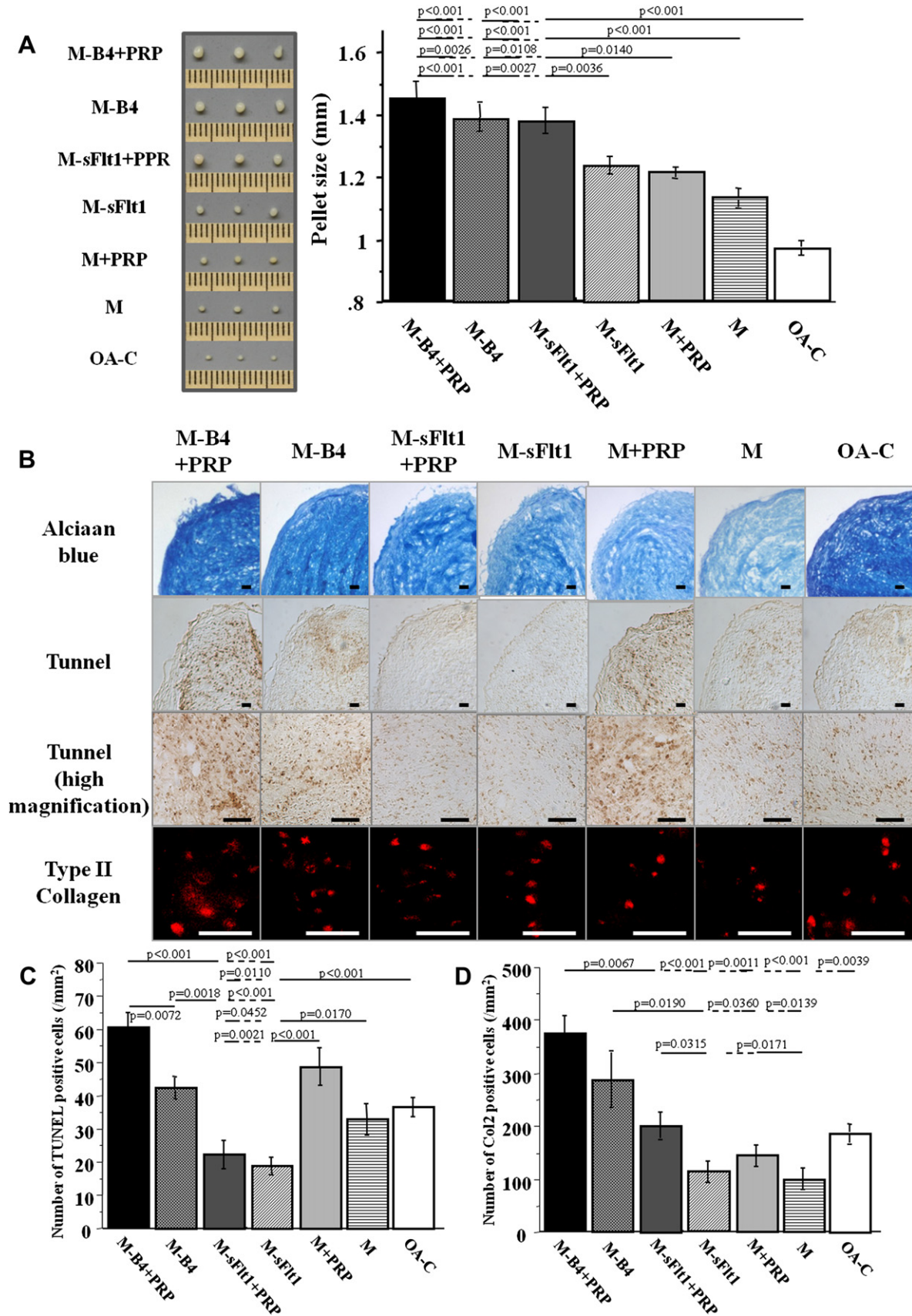


Fig. 5. Mixed pellet co-culture. (A) The pellets in the M–B4 + PRP, M–B4 and M–sFlt1 + PRP groups were significantly larger than the M–sFlt1, M + PRP, M and OA–C groups. The pellets in the M–sFlt1 group were significantly larger than the M and OA–C groups ($n = 3$ in each group). (B) Morphologically pellets were formed in all groups, and the pellets from every group showed hyaline cartilage-like ECM that stained positively for Alcian blue. Cell apoptosis assessed by TUNEL staining was less abundant in the M–sFlt1 + PRP and M–sFlt1 groups. Immunohistochemical staining for Col2 showed that collagen synthesis within the pellets was enhanced in the M–B4 + PRP and M–B4 groups. Bars = 50 μm . (C)

a significant increase in the number of Col2-positive cells was observed in the M + PRP group when compared to the M alone group, no significant difference was noted between these two groups in the histological assessment of AC repair and cell apoptosis analysis (Figs. 1 and 2). These results suggest that PRP provided only a limited effect on the regenerative potential of MDSCs in cartilage repair after OA induction. Since PRP contains angiogenic factors that can have deleterious effects on the AC repair process, and BMP-4 has been found important for MDSC mediated AC repair, we performed additional experiments where sFlt1 (an angiogenic antagonist) and BMP-4 were added to the PRP and MDSCs. Our results indicate that the combination of PRP and BMP-4/sFlt1 expressing MDSCs results in a significantly better beneficial effect on AC repair when compared to all the other groups tested; however, the beneficial effect that PRP has on MDSCs expressing BMP-4 and sFlt1 appears to be transient since the histological assessment of AC repair was found to not be significantly different than the BMP-4 and sFlt1 expressing MDSCs at 12 weeks post-transplantation. From these results, we concluded that PRP could work more effectively for MDSC mediated cartilage repair when combined with BMP-4/sFlt1, and that the influence of PRP may not have lasted over the 12-week period of the experiment since the PRP was injected only once into knee capsule in this study.

Our study also revealed a lack of beneficial effects exhibited by the PRP group on AC repair at both 4 and 12 weeks after injection which is in contrast to prior manuscripts that reported PRP's effectiveness for cartilage repair^{12,26}; however, in these studies the investigators administered PRP multiple times or *via* biodegradable scaffolds. Therefore, we believe that this disparity is related to the frequency of PRP administration and the use of a scaffold. Hence, continuous PRP administration should be considered an important influential factor for future clinical applications of PRP for AC repair.

In the *in vivo* apoptosis assay, chondrocyte apoptosis was significantly reduced in the M + PRP group when compared with the PRP and PBS groups but there was no significant difference between the M + PRP and the M groups (Fig. 2). On the other hand, the M-sFlt1/B4 + PRP group showed a significant reduction in chondrocyte apoptosis compared with the M-sFlt1/B4 group (Fig. 4). In our previous study, we reported that VEGF induced higher levels of chondrocyte apoptosis in the OA knee than all the other groups and that sFlt1-treated OA knees showed the lowest level of chondrocyte apoptosis²³. It has been reported that VEGF increases the catabolic pathway in AC tissue through the stimulation of Matrix metalloproteinases (MMP) activity and the reduction of tissue inhibitors of metalloproteinases (TIMPs), which consequently leads to cartilage destruction by enhancing angiogenesis and vascular invasion^{23,27,28}. Taken together, we posited that the enhancement of chondrocyte apoptosis by PRP was suppressed by sFlt1 in the M-sFlt1/B4 + PRP group and believe this could represent a major mechanism of why PRP's effect is enhanced when used in combination with sFlt1 for AC repair.

In order to clarify the interactions that PRP, BMP-4 and sFlt1 have on MDSC mediated AC repair, we performed *in vitro* studies to assay for cell proliferation, adhesion and migration, as well as with a pellet culture system. The results indicated that PRP induced higher levels of MDSC proliferation, adhesion and migration (Supplementary Fig. A–C). These PRP related effects potentially enhanced the BMP-4/sFlt1 expressing MDSCs direct contribution to the cartilage repair process in our *in vivo* experiments. These results are in agreement with reports showing that PRP enhances cell proliferation and migration of bone marrow MSCs, adipose-derived stem

cells, osteoblasts and chondrocytes^{29–32}. Also, another paper reported that PRP could promote cell adhesion and migration of fibroblasts³³.

In the pellet culture assay, PRP + sFlt1 had a significant effect on the size of the pellets formed, while PRP alone had no significant effect on the pellet size (Fig. 5). Mishra *et al.* reported that PRP could enhance the chondrogenic differentiation of MSCs³⁴, and that TGF-beta and fibroblast growth factor (FGF) signaling, which are factors present within PRP, are important for chondrogenic differentiation of MSCs³⁵. On the other hand, the increased pellet size observed in our experiment may not be entirely related to chondrogenic differentiation of the MDSCs but could also represent an indirect effect that PRP had on cell proliferation and ECM production; therefore, the entire effect that PRP has on the chondrogenic potential of MDSCs is still unclear and further studies are required. This pellet culture assay also demonstrated that PRP had an effect that increased the number of apoptotic cells in the chondrocyte pellets, which in turn was reduced with the addition of sFlt1 (Fig. 5). Interestingly, this finding was inconsistent with our *in vivo* results that showed that PRP did not promote cell apoptosis during cartilage repair. Recently, it was reported that PRP could affect synovial fibroblasts to increase hyaluronic acid production³⁶ and also promote chondrocytes to maintain their phenotype and to inhibit cellular de-differentiation³⁷. In our *in vivo* study, PRP could have some effects, not only on the transplanted MDSCs, but also on the joint tissues including synovium, fat pad, bone marrow as well as the AC, which could potentially suppress the effects of PRP on chondrocyte apoptosis. Additional experiments are required to test the effectiveness of PRP on the healing of these other intra-articular structures.

We also revealed that PRP could promote collagen synthesis (Fig. 5). Some growth factors included in PRP, such as TGF-β1, IGF-I, BMP-2 and -7, have already been reported to have an effect of promoting collagen synthesis^{38,39}, which supports our findings that PRP enhances collagen synthesis. This increase of collagen synthesis could represent another mechanism by which PRP enhances AC healing.

Recently, PRP has been reported to be effective for the treatment of AC. Sanchez *et al.* showed the effectiveness of intra-articular injections of an autologous preparation rich in growth factors for the treatment of OA in the knee, in retrospective matched cohort study⁴⁰. Furthermore, Kon *et al.* reported that the use of PRP injections was safe and had the potential to reduce pain and improve knee function and quality of life in younger patients with low degrees of AC degeneration⁴¹; however, further studies are necessary to clarify the clinical application of PRP and to better understand its mechanism of action. For example, it has been shown that platelets could potentially be involved in the induction of arthritis⁴².

Several limitations should be noted in this study. Firstly, we did not include a group that utilized MDSCs that expressed sFlt1 alone or with sFlt1 and PRP in our *in vivo* studies. Our previous studies have suggested that BMP-4 has a critical effect on MDSC therapy for cartilage repair; therefore we considered the addition of BMP-4 to be indispensable for this type of cell therapy to treat OA. To elucidate the mechanisms of this combined therapy, we performed *in vitro* studies using either BMP-4 or sFlt1 separately. The second limitation to our study was the fact that our OA model was chemically induced which is thought to be less clinically relevant than surgically induced OA models which better resemble the pathophysiology of OA; however, the reproducibility of the grade

Quantification of the TUNEL stained cells showed that the M-sFlt1 + PRP and M-sFlt1 groups showed fewer apoptotic cells compared with the other treatment groups, and that PRP increased TUNEL stained cells ($n = 3$ in each group). (D) Quantitative analysis revealed that there is no significant difference between the M-B4 + PRP and M-B4 groups, as well as between the M + PRP and M groups. Whereas the number of Col2-positive cells was significantly higher in the M-sFlt1 + PRP group than the M-sFlt1 group ($n = 3$ in each group).

and stage of OA achieved using the chemically induced model in immunodeficient rats was highly consistent with previous reports of OA in other rat strains^{43,44}. In this study, we used bilateral knees in a rat, accordingly we cannot exclude the possibility of an influence from the opposite treatment side of the animal. The third limitation to this study was the fact that the PRP used in this study was not characterized for its composition and physical characteristics. Besides variations between donors, there have been a variety of ways to isolate PRP reported; hence the composition of the PRPs isolated will differ from one another in composition. The advantage of PRP is its ready accessibility, simple preparation and that it is an autologously-derived material; however, standardized methods for isolating PRP are required.

In conclusion, our findings indicate that PRP can promote the therapeutic effect of MDSCs expressing BMP-4 and sFlt1 for chondral repair 4 weeks after transplantation via the promotion of collagen synthesis and the suppression of chondrocyte apoptosis; however, the PRP effects are only transient since at 12 weeks the beneficial effect of the PRP is lost.

Author contributions

Yutaka Mifune: the conception and design of the study, or acquisition of data, or analysis and interpretation of data.

Tomoyuki Matsumoto: the conception and design of the study, or acquisition of data, or analysis and interpretation of data.

Koji Takayama: drafting the article or revising it critically for important intellectual content.

Shusuke Ota: the conception and design of the study, or acquisition of data, or analysis and interpretation of data.

Hongshuai Li: drafting the article or revising it critically for important intellectual content.

Laura B Meszaros: drafting the article or revising it critically for important intellectual content.

Arvydas Usas: drafting the article or revising it critically for important intellectual content.

Kouki Nagamune: analysis and interpretation of data.

Burhan Gharaibeh: drafting the article or revising it critically for important intellectual content.

Freddie H Fu: final approval of the version to be submitted.

Johnny Huard: design of experiments and final approval of the version to be submitted.

Role of the funding source

Funding was provided by the Henry Mankin Endowed Chair at the University of Pittsburgh and the Donaldson chair at Children's Hospital of Pittsburgh.

Conflict of interest

All the authors have no conflict of interest in this study.

Acknowledgments

The authors are grateful for the technical and scientific advice provided by James Cummins and for his assistance in editing this manuscript. We are also grateful for the technical assistance provided by Jessica Tebbets and Michele Witt (SCRC). Funding support was provided by the Department of Defense (W81XWH-06-1-0406 awarded to Dr Johnny Huard, Ph.D.), by the William F and Jean W Donaldson Chair at the Children's Hospital of Pittsburgh, and by the Henry J Mankin Endowed Chair in Orthopaedic Surgery at the University of Pittsburgh.

Supplementary information

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.joca.2012.09.018>.

References

- Marx RE. Platelet-rich plasma: evidence to support its use. *J Oral Maxillofac Surg* 2004;62(4):489–96.
- Engebretsen L, Steffen K, Alsousou J, Anitua E, Bachl N, Devilee R, et al. IOC consensus paper on the use of platelet-rich plasma in sports medicine. *Br J Sports Med* 2010;44(15):1072–81.
- Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR. Platelet-rich plasma: growth factor enhancement for bone grafts. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1998;85(6):638–46.
- Sanchez AR, Sheridan PJ, Kupp LI. Is platelet-rich plasma the perfect enhancement factor? A current review. *Int J Oral Maxillofac Implants* 2003;18(1):93–103.
- Mehta S, Watson JT. Platelet rich concentrate: basic science and current clinical applications. *J Orthop Trauma* 2008;22(6):432–8.
- Foster TE, Puskas BL, Mandelbaum BR, Gerhardt MB, Rodeo SA. Platelet-rich plasma: from basic science to clinical applications. *Am J Sports Med* 2009;37(11):2259–72.
- Sanchez M, Anitua E, Orive G, Mujika I, Andia I. Platelet-rich therapies in the treatment of orthopaedic sport injuries. *Sports Med* 2009;39(5):345–54.
- Hall MP, Band PA, Meislin RJ, Jazrawi LM, Cardone DA. Platelet-rich plasma: current concepts and application in sports medicine. *J Am Acad Orthop Surg* 2009;17(10):602–8.
- Camargo PM, Lekovic V, Weinlaender M, Vasilic N, Madzarevic M, Kenney EB. Platelet-rich plasma and bovine porous bone mineral combined with guided tissue regeneration in the treatment of intrabony defects in humans. *J Periodontol Res* 2002;37(4):300–6.
- Drengk A, Zapf A, Sturmer EK, Sturmer KM, Frosch KH. Influence of platelet-rich plasma on chondrogenic differentiation and proliferation of chondrocytes and mesenchymal stem cells. *Cells Tissues Organs* 2009;189(5):317–26.
- Wu W, Chen F, Liu Y, Ma Q, Mao T. Autologous injectable tissue-engineered cartilage by using platelet-rich plasma: experimental study in a rabbit model. *J Oral Maxillofac Surg* 2007;65(10):1951–7.
- Sun Y, Feng Y, Zhang CQ, Chen SB, Cheng XG. The regenerative effect of platelet-rich plasma on healing in large osteochondral defects. *Int Orthop* 2010;34(4):589–97.
- Deasy BM, Gharaibeh BM, Pollett JB, Jones MM, Lucas MA, Kanda Y, et al. Long-term self-renewal of postnatal muscle-derived stem cells. *Mol Biol Cell* 2005;16(7):3323–33.
- Cao B, Zheng B, Jankowski RJ, Kimura S, Ikezawa M, Deasy B, et al. Muscle stem cells differentiate into haematopoietic lineages but retain myogenic potential. *Nat Cell Biol* 2003;5(7):640–6.
- Jankowski RJ, Deasy BM, Cao B, Gates C, Huard J. The role of CD34 expression and cellular fusion in the regeneration capacity of myogenic progenitor cells. *J Cell Sci* 2002;115(Pt 22):4361–74.
- Lee JY, Qu-Petersen Z, Cao B, Kimura S, Jankowski R, Cummins J, et al. Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing. *J Cell Biol* 2000;150(5):1085–100.
- Peng H, Wright V, Usas A, Gearhart B, Shen HC, Cummins J, et al. Synergistic enhancement of bone formation and healing by stem cell-expressed VEGF and bone morphogenetic protein-4. *J Clin Invest* 2002;110(6):751–9.

18. Shen HC, Peng H, Usas A, Gearhart B, Cummins J, Fu FH, *et al.* Ex vivo gene therapy-induced endochondral bone formation: comparison of muscle-derived stem cells and different subpopulations of primary muscle-derived cells. *Bone* 2004;34(6):982–92.
19. Wright V, Peng H, Usas A, Young B, Gearhart B, Cummins J, *et al.* BMP4-expressing muscle-derived stem cells differentiate into osteogenic lineage and improve bone healing in immunocompetent mice. *Mol Ther* 2002;6(2):169–78.
20. Adachi N, Sato K, Usas A, Fu FH, Ochi M, Han CW, *et al.* Muscle derived, cell based ex vivo gene therapy for treatment of full thickness articular cartilage defects. *J Rheumatol* 2002;29(9):1920–30.
21. Kuroda R, Usas A, Kubo S, Corsi K, Peng H, Rose T, *et al.* Cartilage repair using bone morphogenetic protein 4 and muscle-derived stem cells. *Arthritis Rheum* 2006;54(2):433–42.
22. Wakitani S, Yamamoto T. Response of the donor and recipient cells in mesenchymal cell transplantation to cartilage defect. *Microsc Res Tech* 2002;58(1):14–8.
23. Matsumoto T, Cooper GM, Gharraibeh B, Meszaros LB, Li G, Usas A, *et al.* Cartilage repair in a rat model of osteoarthritis through intraarticular transplantation of muscle-derived stem cells expressing bone morphogenetic protein 4 and soluble Flt-1. *Arthritis Rheum* 2009;60(5):1390–405.
24. Plachokova AS, van den Dolder J, Stoeltinga PJ, Jansen JA. The bone regenerative effect of platelet-rich plasma in combination with an osteoconductive material in rat cranial defects. *Clin Oral Implants Res* 2006;17(3):305–11.
25. Pritzker KP, Gay S, Jimenez SA, Ostergaard K, Pelletier JP, Revell PA, *et al.* Osteoarthritis cartilage histopathology: grading and staging. *Osteoarthritis Cartilage* 2006;14(1):13–29.
26. Baltzer AW, Moser C, Jansen SA, Krauspe R. Autologous conditioned serum (Orthokine) is an effective treatment for knee osteoarthritis. *Osteoarthritis Cartilage* 2009;17(2):152–60.
27. Pufe T, Harde V, Petersen W, Goldring MB, Tillmann B, Mentlein R. Vascular endothelial growth factor (VEGF) induces matrix metalloproteinase expression in immortalized chondrocytes. *J Pathol* 2004;202(3):367–74.
28. Enomoto H, Inoki I, Komiya K, Shiomi T, Ikeda E, Obata K, *et al.* Vascular endothelial growth factor isoforms and their receptors are expressed in human osteoarthritic cartilage. *Am J Pathol* 2003;162(1):171–81.
29. Vogel JP, Szalay K, Geiger F, Kramer M, Richter W, Kasten P. Platelet-rich plasma improves expansion of human mesenchymal stem cells and retains differentiation capacity and in vivo bone formation in calcium phosphate ceramics. *Platelets* 2006;17(7):462–9.
30. Kakudo N, Minakata T, Mitsui T, Kushida S, Notodihardjo FZ, Kusumoto K. Proliferation-promoting effect of platelet-rich plasma on human adipose-derived stem cells and human dermal fibroblasts. *Plast Reconstr Surg* 2008;122(5):1352–60.
31. He L, Lin Y, Hu X, Zhang Y, Wu H. A comparative study of platelet-rich fibrin (PRF) and platelet-rich plasma (PRP) on the effect of proliferation and differentiation of rat osteoblasts in vitro. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2009;108(5):707–13.
32. Akeda K, An HS, Okuma M, Attawia M, Miyamoto K, Thonar EJ, *et al.* Platelet-rich plasma stimulates porcine articular chondrocyte proliferation and matrix biosynthesis. *Osteoarthritis Cartilage* 2006;14(12):1272–80.
33. Caceres M, Hidalgo R, Sanz A, Martinez J, Riera P, Smith PC. Effect of platelet-rich plasma on cell adhesion, cell migration, and myofibroblastic differentiation in human gingival fibroblasts. *J Periodontol* 2008;79(4):714–20.
34. Mishra A, Tummala P, King A, Lee B, Kraus M, Tse V, *et al.* Buffered platelet-rich plasma enhances mesenchymal stem cell proliferation and chondrogenic differentiation. *Tissue Eng Part C Methods* 2009;15(3):431–5.
35. Ng F, Boucher S, Koh S, Sastry KS, Chase L, Lakshminpathy U, *et al.* PDGF, TGF-beta, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): transcriptional profiling can identify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages. *Blood* 2008;112(2):295–307.
36. Anitua E, Sanchez M, Zalduendo MM, de la Fuente M, Prado R, Orive G, *et al.* Fibroblastic response to treatment with different preparations rich in growth factors. *Cell Prolif* 2009;42(2):162–70.
37. Spreafico A, Chellini F, Frediani B, Bernardini G, Niccolini S, Serchi T, *et al.* Biochemical investigation of the effects of human platelet releasates on human articular chondrocytes. *J Cell Biochem* 2009;108(5):1153–65.
38. Chen P, Carrington JL, Hammonds RG, Reddi AH. Stimulation of chondrogenesis in limb bud mesoderm cells by recombinant human bone morphogenetic protein 2B (BMP-2B) and modulation by transforming growth factor beta 1 and beta 2. *Exp Cell Res* 1991;195(2):509–15.
39. Goepfert C, Lutz V, Lunse S, Kittel S, Wiegandt K, Kammal M, *et al.* Evaluation of cartilage specific matrix synthesis of human articular chondrocytes after extended propagation on microcarriers by image analysis. *Int J Artif Organs* 2010;33(4):204–18.
40. Sanchez M, Anitua E, Azofra J, Aguirre JJ, Andia I. Intra-articular injection of an autologous preparation rich in growth factors for the treatment of knee OA: a retrospective cohort study. *Clin Exp Rheumatol* 2008;26(5):910–3.
41. Kon E, Buda R, Filardo G, Di Martino A, Timoncini A, Cenacchi A, *et al.* Platelet-rich plasma: intra-articular knee injections produced favorable results on degenerative cartilage lesions. *Knee Surg Sports Traumatol Arthrosc* 2010;18(4):472–9.
42. Zimmerman GA, Weyrich AS. Immunology. Arsonists in rheumatoid arthritis. *Science* 2010;327(5965):528–9.
43. Guingamp C, Gegout-Pottie P, Philippe L, Terlain B, Netter P, Gillet P. Mono-iodoacetate-induced experimental osteoarthritis: a dose-response study of loss of mobility, morphology, and biochemistry. *Arthritis Rheum* 1997;40(9):1670–9.
44. Janusz MJ, Hookfin EB, Heitmeyer SA, Woessner JF, Freemont AJ, Hoyland JA, *et al.* Moderation of iodoacetate-induced experimental osteoarthritis in rats by matrix metalloproteinase inhibitors. *Osteoarthritis Cartilage* 2001;9(8):751–60.