ARTICLE
Journal of Cellular Biochemistry 108:1153–1165 (2009)

Journal of Cellular Biochemistry

Biochemical Investigation of the Effects of Human Platelet Releasates on Human Articular Chondrocytes

Adriano Spreafico,[1,2*] Federico Chellini,[1] Bruno Frediani,[1,2] Giulia Bernardini,[3] Silvia Niccolini,[1] Tommaso Serchi,[3] Giulia Collodel,[4] Alessandro Paffetti,[3] Vittorio Fossombroni,[5] Mauro Galeazzi,[1] Roberto Marcolongo,[2] and Annalisa Santucci[2,3]

[1] *Sezione di Reumatologia, Dipartimento di Medicina Clinica e Scienze Immunologiche, Università di Siena, Policlinico Le Scotte, Siena, Italy*

[2] *Centro Interdipartimentale per lo Studio Biochimico e Clinico delle Patologie Osteoarticolari, Università di Siena, Siena, Italy*

[3] *Dipartimento di Biologia Molecolare, Università di Siena, Via Fiorentina 1, Siena, Italy*

[4] *Dipartimento di Scienze Biomediche, Università di Siena, Policlinico Le Scotte, Siena, Italy*

[5] *U.O.C. Immunoematologia Trasfusionale, Azienda Ospedaliera Universitaria Senese, Siena, Italy*

ABSTRACT

The aim of the present study was to demonstrate the mitogenic and differentiating properties of platelet-rich plasma releasates (PRPr) on human chondrocytes in mono- and three-dimensional cultures. In order to assess if PRPr supplementation could maintain the chondrocyte phenotype or at least inhibit the cell de-differentiation even after several days in culture, we performed a proteomic study on several cell cultures independently grown, for different periods of time, in culture medium with FCS, human serum (HS), and releasates obtained from PRP and platelet-poor plasma (PPP). We found that PRP treatment actually induced in chondrocytes the expression of proteins (some of which novel) involved in differentiation. J. Cell. Biochem. 108: 1153–1165, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: PLATELET RICH PLASMA; CHONDROCYTE; GROWTH FACTORS; PROTEOME; TISSUE ENGINEERING

In recent years, a large number of clinical studies have suggested that platelet (PLT) derivatives, such as PLT gel, PLT-rich plasma (PRP) and similar products, have the potential for a substantial therapeutic role in tissue regeneration [Sanchez et al., 2003a; Marx, 2004; Borzini and Mazzucco, 2005]. In particular, the local administration of PLT derivatives appears to be effective in the healing of tissue lesions in a preponderant number of experiments carried on animal models and human subjects. PLT treatments aimed to significant enhancements of tissue healing have been so far described for bone and soft tissue regeneration in oral and maxillofacial reconstructive surgery [Anitua, 1999; Kassolis et al., 2000; Tozum and Demiralp, 2003] and post-surgery healing and remodelling of ligament grafts and ruptured tendons [Virchenko and Aspenberg, 2006]. However, the general efficacy of PLT treatment in surgery is far to be standardised and universally accepted. In vivo studies are often based on anecdotal cases and their results are sometimes conflicting, highlighting the need for an in-depth analysis of the complex molecular mechanisms playing a role in the regenerative cellular processes. In this direction, an extensive in vitro experimentation has been developed, focused on the biological effects that high concentrated PLT derivatives can exert on cultured cells of mesenchimal origin like fibroblasts, tenocytes, osteoblasts and chondrocytes [Kaps et al., 2002; Gaissmaier et al., 2005; Kanno et al., 2005; de Mos et al., 2008].

Platelets are known to actively participate in healing processes by delivering a broad spectrum of growth factors (GFs) and other active molecules (chemokines, arachidonic acid metabolites, extracellular matrix proteins, nucleotides, ascorbic acid and others) to the injured site by exocytosis following adhesion or their stimulation by thrombin or other strong stimuli-like calcium addition. GFs secreted by platelets include platelet-derived growth factor (PDGF), endothelial growth factor (EGF), insulin-like growth factor (IGF-I),

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Fondazione Monte dei Paschi di Siena; Grant sponsor: Toscana Life Sciences Foundation.

*Correspondence to: Dr. Adriano Spreafico, U.O.C. Reumatologia, Azienda Ospedaliera Universitaria Senese, Viale Bracci 1, 53100 Siena, Italy. E-mail: spreafico@unisi.it

Received 31 October 2008; Accepted 11 August 2009 • DOI 10.1002/jcb.22344 • © 2009 Wiley-Liss, Inc.
Published online 3 September 2009 in Wiley InterScience (www.interscience.wiley.com).

transforming growth factor β-I (TGF β-I), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF) [Fréchette et al., 2005]. These substances act in synergy on local cells inducing specific responses: TGF β-I, PDGF, and IGF-I, promote proliferation, cell migration and synthesis of extracellular matrix proteins including collagen, while VEGF, HGF and bFGF are chemiotactic and mitogenic for endothelial cells promoting angiogenesis and vascularisation.

Articular cartilage is an avascular tissue that has a limited capacity for self-repair. Recently proposed cell-based cartilage repair procedures require that autologous articular chondrocytes are isolated from a small biopsy, expanded in vitro, and then either directly injected into the defect [Brittberg et al., 2001; Giannini et al., 2005], or used to engineer implantable grafts [Sittinger et al., 1999; Hunter and Levenston, 2004]. Generally, several technical problems have limited the wide clinical application of these techniques, in particular: (i) the low yield of chondrocytes that can be obtained from a biopsy; (ii) the chondrocyte trend to lose their chondrogenic potential during the expansion phase in conventional culture systems based on foetal calf serum (FCS) addition to the culture medium; (iii) the low capacity of chondrocytes to re-differentiate towards cartilage-forming cells after the expansion phase. In the last years, many of these aspects have been ameliorated thanks to the development of cultural conditions based on the introduction of mixtures of recombinant GFs and the employment of a variety of biocompatible matrices supporting an appropriate tissue formation in vitro and in vivo [Arévalo-Silva et al., 2001; Schmidt et al., 2006]. Although it is demonstrated that many GFs are able to induce chondrocyte proliferation and differentiation, their biological effects strongly vary in function of their synergy and combination [Harrison et al., 1992; Chopra and Anastassiades, 1998; Jakob et al., 2001]. Moreover, the stimulating properties of distinct GFs may depend on the physiological status of the cultured chondrocytes, including the differentiation potential and the age of donor [Barbero et al., 2004]. These cultural aspects suggested the use of concentrated autologous or homologous PLT preparations as rich, safe and economic sources of GFs to be additioned to cultural medium [Kaps et al., 2002]. In some applications, platelets have been used directly as PRP, while in other cases, platelet gels and releasates were prepared starting from PRP.

The aim of the present study was to demonstrate the mitogenic and differentiating properties of PRP releasates (PRPr, obtained by means an optimised and standardised protocol) on human chondrocytes in mono- and three-dimensional cultures. We monitored the gene expression of some differentiation markers and we found that PRPr treatment actually induced in chondrocytes the expression of proteins involved in differentiation. We also found that PRPr supplementation was able to maintain the chondrocyte phenotype and inhibit cell de-differentiation even after several days of culture. Moreover, we performed a proteomic study on several chondrocytic cultures independently grown for different periods of time in culture medium with FCS, human serum (HS), PRPr and PPPr. The analysis of protein repertoires confirmed significant differences of gene expression and assessed the involvement of additional proteins in chondrocytic differentiation and anabolic activity. Our in vitro results strongly support the efficacy of PRPr supplementation to ameliorate the differentiation status of human articular chondrocytes.

MATERIALS AND METHODS

PREPARATION OF PLATELET RELEASATES

Platelet concentrates were prepared employing a conventional bag system following the protocol, with minor modifications, proposed by Dugrillon et al. [2002]. A pool of venous blood from three healthy volunteers of the same blood group (250 ml) was collected into a 500 ml bag containing a citrate phosphate dextrose adenine (CPDA) solution as anticoagulant (1 ml CPDA/7 ml blood). Blood was centrifuged once at 205*g* for 20 min to remove red blood cells. The buffy coat was transferred into a 150 ml bag placed at room temperature for 3–4 h under mild agitation and then centrifuged at 800*g* for 15 min. The obtained supernatant, constituted by platelet-poor plasma (PPP), was removed and collected, while the pelleted platelets were resuspended with 10 ml of remaining autologous plasma in order to yield a platelet-rich plasma (PRP) suspension. The PRP product was then aliquoted into sterile vials. During the process, the platelet content has been determined for the whole blood, PPP and PRP fractions.

Platelet releasates (PPPr, PRPr) were then obtained by two cycles of freezing (−20 and −80°C, respectively) and thawing (37°C) followed by a final centrifugation step (1,500*g* for 15 min at 4°C) to remove debris. This method causes the rupture of the platelet membranes, leading to a massive release in the supernatant of the GFs contained within the platelet alpha granules. To avoid the formation of a fibrin clot in the cell cultures grown with PRPr and PPPr, probably induced by the activity of cellular endogenous thrombin, the platelet releasates were separately clotted by adding calcium gluconate alone. The clotted preparations were then centrifuged (1,500*g* for 5 min) and the isolated supernatants were added to the cultures. The protein concentrations of the PRP and PPP releasate preparations were equal and in the same range of the related serum. However, the preparations were adjusted, with a minimal addition of physiological solution, to the concentration of 40 mg/ml.

In our experiments, the cell cultures were supplemented with 10% FCS or with HS, PRPr and PPPr at an equivalent protein concentration (about 2 mg/ml). This cultural condition was achieved when our preparations of HS, PRPr and PPPr were conveniently diluted in physiological solution and added at 5% to the culture medium.

TGF-β1 QUANTIFICATION

TGF-β1 levels were assayed in serum and in the PPPr and PRPr preparations obtained from the same blood, by means of a quantitative sandwich enzyme immunoassay (TGF-β1 detection kit, ELISA, Biosource International, Camarillo, CA) following the instructions of the manufacturer.

ISOLATION AND CULTURE OF HUMAN ARTICULAR CHONDROCYTES IN VITRO

Human articular cartilage fragments were obtained after informed consent from the femoral heads of patients with OA, defined by the

clinical and radiological criteria of the American College of Rheumatology (ACR), undergone surgery for total hip replacement. Immediately after surgery, macroscopically healthy cartilage was cut aseptically and minced in small pieces. The fragments were washed in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 2% penicillin/streptomycin solution (Life Technologies, GIBCO BRL, Grand Island, NY) and 0.2% Amphoterycin B (Sigma). Chondrocytes were isolated from articular cartilage by sequential enzymatic digestion: 30 min with 0.1% hyaluronidase (Sigma), 1 h with 0.5% pronase (Sigma) and 1 h with 0.2% collagenase (Sigma) at 37°C in wash solution (DMEM + penicillin/streptomycin solution + Amphoterycin B). The cells suspension was then filtered twice using 70 μm nylon meshes, washed and centrifuged for 10 min at 700*g*. The cell pellets were then re-suspended in DMEM containing 10% FCS and expanded in monolayer culture. Cells from individual donors were cultured separately at 37°C in 95% relative humidity and 5% CO_2 atmosphere. Chondrocytes were then seeded in six-well plates at a starting density of 4×10^4 cells/well. Different cell cultures were then separately grown in DMEM supplemented with 5% PPPr, 5% PRPr, 5% HS or 10% FCS. The medium was changed 2 days a week.

CELL PROLIFERATION

The proliferative response of chondrocytes to PRPr, PPPr, FCS and HS, was evaluated in monolayer cultures, in two sets of experiments, using a colorimetric assay system based on tetrazolium salts reduction exerted by cellular mitochondrial dehydrogenases (MTT assay, Boehringer, Mannheim, Germany). The experiments were carried out on pre-confluent cell cultures to avoid that contact inhibition would influence the results. Briefly, each culture was incubated with 0.5 mg/ml of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide labelling reagent for 4 h in a humidified atmosphere. The resultant purple formazan salt crystals were solubilised with DMSO. After 15 min of incubation, the solubilised formazan product was spectrophotometrically quantified. An increase in the number of living cells resulted in the increased total metabolic activity in the sample. This increase was directly correlated to the amount of purple formazan crystals formed, as monitored by the absorbance. In a first series of experiments we investigated the mitogenic effect of different PRPr concentrations in comparison with equal amounts of PPPr. Twenty-four hours after plating, the cultural medium was changed and the cells were grown for 48 h in culture medium supplemented with 1%, 5% and 10% PRPr or PPPr. Successively, time course experiments were performed to establish a growth curve of cells cultured in PRPr, PPPr, FCS and HS, at equivalent protein concentrations (about 2 mg/ml). The cultural medium supplemented with the different biological compounds was replaced every 3 days. The cellular proliferation was assayed at days 2, 9 and 20 after the beginning of the treatment. The MTT measurements were repeated for four chondrocyte cultures derived from different human specimens.

GENE EXPRESSION ANALYSIS

To investigate the transcriptional expression of chondrocyte-specific genes, we used a real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) protocol [Bustin, 2000], according to the instructions of the manufacturer (LightCycler® PCR, Roche Diagnostics, Mannheim, Germany). RNA was isolated from chondrocytes using a Totally RNA Kit (Ambion, Inc., Austin, TX) and cDNA was generated from 1 μg RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) and oligo$(dT)_{18}$ primers (Table I). The Real Time PCR analyses were performed with a LightCycler™ Version 3.5 using a FastStart DNA Master^{PLUS} SYBR Green I Kit (Roche Diagnostics). Human collagen-type I, human collagen-type II, Sox9, aggrecan and alkaline phosphatase gene sequences were amplified by using target-specific primer pairs obtained from MWG Biotech AG (Ebersberg, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for internal control and gene expressions in each reaction was normalised by GAPDH expression. The primer sequences and the corresponding annealing temperatures are shown in Table I. The yield of the PCR products was enumerated for individual cDNAs by monitoring the SYBR-Green fluorescence intensities. The analysis of the data was performed using a LightCycler Relative Quantification Software, Version 1.0 (Roche Diagnostics). For each sample the quality of the PCR product was tested by melting curve analysis. The size of the product was confirmed by gel electrophoresis.

PROTEOGLYCANS (PGs) ASSAY

PGs release was assayed by an immunoenzymatic method (PG-EASIA kit, BioSource Europe, Belgium). The assay sensitivity was 0.9 ng/ml. The results obtained for the different cultural supernatants were normalised to the related DNA content of each assayed culture. The DNA was extracted with a high pure PCR template preparation kit (Roche Diagnostics) and measured with the high sensitive Qubit™ quantitation system (Invitrogen, USA).

GEL ASSEMBLY FOR THREE-DIMENSIONAL CULTURE

The maintenance of the chondrogenic potential in monolayer de-differentiated chondrocytes treated with PRPr was assessed by means of an in vitro assay for cartilage formation in three-dimensional cultures based on fibrin gel [Hunter et al., 2004].

TABLE I. Primer Sequences and PCR Conditions

RT-PCR primers	Sense primer (5′–3′)	Antisense primer (5′–3′)	Annealing temp. (°C),	Product size (bp)
GAPDH	AGCCACATCGCTCAGACA	GCCCAATACGACCAAATCC	60	66
COL1	CAGCCGCTTCACCTACAGC	TTTTGTATTCAATCACTGTCTTGCC	60	83
COL2	GTGCTAACGGCGAGAAGG	CCAGTCTCTCCACGTTCACC	60	122
SOX9	AACGCCTTCATGGTGTGG	TCGCTCTCGTTCAGAAGTCTC	60	91
ALPL	GGGTCAGCTCCACCACAA	GGCATTGGTGTTGTACGTCTT	60	94
AGC1	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA	60	85

For each cell culture, a suspension of 5×10^5 cells in 100 μl of DMEM was added to 500 μl of PRP previously placed in a well of a polycarbonate mould (11 mm diameter by 5 mm depth). Immediately after the seeding of the cells, about 30 μl of a commercial 10% calcium gluconate solution were added. The fibrin gel was allowed to polymerise in a 5% CO_2 incubator for 30 min at 37°C. The gel cultures were then released from the mould and transferred into wells of culture plates (24 wells) each filled with 2 ml of DMEM supplemented with 10% FCS, 5% PPPr or 5% PRPr. Gels were cultured in free-swelling conditions in a 37°C, 5% CO_2 incubator, changing the medium every 3 days.

HISTOLOGICAL AND IMMUNOFLUORESCENCE ANALYSES

The deposition of cartilage matrix components in the fibrin gel masses was evaluated by means of histological examination for PG accumulation via toluidine blue staining. Moreover, collagen type II deposition was determined by immunofluorescence.

Gel masses containing chondrocytes entrapped in the fibrin scaffold and cultured with culture medium containing FCS or PPPr or PRPr, were shock-frozen in liquid nitrogen and embedded in 'TissueTec' (Jung/Leica Instruments GmbH, Nussloch, Germany). Sections of 7–15 μm thickness were cut with a cryotome. The cryosections were then fixed with 4% formaldehyde for 20 min, washed and stained with toluidine blue to visualise cell morphology and PG distribution by light microscopy.

In order to detect collagen type II deposition, fixed sections were rinsed, incubated with 1% BSA/PBS, and then incubated again with a mouse anti-collagen type II monoclonal antibody (Sigma) for 60 min at room temperature in a humidified atmosphere. After binding of the primary antibodies, the samples were washed in 1% BSA/PBS and then incubated for 45 min at room temperature with secondary fluorescence-labelled goat anti-mouse antibodies (Invitrogen). Finally, the cryosections were embedded in mounting medium and observed with a Leica DM/LB Fluorescent Microscopy System.

PROTEIN EXTRACTS PREPARATION

Cellular suspensions (undisturbed monolayers lysed directly on the culture plates and immediately additionated with a cocktail of proteases inhibitors) were centrifuged at 700*g* for 5 min in a Beckman model J2-21 centrifuge equipped with a JA10 rotor. The supernatant of each culture was discarded and the pellet was washed three times with PBS. The cell pellets were immediately resuspended and denatured in the buffer for 2-DE analysis consisting of a solution containing 8 M urea, 4% (w/v) CHAPS, 40 mM Tris, 65 mM dithioerythritol and a trace of bromophenol blue. Total protein content was determined in cell cultures lysates using the BioRad protein assay. The total protein values were normalised to the relative number of viable cells.

TWO-DIMENSIONAL ELECTROPHORESIS

2-DE was carried out according to procedures detailed elsewhere [Bernardini et al., 2004]. Forty-five micrograms of protein sample was applied to an Immobiline strip (IPG, Immobilized pH Gradient, Amersham Bioscience) consisting of a nonlinear gradient, pH range 3.5–10, previously rehydrated. Isoelectric focusing was carried out on Multiphor II (Amersham Bioscience). The voltage was linearly increased from 300 to 3,500 V during the first 3 h and then stabilised at 5,000 V for 22 h (total 110 kV × h). The IPG strip was then equilibrated in 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, 0.05 M Tris–HCl pH 6.8, 2% (w/v) dithioerythritol and then with 2.5% (w/v) iodoacetamide.

Electrophoresis in the second dimension was carried out on a 9–16% polyacrylamide nonlinear gradient gel (18×20 cm^2 × 1.5 mm), at a constant current of 40 mA. Gels were stained with silver nitrate as previously described [Bernardini et al., 2004]. Analysis was performed in triplicate. The digitalised images were obtained by scanning of the gels (Image Scanner, Amersham Bioscience) and then analysed qualitatively and quantitatively by the Melanie II 2D-PAGE and PDQuest softwares (Bio-Rad, Hercules, CA).

Spot volumes were obtained in pixel units and normalised to the total absorbance of the gel. The increasing/decreasing index (fold change) was calculated as the ratio of spot volume (relative volumes) between the different gel maps.

Protein spot identification was obtained by gel matching with reference gels [Ruiz-Romero et al., 2005, 2006; Spreafico et al., 2006].

STATISTICS

Data are expressed as the mean ± standard deviation. Student's *t*-test and multiple-measurement ANOVA analysis followed by the Bonferroni-type multiple comparison were used. Differences with *P*-value <0.05 were considered significant.

RESULTS

PLATELET RELEASATES

Platelet count was performed on whole pooled blood and its PPP and PRP fractions (Table IIa). The concentration of platelets of whole blood was 234k/μl. Our platelet concentration protocol allowed obtaining a 6.23-fold increase in the platelet count of the PRP fraction (1,460k/μl) in comparison with the whole blood.

As reported in Table IIb, the concentration of TGF-β in the PRP releasate after activation was about 126 ng/ml. The level of TGF-β was considerably higher in the PRP releasate compared to the serum and/or PPP releasate obtained from the same blood.

TABLE II. PRP Platelet (a) and TGF-β (b) Enrichment

	No. of platelets (10^3/μl)	Platelet enrichment vs. blood
(a)		
Pooled blood	234 ± 46.8	1
PRP fraction	1460 ± 292.0	6.23
PPP fraction	28 ± 5.6	0.12
	TGF-β content (ng/ml)	**TGF-β enrichment vs. serum**
(b)		
Serum	35.7 ± 7.1	1
PRP releasate	126.2 ± 25.2	3.5
PPP releasate	10.6 ± 2.1	0.29

Mean values (±SD) from three donors.

TWO-DIMENSIONAL MONOLAYER CULTURES

Cell proliferation. As observed in similar experiments proposed by other authors [Kaps et al., 2002] the PRP releasates enhanced chondrocyte proliferation in a dose- and time-dependent manner.

MTT assays performed on four different chondrocyte cultures revealed that, after 48 h of treatment, the proliferation of cells grownwith 5% PRPr increased of +96% in comparison with cells grown with 5% PPPr. The treatment with higher PRPr and PPPr concentrations (10%) did not induce a significant enhancement of cell proliferation (Fig. 1A).

Monolayer cultures were also treated for 2, 9 and 20 days [Girotto et al., 2003] with PRPr, PPPr, FCS and HS at the same protein concentration. As revealed by MTT assays (Fig. 1B), treatment with PRPr resulted in a significant increase in cell proliferation when compared to the PPPr, FCS and HS groups (PRPr: +91%, $P < 0.001$ vs. PPPr; +41%, $P < 0.001$ vs. FCS; +35%, $P < 0.005$ vs. HS).

VALIDATION OF CHONDROCYTIC PHENOTYPE: EXTRACELLULAR MATRIX SYNTHESIS AND CELL APPEARANCE

Gene expression of chondrogenic markers. During the expansion phase, the levels of different transcripts for Sox9 and aggrecan were assessed in the first 24 h of treatment by means of Real Time PCR. In PRPr treated cultures, after 4 h, the mRNA expression for Sox9 was increased at least sixfold in comparison with the other types of treatment (Fig. 2a). An increase in aggrecan expression (about 2.5-fold in comparison with the induction by HS and FCS) was also observed after 4 h in PRPr treated cultures (Fig. 2b). In order to evaluate the degree of de-differentiation of our culture systems, we performed real-time PCR assays for the gene expression of collagen type I and collagen type II after 2 and 20 days of treatment. Collagen type I, a well-known marker of de-differentiation, showed quite a constant expression level amongst the cultures grown with the same cultural supplementation (Fig. 2c). On the contrary, a substantial down-regulation of collagen type II expression was noted in all the tested cultures (Fig. 2d). Anyway, the addition of PRPr seemed effective in sustaining collagen II type transcription at a higher level than that obtained by the other types of treatment.

We assessed the levels of alkaline phosphatase (Fig. 2e). Our results showed that after 21 days of treatment with PRP, alkaline phosphatase levels were equal or lower than those obtained with other cell culture supplementations. This could be a good feature for cartilage repair systems where prevention of the hypertrophy of chondrogenic cells is desirable.

Collagen type X was undetectable under our conditions. This is in agreement with the common observation that its synthesis is generally very low during the formation of articular hyaline cartilage.

Proteoglycan assay. As shown in Figure 3, chondrocytes cultured in the presence of PRPr significantly released more PGs (per μg of DNA) than chondrocytes cultured in FCS (+87% after 2 and 9 days, +335% after 20 days; $P < 0.001$). PRPr-treated chondrocytes showed an increase of PG secretion in the first 2 days of culture in comparison with PPPr or HS (+25% vs. PPPr, +32% vs. HS; $P < 0.001$), while no significant treatment-related differences were observed after 9 days. However, after 20 days of treatment the PG release in PPPr and HS treated cultures dropped to basal values, while PRP-treated chondrocytes seemed to be eventually quite high (+625% vs. PPP, +304% vs. HS; both $P < 0.001$).

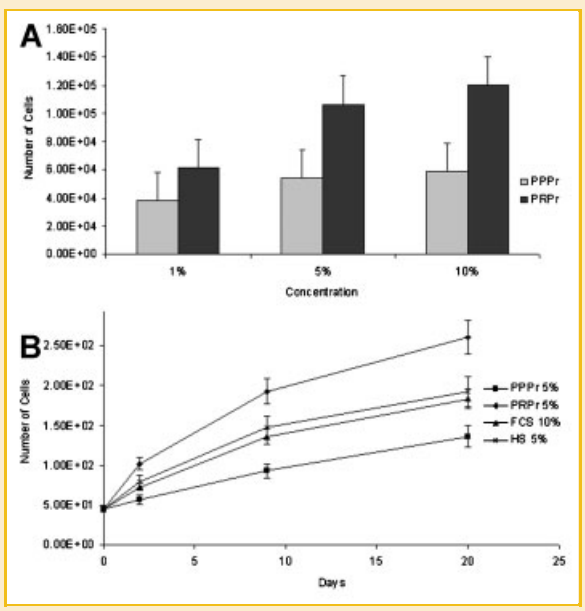

Fig. 1. A: Effects of different concentrations of PPPr and PRPr on human chondrocytes' proliferation. MTT assay was performed after 48 h of treatments. Reported results are the average of experiments performed in triplicate on four different cell cultures. For all the PRPr concentrations *P*-value was <0.05. B: Comparison of the effects of PRPr, PPPr, FCS and HS at the same protein concentration. MTT assay was performed in three independent experiments (see Results Section for statistical analysis).

COMPARATIVE PROTEOMIC ANALYSIS

2-DE was applied to analyse the proteomes of human chondrocytes cultured in 10% FCS or in 5% PRPr for 21 days or in 5% PRPr for 21 days followed by culture in 10% FCS for further 11 days (Fig. 1S). Whole cell protein extracts were separated on a 2-DE gel covering the pH 3-10 (IPG nonlinear gradient) and M_r 200-8 kDa (linear gradient) ranges. Gels loaded with 50 μg protein followed by silver staining were also produced. Performing culture experiments three times assessed reproducibility and sets of 12 gels were produced for each culture; all the gels associated to the same collection were completely superimposable. Proteins were annotated only if detectable in all identical gels. Determination of the p*I* and M_r scales on the gels was performed by gel matching with the calibrated homemade master gel previously produced [Spreafico et al., 2006]. The total number of protein spots revealed in 2-DE gels was of 1,863 for 10% FCS-, 1,602 for 5% PRPr- and 1,877 for 5% PRPr + 10% FCS-cultured chondrocytes. The computer-assisted comparison between the proteomes of cells grown in different media revealed an increasing/decreasing intensity of many spots. A quantitative evaluation was carried out by laser densitometry and the relative abundance of individual polypeptides was calculated. In Table III, the protein species whose intensities showed differences between the maps by a factor greater than 1.5 were reported.

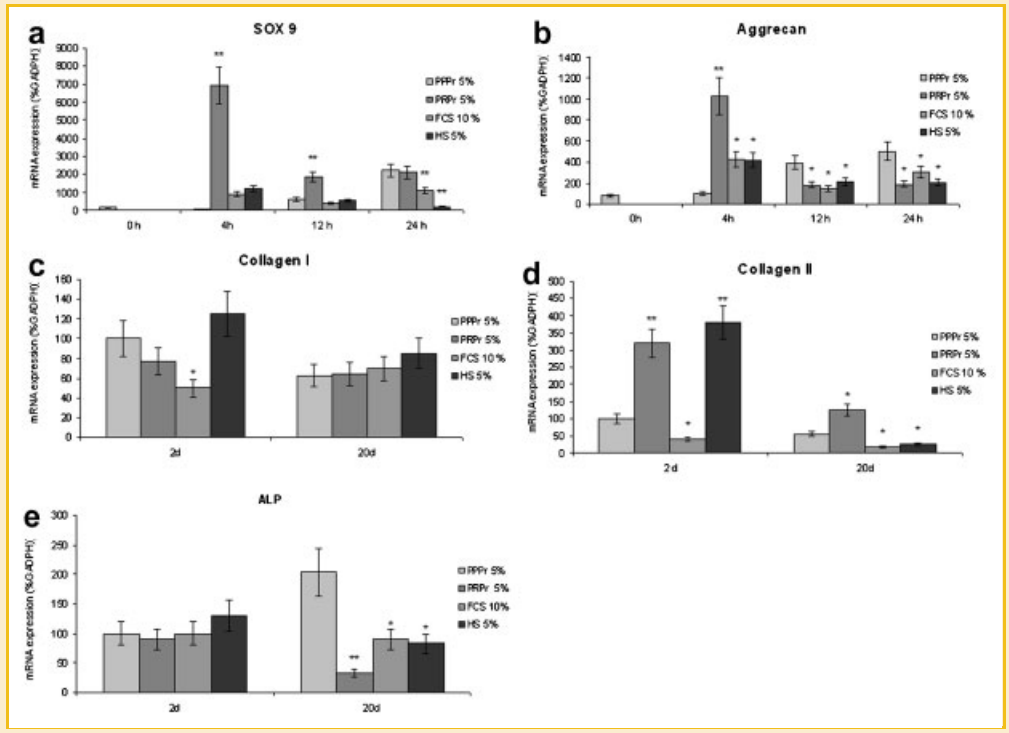

Fig. 2. Time-course gene expression of Sox9 (a) and Aggrecan (b) in human chondrocytes cultured for 4, 12 and 24 h in the presence of 5% PPPr, 5% PRPr, 10% FCS and 5% HS. Levels of mRNA expression of collagen type I (c), collagen type II (d) and alkaline phosphatase (e) after 2 and 20 days of treatment with 5% PPPr, 5% PRPr, 10% FCS and 5% HS. Reported results are the average of three independent experiments using cells from three individuals. $^{*}P < 0.05$, $^{**}P < 0.001$ versus 5% PRPr.

We analysed and compared the proteomes of chondrocyte cultures, derived from the same patient, and independently grown in monolayer for 21 days with 10% FCS or with 5% PRPr. In order to evaluate if the PRPr treatment could induce reversible effects on cellular proteome, we carried on further analyses on cultures initially treated for 21 days with 5% PRPr and grown for further 11 days with 10% FCS. In comparison with the proteome of FCS-treated cells, the number of protein spots of the PRPr-treated cells seemed to be reduced of about −14%. This proteomic change seemed to be almost completely reverted when the 5% PRPr supplementation in the culture medium is suspended and replaced with 10% FCS.

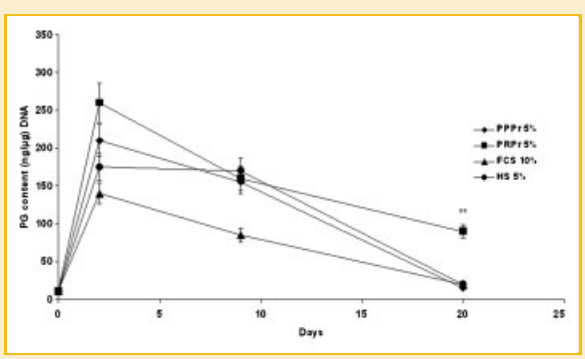

Fig. 3. Proteoglycan secretion of human chondrocytes cultured in the presence of 5% PPPr, 5% PRPr, 10% FCS and 5% HS. Reported results are the average of three independent experiments. $^{*}P < 0.001$ versus 5% PRPr.

After 21 days in culture, PRPr supplementation induced the increase of some protein molecular species while other proteins were remarkably under-expressed in chondrocytes. These proteins may be important to understand biochemical mechanisms associated with chondrocyte differentiation.

DIFFERENTIALLY EXPRESSED PROTEINS

Energy. We observed a slight increase of some of the main high-abundant glycolytic enzymes, typical of the energetic demand of differentiating chondrocytes [Hadhàzy et al., 1973], living in an original hypoxic milieu. Interestingly, we didn't find an increase of glyceraldheyde-3-phosphate dehydrogenase that is in fact considered a negative marker of chondrocytes differentiation [Haudenschild et al., 2001].

Protein synthesis, folding, assembly and cytoskeleton. Proteins involved in protein synthesis were found quite over-expressed in PRPr-treated cells as wells as proteins playing a role in the subsequent protein folding and assembly, confirming the very anabolic status of the cells.

Elongation factor 2 is over-expressed during chondrocyte differentiation [Jefferies et al., 1998]. An enhanced expression of the T-complex protein 1 beta and zeta subunits of the machinery deputed to proper folding of actin and tubulin was observed [Llorca et al., 2001].

TABLE III. Identification of Human Chondrocyte Proteins Whose Relative Abundance Was Affected by Different Cultural Conditions, in 10% FCS or in 5% PRPr for 21 days or in 5% PRPr for 21 days Followed by Culture in 10% FCS for Further 11 days (REV)

Spot	Protein name	Swiss Prot	Gene name	Function	Subcellular localisation	p*I*/M_r theoretical	p*I*/M_r experim.	FCS 10%	PRPr 5%	Fold change PRP vs. FCS	REV
PGAM1	Phosphoglycerate mutase 1	P18669	*pgam1*	Glycolysis	Cytoplasm	6.67/28803	6.43/27956	0.300	0.473	1.57	0.271
LDHB	L-lactate dehydrogenase B chain	P07195	*ldhb*	Glycolysis	Cytoplasm	5.71/36638	5.87/33733	0.222	0.371	1.67	0.335
ENOA	Alpha-enolase	P06733	*eno1*	Glycolysis	Cytoplasm, cell membrane	7.01/47168	6.06/37367	1.403	2.464	1.75	1.247
ACON	Aconitate hydratase	Q99798	*aco2*	Tricarboxylic acid cycle	Mitochondrion	7.36/85425	6.90/84115	0.044	0.241	5.47	0.057
TKT	Transketolase	P29401	*tkt*	Pentose phosphate pathway	Cytoplasm	7.58/67877	7.16/68771	0.117	0.257	2.19	0.179
EF2	Elongation factor 2	P13639	*eef2*	Protein synthesis	Cytoplasm	6.41/95338	6.64/92735	0.060	0.270	4.50	0.020
EFTU	Elongation factor Tu	P49411	*tufm*	Protein synthesis	Mitochondrion	7.26/49541	6.59/44979	0.107	0.254	2.37	0.10
RLA0	60S acidic ribosomal protein P0	P05388	*rlpp0*	Protein synthesis	Cytoplasm, nucleus	5.72/34273	5.78/34430	0.101	0.194	1.92	0.185
HSP74	Heat shock 70 kDa protein 4	P34932	*hspa4*	Protein folding and assembly	Cytoplasm	5.18/94299	4.97/95699	0.115	0.185	1.60	0.112
LAMA	Lamin-A/C	P02545	*lmna*	Regulation of differentiation	Nucleus	6.57/74139	6.29/74870	0.78	1.616	2.07	0.36
DPY2	Dihydropyrimidinase-related protein 2	Q16555	*dpysl2*	Regulation of differentiation; remodelling of the cytoskeleton	Cytoplasm	5.95/62293	6.31/65394	0.320	0.602	1.88	0.161
TCPB	T-complex protein 1 subunit beta	P78371	*cct2*	Folding of actin and tubulin	Cytoplasm	6.01/57488	6.26/57298	0.090	0.144	1.60	0.062
TCPZ	T-complex protein 1 subunit zeta	P40227	*cct6a*	Folding of actin and tubulin	Cytoplasm	6.24/58024	6.47/62379	0.071	0.136	1.91	0.015
VINC	Vinculin	P18206	*vcl*	Cell adhesion, morphology and motility	Cytoplasm, cytoskeleton	5.50/123799	5.84/102559	0.481	0.725	1.50	0.314
SEP2	Septin-2	Q15019	*sept2*	Cytokinesis	Cytoplasm	6.15/41487	6.31/39000	0.061	0.126	2.06	0.062
ANXA2	Annexin A2	P07355	*anxa2*	Heat-stress response	Secreted	7.56/38472	6.93/32988	1.464	3.213	2.19	2.17
AOP2	Peroxiredoxin-6	P30041	*prdx6*	Redox regulation; regulation of phospholipid turnover	Cytoplasm, Lysosome, Cytoplasmic vesicle	6.02/24903	6.33/27078	0.192	0.284	1.50	0.177
CATA	Catalase	P04040	*cat*	Redox regulation	Peroxisome, secreted	6.90/59756	6.88/62971	2.253	4.210	1.86	2.011
DJ1	Protein DJ-1	Q99497	*park7*	Redox regulation	Nucleus, cytoplasm	6.33/19891	6.19/23609	0.110	0.166	1.51	0.141
PD6I	ALG2-interacting protein	Q8WUM4	*pdcd6ip*	Targeting the cargo proteins to the vacuolar lumen	Cytoplasm	6.13/96023	6.31/91863	0.008	0.05	5.87	0.007
SIH2	Serpin H1	P50454	*serpinh1*	Chaperone in the biosynthetic pathway of collagen	ER lumen	8.75/46440	8.61/42771	0.386	0.611	1.58	0.531
CATD	Cathepsin D	P07339	*ctsd*	Acid protease active in intracellular protein breakdown	Lysosome	6.10/44552	5.32/28854	1.318	0.748	0.56	1.148
CH60	60 kDa heat shock protein	P10809	*hspd1*	Protein folding and assembly	Mitochondrion matrix	5.70/61054	5.08/61405	1.002	0.518	0.50	0.903
GPR75	Stress-70 protein	P38646	*hspa99*	Protein folding and assembly; regulation of proliferation	Mitochondrion	5.87/73680	5.65/71418	0.634	0.419	0.66	0.532
LEG1	Galectin-1	P09382	*lgals1*	Regulation of differentiation	Secreted	5.33/14715	4.93/11156	0.551	0.333	0.60	0.479
TBB5	Tubulin beta chain	P07437	*tubb5*	Major constituent of microtubules	Cytoplasm, nucleus	4.78/49670	4.80/51484	0.824	0.490	0.59	0.676
ATPD	ATP synthase subunit delta	P30049	*atp5d*	Produces ATP from ADP in the presence of a proton gradient across the membrane	Mitochondrion	5.38/17489	4.39/12536	0.095	0.022	0.23	0.077
ATPQ	ATP synthase subunit	O75947	*atp5h*	One of the chains of the nonenzymatic component of the mitochondrial ATPase complex	Mitochondrion	5.21/18491	5.13/21736	0.081	0.013	0.16	0.08
NDKA	Nucleoside diphosphate kinase A	P15531	*nme1*	Synthesis of nucleoside triphosphates other than ATP	Cytoplasm, nucleus	5.83/17148	5.9/18100	0.101	0.036	0.35	0.156
IF5A	Eukaryotic translation initiation factor 5A-1	P63241	*eif5a*	Protein biosynthesis	Cytoplasm	5.07/16832	5.05/15277	0.114	0.023	0.20	0.12
CALR	Calreticulin	P27797	*calr*	Protein folding and assembly	ER lumen, cytoplasm, secreted	4.29/48141	4.2959503	0.435	0.188	0.43	0.662
SIAL	Bone sialoprotein 2	P21815	*ibsp*	Cell–matrix interaction	Secreted	4.12/35018	4.68/29836	0.388	Absent	Absent	0.158
ANX5	Annexin A5	P08758	*anxa5*	Anticoagulant protein that acts as an indirect inhibitor of the thromboplastin-specific complex.	Cytoplasm	4.94/35805	4.84/30340	0.624	0.268	0.42	0.508
CALU	Calumenin	O43852	*calu*	Involved in regulation of vitamin K-dependent carboxylation of multiple amino-terminal glutamate residues	ER lumen, secreted	4.47/37106	4.42/39329	0.126	0.038	0.30	0.144
CRAA	Alpha-crystallin A chain	P02489	*cryaa*	Regulation of apoptosis; protein folding	Nucleus	5.77/19909	5.67/18358	0.166	0.008	0.04	0.082
CRAB	Alpha-crystallin B chain	P02511	*cryab*	Regulation of apoptosis; protein folding	Mitochondrion	6.76/20158	6.9720700	0.204	0.056	0.27	0.037
FRIL	Ferritin light chain	P02792	*ftl*	Iron homeostasis and transport	Cytoplasm	5.51/20019	5.64/20482	0.018	0.001	0.05	0.014

TABLE III. (*Continued*)

Spot	Protein name	Swiss Prot	Gene name	Function	Subcellular localisation	pI/M_r theoretical	pI/M_r experim.	FCS 10%	PRPr 5%	Fold change PRP vs. FCS	REV
GSTP1	Glutathione S-transferase P	P09211	*gstp1*	Redox regulation	Mitochondrion	5.43/23355	5.7/24697	0.240	0.120	0.50	0.275
THIO	Thioredoxin	P10599	*txn*	Redox regulation	Cytoplasm	4.82/11737	4.74/10581	0.140	0.050	0.35	0.184
PDX2	Peroxiredoxin-2	P32119	*prdx2*	Redox regulation	Cytoplasm	5.66/21891	5.37/20410	0.011	Absent	Absent	0.007
PHB	Prohibitin	P35232	*phb*	Regulation of proliferation	Mitochondrion inner membrane	5.57/29804	5.70/29015	0.142	0.044	0.30	0.142
PSA5	Proteasome subunit alpha type-5	P28066	*psma5*	Protein degradation	Cytoplasm, nucleus	4.74/26411	4.56/26031	0.101	0.004	0.03	0.11
PSE1	Proteasome activator complex subunit 1	Q06323	*psme1*	Protein degradation	Nucleus	5.78/28723	5.54/30171	0.061	0.029	0.47	0.06
UBC1	Ubiquitin-conjugating enzyme E2-25 kDa	P61086	*hip2*	Protein degradation	Cytoplasm	5.33/22406	5.23/24329	0.073	0.006	0.08	0.05
RANG	Ran-specific GTPase-activating protein	P43487	*ranbp1*	Regulation of cell cycle	Cytoplasm	5.19/23310	4.94/29069	0.456	0.133	0.29	0.334
HSB1	Heat shock protein beta-1	P04792	*hspb1*	Stress resistance and actin organisation	Cytoplasm, nucleus	5.98/22782	6.10/27078	0.244	0.155	0.63	0.294
GDIR	Rho GDP-dissociation inhibitor 1	P52565	*arhgdia*	Stabilisation of actin cytoskeleton	Cytoplasm, cytoskeleton	5.03/23207	4.89/26326	0.230	0.034	0.14	0.181
TCTP	Translationally controlled tumour protein	P13693	*tpt1*	Microtubule stabilisation Interacts with STEAP3	Cytoplasm, secreted	4.84/ 19595	4.72/21492	0.095	0.002	0.02	0.065
TPNM	Tropomyosin alpha-3 chain	P06753	*tpm3*	Stabilisation of cytoskeleton	Cytoplasm	4.68/32818	4.50/28164	0.156	0.020	0.12	0.187
VIME	Vimentin	P08670	*vim*	Cytoskeletal class-III filaments intermediate	Nucleus, cytoplasm	4.83/46723	5.06/53520	2.125	1.359	0.63	1.976

The protein fold-change values were calculated by the ratio of percentage relative abundance of proteins in the differently cultured cells *versus* the control (10% FCS). Grey background indicates over-expressed proteins (P-value ≤ 0.05), while blank background indicates under-expressed proteins ($P < 0.05$) in PRPr; in both cases the fold change was equal or above 1.5.
Swiss Prot (http://www.expasy.org/sprot/) accession numbers are reported.

Septins often co-localise with actin bundles and may be important components of cytoskeleton. Particularly, Septin-2 that we found increased, is down-regulated in tumours compared to normal tissues [Khalil, 2007].

Annexin A2 is associated to protein glycosilation processes for epithelial regeneration and it is a marker for terminally differentiated chondrocytes [Patchell et al., 2007].

Serpins are cartilage-relevant differentiation markers [Boeuf et al., 2008] and particularly Serpin H1 binds specifically to collagen and could be involved as a chaperone in the biosynthetic pathway of collagen.

Differentiation. Lamin-A/C is associated to the differentiation of human stem cells [Constantinescu et al., 2006] and its high expression is related to a good osteoarticular functioning [Duque and Rivas, 2006].

Vinculin combines with integrins and other proteins to form the focal adhesions that mediate cell adhesion. Formation of these structures activates signalling pathways that ultimately regulate gene expression [Knudson and Loeser, 2002] and could influence whether these cells will maintain their phenotype. Changes in vinculin expression in response to GF stimulation may reflect either a necessary step in the progression through the cell cycle or a response related to complex cellular processes such as cartilage repair.

Redox regulation. Reactive oxygen species (ROS) are the main biochemical factors of cartilage degradation. However, the endogenous production of ROS is also required for stimulation of human articular chondrocyte to produce matrix [Del Carlo et al., 2007]. So the ROS play a double role mediating on one side the loss of cartilage and on the other one the production of new matrix. To prevent ROS toxicity, chondrocytes possess a well-coordinated enzymatic antioxidant system formed principally by superoxide dismutases, catalase and glutathione peroxidase.

Protein DJ1 has cell-growth promoting activity and transforming activity but mainly it is a member of the dj1-hsp70 chaperone pair effective in preventing NO-mediated apoptosis [Gotoh et al., 2004].

Peroxideroxins are peroxidases involved in antioxidant defence and intracellular signalling with important roles in cell differentiation, proliferation and apoptosis. In particular peroxideroxin 6 has been found upregulated during bovine cell maturation [Leyens et al., 2004].

Catalase, when over-expressed and even secreted in the medium, plays a critical role for thiols levels in modulating events linked to chondrocyte maturation and cartilage synthesis and mineralisation [Teixeira et al., 1996].

Protein degradation. Due to the anabolic status of the cell, catabolism, characterised by decreased protein synthesis and accelerated proteolysis, is obviously repressed, as witnessed by the decreased expression of proteasome subunit alpha type-5, proteasome activator complex subunit 1 and the ubiquitin-conjugating enzyme E2-25 kDa. Moreover, it is known that GFs (i.e. IGF-1) oppose catabolism acting on the Ub/proteasome system decreasing the transcription rate and/or stability of mRNAs [Chrysis and Underwood, 1999].

Translationally controlled tumour protein (TCTP) had the most striking differential expression. It is a highly conserved GDP/GTP

exchange factor involved in a wide range of cellular processes, mainly related to cell growth and proliferation: anti-apoptotic, tubulin binding, Ca^{2+} binding. Moreover, being also secreted, it has extracellular functions as histamine-releasing factor and cytokine. TCTP is expressed at a low rate in post-mitotic tissues while at high levels in cells undergoing active division like tumour cells [Schmidt et al., 2007], because it specifically antagonizes the elongation reaction of protein synthesis, functioning as an eEF1Bβ-specific guanine nucleotide dissociation inhibitory factor (GDI) during translation.

TCTP decrease is congruent with the diminished expression of Rho GDP-dissociation inhibitor 1, or Rho GDI 1, (GIDR) whose over-expression leads to disruption of the actin cytoskeleton and negative regulation of cell adhesion. This protein is involved in the myofibroblast differentiation [Lakhe-Reddy et al., 2006].

Annexin V, an early marker of apoptosis, has been observed to decrease in re-differentiating chondrocyte cultures [Haudenschild et al., 2001].

THREE-DIMENSIONAL CULTURE ON FIBRIN SCAFFOLD OF HUMAN CHONDROCYTES

PRPr effects on matrix accumulation of chondrocyte-seeded fibrin gels. The fibrin gel is known to be a tenacious polymer that, resembling the cartilage matrix environment, supports chondrocyte phenotypic expression and matrix elaboration [Perka et al., 2001; Hunter et al., 2004; Lee et al., 2005]. Figure 4 shows the effects of different cultural supplementations on fibrin gels obtained from PRP fractions and seeded with de-differentiated chondrocytes previously expanded with conventional methods. Chondrocytes were three-dimensionally reassembled in fibrin gel and cultured in the presence of 10% FCS or equal protein amounts of PPPr and PRPr preparations. In our experiments, the cellular distribution in all dimensions of the gels appeared to be uniform, independently from the applied experimental conditions. The cells encapsulated in fibrin gel were spherical resembling the typical morphology of the differentiated chondrocytes. After the toluidine blue staining, the rough comparison of several optical fields sampled from differentially treated cultures with a similar cell density, suggests that PRPr supplementation induced a major deposition of PGs in the extracellular compartment.

Moreover, since the different fibrin cultures had been prepared from the same PRP batch, the observed anabolic effects seemed to be prevalently due to GFs contained in the PRPr supplementation and not from those potentially released by the fibrin clot.

IMMUNOFLUORESCENCE ANALYSIS FOR COLLAGEN TYPE II SYNTHESIS AND DEPOSITION

Figure 5 shows different fibrin-gel chondrocyte cultures supplemented with PPPr (A, B), FCS (C, D) or PRPr (E, F) for different periods of time (14 and 21 days). A prominent immunofluorescence staining for collagen type II, especially in the pericellular compartment, was observed for chondrocytes grown in presence of PRPr, in comparison with other fibrin gel cultures treated for the same period of time. The staining intensity appeared to be weaker and not so largely diffused in chondrocyte cultures grown with FCS,

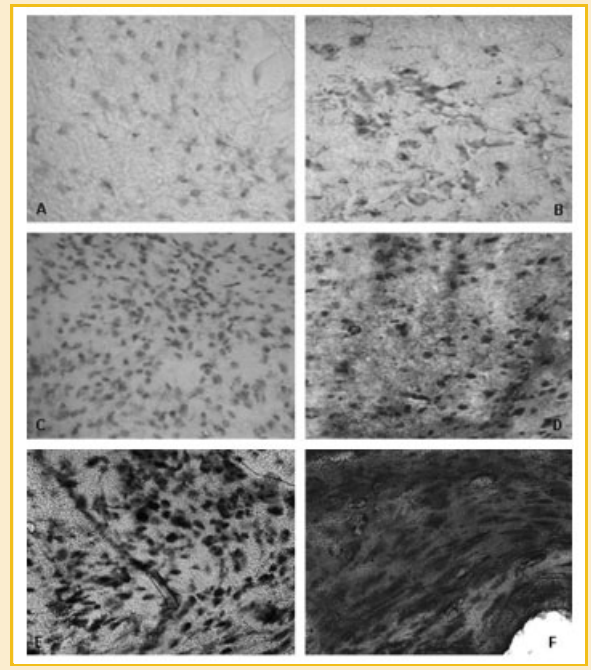

Fig. 4. Histological staining. Toluidine blue staining of fibrin gels seeded with human chondrocytes and cultured for 14 (left panel) and 21 (right panel) days. Different sections were obtained from cultures grown with 5% PPPr (A, B), 10% FCS (C, D) and 5% PRPr (E, F) as cultural supplements.

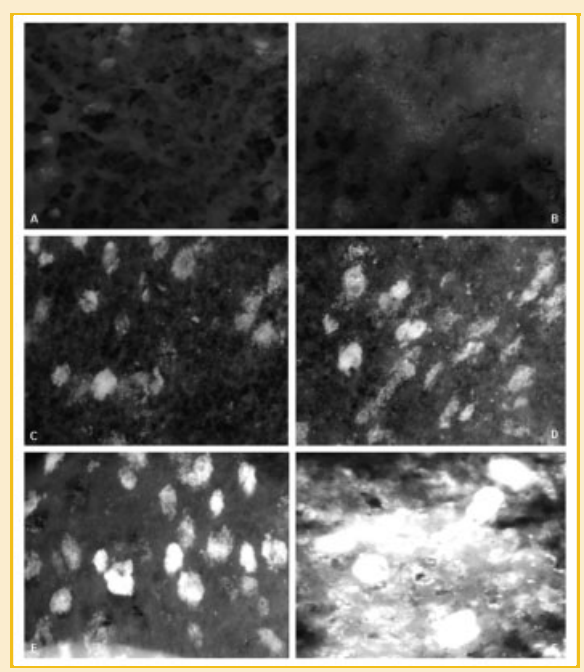

Fig. 5. Immunofluorescence microscopy. Immunolocalisation of collagen type II (green fluorescence) in fibrin gels seeded with human chondrocytes and cultured for 14 (left panel) and 21 (right panel) days with 5% PPPr (A, B), 10% FCS (C, D) and 5% PRPr (E, F) as different cultural supplements.

while only a very low fluorescence was observed for cultures supplemented with PPPr.

DISCUSSION

In the last years, several studies evaluated the possible employment of platelet-derived extracts in clinical applications and tissue engineering. In particular, it has been suggested that platelet extracts could be a valuable source for autologous growth and differentiation factors needed for tissue-engineering approaches in cartilage regeneration [Sanchez et al., 2003b; Wu et al., 2007]. At the moment, the modern autologous chondrocyte transplantation techniques are seriously limited in their clinical use by the elevated cost of purified genetically engineered GFs that are essential for the cellular expansion and differentiation. Moreover, the supplemented medium currently used for cell growth would be qualified to be free of adventitious agents and adverse genetic material [Doucet et al., 2005].

Although the biological effects of PRP-derived preparations on chondrocyte proliferation have been well demonstrated, an effective action on chondrocyte differentiation is still lacking of molecular evidences. Akeda et al. [2006] have suggested that, in a cellular model based on porcine articular chondrocytes grown on alginate beads, PRP releasates can be used as tissue culture supplements for their positive effects on matrix synthesis and accumulation. The aim of our work was to provide further biochemical evidences that the PRPr supplementation to human chondrocytes could support their chondrogenic properties.

Platelet extracts were obtained by means of an optimised method that, on the basis of previous studies [Choi et al., 1980; Akeda et al., 2006], has allowed recovering high concentrations of GFs as revealed by the high amount of TGF-β1 contained in our preparations. In our experimental procedure, we observed that the release of GFs from platelet preparations was generally higher if the cells were submitted to sudden changes in temperature rather than by a physiological degranulation induced by thrombin and calcium addition. This difference in GF yield could be relevant when technical and logistic problems hamper the preparation of platelet releasates immediately after the platelet enrichment procedure. The introduction of a clot formation step after the freeze/thawing platelet treatment was necessary in order to reduce the formation of a fibrin gel in the cultures grown with 5% PRP or 5% PPP. In our procedure, differently from other similar studies, the clot formation was achieved without the use of exogenous thrombin, which has been shown to induce biological effects on cell proliferation and differentiation by itself [Pagel et al., 2003; Nowak et al., 2007]. An alternative method based on the cultural addition of heparine, a fibrinolytic agent, was considered not suitable since the activity of the enzyme could interfere with several cellular physiological processes.

In agreement with previous studies [Kaps et al., 2002; Gaissmaier et al., 2005; Akeda et al., 2006], we observed a strong effect of PRPr preparations on chondrocyte proliferation. Gene expression analyses also supported a stimulating action of PRPr on Sox9 and aggrecan expression at least in the first days of treatment. An increased transcription of Sox9 has been shown to be associated with the chondrocytic re-differentiation process [Stokes et al., 2001]. On the contrary, collagen type II mRNA expression, strongly reduced when chondrocytes are grown in FCS in monolayer cultures for a long time, decreased at a lower extent when cells had been supplemented with PRPr. We also observed that PRPr-treated chondrocytes acquired, on the basis of morphological analysis and PG release, a phenotype suggesting a high anabolic activity, at least in the first days of treatment, even if the proliferation rate is still quite high, as reported also by other authors [Grigolo et al., 2002; Akeda et al., 2006].

A particular interest in regenerative medicine and tissue engineering is focused in the development of well-defined and efficient protocols for directing and sustaining the cultured chondrocyte into an active chondrogenic state. In the ACT procedures, it is highly desirable to define a balanced combination of cytokines and GFs that promotes the synthesis of hyaline cartilage matrix proteins concomitantly delaying the cellular progression to terminal hypertrophy. Hypertrophic chondrocytes are in fact characterised by a strong expression of type X collagen and osteogenic marker genes, and by a substantial down-regulation of many genes for ECM proteins [Col2a1, Col11a2 and aggrecan; Lefebvre and Smits, 2005].

The transcription factor Sox9 plays a critical role in cartilage development by initiating chondrogenesis and preventing the subsequent maturation process leading to chondrocyte hypertrophy. This suppression mechanism by Sox9 on late-stage chondrogenesis partially results from the direct [Zhou et al., 2006] and indirect [Yamashita et al., 2009] inhibition of the Runx2 transcription factor, the main activator of hypertrophic chondrocyte differentiation. In our experiments, we have observed a major transient increase in Sox9 and aggrecan expression just few hours after the addition of fresh culture medium supplemented with PRPr. Since the expression of other markers for chondrocyte terminal differentiation seems to be inhibited or at least delayed by PRPr treatment, we speculate that the increase in Sox9 levels induced by PRPr could at the same time stimulate the chondrogenic activity and prevent hypertrophy during the autologous chondrocyte transplantation (ACT) culture.

In the present work we have mainly focused our research on a limited experimental model based on two-dimensional chondrocyte cultures. Such experimental condition has allowed better standardising our experiments, drastically reducing many methodological variables due to the different nature of the cultural scaffolds and allowing obtaining large amounts of cellular lysates minimising further complex procedures for protein extraction. Although chondrocytes grown under our experimental conditions would inevitably go towards a progressive state of de-differentiation, we obtained, both at the cellular and molecular level, many evidences supporting the differentiating effects PRPr on chondrocytes.

In order to better define the efficacy of the PRPr as preparations rich of GFs to be used in ACT techniques, we have also performed preliminary experiments on human chondrocytes cultured in fibrin gels, an important three-dimensional validation system where the chondrogenic differentiation was ascertained.

The cultures of allograft and autograft chondrocytes transplanted in fibrin have been shown to stimulate hyaline cartilage repair in

various experimental models [Hunter et al., 2004; Lee et al., 2005]. The use of fibrin-based vehicles is appealing because of the ready source of autogenous fibrinogen from plasma, its application as a self-polymerising liquid, and the inherent 'glue'-like properties of thrombin-activated fibrinogen. Moreover, fibrin gel seems to provide a good cell seeding efficiency and allows the maintenance of the chondrocytic phenotype [Malicev et al., 2007].

In our experiments the source of the fibrin gel consisted of a limited volume of PRP. According to the method described by Anitua et al. [2004], once the platelet concentrate is activated by way of thrombin generation with calcium, a three-dimensional and biocompatible fibrin scaffold is formed, and a lot of GFs and proteins are released, progressively, to the local environment.

Our preliminary experiments further support the in vitro efficacy of the PRPr supplementation in the enhancement of the chondrocyte synthesis and deposition of cartilage matrix components. Further experiments will be performed in order to demonstrate a significant efficacy and convenience of PRPr use in comparison with autologous or homologous serum.

The different extents with which PRPr and HS may act on chondrocyte differentiation were only briefly analysed in the present work and needs to be investigated by means of further proteomic analyses. However, in comparison with HS, the whole of the differentiating PRPr activity in addition to its stronger effect on cell proliferation, should allow to consider this preparation very useful to the safe and economic engineering of cartilage constructs.

Proteomic analysis of protein repertoires of chondrocytes differentially cultured clearly indicated the PRPr supplementation positively regulated anabolic pathways towards a differentiation status. The positive and specific effect of PRPr was confirmed when the proteome of 5% PRPr + 10% FCS-treated chondrocytes ('reverted' phenotype) was analysed as well, showing the reversibility of the treatment through the restoration of a protein expression profile of cells typically grown in FCS. Finally, a validation of PRPr effects at the proteome level was obtained when that human articular chondrocytes cultured in PGA three-dimensional scaffolds (where cells are universally considered at a higher differentiation level) in 5% PRPr supplemented medium: in this case over- and under-express proteins that we found analogously expressed as in PRPr-treated mono-dimensional cultures (data not shown).

ACKNOWLEDGMENTS

Authors would like to thank Fondazione Monte dei Paschi di Siena (Traslazione in ambito clinico delle più recenti conoscenze precliniche nell'ambito dell'ingegneria tessutale muscolo-scheletrica per ricostruzioni biologiche da perdita di sostanza traumatiche o patologiche; 2008—Studio proteomico e clinico della patofisiologia dell'alcaptonuria e creazione di un'associazione di pazienti) and Toscana Life Sciences Foundation (Orph0108—Studio proteomico e clinico della patofisiologia dell'alcaptonuria ed allestimento di un trattamento terapeutico) for financial support.

REFERENCES

Akeda K, An HS, Okuma M, Attawia M, Miyamoto K, Thonar EJ, Lenz ME, Sah RL, Masuda K. 2006. Platelet-rich plasma stimulates porcine articular chondrocyte proliferation and matrix biosynthesis. Osteoarthritis Cartilage 14(12):1272–1280.

Anitua E. 1999. Plasma rich in growth factors: Preliminary results of use in the preparation of future sites for implants. Int J Oral Maxillofac Implants 14(4):529–535.

Anitua E, Andia I, Ardanza B, Nurden P, Nurden AT. 2004. Autologous platelets as a source of proteins for healing and tissue regeneration. Thromb Haemost 91(1):4–15.

Arévalo-Silva CA, Cao Y, Weng Y, Vacanti M, Rodríguez A, Vacanti CA, Eavey RD. 2001. The effect of fibroblast growth factor and transforming growth factor-beta on porcine chondrocytes and tissue-engineered autologous elastic cartilage. Tissue Eng 7(1):81–88.

Barbero A, Grogan S, Schäfer D, Heberer M, Mainil-Varlet P, Martin I. 2004. Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity. Ostheoarthritis Cartilage 12(6):476–484.

Bernardini G, Renzone G, Comanducci M, Mini R, Arena S, D'Ambrosio C, Bambini S, Trabalzini L, Grandi G, Martelli P, Achtman M, Scaloni A, Ratti G, Santucci A. 2004. Proteome analysis of Neisseria meningitidis serogroup A. Proteomics 4(10):2893–2926.

Boeuf S, Steck E, Pelttari K, Hennig T, Buneb A, Benz K, Witte D, Sültmann H, Poustka A, Richter W. 2008. Subtractive gene expression profiling of articular cartilage and mesenchymal stem cells: Serpins as cartilage-relevant differentiation markers. Osteoarthritis Cartilage 16(1):48–60.

Borzini P, Mazzucco L. 2005. Platelet gels and releasates. Curr Opin Hematol 12(6):473–479.

Brittberg M, Tallheden T, Sjögren-Jansson B, Lindahl A, Peterson L. 2001. Autologous chondrocytes used for articular cartilage repair: An update. Clin Orthop Relat Res (Suppl 391):S337–S348.

Bustin SA. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25(2):169–193.

Choi YC, Morris GM, Sokoloff L. 1980. Effect of platelet lysate on growth and sulfated glycosaminoglycan synthesis in articular chondrocyte cultures. Arthritis Rheum 23(2):220–224.

Chopra R, Anastassiades T. 1998. Specificity and synergism of polypeptide growth factors in stimulating the synthesis of proteoglycans and a novel high molecular weight anionic glycoprotein by articular chondrocyte cultures. J Rheumatol 25(8):1578–1584.

Chrysis D, Underwood LE. 1999. Regulation of components of the ubiquitin system by insulin-like growth factor I and growth hormone in skeletal muscle of rats made catabolic with dexamethasone. Endocrinology 140(12):5635–5641.

Constantinescu D, Gray HL, Sammak PJ, Schatten GP, Csoka AB. 2006. Lamin A/C expression is a marker of mouse and human embryonic stem cell differentiation. Stem Cells 24(1):177–185.

de Mos M, van der Windt AE, Jahr H, van Schie HT, Weinans H, Verhaar JA, van Osch GJ. 2008. Can platelet-rich plasma enhance tendon repair? A cell culture study. Am J Sports Med 36(6):1171–1178.

Del Carlo M, Schwartz D, Erickson EA, Loeser RF. 2007. Endogenous production of reactive oxygen species is required for stimulation of human articular chondrocyte matrix metalloproteinase production by fibronectin fragments. Free Radic Biol Med 42(9):1350–1358.

Doucet C, Ernou I, Zhang Y, Llense JR, Begot L, Holy X, Lataillade JJ. 2005. Platelet lysates promote mesenchymal stem cell expansion: A safety substitute for animal serum in cell-based therapy applications. J Cell Physiol 205(2):228–236.

Dugrillon A, Eichler H, Kern S, Klüter H. 2002. Autologous concentrated platelet-rich plasma (cPRP) for local application in bone regeneration. Int J Oral Maxillofac Surg 31(6):615–619.

Duque G, Rivas D. 2006. Age-related changes in lamin A/C expression in the osteoarticular system: Laminopathies as a potential new aging mechanism. Mech Ageing Dev 127(4):378–383.

Fréchette JP, Martineau I, Gagnon G. 2005. Platelet-rich plasmas: Growth factor content and roles in wound healing. J Dent Res 84(5):434–439.

Gaissmaier C, Fritz J, Krackhardt T, Flesch I, Aicher WK, Ashammakhi N. 2005. Effect of human platelet supernatant on proliferation and matrix synthesis of human articular chondrocytes in monolayer and three-dimensional alginate culture. Biomaterials 26(14):1953–1960.

Giannini S, Buda R, Grigolo B, Vannini F, De Franceschi L, Facchini A. 2005. The detached osteochondral fragment as a source of cells for autologous chondrocyte implantation (ACI) in the ankle joint. Osteoarthritis Cartilage 13(7):601–607.

Girotto D, Urbani S, Brun P, Renier D, Barbucci R, Abatangelo G. 2003. Tissue-specific gene expression in chondrocytes grown on three-dimensional hyaluronic acid scaffolds. Biomaterials 24(19):3265–3275.

Gotoh T, Terada K, Oyadomari S, Mori M. 2004. hsp70-DnaJ chaperone pair prevents nitric oxide- and CHOP-induced apoptosis by inhibiting translocation of Bax to mitochondria. Cell Death Differ 11(4):390–402.

Grigolo B, Lisignoli G, Piacentini A, Fiorini M, Gobbi P, Mazzotti G, Duca M, Pavesio A, Facchini A. 2002. Evidence for redifferentiation of human chondrocytes grown on a hyaluronan-based biomaterial (HYAff 11): Molecular, immunohistochemical and ultrastructural analysis. Biomaterials 23(4): 1187–1195.

Hadhàzy C, Glant T, Màndi B, Tóth Z, Vereckei L, Boross A. 1973. Studies on cartilage formation. XV. Enzyme histochemical investigation on Krebs cycle, terminal oxidation and hexose monophosphate shunt. Acta Morphol Acad Sci Hung 21(1):13–25.

Harrison ET, Jr., Luyten FP, Reddi AH. 1992. Transforming growth factor-beta: Its effect on phenotype reexpression by dedifferentiated chondrocytes in the presence and absence of osteogenin. In Vitro Cell Dev Biol 28(6):445–448.

Haudenschild DR, McPherson JM, Tubo R, Binette F. 2001. Differential expression of multiple genes during articular chondrocytes redifferentiation. Anat Rec 263(1):91–98.

Hunter CJ, Levenston ME. 2004. Maturation and integration of tissue-engineered cartilages within an in vitro defect repair model. Tissue Eng 10(5–6):736–746.

Hunter CJ, Mouw JK, Levenston ME. 2004. Dynamic compression of chondrocyte-seeded fibrin gels: Effects on matrix accumulation and mechanical stiffness. Osteoarthritis Cartilage 12(2):117–130.

Jakob M, Démarteau O, Schäfer D, Hintermann B, Dick W, Heberer M, Martin I. 2001. Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes enhance chondrogenesis and cartilaginous tissue formation in vitro. J Cell Biochem 81(2):368–377.

Jefferies D, Botman M, Farquharson C, Lester D, Whitehead CC, Thorp BH, Houston B. 1998. Cloning differentially regulated genes from chondrocytes using agarose gel differential display. Biochim Biophys Acta 1396(3):237–241.

Kanno T, Takahashi T, Tsujisawa T, Ariyoshi W, Nishihara T. 2005. Platelet-rich plasma enhances human osteoblast-like cell proliferation and differentiation. J Oral Maxillofac Surg 63(3):362–369.

Kaps C, Loch A, Haisch A, Smolian H, Burmester GR, Häupl T, Sittinger M. 2002. Human platelet supernatant promotes proliferation but not differentiation of articular chondrocytes. Med Biol Eng Comput 40(4):485–490.

Kassolis JD, Rosen PS, Reynolds MA. 2000. Alveolar ridge and sinus augmentation utilizing platelet-rich plasma in combination with freeze-dried bone allograft: Case series. J Periodontol 71(10):1654–1661.

Khalil AA. 2007. Biomarker discovery: A proteomic approach for brain cancer profiling. Cancer Sci 98(2):201–213.

Knudson W, Loeser RF. 2002. CD44 and integrin matrix receptors participate in cartilage homeostasis. Cell Mol Life Sci 59(1):36–44.

Lakhe-Reddy S, Khan S, Konieczkowski M, Jarad G, Wu KL, Reichardt LF, Takai Y, Bruggeman LA, Wang B, Sedor JR, Schelling JR. 2006. Beta8 integrin binds Rho GDP dissociation inhibitor-1 and activates Rac1 to inhibit mesangial cell myofibroblast differentiation. J Biol Chem 281(28):19688–19699.

Lee CR, Grad S, Gorna K, Gogolewski S, Goessl A, Alini M. 2005. Fibrin-polyurethane composites for articular cartilage tissue engineering: A preliminary analysis. Tissue Eng 11(9–10):1562–1573.

Lefebvre V, Smits P. 2005. Transcriptional control of chondrocyte fate and differentiation. Birth Defects Res C Embryo Today 75(3):200–212.

Leyens G, Verhaeghe B, Landtmeters M, Marchandise J, Knoops B, Donnay I. 2004. Peroxiredoxin 6 is upregulated in bovine oocytes and cumulus cells during in vitro maturation: Role of intercellular communication. Biol Reprod 71(5):1646–1651.

Llorca O, Martín-Benito J, Grantham J, Ritco-Vonsovici M, Willison KR, Carrascosa JL, Valpuesta JM. 2001. The 'sequential allosteric ring' mechanism in the eukaryotic chaperonin-assisted folding of actin and tubulin. EMBO J 20(15):4065–4075.

Malicev E, Radosavljevic D, Velikonja NK. 2007. Fibrin gel improved the spatial uniformity and phenotype of human chondrocytes seeded on collagen scaffolds. Biotechnol Bioeng 96(2):364–370.

Marx RE. 2004. Platelet-rich plasma: Evidence to support its use. J Oral Maxillofac Surg 62(4):489–496.

Nowak G, Lopez M, Zieger M. 2007. Thrombin induced tumour growth—Pharmacological control. Hamostaseologie 27(2):105–110.

Pagel CN, de Niese MR, Abraham LA, Chinni C, Song SJ, Pike RN, Mackie EJ. 2003. Inhibition of osteoblast apoptosis by thrombin. Bone 33(4):733–743.

Patchell BJ, Wojcik KR, Yang TL, White SR, Dorscheid DR. 2007. Glycosylation and annexin II cell surface translocation mediate airway epithelial wound repair. Am J Physiol Lung Cell Mol Physiol 293(2):L354–363.

Perka C, Arnold U, Spitzer RS, Lindenhayn K. 2001. The use of fibrin beads for tissue engineering and subsequential transplantation. Tissue Eng 7(3):359–361.

Ruiz-Romero C, López-Armada MJ, Blanco FJ. 2005. Proteomic characterization of human normal articular chondrocytes: A novel tool for the study of osteoarthritis and other rheumatic diseases. Proteomcis 5(12):3048–3059.

Ruiz-Romero C, López-Armada MJ, Blanco FJ. 2006. Mitochondrial proteomic characterization of human normal articular chondrocytes. Osteoarthritis Cartilage 14(6):507–518.

Sanchez AR, Sheridan PJ, Kupp LI. 2003a. Is platelet-rich plasma the perfect enhancement factor? A current review. Int J Oral Maxillofac Implants 18(1): 93–103.

Sanchez M, Azofra J, Anitua E, Andia I, Padilla S, Santisteban J, Mujika I. 2003b. Plasma rich in growth factors to treat an articular cartilage avulsion: A case report. Med Sci Sports Exerc 35(10):1648–1652.

Schmidt MB, Chen EH, Lynch SE. 2006. A review of the effects of insulin-like growth factor and platelet derived growth factor on in vivo cartilage healing and repair. Osteoarthritis Cartilage 14(5):403–412.

Schmidt I, Fähling M, Nafz B, Skalweit A, Thiele BJ. 2007. Induction of translationally controlled tumor protein (TCTP) by transcriptional and post-transcriptional mechanisms. FEBS J 274(20):5416–5424.

Sittinger M, Perka C, Schultz O, Häupl T, Burmester GR. 1999. Joint cartilage regeneration by tissue engineering. Z Rheumatol 58(3):130–135.

Spreafico A, Frediani B, Capperucci C, Chellini F, Paffetti A, D'Ambrosio C, Bernardini G, Mini R, Collodel G, Scaloni A, Marcolongo R, Santucci A. 2006. A proteomic study on human osteoblastic cells proliferation and differentiation. Proteomics 6(12):3520–3532.

Stokes DG, Liu G, Dharmavaran R, Hawkins D, Piera-Velazquez S, Jimenez SA. 2001. Regulation of type-II collagen gene expression during human chondrocyte de-differentiation and recovery of chondrocyte-specific phenotype in cultures involves Sry-type high-mobility-group box (SOX) transcription factors. Biochem J 360(Pt 2):461–470.

Teixeira CC, Shapiro IM, Hatori M, Rajpurohit R, Koch C. 1996. Retinoic acid modulation of glutathione and cysteine metabolism in chondrocytes. Biochem J 314(Pt 1):21–26.

Tozum TF, Demiralp B. 2003. Platelet-rich plasma: A promising innovation in dentistry. J Can Dent Assoc 69(10):664.

Virchenko O, Aspenberg P. 2006. How can one platelet injection after tendon injury lead to a stronger tendon after 4 weeks? Interplay between early regeneration and mechanical stimulation. Acta Orthop 77(5):806–812.

Wu W, Chen F, Liu Y, Ma Q, Mao T. 2007. Autologous injectable tissue-engineered cartilage by using platelet-rich plasma: Experimental study in a rabbit model. J Oral Maxillofac Surg 65(10):1951–1957.

Yamashita S, Andoh M, Ueno-Kudoh H, Sato T, Miyaki S, Asahara H. 2009. Sox 9 directly promote Bapx1 gene expression to repress Runx2 in chondrocytes. Exp Cell Res 315(13):2231–2240.

Zhou G, Zheng Q, Engin F, Munivez E, Chen Y, Sebald E, Krakow D, Lee B. 2006. Dominance of SOX9 function over RUNX2 during skeletogenesis. Proc Natl Acad Sci USA 103(50):19004–19009.