

Up-regulation of the chemo-attractive receptor ChemR23 and occurrence of apoptosis in human chondrocytes isolated from fractured calcaneal osteochondral fragments

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Abstract

To study the expression level of a panel of pro/anti-apoptotic factors and inflammation-related receptors in chondral fragments from patients undergoing surgical treatment for intra-articular calcaneal fractures, cartilage fragments were retrieved from calcaneal fractures of 20 patients subjected to surgical treatment. Primary cultures were performed using chondral fragments from fractured and control patients. Chondrocyte cultures from each patient of the fractured and control groups were subjected to immunofluorescence staining and quantitatively analyzed under confocal microscopy. Proteins extracted from the cultured chondrocytes taken from the fractured and control groups were processed for Western blot experiments and densitometric analysis. The percentage of apoptotic cells was determined using the cleaved PARP-1 antibody. The proportion of labelled cells was 35% for fractured specimens, compared with 7% for control samples. Quantification of caspase-3 active and Bcl-2 proteins in chondrocyte cultures showed a significant increase of the apoptotic process in fractured specimens compared with control ones. Fractured chondrocytes were positively stained for ChemR23 with statistically significant differences with respect to control samples. Densitometric evaluation of the immunoreactive bands confirmed these observations. Human articular chondrocytes obtained from patients with intra-articular calcaneal fractures express higher levels of pivotal pro-apoptotic factors, and of the chemo-attractive receptor ChemR23, compared with control cultures. On the basis of these observations, the authors hypothesize that consistent prolonged chondrocyte death, associated with the persistence of high levels of pro-inflammatory factors, could enhance the deterioration of cartilage tissue with consequent development of post-traumatic arthritis following intra-articular bone fracture.

Key words: bone fracture; chondrocytes – apoptosis; inflammation; joint injury; osteoarthritis.

Introduction

Post-traumatic osteoarthritis (PTOA) is a complex debilitating disease following acute joint injury that accounts for

12% of the total OA population (Brown et al. 2006). The development of PTOA after bone fractures remains one of the major unsolved clinical problems associated with various types of fractures in near proximity of diarthrotic joints, despite the current treatments that effectively reduce and stabilize the fractured surfaces. As far as calcaneal fractures are concerned, since the 1950s PTOA frequency has been estimated at about 2% of all fractures and approximately 75% of intra-articular calcaneal fractures with involvement of the posterior subtalar joint (Soeur & Remy, 1975; Slatk et al. 1979). Intra-articular fractures have a high morbidity, with 40–85% of patients able to return to work within 9 months of the trauma but approximately 20% unable to do so within 1 year (Pozo et al. 1984; Brauer et al. 2005).

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Furthermore, these injuries affect the physical mobility of patients, thus frequently resulting in psychosocial impairment and often requiring multiple surgical operations and extended rehabilitation. The percentage of patients that develop osteoarthritis after a joint trauma is very high, notwithstanding the achievement of anatomic reduction (Sanders et al. 1993; Thomas et al. 2010; Gurkan et al. 2011). Several factors can contribute to cartilage degeneration in fracture-injured joints (Buckwalter & Brown, 2004; Furman et al. 2006), including acute mechanical damage of the articular cartilage as in compressive injuries (Rolauffs et al. 2010) and various biological stimuli associated with joint bleeding and inflammatory responses.

Over the past few years, the possibility that extensive chondrocyte apoptosis occurs following intra-articular fracture and may contribute to the development of post-traumatic arthritis has received increasing attention (Kim et al. 2002; Murray et al. 2004; Hembree et al. 2007). The decrease in the viable chondrocyte number is accelerated in patients with osteoarthritis and a direct correlation between higher rates of apoptosis and greater severity of osteoarthritis has been demonstrated (Kim et al. 2000). It is not possible to evaluate chondrocyte viability *in situ* in patients following articular fractures, but small osteochondral fragments taken from the zone of injury, which cannot be used in articular reconstruction, can be assessed for cell viability. Indeed, the possibility of detecting *in situ* cell death in live tissues was recently reported in literature, but this requires quantitative multiphoton microscopy (Novakofski et al. 2014). Previous reports have shown a generalized decrease in chondrocyte viability or increased rates of apoptosis in such fragments; however, few authors have critically evaluated the various cell death parameters, and which pathways become activated and are responsible for cell death after calcaneal fractures (Ball et al. 2007). In this regard, particular attention should be given to the different types of cell death recently identified by some authors (Kaczmarek et al. 2013). Knowing the causes as well as the pathways of cell death is extremely important because these determine different *in vivo* consequences. Pharmacological enzyme inhibitors involved in the apoptotic process have been explored as potential therapeutic agents in animal models of osteoarthritis (D'Lima et al. 2006). Moreover, the release of intracellular contents from apoptotic cells is thought to provoke an inflammatory response (Ditsworth et al. 2007). Another crucial aspect of osteoarthritis is that local production of inflammatory mediators is well known to contribute to cartilage degradation. Over the last 10 years, attention has been increasingly focused on the pivotal role played by chondrocytes in mediating inflammatory signalling in the early phases of the arthritic disease (Buckwalter et al. 2005; Lee et al. 2005; Anderson et al. 2011; Berenbaum, 2013; Byun et al. 2013; Li et al. 2013). Indeed, Berg et al. (2010) demonstrated that human chondrocytes express the receptor ChemR23 and hypothesize

that this receptor could serve as a central bridge for the onset and maintenance of joint inflammation. ChemR23 is a G protein-coupled receptor binding several different ligands (Meder et al. 2003), which directs the migration of leukocytes towards the sites of inflammation. In cartilage, the protein expressed by chondrocytes promotes the secretion of pro-inflammatory cytokines and matrix metalloproteinases, acting as a central bridge for the onset and maintenance of joint inflammation (Wittamer et al. 2005). In the present study it is hypothesized that in human chondrocytes derived from calcaneal cartilage fragments, pro-apoptotic factors and the chemo-attractive receptor ChemR23 are increased after intra-articular fractures.

For this purpose, the expression level of a panel of pro/anti-apoptotic factors (PARP-1, caspase 3, Bcl-2) as well as the inflammation-related receptor (ChemR23) were analyzed in chondrocytes isolated from chondral fragments of patients undergoing surgical treatment for intra-articular calcaneal fractures.

Materials and methods

Study population

Institutional review board approval was obtained before beginning the study and adherence to the established guidelines was maintained throughout the study. Informed consent was obtained from each patient. Irreducible chondral fragments are frequently encountered at the time of surgical treatment for intra-articular calcaneal fractures. These fragments are normally removed and, when not used for articular chondrocyte implantation (ACI), discarded to prevent loose bodies and resultant third body wear within the subtalar joint. For this study, cartilage fragments were retrieved from calcaneal fractures in 20 patients subjected to surgical treatment at the Orthopedic Clinic of Policlinico of Modena. Exclusion criteria were: patient age > 60 years, radiographic evidence of pre-existing joint pathology or evidence of concurrent joint infection. The average age of the patients was 35 (range 19–58) years. The patients were relatively healthy with no relevant comorbidity conditions. The average time between injury and surgery was 5 (range 2–7) days. Each fracture was classified according to the Sanders classification (Sanders et al. 1993) based on pre-operative CT and intra-operative findings. Fractures were classified as Sanders type II in eight patients, Sanders type III in seven, and Sanders type IV in five. The follow-up, as far as the clinical evaluation is concerned, ranged from 12 to 24 months. Computed tomography scans were done for all patients pre-operatively, post-operatively, and at the 1/2-year follow-up evaluation. Classification of the clinical outcome was based on the Maryland Foot Score (Sanders et al. 1993), with feet that needed a subsequent subtalar arthrodesis because of post-traumatic arthritis considered a failure of surgical treatment. Five control specimens (male subjects, aged 21–50 years) were retrieved from tissue donors without osteoarthritis who died from unrelated causes.

Chondrocyte monolayer culture

Upon removal, irreducible chondral fragments from fractured and control patients were immediately placed into saline buffer to prevent desiccation. Primary cultures were performed incubating the

cartilage pieces in modified Eagle's Medium (DMEM, GIBCO, Grand Island, NY, USA) containing 0.2% collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 5% fetal bovine serum (GIBCO) for 14–16 h at 37 °C and 5% CO₂. The resulting cell suspension was then filtered through 70- μ m nylon filters (Cell Strainer; Falcon, Franklin Lakes, NJ, USA) and washed three times with phosphate-buffered saline (PBS) containing 100 U mL⁻¹ penicillin and 100 μ g mL⁻¹ streptomycin. The number and size distribution of the isolated cells were then determined with a Z2 Coulter Counter and Size Analyzer (Beckman Coulter, Inc., Palo Alto, CA, USA). After isolation, the chondrocytes were plated onto separate 10-cm tissue culture dishes at a density of 10 000 cells cm⁻². Cells were incubated at 37 °C and 5% CO₂ in chondrocyte medium composed of DMEM containing 10% fetal bovine serum, 0.4 mM proline, 50 μ g mL⁻¹ ascorbic acid, 10 mM HEPES, 0.1 mM non-essential amino acid, with 100 U mL⁻¹ penicillin and 100 μ g mL⁻¹ streptomycin. The cells were cultured in monolayer after seeding in four multi-well culture plates at low density (4000 cell cm⁻²). The medium was changed three times per week. The cell morphology was examined daily under an inverted microscope (Axiovert 40 CFL, Zeiss). When chondrocytes reached 70–90% confluence, the cells were detached by trypsinization (0.25% w/v in Ca²⁺- and Mg²⁺-free PBS). Then cultured chondrocytes from each plate were plated three times for up to 12–16 days to avoid the dedifferentiation of expanded chondrocytes and to preserve their phenotypic stability (Marlovits et al. 2004).

Immunofluorescence confocal microscopy

Chondrocyte cultures from each patient of the fractured and control groups were grown on multi-well chamber slides, washed three times with PBS, fixed in methanol at 4 °C for 20 min, and further washed with PBS three times. The fixed cells were subjected to immunofluorescence staining and analyzed by two investigators in blinded conditions. Immunofluorescence analysis was carried out to evaluate the expression of cleaved PARP-1, caspase-3 active, Bcl-2 and ChemR23 proteins. After treatment with 3% bovine serum albumin (BSA) in PBS for 30 min at room temperature, cells were incubated with the primary antibody (mouse anti-c PARP-1, Cell Signalling; rabbit anti-caspase-3 active SIGMA; anti-mouse Bcl-2, BD Transduction Laboratories; rabbit anti-ChemR23, Santa Cruz) diluted 1 : 25 in PBS containing 3% BSA for 1 h at room temperature. After washing in PBS, the samples were incubated for 1 h at room temperature with the secondary antibodies diluted 1 : 20 in PBS containing 3% BSA (sheep anti-mouse FITC conjugated, goat anti-mouse-Cy3 conjugated, sheep anti-rabbit FITC conjugated; Sigma). After washing in PBS and in H₂O, the samples were counter-stained with 1 μ g mL⁻¹ 4',6-diamidino-2-phenylindole (DAPI) in H₂O and then mounted with anti-fading medium (0.21 M DABCO and 90% glycerol in 0.02 M Tris, pH 8.0).

Negative control samples were not incubated with the primary antibody. The phenotype of cultured cells was assessed at the time of experiments by immune-labelling for collagen type II, using the specific antibody, mouse anti-human collagen II (Millipore) as primary and sheep anti-mouse FITC conjugated (SIGMA) as secondary.

Confocal imaging was performed on a Leica TCS SP2 AOBs confocal laser scanning microscope. For DAPI and fluorescein isothiocyanate (FITC) double detection, samples were sequentially excited with the 405-nm/25-mW line of a blue diode laser and the 488-nm/20-mW line of an Argon laser. The emission signals from DAPI and FITC were detected by two photomultiplier tubes. For DAPI and Cy3 double detection, samples were sequentially excited with the

405 nm per 25-mW line of a blue diode laser and the 543 nm per 1.2-mW line of a HeNe laser. The emission signals from DAPI and Cy3 were detected using two photomultiplier tubes. The samples, processed for triple fluorescence (DAPI, FITC and Cy3), were sequentially excited with the 405-nm per 25-mW line of a blue diode laser, the 488-nm per 20-mW line of an Argon laser and the 543-nm per 1.2-mW line of a HeNe laser.

Excitation and detection of the samples were carried out in sequential mode to avoid overlapping of signals. Sections were scanned with laser intensity, confocal aperture, gain and black-level setting kept constant for all samples. Optical sections were obtained at increments of 0.3 μ m in the z-axis and digitized with a scanning mode format of 512 \times 512 or 1024 \times 1024 pixels and 256 shades of grey. The confocal serial sections were processed with the Leica LCS software to obtain three-dimensional projections. Image rendering was performed using ADOBE PHOTOSHOP software.

Evaluation of cleaved PARP-1 positive cells

The original green fluorescent confocal images were converted to grey-scale (as previously published: Sena et al. 2012; Mariani et al. 2013) and median filtering was performed. An intensity value ranging from 0 (black) to 255 (white) was assigned to each pixel. Background fluorescence was subtracted and the immunofluorescence intensity was calculated as the average for each selected area. The percentage of apoptotic cells was determined using cleaved PARP-1 antibody, which detects endogenous levels of the large fragment (89 kDa) of the human protein resulting from cleavage of the native protein and does not recognize the full length PARP-1 or other isoforms. The cleaved PARP-1 positive rate was determined by observing more than 1000 nuclei for each experimental sample at \times 40 magnification. At least three sets of experiments were performed for each patient. Each sample was assigned a code number, determined by an observer who was blind during analysis.

Evaluation of caspase-3 active, Bcl-2 and ChemR23 immunofluorescence

Evaluation was carried out for all fractured and control samples. The original green fluorescent confocal images were converted to grey-scale and median filtering was conducted (as previously published: Sena et al. 2012; Mariani et al. 2013). An intensity value ranging from 0 (black) to 255 (white) was assigned to each pixel. Background fluorescence was subtracted and the immunofluorescence intensity was calculated as the average for each selected area. To quantify caspase-3 active, Bcl-2 and ChemR23 expression for each patient, all multi-well chamber slides were examined at \times 40 magnification. Starting randomly, three microscopic fields for each section were used for sampling all sections within the unbiased sampling frame. The fluorescence intensity in the selected areas, linearly correlated with the number of pixels, was quantitatively analyzed using the standard imaging analysis software of the NIS-Elements System. Each sample was assigned a code number and the score, the immunofluorescence intensity score (IFIS), was determined by an observer who was blind to sample groups during analysis (Sena et al. 2012).

Western blot analysis

Proteins were extracted from cultured chondrocytes taken from the fractured and control groups. At the end of the culture

periods, cells were lysed at 4 °C for 20 min in the presence of a lysis buffer [50 mM Tris-Cl, pH 7.8, containing 1% Nonidet P40, 140 mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 0.1% Na deoxycholate, 1 mM Na₃VO₄, and freshly added protease inhibitor cocktail]. Lysates were then cleared by centrifugation for 15 min in a refrigerated centrifuge, max speed (200 g), and immediately boiled in SDS sample buffer. Protein content in the supernatants was determined by Bradford assay. Protein extract (40 µg) from each sample was electrophoresed on SDS-PAGE and transferred to a nitrocellulose membrane. The protocols of the Western blotting were performed as previously described (Mariani et al. 2013). The membrane was blocked with 3% dry milk and 2% BSA in PBS-T and was incubated with the following antibodies, diluted 1 : 1000 overnight at 4 °C under agitation: mouse anti-cleaved PARP-1 (Cell Signalling), rabbit anti-caspase-3 active (Sigma), mouse anti-Bcl-2 (BD Transduction Laboratories), rabbit anti-ChemR23 (Santa Cruz). After washing, the membranes were incubated with secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (1 : 10 000) or HRP-conjugated sheep anti-mouse IgG antibody (1 : 3000) for 30 min at room temperature. Immunoreactive proteins were detected with ECL (Amersham). The membranes were then stripped and incubated with anti-mouse β-tubulin (Sigma) to control and correct for loading error. Densitometry analysis was performed using a Kodak (Rochester, NY) Image Station 440cf system.

Statistics

The cleaved PARP-1, caspase-3 active, Bcl-2, ChemR23 expression was tested for statistical significance using one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls tests. All quantitative data are reported as mean ± SD. A *P*-value less than 0.05 was chosen to indicate statistically significant differences between fractured and control groups.

Results

Chondrocyte cultures

To check the chondrocyte phenotype, cells were immunolabelled for collagen type II after the second week of proliferation in culture preceding *in vitro* experiments. As shown in Fig. 1(A,B) the phenotype was preserved, although the chondrocyte adhesion to the culture plates implied some changes in the characteristic rounded shape of *in vivo* chondrocytes. This modification of cell morphology was evident in all cultures from both control and fractured samples, with no significant differences.

Morphological evidence of apoptosis

The typical features of cell apoptosis, such as chromatin condensation and nuclear fragmentation, can be clearly observed by staining with DAPI with simultaneous transmission of light performed by confocal microscope analysis. Figure 2 clearly shows the morphological signs of apoptosis, i.e. the nuclear morphology alterations in advanced stages

of apoptotic progression, appearing to shrink into little single balls or grape-like clusters.

Evaluation of apoptotic pattern in chondrocyte cultures

Quantitative differences were evaluated in the amounts of apoptosis between the chondrocyte cultures obtained from patients with intra-articular calcaneal fractures and those from control patients. This was achieved in immunofluorescence experiments using confocal microscopy, which provides good resolution of subcellular structures in very thick samples while also enabling quantification of the detected proteins. The percentage of apoptotic cells was determined using cleaved PARP-1 antibody. Some representative staining patterns are shown in Fig. 1(C,D), in which nuclear labelling of apoptotic cells is evident, as revealed by DAPI/FITC co-immunostaining. In all chondrocyte cultures obtained from fractured patients, PARP-1-positive cells were detected in large quantities (Fig. 1D), whereas the number of positive cells was very low in control chondrocyte cultures (Fig. 1C). The percentage of labelled cells was 35% in fractured specimens, with only 7% detected in control samples (Table 1). Confocal analysis of caspase-3 active and Bcl-2 allowed evaluation of distribution patterns and quantification of these proteins in chondrocyte cultures obtained from patients with intra-articular calcaneal fractures and from control patients.

Caspase-3 protein appeared aggregated in large clumps distributed in the cytoplasm of chondrocytes and this were particularly evident in the perinuclear area of fractured samples (Fig. 1F). These samples were strongly stained, whereas control samples showed lower staining, appearing as smaller clumps scattered in the cytoplasm (Fig. 1E). The same features were confirmed in the measurements of the protein expression levels with an IFIS of 100 for fractured specimens and of 40 for control specimens (Table 1). Figure 1(G,H) shows the localization of cleaved PARP-1 antibody coupled with caspase-3 staining in fractured samples. The overlapping expression pattern of the two proteins of interest is very evident and emphasizes the presence of apoptotic processes in the samples. Bcl-2 protein is evenly distributed in the cytoplasm of chondrocytes and very evident in control samples, whereas it is hardly detectable in fractured samples (Fig. 1I,J). The Bcl-2 expression level trend was the inverse of that of caspase-3: fractured samples had an IFIS value of 32, whereas control sample had a value of 81 (Table 1).

To confirm the previously described immunofluorescence observations of some regulatory proteins involved in the apoptotic process, cell lysates of chondrocytes obtained from patients with intra-articular calcaneal fractures and from control patients were analyzed by Western blotting. A panel of antibodies was evaluated to investigate the apoptotic process to avoid the pitfalls of relying on a single

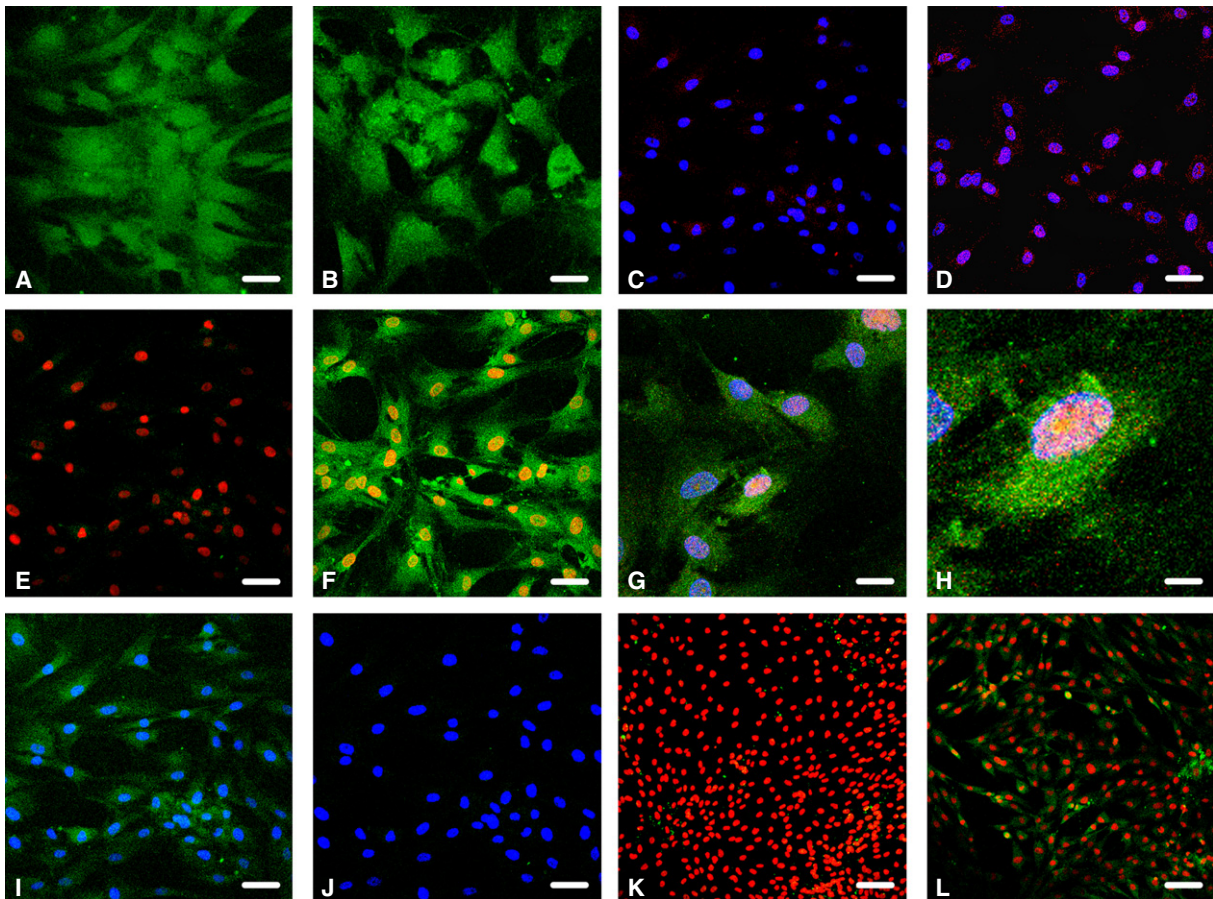


Fig. 1 Confocal microscope images of human chondrocyte cultures labelled by anti-collagen type II (green), DAPI (blue in C,D and G–J; red in E,F, K,L), anti-cleaved PARP-1 (red), anti-caspase-3 active (green), anti-Bcl-2 (green), and anti ChemR23 (green). (A,B) Chondrocyte labelled with anti-collagen type II. The immunostaining was evenly distributed in cells obtained from both control (A) and fractured (B) patients after 2 weeks of culture. (C,D) Chondrocyte labelled with anti-cleaved PARP-1. Control specimens do not show an appreciable PARP-1 staining (C). The presence of several apoptotic cells is revealed by the merging signals at nuclear levels of both PARP-1 labelling and DAPI, clearly co-immunostaining (red plus blue = violet) in fracture specimens (D). (E,F) Caspase-3 active protein expression is poorly detectable in control cultures (E) but is clearly evident in the perinuclear area of chondrocytes from fractured patients (F). (G,H) Co-immunostaining of anti-cleaved PARP-1 and anti-caspase-3 active in samples from fractured patients; the cell showed in (H) is enlarged with respect to (G). (I,J) Staining of Bcl-2 [the amount results are inverse to those for caspase-3 expression, shown in (E,F)] – Control specimens (I), fractured specimens (J). (K,L) Control samples were slightly marked with anti-ChemR23 (K), whereas fractured samples showed a strong immunostaining (L). Scale bars = 10 μm (A,B), 20 μm (C–F, I,J), 15 μm (G), 5 μm (H), 40 μm (K,L).

antibody. The results clearly showed a single intense band at the expected molecular weight (89 kDa) for cleaved PARP-1 in fractured specimens, whereas a lower band was identified in control samples (Fig. 2A). This was also confirmed by the evaluation of active caspase-3: the corresponding band at 17 kDa was clearly recognizable in fractured samples but difficult to detect in control samples (Fig. 3A). The Bcl-2 expression profile was also assessed: this anti-apoptotic protein produced an evident band at the expected molecular weight (25 kDa) in control samples and a faint band in fractured samples (Fig. 3A). Densitometric analysis and normalization (with equal amounts of protein loading) of the immunoreactive band showed that the levels of cleaved PARP-1 and caspase-3 active are both the

inverse of those of Bcl-2, with the different expressions being statistically significant (Fig. 3B).

Evaluation of ChemR23 expression in chondrocyte cultures

The presence of ChemR23 was investigated by immunofluorescence coupled with confocal analysis on chondrocyte cultures harvested from patients with intra-articular calcaneal fractures and from control patients. Western blotting was also performed, followed by densitometric evaluation of immunoreactive bands. In all cases, cultured chondrocytes were positively stained for ChemR23 with statistically significant differences. Fractured samples showed an intense

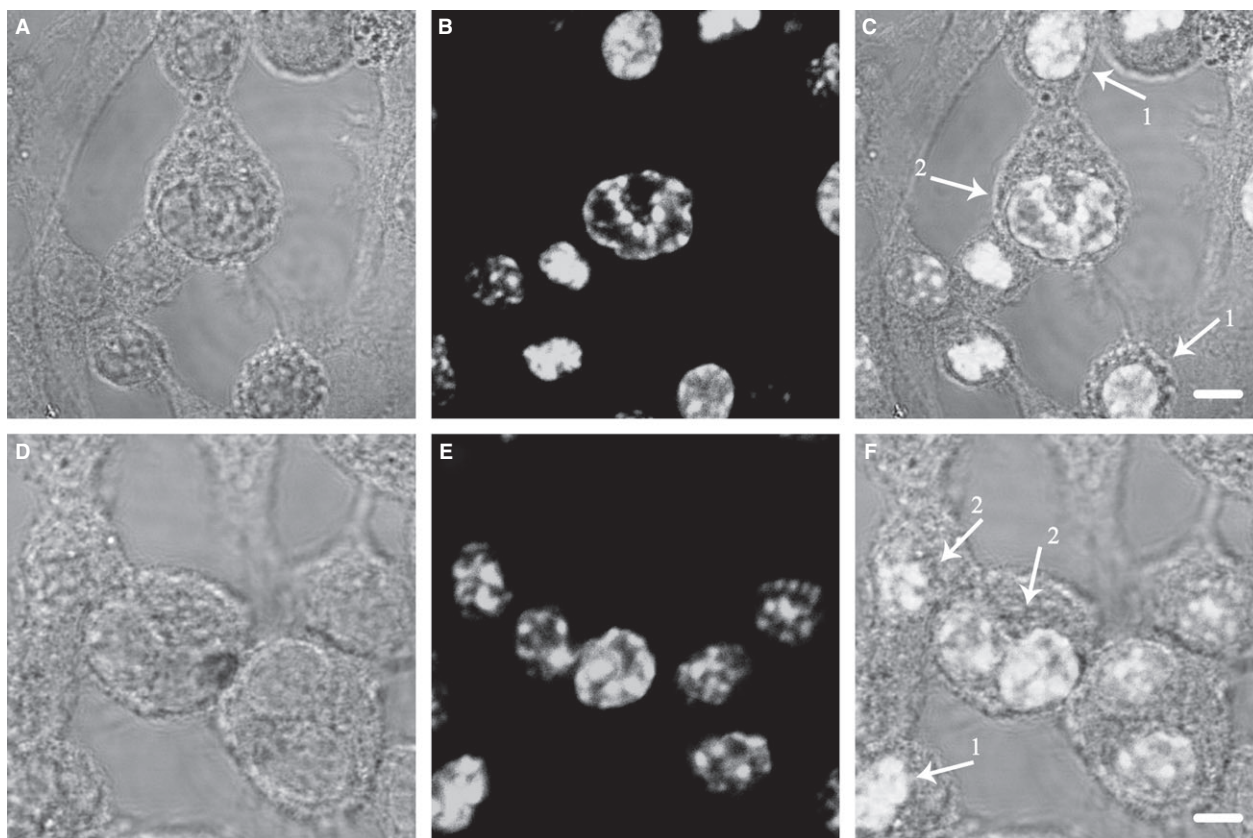


Fig. 2 Confocal microscope images of human chondrocyte cultures labelled by DAPI (white in B and E). (A,D) Transmitted light. (B,E) DAPI immunofluorescence. (C,F) Merging. (C,F) Arrows: 1 indicates viable cells with normal nuclei, 2 indicates cells presenting large apoptotic nuclei tending to shrink into little single balls or clusters of grapes, as well as initial nuclear envelope fragmentation. Scale bars: 5 μm (C), 4 μm (F).

Table 1 Percentage of cleaved PARP-1 positive cells and immunofluorescence intensity score (IFIS: mean \pm SD) in fractured and control samples.

Groups	Positive cells (%) Cleaved PARP-1	IFIS		
		Caspase-3	Bcl-2	ChemR23
Control samples	7.00 \pm 0.37*	40.0 \pm 2.5*	81.0 \pm 3.4*	24.0 \pm 3.5*
Fractured samples	35.00 \pm 0.40*	100 \pm 3*	32.0 \pm 1.2*	78.0 \pm 6.4*

* $P < 0.05$ by one-way ANOVA, followed by Student–Newman–Keuls tests. Percentage of cleaved PARP-1-positive cells and differences in IFIS between groups are always significant, comparing control and treated groups.

immunostaining extending throughout the cytoplasm, whereas control samples were only slightly labelled (Fig. 1K,L). The immunofluorescence intensity score confirmed these features: fractured specimens had an IFIS score of 78, whereas control specimens scored 24 (Table 1). Western blot analysis confirmed the expression pattern indicated by immunofluorescence: cell lysates of chondrocytes obtained from patients with intra-articular calcaneal fractures showed a very intense band at 42 kDa corresponding with the molecular weight of ChemR23; cell lysates from control patients, instead, had a narrow band at the same molecular weight (Fig. 3A). The results of densitometric evaluation and normalization (with equal amounts of

protein loading) of the immunoreactive band, clearly demonstrated that there was a statistically significant difference in the expression levels of ChemR23 between fractured and control patients (Fig. 3B).

Discussion

Post-traumatic arthritis of the subtalar joint is a common consequence of human intra-articular calcaneal fractures. This pathology affects patients' physical mobility, frequently resulting in psychosocial impairment and represents the major unsolved clinical problem associated with calcaneal fractures.

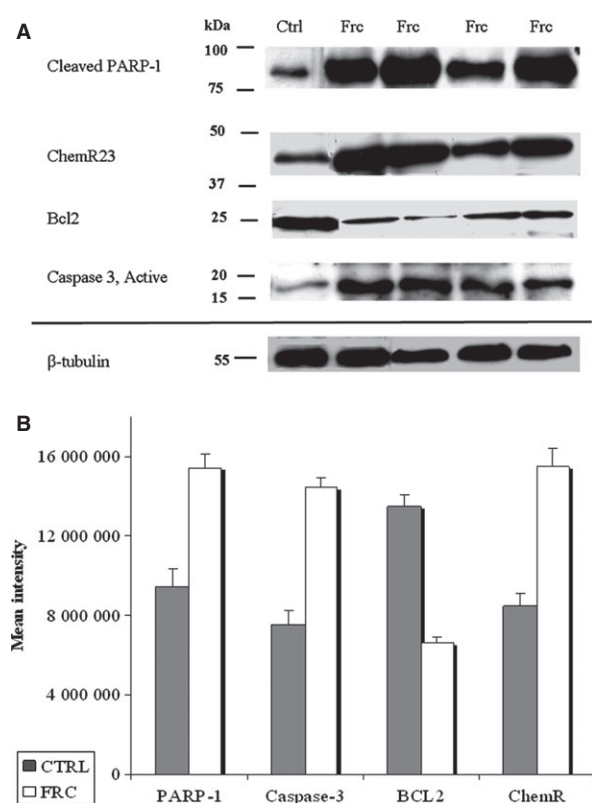


Fig. 3 Western blot of pro-apoptotic (cleaved PARP-1, caspase-3 active), anti-apoptotic (Bcl-2) and pro-inflammatory (ChemR23) proteins in control patients (Ctrl) and fractured patients (Frc) (A). Frc lines correspond to patients of different ages (49, 25, 19, 56 years, respectively, from left to right). Anti-cleaved PARP-1 antibody recognizes a very low band in control patients, and a single intense band in fractured samples; ChemR23 showed the same expression pattern of cleaved PARP-1; anti-Bcl-2 antibody recognizes a strong band in control specimens and a slightly appreciable band in fractured ones; the band corresponding to caspase-3 active is difficult to detect in control patients but clearly evident in fractured ones. Beta-tubulin is used as loading control (A). (B) Mean densitometry values of protein expression level. $P < 0.05$ between all group pairs of samples. Bar = SD.

As cartilage has poor intrinsic healing capacity, it seems logical to assume that patients with low chondrocyte viability after bone fracture would be more prone to developing post-traumatic arthritis compared with patients with high chondrocyte viability. Many reports have shown increased levels of apoptosis in biopsies of human cartilage obtained after acute injury (Kim et al. 2002; Murray et al. 2004; Hembree et al. 2007). Tochigi et al. (2011) assessed the distribution of cell cartilage damage associated with fractures using a human ankle intra-articular fracture model. Other authors proposed different experimental injury/impact models (Loening et al. 2000; Marsh et al. 2002; Kurz et al. 2005; Anderson et al. 2011) to investigate the role of apoptosis. Ball et al. (2007) investigated the significant decline in chondrocyte viability occurring after intra-articular fractures of the calcaneus, compared with control specimens.

The authors have also found a correlation between decreased chondrocyte viability and both the degree of injury and the time elapsed between injury and surgical reduction. Taking this evidence into account, it was hypothesized that a possible mechanism for development of post-traumatic osteoarthritis could be the protracted expression of pro-apoptotic factors in chondrocytes. To test this hypothesis, chondrocyte cultures were performed on samples from patients with intra-articular calcaneal fractures, and from control patients, taken not more than 3 weeks previously, this being the maximum period of time to assess the persistence of pro-apoptotic signalling before the occurrence of chondrocyte dedifferentiation. Culturing chondrocytes from lesioned cartilage offers the possibility to studying cellular behaviour in the short to medium term after the trauma, unlike a 'static' analysis of whole chondral fragments at the moment of removal. In this way, it was demonstrated that human chondrocytes cultured in monolayer, although proliferating, are gradually more impeded in their expression of the typical specialized chondrocyte proteins, and thus increasingly tend to assume the fibroblastic phenotype (Lefebvre et al. 1990; Munirah et al. 2010). This suggests that under such conditions, the chondrocyte phenotype is not stable. To establish the chondrogenic phenotype, cells were immunolabelled for collagen type II after propagation, during the culture time preceding *in vitro* experiments. As was clearly observed, the phenotype of chondrocytes cultured in monolayer was preserved.

As far as the authors are aware, this is the first study that has critically evaluated the various cell death parameters and which pathways become activated and are responsible for cell death of chondrocytes in human intra-articular calcaneal fractures. Existing literature data mostly regard ankle and knee fractures (Kerin et al. 2002; Pascual Garrido et al. 2009; Stufkens et al. 2010). It is important to note that the different cell death subroutines often run in parallel to each other and are not mutually exclusive. It is also important to be aware that the detection of the activation of a cell death pathway does not necessarily mean that it is the predominant pathway mediating cell death in a particular cellular model. Thus, a careful analysis of various cell death parameters is always necessary to reveal which pathways become activated and which of the active pathways are responsible for cell death.

Morphological analysis alone is not enough to establish the occurrence of apoptosis, particularly in the early stages because, as is well known, apoptosis proceeds through typical progressive alterations of nuclear morphology (Robertson et al. 2000) characterized by increasing condensation of chromatin, which induces nuclei to shrink into little single balls or grape-like clusters. The analysis thus also included some critical apoptotic factors.

It has been observed that PARP-1 plays an important role in many of cell death pathways. A key finding that ascribed a pivotal role to PARP-1 in the apoptotic process was the

demonstration that PARP-1 is the substrate of caspase-3 and 7 (Oliver et al. 1998), so that the detection of its cleavage has been widely used as a hallmark of apoptosis. PARP-1 was also investigated in transgenic mice with inhibition of β -catenin signalling in chondrocytes (Zhu et al. 2008). In these conditions, the observed high levels of both PARP-1 and cleaved caspase-3 indicate that apoptosis of articular chondrocytes is indeed increased and the results shown by the authors are consistent with the observed TUNEL staining, thus indicating that articular cartilage destruction in these mice may be caused by an increase in articular chondrocyte apoptosis. Recently, poly(ADP-ribosyl)ation was discovered to play at most only an accessory role in apoptosis, with the PARP-mediated cell death displaying the features of *necroptosis* (Galluzzi et al. 2012). More recently, PARP-1 has been implicated in autophagy resulting from DNA damage or nutrient deprivation. Activation of PARP may cause regulated necrosis via three major pathways that are likely to be closely intertwined (Virág et al. 2013): (i) compromised cellular energetic balance mainly due to depletion of NAD, the substrate of PARPs; (ii) PAR-mediated translocation of apoptosis inducing factor (AIF) from mitochondria to nucleus (*parthanatos*); and (iii) a mostly elusive interplay between PARylation and cell death/survival kinases and phosphatases.

As cleavage of PARP-1 by caspases has been identified as one of the first biochemical markers of apoptosis, further analysis was conducted on the expression profile of caspase-3, which is activated through proteolytic cleavage and is responsible for causing cell death, particularly in mitochondrial-mediated apoptosis (Nunez et al. 1998). The results of the present study clearly demonstrate a significant increase in the apoptotic rate in chondrocyte cultures obtained from patients with intra-articular calcaneal fractures compared with control patient cultures. The findings also exhibited a very low degree of variability between patients, and although older patients in general were found to have a higher average percentage of apoptotic cells, the apoptotic rate appears to be unrelated to patient age.

The relevance of the cell death pathway is particularly important because it has *in vivo* consequences. According to Lotz et al. (1999), chondrocyte death, in particular by apoptosis, associated with the absence in cartilage of both mononuclear phagocytes and mesenchymal stem cells, implies, respectively, that remnants of dead cells will remain in the cartilage matrix, thus probably affecting the fate of viable chondrocytes; and that precursors of chondrocytes cannot be directly recruited from non-vascularized tissue. Necrotic/necroptotic cell death is considered a potentially dangerous form of cell demise because it is accompanied by the release of the cell contents into the environment, causing further inflammation. This is a key difference between apoptotic and necrotic cells, as the former are efficiently cleared from the tissue by professional phagocytes as well as by non-professional neighbouring cells before their plasma

membrane loses its integrity, in a process called *efferocytosis* (Elliott & Ravichandran, 2010). Apoptotic cells also release the 'find me' and 'eat me' signals that facilitate the migration of phagocytic cells towards the apoptotic cells (chemotaxis), followed by the engulfment of the latter (Elliott & Ravichandran, 2010). Impaired clearance of apoptotic cells has been implicated in autoimmune diseases, atherosclerosis, cystic fibrosis and neurological diseases (Elliott & Ravichandran, 2010). A further confirmation of activation of the apoptotic process in chondrocyte cultures taken from fractured patients after injury, is the significant lower expression of Bcl-2 protein in these specimens than in control cultures. Bcl-2 protein is a pro-survival factor and its overexpression efficiently protects cells from intrinsic apoptosis (Wang & Youle, 2009). Another possible condition that increases chondrocyte damage is the cytotoxic effect of biochemical mediators released from the cells themselves and/or from the disrupted extracellular matrix, as demonstrated by Sui et al. (2009). In intra-articular fractures *in vivo*, the acutely damaged cartilage is subjected to biological stimuli associated with joint bleeding and/or inflammatory responses such as the release of inflammatory cytokines (Goldring & Goldring, 2004). Recently, Berg et al. (2010) demonstrated that human chondrocytes express the receptor ChemR23, a G protein-coupled receptor binding several different ligands (Meder et al. 2003), which directs the migration of leucocytes towards inflammation sites (Wittamer et al. 2005). The authors also showed that ChemR23 expressed by chondrocytes promotes the secretion of pro-inflammatory cytokines and matrix metalloproteinases, acting as a central link for the onset and maintenance of joint inflammation. The present study aimed to investigate whether cultures of human articular chondrocytes obtained from patients with intra-articular fractures express ChemR23 in different amounts compared with control cultures. The results clearly demonstrated that the amount of ChemR23 markedly increases in fractured specimens compared with controls, with statistical relevance. This finding is intriguing because ChemR23 binds not only the ligand Chemerin, mediating pro-inflammatory effects, but also resolvin-E1 (RvE1), a potent anti-inflammatory and pro-resolving mediator derived from omega-3 eicosapentaenoic acid (EPA), which is generated during the resolution phase of inflammation (Arita et al. 2005). There is compelling evidence of the beneficial effects of dietary supplements of EPA as well as the potential therapeutic utility of RvE1 and its mimetics in a wide range of human inflammatory conditions including arthritis (Zainal et al. 2009; Wann et al. 2010).

In summary, this study demonstrated that cultures of human articular chondrocytes obtained from patients with intra-articular calcaneal fractures express high levels of pivotal pro-apoptotic factors, and of the chemo-attractive receptor ChemR23, compared with control cultures. These findings support the hypothesis that concomitant processes, such as consistent prolonged chondrocyte death associated

with the persistence of high levels of pro-inflammatory factors, could explain the deterioration of cartilage tissue with consequent development of post-traumatic arthritis following intra-articular fracture. Evidences from *in vitro* and animal model studies suggest the existence of a 'time window' after injury in which cartilage cells can be rescued or protected from cell death, resulting in the maintenance of viable and functional cells and reducing structural damage to cartilage (Kuhn et al. 2004). Pharmacological strategies for cell death prevention need to inhibit post-traumatic inflammatory responses, in order to avoid matrix degradation and to stimulate the production of new cartilage extracellular matrix. The optimal therapy should address *several* or, more appropriately, *all* the pathogenesis pathways. Separate approaches are required from the onset, first to interfere with early catabolic and inflammatory events, and then to promote anabolic responses. Therapeutic approaches that effectively stimulate proper cartilage repair at the appropriate time after trauma need to be clearly determined. Over the past few years, a series of *in vitro* studies have demonstrated that caspase inhibitors are effective in protecting against chondrocyte apoptosis, maintaining viable cells and physiologically functional tissue (Nuttall et al. 2000). Reduction of cartilage degeneration following intra-articular injection of caspase inhibitor has also been reported for a rabbit model of osteoarthritis (D'Lima et al. 2006). Although several candidate markers have been identified, they need further validation.

In conclusion, considering the inflammatory properties of ChemR23, the novelty of this study is the identification of the up-regulation of ChemR23 in post-traumatic injury of calcaneal articular fracture. The authors are preparing further studies to investigate the molecular pathway involving ChemR23, which could be useful for testing newly proposed inhibitors in order to reduce cartilage inflammation and degradation.

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Conflict of interest

All authors declare no conflicts of interest.

References

Anderson DD, Chubinskaya S, Guilak F, et al. (2011) Post-traumatic osteoarthritis: improved understanding and opportunities for early intervention. *J Orthop Res* **29**, 802–809.

- Arita M, Bianchini F, Aliberti J, et al. (2005) Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. *J Exp Med* **201**, 713–722.
- Ball ST, Jadin K, Allen RT, et al. (2007) Chondrocyte viability after intra-articular calcaneal fractures in humans. *Foot Ankle Int* **28**, 665–668.
- Berenbaum F (2013) Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). *Osteoarthritis Cartilage* **21**, 16–21.
- Berg V, Sveinbjörnsson B, Bendixsen S, et al. (2010) Human articular chondrocytes express ChemR23 and chemerin; ChemR23 promotes inflammatory signalling upon binding the ligand chemerin (21–157). *Arthritis Res Ther* **12**, R228.
- Brauer CA, Manns BJ, Ko M, et al. (2005) An economic evaluation of operative compared with nonoperative management of displaced intra-articular calcaneal fractures. *J Bone Joint Surg Am* **87**, 2741–2749.
- Brown TD, Johnston RC, Saltzman CL, et al. (2006) Posttraumatic osteoarthritis: a first estimate of incidence, prevalence, and burden of disease. *J Orthop Trauma* **20**, 739–744.
- Buckwalter JA, Brown TD (2004) Joint injury, repair, and remodeling: roles in post-traumatic osteoarthritis. *Clin Orthop Relat Res* **423**, 7–16.
- Buckwalter JA, Mankin HJ, Grodzinsky AJ (2005) Articular cartilage and osteoarthritis. *Instr Course Lect* **54**, 465–480.
- Byun S, Sinskey YL, Lu YC, et al. (2013) Transport of anti-IL-6 antigen binding fragments into cartilage and the effects of injury. *Arch Biochem Biophys* **532**, 15–22.
- Ditsworth D, Zong WX, Thompson CB (2007) Activation of poly (ADP)-ribose polymerase (PARP-1) induces release of the pro-inflammatory mediator HMGB1 from the nucleus. *J Biol Chem* **282**, 17845–17854.
- D'Lima D, Hermida J, Hashimoto S, et al. (2006) Caspase inhibitors reduce severity of cartilage lesions in experimental osteoarthritis. *Arthritis Rheum* **54**, 1814–1821.
- Elliott MR, Ravichandran KS (2010) Clearance of apoptotic cells: implications in health and disease. *J Cell Biol* **189**, 1059–1070.
- Furman BD, Olson SA, Guilak F (2006) The development of post-traumatic arthritis after articular fracture. *J Orthop Trauma* **20**, 719–725.
- Galluzzi L, Vitale I, Abrams JM, et al. (2012) Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death. *Cell Death Differ* **19**, 107–120.
- Goldring SR, Goldring MB (2004) The role of cytokines in cartilage matrix degeneration in osteoarthritis. *Clin Orthop Relat Res* **427**(Suppl), S27–S36.
- Gurkan V, Dursun M, Orhun H, et al. (2011) Long-term results of conservative treatment of Sanders type 4 fractures of the calcaneum: a series of 64 cases. *J Bone Joint Surg Br* **93**, 975–979.
- Hembree WC, Ward BD, Furman BD, et al. (2007) Viability and apoptosis of human chondrocytes in osteochondral fragments following joint trauma. *J Bone Joint Surg Br* **89**, 1388–1395.
- Kaczmarek A, Vandenabeele P, Krysko DV (2013) Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. *Immunity* **38**, 209–223.
- Kerin A, Patwari P, Kuettner K, et al. (2002) Molecular basis of osteoarthritis: biomechanical aspects. *Cell Mol Life Sci* **59**, 27–35.
- Kim HA, Lee YJ, Seong SC, et al. (2000) Apoptotic chondrocyte death in human osteoarthritis. *J Rheumatol* **27**, 455–462.

- Kim HT, Lo MY, Pillarisetty R (2002) Chondrocyte apoptosis following intraarticular fracture in humans. *Osteoarthritis Cartilage* **10**, 747–749.
- Kuhn K, D'Lima DD, Hashimoto S, et al. (2004) Cell death in cartilage. *Osteoarthritis Cartilage* **12**, 1–16.
- Kurz B, Lemke AK, Fay J, et al. (2005) Pathomechanisms of cartilage destruction by mechanical injury. *Ann Anat* **187**, 473–485.
- Lee JH, Fitzgerald JB, Dimicco MA, et al. (2005) Mechanical injury of cartilage explants causes specific time-dependent changes in chondrocyte gene expression. *Arthritis Rheum* **52**, 2386–2395.
- Lefebvre V, Peeters-Joris C, Vaes G (1990) Production of collagens, collagenase and collagenase inhibitor during the dedifferentiation of articular chondrocytes by serial subcultures. *Biochim Biophys Acta* **1051**, 266–275.
- Li Y, Frank EH, Wang Y, et al. (2013) Moderate dynamic compression inhibits pro-catabolic response of cartilage to mechanical injury, tumor necrosis factor- α and interleukin-6, but accentuates degradation above a strain threshold. *Osteoarthritis Cartilage* **21**, 1933–1941.
- Loening AM, James IE, Levenston ME, et al. (2000) Injurious mechanical compression of bovine articular cartilage induces chondrocyte apoptosis. *Arch Biochem Biophys* **381**, 205–212.
- Lotz M, Hashimoto S, Kühn K (1999) Mechanisms of chondrocyte apoptosis. *Osteoarthritis Cartilage* **7**, 389–391.
- Mariani F, Sena P, Pedroni M, et al. (2013) Th inducing POZ-Kruppel Factor (ThPOK) is a key regulator of the immune response since the early steps of colorectal carcinogenesis. *PLoS ONE* **8**, e54488.
- Marlovits S, Hombauer M, Truppe M, et al. (2004) Changes in the ratio of type-I and type-II collagen expression during monolayer culture of human chondrocytes. *J Bone Joint Surg Br* **86**, 286–295.
- Marsh JL, Buckwalter J, Gelberman R, et al. (2002) Articular fractures: does an anatomic reduction really change the result? *J Bone Joint Surg Am* **84-A**, 1259–1271.
- Meder W, Wendland M, Busmann A, et al. (2003) Characterization of human circulating TIG2 as a ligand for the orphan receptor ChemR23. *FEBS Lett* **555**, 495–499.
- Munirah S, Samsudin OC, Aminuddin BS, et al. (2010) Expansion of human articular chondrocytes and formation of tissue-engineered cartilage: a step towards exploring a potential use of matrix-induced cell therapy. *Tissue Cell* **42**, 282–292.
- Murray MM, Zurakowski D, Vrahas MS (2004) The death of articular chondrocytes after intra-articular fracture in humans. *J Trauma* **56**, 128–131.
- Novakofski KD, Williams RM, Fortier LA, et al. (2014) Identification of cartilage injury using quantitative multiphoton microscopy. *Osteoarthritis Cartilage* **22**, 355–362.
- Nunez G, Benedict MA, Hu Y, et al. (1998) Caspases: the proteases of the apoptotic pathway. *Oncogene* **17**, 3237–3245.
- Nuttall ME, Nadeau DP, Fisher PW, et al. (2000) Inhibition of caspase-3-like activity prevents apoptosis while retaining functionality of human chondrocytes *in vitro*. *J Orthop Res* **18**, 356–363.
- Oliver FJ, de la Rubia G, Rolli V, et al. (1998) Importance of poly (ADP-ribose) polymerase and its cleavage in apoptosis. Lesson from an uncleavable mutant. *J Biol Chem* **273**, 33533–33539.
- Pascual Garrido C, Hakimiyan AA, Rappoport L, et al. (2009) Anti-apoptotic treatments prevent cartilage degradation after acute trauma to human ankle cartilage. *Osteoarthritis Cartilage* **17**, 1244–1251.
- Pozo JL, Kirwan EO, Jackson AM (1984) The long-term results of conservative management of severely displaced fractures of the calcaneus. *J Bone Joint Surg Br* **66**, 386–390.
- Robertson JD, Orrenius S, Zhivotovsky B (2000) Review: nuclear events in apoptosis. *J Struct Biol* **129**, 346–358.
- Rolauffs B, Muehleman C, Li J, et al. (2010) Vulnerability of the superficial zone of immature articular cartilage to compressive injury. *Arthritis Rheum* **62**, 3016–3027.
- Sanders R, Fortin P, Di Pasquale T, et al. (1993) Operative treatment in 120 displaced intraarticular calcaneal fractures. Results using a prognostic computed tomography scan classification. *Clin Orthop Relat Res* **290**, 87–95.
- Sena P, Mariani F, Marzona L, et al. (2012) Matrix metalloproteinases 15 and 19 are stromal regulators of colorectal cancer development from the early stages. *Int J Oncol* **41**, 260–266.
- Slatis P, Kiviluoto O, Santavirta S, et al. (1979) Fractures of the calcaneum. *J Trauma* **19**, 939–943.
- Soeur R, Remy R (1975) Fractures of the calcaneus with displacement of the thalamic portion. *J Bone Joint Surg Br* **57**, 413–421.
- Stufkens SA, Knupp M, Horisberger M, et al. (2010) Cartilage lesions and the development of osteoarthritis after internal fixation of ankle fractures: a prospective study. *J Bone Joint Surg Am* **92**, 279–286.
- Sui Y, Lee JH, DiMicco MA, et al. (2009) Mechanical injury potentiates proteoglycan catabolism induced by interleukin-6 with soluble interleukin-6 receptor and tumor necrosis factor alpha in immature bovine and adult human articular cartilage. *Arthritis Rheum* **60**, 2985–2996.
- Thomas TP, Anderson DD, Mosqueda TV, et al. (2010) CT-based metrics of articular fracture severity to assess risk for posttraumatic osteoarthritis. *J Orthop Trauma* **24**, 764–769.
- Tochigi Y, Buckwalter JA, Martin JA, et al. (2011) Distribution and progression of chondrocyte damage in a whole-organ model of human ankle intra-articular fracture. *J Bone Joint Surg Am* **93**, 533–539.
- Virág L, Robaszkiewicz A, Vargas JM, et al. (2013) Poly(ADP-ribose) signaling in cell death. *Mol Aspects Med* **34**, 1153–1167.
- Wang C, Youle RJ (2009) The role of mitochondria in apoptosis. *Annu Rev Genet* **43**, 95–118.
- Wann AK, Mistry J, Blain EJ, et al. (2010) Eicosapentaenoic acid and docosahexaenoic acid reduce interleukin-1 β -mediated cartilage degradation. *Arthritis Res Ther* **12**, R207.
- Wittamer V, Bondue B, Guillaibert A, et al. (2005) Neutrophil-mediated maturation of chemerin: a link between innate and adaptive immunity. *J Immunol* **175**, 487–493.
- Zainal Z, Longman AJ, Hurst S, et al. (2009) Relative efficacies of omega-3 polyunsaturated fatty acids in reducing expression of key proteins in a model system for studying osteoarthritis. *Osteoarthritis Cartilage* **17**, 896–905.
- Zhu M, Chen M, Zuscik M, et al. (2008) Inhibition of β -catenin signaling in articular chondrocytes results in articular cartilage destruction. *Arthritis Rheum* **58**, 2053–2064.