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Original article

Role of peroxiredoxin 6 in the chondroprotective effects of microvesicles from human adipose tissue-derived mesenchymal stem cells

María Isabel Guillén [a,b], Miguel Tofiño-Vian [a], Antonio Silvestre [c], Miguel Angel Castejón [d], María José Alcaraz [a,*]

[a] Interuniversity Research Institute for Molecular Recognition and Technological Development (IDM), University of Valencia, Polytechnic University of Valencia, Av. Vicent A. Estellés s/n, 46100, Burjassot, Valencia, Spain
[b] Department of Pharmacy, Faculty of Health Sciences, Cardenal Herrera-CEU University, 46115, Alfara del Patriarca, Valencia, Spain
[c] Department of Surgery, Faculty of Medicine, University of Valencia, Av. Blasco Ibáñez 15, 4610, Valencia, Spain
[d] Department of Orthopaedic Surgery and Traumatology, De la Ribera University Hospital, Alzira, 46600, Valencia, Spain

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ABSTRACT

Background: Osteoarthritis (OA) is a joint disease characterized by cartilage degradation, low-grade synovitis and subchondral bone alterations. In the damaged joint, there is a progressive increase of oxidative stress leading to disruption of chondrocyte homeostasis. The modulation of oxidative stress could control the expression of inflammatory and catabolic mediators involved in OA. We have previously demonstrated that extracellular vesicles (EVs) present in the secretome of human mesenchymal stem cells from adipose tissue (AD-MSCs) exert anti-inflammatory and anti-catabolic effects in OA chondrocytes. In the current work, we have investigated whether AD-MSC EVs could regulate oxidative stress in OA chondrocytes as well as the possible contribution of peroxiredoxin 6 (Prdx6).

Methods: Microvesicles (MV) and exosomes (EX) were isolated from AD-MSC conditioned medium by differential centrifugation with size filtration. The size and concentration of EVs were determined by resistive pulse sensing. OA chondrocytes were isolated from knee articular cartilage of advanced OA patients. 4-Hydroxynonenal adducts, IL-6 and MMP-13 were determined by enzyme-linked immunosorbent assay. Expression of Prdx6 and autophagic markers was assessed by immunofluorescence and Western blotting. Prdx6 was downregulated in AD-MSCs by transfection with a specific siRNA.

Results: MV and to a lesser extent EX significantly reduced the production of oxidative stress in OA chondrocytes stimulated with IL-1β. Treatment with MV resulted in a dramatic upregulation of Prdx6. MV also enhanced the expression of autophagy marker LC3B. We downregulated Prdx6 in AD-MSCs by using a specific siRNA and then MV were isolated. These Prdx6-silenced MV failed to modify oxidative stress and the expression of autophagy markers. We also assessed the possible contribution of Prdx6 to the effects of MV on IL-6 and MMP-13 production. The reduction in the levels of both mediators induced by MV was partly reverted after Prdx6 silencing.

Conclusion: Our results indicate that EVs from AD-MSCs regulate the production of oxidative stress in OA chondrocytes during inflammation. Prdx6 may mediate the antioxidant and protective effects of MV.

The translational potential of this article: This study gives insight into the protective properties of EVs from AD-MSCs in OA chondrocytes. Our findings support the development of novel therapies based on EVs to prevent or treat cartilage degradation.

1. Introduction

Osteoarthritis (OA) is a disabling disease with a high prevalence. Symptoms frequently include pain, stiffness and loss of movement and function. This chronic condition affects the whole joint and is characterized by degradation of articular cartilage, subchondral bone sclerosis,

* Corresponding author. Interuniversity Research Institute for Molecular Recognition and Technological Development (IDM), University of Valencia, Polytechnic University of Valencia, Burjassot, Valencia, Spain.
E-mail addresses: isabel.guillen@uv.es (M.I. Guillén), miguel.tofino@uv.es (M. Tofiño-Vian), antonio.silvestre@uv.es (A. Silvestre), macastejon@hospital-ribera.com (M.A. Castejón), maria.j.alcaraz@uv.es (M.J. Alcaraz).

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osteophyte formation, synovitis, etc. [1]. Cartilage degradation may be a consequence of aging, inappropriate mechanical loading and low-grade inflammation which promote disease symptomatology and accelerate disease progression. Considerable evidence has accumulated to indicate that inflammatory mediators induce oxidative stress which damages the mitochondrial respiratory chain protein complex contributing to alterations in chondrocyte viability and function. Therefore, OA chondrocytes show phenotypic changes leading to a reduced repair ability [2]. As there is no treatment able to inhibit joint destruction there is a need for novel approaches directed at protecting joint tissues with the potential to halt the progression of OA.

Mesenchymal stem/stromal cells (MSCs) act on different cell populations to promote immune modulation, angiogenesis, MSC and progenitor cell recruitment and differentiation, inhibition of apoptosis and tissue repair. Thus, MSCs have potential applications in joint conditions due to their regenerative and immunomodulatory properties. Clinical trials have shown that intra-articular MSC therapy in knee OA patients controls pain and improves function with the potential to modify disease progression [3]. A wide range of evidence indicates that MSCs exert their beneficial actions mainly through paracrine mechanisms. These cells secrete a variety of bioactive molecules and extracellular vesicles (EVs) responsible for their immunomodulatory and trophic effects. This secretome can vary depending on cell phenotype and function, as well as on the local microenvironment [4]. EVs are generally classified into large EVs or microvesicles (MV, 150 nm to 1,000 nm in diameter) derived from the outward budding and fission of the plasma membrane, and small EVs or exosomes (EX, 30–100 nm in diameter) which are generated by the inward budding of endosomal membrane. Different studies have shown that EVs can transfer their content (nucleic acids, lipids, proteins or mitochondria) to recipient cells which may mediate the effects exerted by these microparticles [5,6]. Increasing evidence also suggests that cell-free therapy can offer some advantages over MSC therapy and it may represent a safer alternative in cartilage regeneration and OA (reviewed in Ref. [7]).

MSC EVs can be internalized by OA articular cells to downregulate the production of inflammatory and catabolic mediators, and improve the synthesis of cartilage extracellular matrix components [8]. Recent investigations have demonstrated the protective effects of MV and EX from human adipose tissue-derived MSCs (AD-MSCs) in OA chondrocytes and cartilage explants stimulated with interleukin(IL)-1β [9]. This cytokine induces in chondrocytes gene expression changes similar to OA [10]. Both types of EVs significantly reduced the production of pro-inflammatory and catabolic mediators whereas they increased the production of IL-10 and the expression of type II collagen [9].

Oxidative stress contributes to the disruption of chondrocyte homeostasis and OA pathogenesis leading to the activation of inflammatory and catabolic signaling pathways [11]. Therefore, induction of proteinase expression by IL-1β is reactive oxygen species (ROS) dependent [12]. In particular, the lipid peroxidation product 4-hydroxynonenal (HNE) can form multiple protein adducts thus affecting cellular activity and function during OA. The levels of these adducts are higher in cartilage and other articular tissues of OA patients compared with healthy subjects [13]. In this regard, the modification of collagen II and matrix metalloproteinase-13 (MMP-13) by HNE is relevant for OA pathology [14]. Although low levels of ROS play a physiological role, excessive oxidative stress is associated with tissue damage and OA progression. Different endogenous mechanisms are activated by oxidative stress to counteract its effects. Therefore, antioxidant response and autophagy are simultaneously induced by oxidative stress to decrease the excessive production of reactive species and the oxidative damage to macromolecules [15]. It is also known that autophagy deficiency results in pro-inflammatory cytokines and oxidative stress production [16] whereas the modulation of ROS by autophagy downregulates OA-like gene expression induced by IL-1β in human chondrocytes [17].

Peroxiredoxin (Prdx) family members are induced as part of a defensive response against cell injury and they could be involved in protection against cartilage damage induced by ROS. In particular, the expression of Prdx6 is reduced in OA cartilage compared with controls [18]. Nevertheless, the precise mechanism of antioxidant protection by Prdx6 has not been elucidated although some possibilities can be the direct scavenging of low molecular weight hydroperoxides and the reduction of phospholipid hydroperoxides. This protein expresses three enzymatic activities (peroxidase, phospholipase A_2 and lysophosphatidylcholine acyltransferase) involved in the repair of peroxidized cell membranes which is crucial for cell survival [19].

In the present study, we investigated whether AD-MSC EVs could protect OA chondrocytes against oxidative stress induced by IL-1β, which represents an important element of pro-inflammatory cytokine actions during OA. We also examined the effects of these microparticles on autophagy markers as well as the possible role of Prdx6 in mediating the effects of AD-MSC MV.

2. Materials and methods

2.1. Adipose tissue-derived mesenchymal stem cells

AD-MSCs were obtained from the adipose tissue of 11 healthy non-obese donors (11 women, aged 51.3 ± 6.8 years, mean ± SEM) who had undergone abdominoplasty. The experimental design was approved by the Institutional Ethical Committees (University of Valencia and La Fe Polytechnic University Hospital, Valencia, Spain). Samples were obtained from donors after they provided informed consent according to the Helsinki Declaration.

Samples were washed with phosphate-buffered saline (PBS), minced and digested at 37 °C for 1 h with 2% of type I collagenase (Gibco, Life Technologies, Madrid, Spain). Tissue remains were filtered through a 100 μm cell strainer (BD Biosciences, Bedford, MA, USA). Cells were washed with DMEM/HAM F12 (Sigma–Aldrich, St. Louis, MO, USA) containing penicillin and streptomycin (1%), seeded onto tissue culture flasks (1–2 × 10^6 cells/mL) in DMEM/HAM F12 medium with penicillin and streptomycin (1%) and EV-free human serum (15%), and incubated at 37 °C in a 5% CO_2 atmosphere. Human serum was obtained from Biowest (Maine-et-Loire, France) from human male AB group. To eliminate the EV fraction, serum was centrifuged for 18 h at 120,000×*g* and 4 °C using the SW-28 swinging-bucket rotor (Beckman Coulter, Brea, CA, USA). At 24 h, when cells reached semiconfluence, culture plates were washed and the AD-MSC phenotype confirmed by flow cytometry (Flow Cytometer II, BD Biosciences, San Jose, CA, USA) by means of positive (CD105, CD90) and negative (CD34, CD45) markers (Fig. 1) using specific antibodies: anti-CD105-PE, antiCD90PerCP-eFluo 710, anti-CD34APC (eBioscience, Inc., San Diego, CA, USA) and anti-CD45-FITC (BDPharmingen™, BD Biosciences), and cellular viability was assessed with propidium iodide.

CM was collected from AD-MSCs (passage 0) at 48 h of culture. CM was centrifuged and stored in sterile conditions at −80 °C until use [9]. For Prdx6 silencing, AD-MSCs were obtained as indicated above. At 24 h, the culture medium was changed, and the cells were transfected with human Prdx6 siRNA or negative control siRNA (Ambion Silencer® Select validated siRNA, Life Technologies) using the kit Lipofectamine® RNAiMAX reagent (Life Technologies), following the manufacturer's recommendations. CM was collected at 48 h to isolate EVs.

2.2. Isolation and characterization of EVs

CM from AD-MSCs was centrifuged at 300×*g* for 10 min at 4 °C. The supernatant was collected, filtered through an 800 nm filter (Merck, Darmstadt, Germany) and centrifuged at 12,200×*g* for 20 min at 4 °C to pellet MV. Then, supernatants were filtered through a 200 nm filter (Merck, Darmstadt, Germany) and centrifuged at 100,000×*g* for 90 min at 4 °C to collect EX. MV and EX were washed once with sterile PBS, resuspended in 15 μL of PBS and stored at −80 °C until further use.

EV preparations were analyzed by Tunable resistive pulse sensing

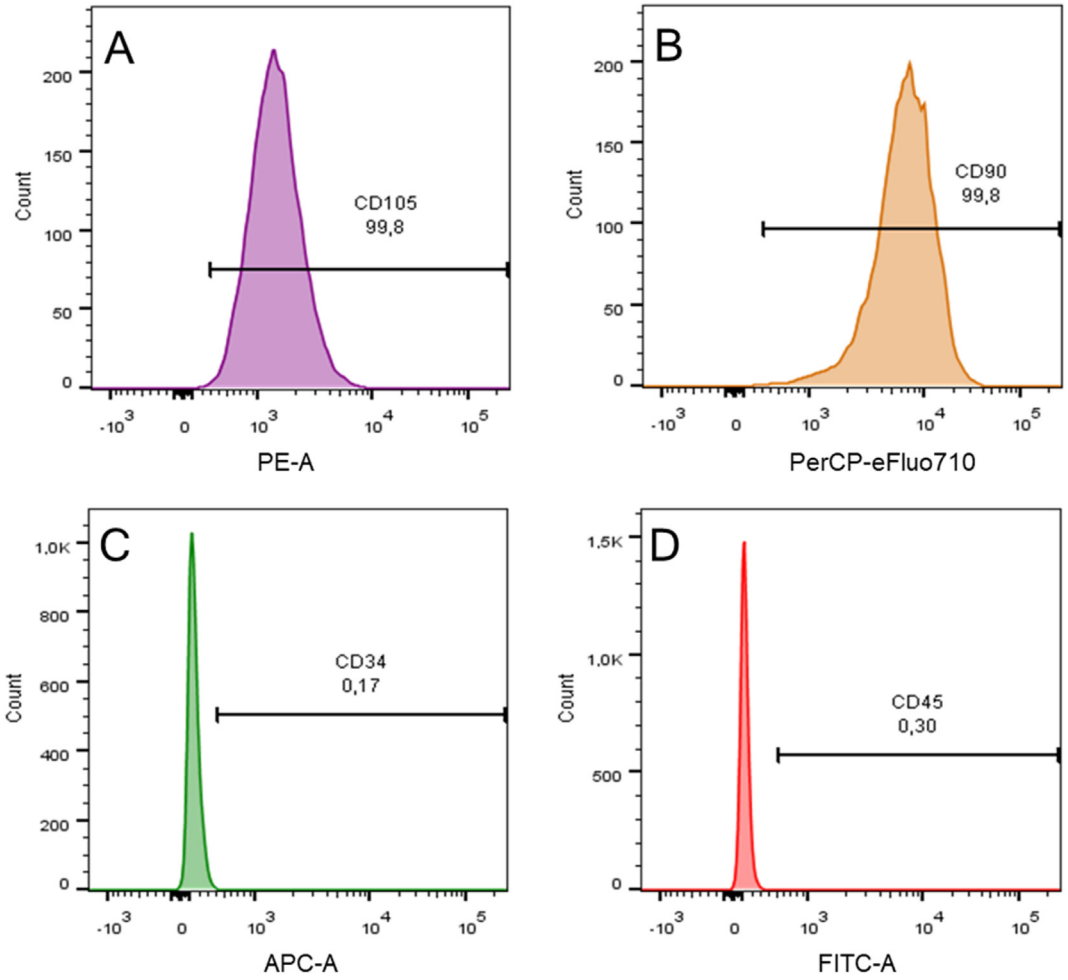

Fig. 1. Detection of surface antigens in human AD-MSCs by Flow Cytometry The data show positive expression of MSC-associated surface antigens CD105 (A) and CD90 (B). In contrast, minimal expression of CD34 (C) and CD45 (D) antigens was detected.

(TRPS) using a qNano instrument (IZON Sciences Ltd., Oxford, UK) to measure the concentration and size distribution. Calibration was performed using calibration beads SKP200 and SKP400. NP100 and NP300 nanopore membranes were used to measure EX and MV samples, respectively. At least 500 events/sample were counted. TRPS analysis indicated mean diameter values of 295 nm and 115 nm for MV and EX, respectively. The morphology characterization was performed using transmission electron microscopy (TEM) by the Microscopy Service (SCSIE, University of Valencia, Spain). The pellets of EVs were fixed in Karnovsky's solution. After rinsing the samples with PBS and post-fixed in osmium tetroxide, they were dehydrated in graded ethanol and embedded in LR-White resin. Following overnight polymerization of samples at 60 °C, resin blocks were cut with the ultra-microtome Ultracut UC6 (Leica, Wetzlar, Germany). The ultrathin sections (60 nm) obtained were contrasted with uranyl acetate 2% for 25 min and lead citrate 3% for another 12 min and observed using Jeol JEM-1010 transmission electron microscope (JEOL Ltd. Tokyo, Japan) at 80 kV. For gold-immunostaining, mouse anti-human CD63 monoclonal and goat anti-mouse IgG H&L (10 nm Gold) polyclonal antibodies from Abcam (Cambridge, MA, USA) were used. Images were acquired with a digital camera MegaView III with Olympus Image Analysis Software (Olympus, Tokyo, Japan). Fig. 2 shows representative TEM images of these EVs which were also characterized for protein expression by flow cytometry, and proteomic analysis (mass spectrometry), as reported [9].

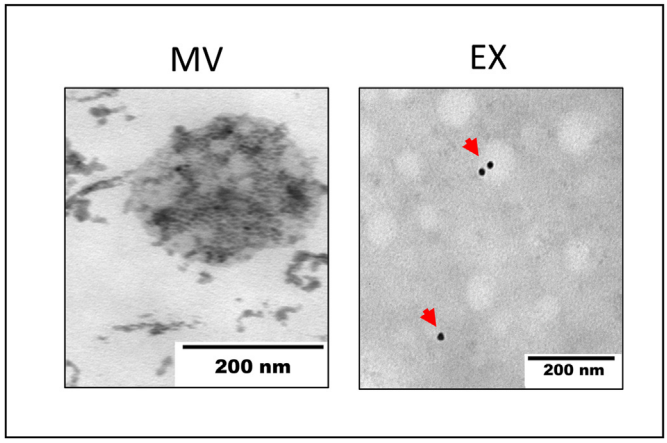

Fig. 2. Representative TEM images of MV and EX. Immunostaining with gold-labeled anti-CD63 antibodies: red arrows show positive staining.

2.3. OA chondrocytes

Knee cartilage samples were obtained from patients diagnosed with advanced OA (25 women and 12 men, aged 67.8 ± 8.4 years, mean ± SEM) undergoing total joint replacement. The experimental design was approved by the Institutional Ethical Committees as indicated

above. Small pieces of cartilage were prepared from the femoral condyles and tibial plateau. Chondrocytes were isolated by sequential digestion: 1 h with 0.1 mg/mL hyaluronidase (Sigma–Aldrich) followed by 3–5 h with 1 mg/mL type IA collagenase (Sigma–Aldrich) in DMEM/HAM F12 containing penicillin and streptomycin (1%) at 37 °C in 5% CO_2. The digested tissue was filtered through a 70 μm nylon mesh (BD Biosciences), washed, and resuspended in DMEM/HAM F12 containing 1% antibiotics and 10% fetal bovine serum (Sigma–Aldrich). Cell viability was greater than 95% as assessed by the Trypan blue exclusion test. Chondrocytes were seeded at 270 × 10^3 cells/well in 6-well plates or 20x10^3 cells/well in Lab-tek chambers (Thermo Scientific, Rochester, NY, USA), and cultured with 5% CO_2 at 37 °C in DMEM/HAM F12 with penicillin and streptomycin (1%) and 10% fetal bovine serum (Sigma–Aldrich). All experiments were performed with chondrocyte primary cultures at semiconfluence. For cell stimulation, media was replaced with DMEM/HAM F12 containing penicillin and streptomycin (1%) supplemented with 10% EV-free human serum and chondrocytes were incubated for 24 h with IL-1β (10 ng/mL) in the presence or absence of AD-MSC MV (3.6x10^7 particles/mL), EX (7.2x10^7 particles/mL) or the same volume of CM. In these conditions, the amount of EVs in isolated fractions and CM was comparable.

2.4. Chondrocyte culture viability assay

The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) to formazan was performed in OA chondrocytes stimulated with IL-1β (10 ng/mL) and treated with MV (3.6x10^7 particles/mL, 1 mL/well), EX (7.2x10^7 particles/mL, 1 mL/well) or CM (1 mL/well) for 24 h in 6-well plates. Cells were incubated with MTT (200 μg/mL) for 2 h. Then, the medium was removed and cells were solubilized in dimethyl sulfoxide (100 μL) to measure formazan at 550 nm using a Victor3 microplate reader (PerkinElmer España, Madrid, Spain).

2.5. Enzyme-linked immunosorbent assay

Chondrocytes were stimulated with IL-1β (10 ng/mL) in presence or absence of MV (3.6x10^7 particles/mL, 1 mL/well), EX (7.2x10^7 particles/mL, 1 mL/well) or CM (1 mL/well) for 24 h in 6-well plates. Supernatants were centrifuged and stored at −80 °C until analysis of IL-6 and MMP-13 using enzyme-linked immunosorbent assay (ELISA) kits with a sensitivity of 2.0 pg/mL for IL-6 (eBioscience, San Diego, CA, USA) and 6.0 pg/mL for MMP-13 (Invitrogen, Life Technologies). To measure the levels of HNE-protein adducts, cells were lysed with buffer (1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl and 25 mM Tris, pH 7.4). Lysates were centrifuged at 4 °C for 10 min at 10,000×*g* and HNE-adducts were measured by ELISA using the kit from Cell Biolabs (San Diego, CA, USA) with a sensitivity of 1.56 μg/mL, according to manufacturer's recommendations.

2.6. Immunofluorescence

Chondrocytes were stimulated with IL-1β (10 ng/mL) and treated with MV (3.6x10^7 particles/mL, 0.2 mL/well), EX (7.2x10^7 particles/mL, 0.2 mL/well) or CM (0.2 mL/well) for 24 h in Lab-tek chambers. Cells were fixed with 4% formaldehyde in PBS for 30 min at 4 °C, blocked with 1% BSA in PBS for 20 min at room temperature and incubated with phycoerythrin-conjugated rabbit anti-human Prdx6 monoclonal antibodies from Abcam (Cambridge, MA, USA) or rabbit anti-human LC3B polyclonal antibody (Invitrogen, Thermo Fisher Scientific, MA, USA) and subsequently incubated with goat anti-rabbit IgG-FITC (R&D Biosystems, Abingdon, UK). The samples were mounted in Prolong Gold antifade reagent with DAPI (Molecular Probes, Invitrogen, Life Technologies) and examined under a confocal microscope (Olympus FV1000, Tokyo, Japan). Fluorescence intensity per pixel per cell in 6 microscopic fields of each well was taken as a measure of Prdx6 positivity. Perinuclear positive endosomes (puncta) per cell were counted in 6 microscopic fields of each well. Fluorescence was quantified using ImageJ software (National Institutes of Health, USA).

2.7. Western blot

Protein analysis was performed by exponential Bio-Rad Mini-PROTEAN® TGX Gels (Bio-Rad, Hercules, CA, USA), followed by a semi-dry transfer (Bio-Rad) on nylon membrane Hybond™-P (GE Healthcare Amersham, Thermo Fisher Scientific). Protein expression was detected by incubation with different primary polyclonal antibodies: rabbit anti-peroxiredoxin 6™ Picoband (Boster, Pleasanton, CA, USA), rabbit anti-p62/SQTM1 and rabbit anti-atg5 (Novus Biologicals, Centennials, CO, USA), followed by incubation with polyclonal goat anti-rabbit immunoglobulin/HRP (Dako Agilent, Santa Clara, CA, USA), followed by an enhanced chemiluminescent substrate ECL (Pierce™ ECL Western Blotting Substrate, Thermo Fisher). Bands were visualized using an AutoChemi Imaging System (BioImaging Systems, UPV Inc., Upland, CA, USA).

2.8. Statistical analysis

Data are expressed as the mean and standard error of the mean (mean ± SEM). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-test using the GraphPad Prism 7.0 software (Graph Pad Software, La Jolla, CA, USA). A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of EVs on oxidative stress and Prdx6 in OA chondrocytes

To determine whether EVs protected OA chondrocytes against oxidative stress, AD-MSC-derived MV, EX and CM were incubated with these cells in the presence of IL-1β for 24 h. Previously, we confirmed that none of the treatments significantly affected cell viability assessed by the MTT method in comparison with nonstimulated chondrocytes (data not shown). Determination of HNE-modified proteins demonstrated that IL-1β enhanced oxidative stress in chondrocytes and all treatments decreased it significantly (Fig. 3A). The protective effect of MV was similar to that of CM and higher than EX suggesting that MV may be the main agent present in CM responsible for the control of oxidative stress. Thus, MV restored basal levels of HNE adducts, preventing the effect of IL-1β. Prdx6 represents a defensive mechanism to protect cells against injury by oxidative stress [19]. We determined whether EVs contained in AD-MSC-derived CM were able to regulate Prdx6 expression in OA chondrocytes. Fig. 3B shows that Prdx6 protein was present in non-stimulated cells and IL-1β stimulation did not modify it. Interestingly, treatment with MV resulted in a dramatic upregulation of this protein in OA chondrocytes whereas EX or CM did not affect Prdx6 expression.

3.2. Effects of EVs on LC3B autophagy marker in OA chondrocytes

Stimulation of human OA chondrocytes with IL-1β increases the autophagic flux as part of a mechanism to clear dysfunctional mitochondria thus preventing further oxidative stress [20]. To evaluate the effects of EVs on autophagy marker LC3B, OA chondrocytes were stimulated with IL-1β for 24 h in the presence or absence of EVs or CM, and then LC3B-positive vesicles were studied by immunofluorescence staining followed by confocal microscopy. We found a small increase in the expression of LC3B in the cytoplasm of OA chondrocytes stimulated with IL-1β compared with control chondrocytes (Fig. 4). Our results indicate that treatment with EX was ineffective whereas CM and MV significantly enhanced LC3B expression and the number of associated autophagosomes in IL-1β-stimulated chondrocytes.

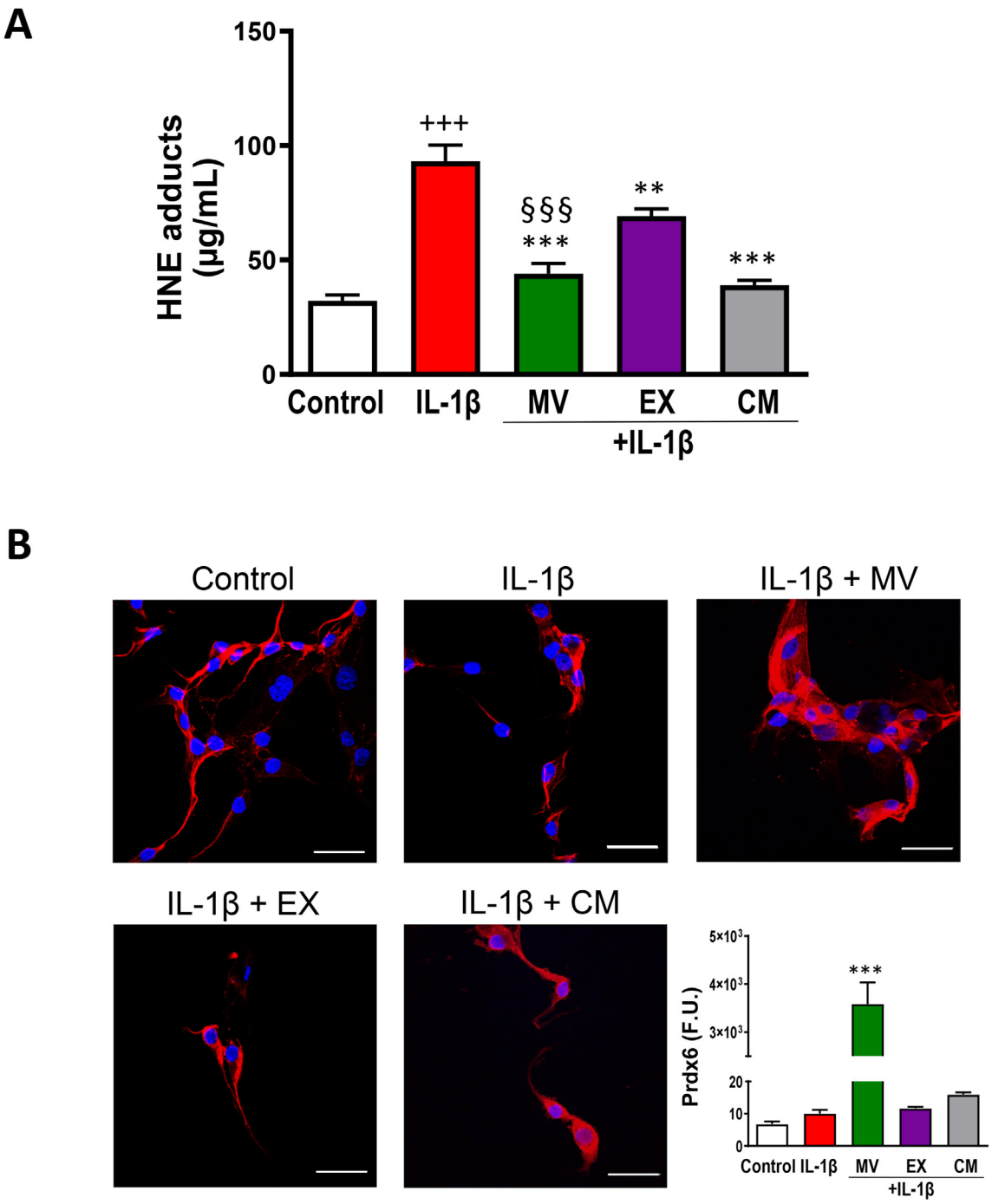

Fig. 3. Effects of EVs on oxidative stress (A) and Prdx6 protein expression (B) in OA chondrocytes. Cells were incubated with MV, EX, or CM in the presence of IL-1β for 24 h. A: HNE adducts formation was determined by ELISA. Data represent mean ± SEM of independent cultures of chondrocytes from six different donors. +++ $p < 0.001$ versus nonstimulated cells (control), ** $p < 0.01$, *** $p < 0.001$ versus IL-1β; §§§ $p < 0.001$ versus EX. B: Intensity of fluorescence (red channel) was measured in independent cultures of chondrocytes from six different donors. DAPI was used to stain the nuclei. F.U., fluorescence units. Bars: 30 µm. The figure shows representative images and the mean ± SEM of data. *** $p < 0.001$ versus IL-1β.

3.3. Contribution of Prdx6 to MV effects on oxidative stress and inflammatory mediators

Since our results showed that MV treatment induces a dramatic increase in the expression of Prdx6 in IL-1β-stimulated OA chondrocytes, we assessed the possible contribution of Prdx6 to the protective effects of MV on oxidative stress. For that purpose, we downregulated Prdx6 in AD-MSCs by using a specific siRNA and then we isolated the MV present in their CM. Prdx6 siRNA markedly reduced the expression of Prdx6 in AD-MSCs and MV (Fig. 5A). As shown in Fig. 5B, MV from Prdx6-silenced AD-MSC failed to reduce the levels of HNE adducts induced by IL-1β stimulation. As MV reduces the production of IL-6 and MMP-13 in OA chondrocytes stimulated with IL-1β [9] we also assessed the possible role of Prdx6 in the anti-inflammatory effects of these EVs. Fig. 4C and D shows that Prdx6 silencing partly prevented the effects of MV on IL-6 and MMP-13 levels.

3.4. Contribution of Prdx6 to MV effects on autophagy markers

Our previous experiments indicated that MV enhance the expression

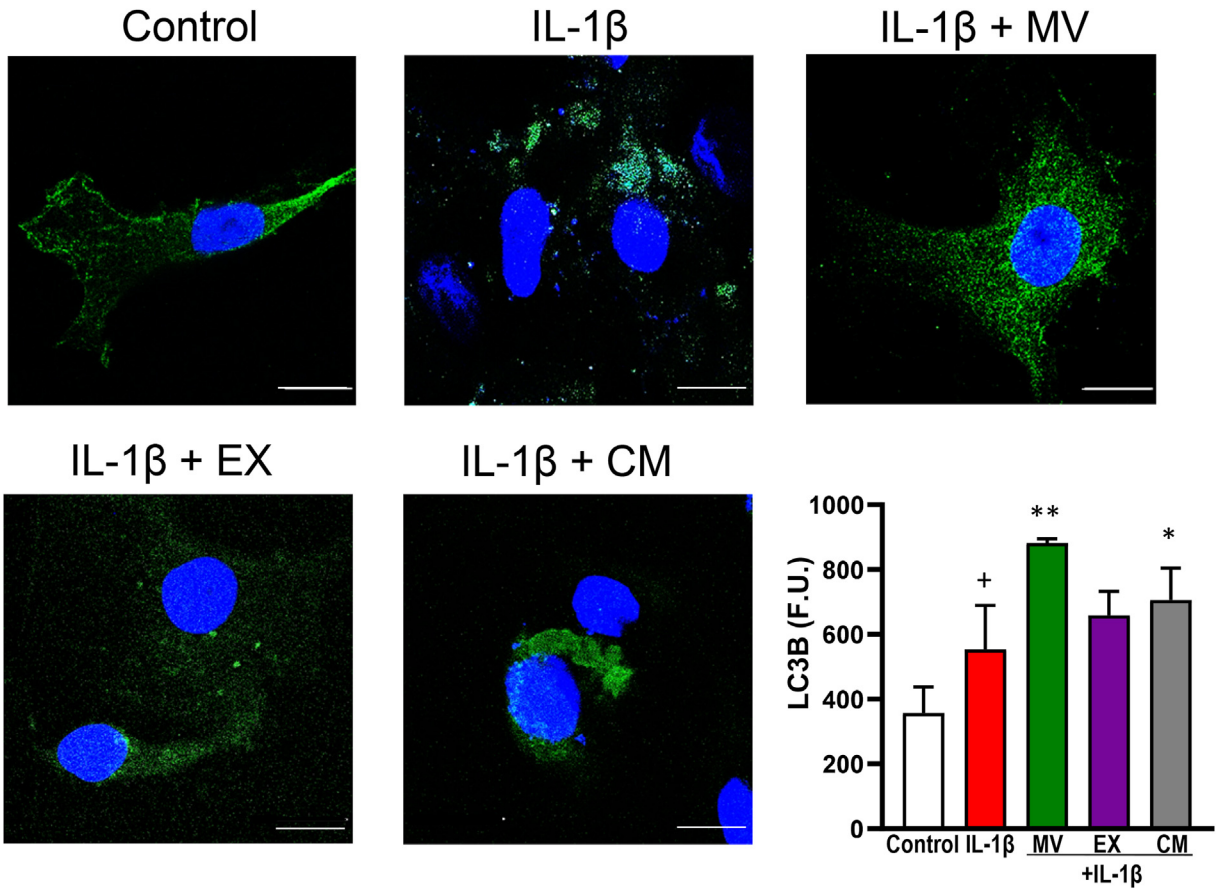

Fig. 4. Effects of EVs on LC3B autophagy marker in OA chondrocytes. Immunocytochemistry analysis for LC3B was performed in independent cultures of chondrocytes from six different donors. Cells were incubated with MV, EX or, CM in the presence of IL-1β for 24 h, and subsequently fixed with 4% formaldehyde in PBS for 15 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS. Then, cells were incubated with primary and FITC-conjugated antibodies as specified in Material and methods. DAPI was used to stain the nuclei. F.U., fluorescence units. Bars: 10 μm. The figure shows representative images and the mean ± SEM of data. $+p < 0.05$ versus nonstimulated cells (control), $*p < 0.05$, $**p < 0.01$ versus IL-1β.

of autophagy component LC3B in OA chondrocytes. To extend these findings and assess the possible contribution of Prdx6, we incubated OA chondrocytes with IL-1β and MV from normal AD-MSCs or Prdx6-deficient AD-MSCs. After 24 h, the expression of relevant autophagic proteins atg5 and p62 was evaluated by Western blotting. The autophagic process is dependent on atg5 expression in OA chondrocytes stimulated with IL-1β [17]. As shown in Fig. 6A and B, IL-1β increased the expression of these proteins compared with control (nonstimulated chondrocytes). Chondrocyte treatment with MV from AD-MSCs (control siRNA) significantly increased the expression of atg5 and p62 relative to IL-1β. Interestingly, this positive effect of MV on autophagic proteins was abolished if Prdx6 was silenced.

4. Discussion

It is well established that ROS contribute to the regulation of many basic chondrocyte activities such as cell activation, proliferation, and matrix remodeling besides their role as mediators of signaling induced by pro-inflammatory cytokines [21,22]. Nevertheless, considerable evidence supports a central role of ROS in triggering harmful events during OA progression. In this regard, oxidative stress is related to mitochondrial dysfunction and DNA damage present in OA chondrocytes [23,24]. Besides, ROS are involved in MMP and aggrecanase transcription, MMP activation, and enhancement of the rate MMP/tissue inhibitors of metalloproteinases [22]. Articular cartilage components are sensitive to modifications by oxidative stress. In particular, the lipid peroxidation product HNE is generated in joint tissues and may contribute to OA pathogenesis. HNE can induce cartilage lesion in animal models of OA [25], the cleavage of collagen II, and the expression of MMP-13, aggrecanase-2 and cyclooxygenase-2. Besides, the formation of HNE adducts with MMP-13 leads to the activation of this enzyme without proteolytic modification [13,25] while HNE binding to collagen II leads to alterations of chondrocyte phenotype and function [26]. Pro-inflammatory cytokines induce oxidative stress as shown by IL-1β in OA chondrocytes. We revealed a significant reduction of HNE-adducts formation by treatment of OA chondrocytes with EVs or CM. Thus, EVs from AD-MSCs inhibit oxidative stress induced by inflammation in OA chondrocytes with a higher effect of MV compared with EX.

Pro-inflammatory cytokines are produced by different articular cells and contribute to cartilage degradation during OA progression. We have shown previously that EVs from AD-MSCs exert anti-inflammatory and anti-catabolic effects in OA cartilage and chondrocytes stimulated with IL-1β. Proteomic analysis of EVs and functional studies suggested a role for annexin A1 in the anti-inflammatory effects of MV [9]. In particular, EVs decrease the release of IL-6 which synergizes with different cytokines to induce collagenase production, cartilage degradation [27] and inhibition of type II collagen expression [28]. It is also known that elevated serum levels of IL-6 are correlated with the development of radiographic knee OA [29]. EVs from AD-MSCs reduce the release of MMP-13 which is the main collagenase activity degrading type II collagen in OA chondrocytes and facilitates the progression to a hypertrophic-like differentiated state [30]. Therefore, EVs may be able to control not only the production of inflammatory mediators but also the consequences of cell activation by these agents.

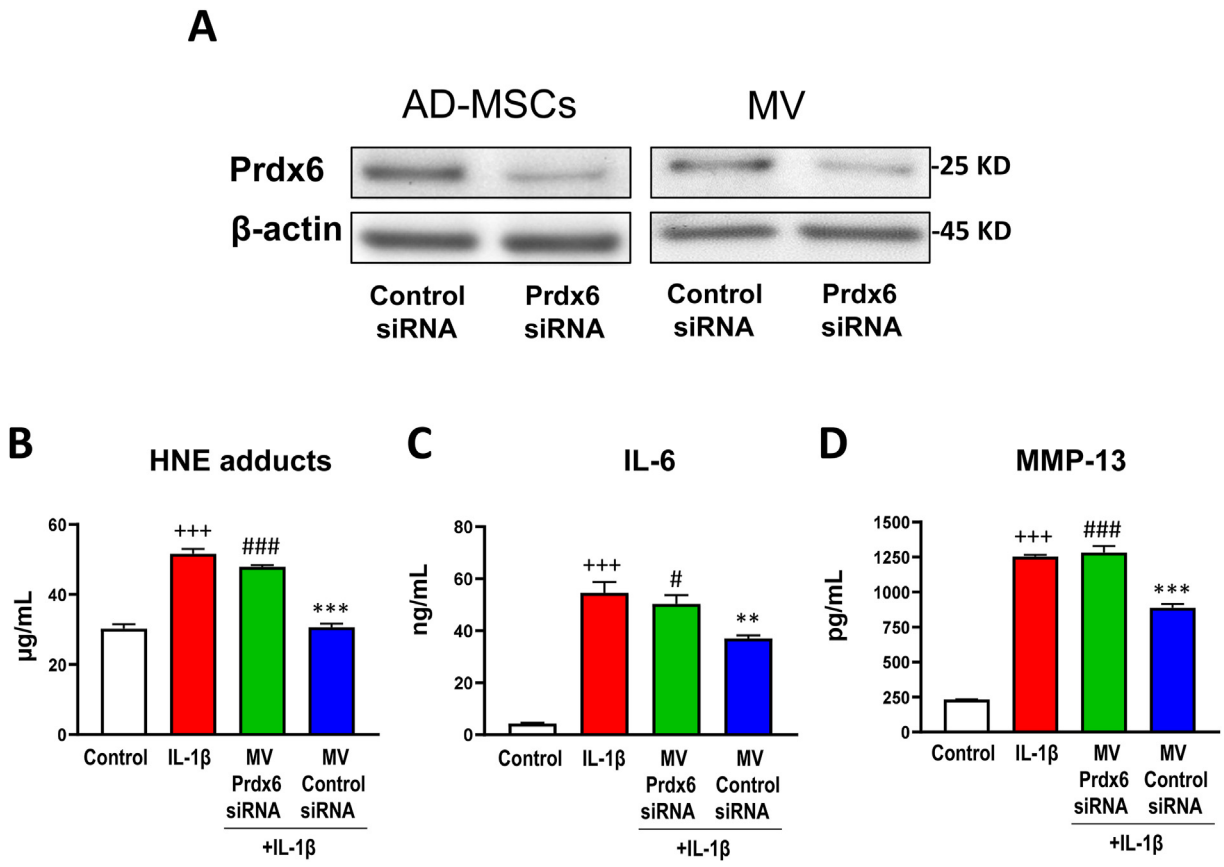

Fig. 5. Contribution of Prdx6 to MV effects on oxidative stress and inflammation. Expression of Prdx6 was determined by Western blotting in AD-MSCs treated with control siRNA or Prdx6 siRNA (A). OA chondrocytes were incubated with MV from AD-MSCs or Prdx6-silenced AD-MSCs in the presence of IL-1β for 24 h. HNE adducts (B), IL-6 (C) and MMP-13 (D) were determined by ELISA in independent cultures of chondrocytes from six different donors, as indicated in Materials and methods. Data show the mean ± SEM. $+++p < 0.001$ versus nonstimulated cells (control); $**p < 0.01$, $***p < 0.001$ versus IL-1β; $\#p < 0.05$, $\#\#\#p < 0.001$ versus MV from AD-MSCs treated with control siRNA.

The development of OA is a multifactorial process with the contribution of mechanical factors, inflammation, oxidative stress, etc. [31] which has been associated with a reduction in key regulators of autophagy in articular cartilage [32]. There is evidence showing that inhibition of autophagy promotes mitochondrial dysfunction and ROS production induced by IL-1β leading to OA-like gene expression changes whereas induction of autophagy has the opposite effect [17,20]. We have shown that MV from AD-MSCs enhance the expression of autophagy markers in OA chondrocytes stimulated with IL-1β. This is the first observation of an effect of these EVs on the autophagy pathway although further studies would be necessary to determine whether MV may regulate autophagy in OA chondrocytes.

A wide range of evidence has demonstrated that EVs represent an important way of cell communication [33]. MSCs could exert protective actions via the secretion of antioxidant enzymes in EVs [34]. It is also known that EVs can deliver DNA and RNA to recipient cells [6]. Nevertheless, little is known of the actions and mechanisms of these microparticles in joint conditions such as OA. Chondrocytes from OA patients show a reduction in the expression of antioxidant enzymes such as Prdx6 and Cu/Zn superoxide dismutase, compared with healthy cells [18,35] suggesting that Prdx6 may play a protective role in these cells by controlling ROS homeostasis [36]. Interestingly, Prdx6 is found in a high concentration in AD-MSC MV but not in EX as we determined previously by proteomic analysis [9]. In the current work, we have shown that MV from AD-MSCs strongly increase Prdx6 expression in OA chondrocytes stimulated with IL-1β and our data suggest that Prdx6 mediates at least in part the capacity of AD-MSC-derived MV to protect OA chondrocytes against the consequences of IL-1β stimulation. Therefore, our findings could pave the way for mechanistic research on the role of Prdx6 in chondrocyte homeostasis.

Different therapeutic strategies are under investigation to prevent or delay the destructive process that occurs in OA (reviewed in Ref. [37]). In particular, the therapeutic potential of MSCs from diverse sources has been investigated in OA models and clinical studies. The cytoprotective effects of MSCs may depend on several complementary factors including soluble mediators and the release of EVs [7]. A number of factors can modify the properties of MSCs and their secretome and need to be considered in relation with potential therapeutic applications. For instance, increasing donor age is related with the production of oxidative stress and inflammatory mediators which may reduce their immunomodulatory function [38]. Besides, female AD-MSCs induce greater immunomodulatory effects than male AD-MSCs [39]. It is likely that these changes in MSCs modify the properties of their EV although more studies are needed to know the exact impact on EV composition and actions. The use of EVs may have some advantages compared with MSC therapy as EVs are not modified by the environment thus resulting in more predictable effects. Also, EVs have a lower immunogenic potential allowing the administration of allogeneic EVs which can be suitable for large-scale production [40]. Nevertheless, to ensure the reproducibility of EV effects, strict standardized protocols must be followed to control all steps in EV isolation and application. However, the mechanisms by which EVs can protect OA chondrocytes have not been still elucidated. The downregulation of oxidative stress and inflammation can be an important component of the protective actions of AD-MSC MV in OA chondrocytes. Our data reinforce the view that MV from AD-MSCs could provide a novel and effective therapeutic approach for the control of cartilage

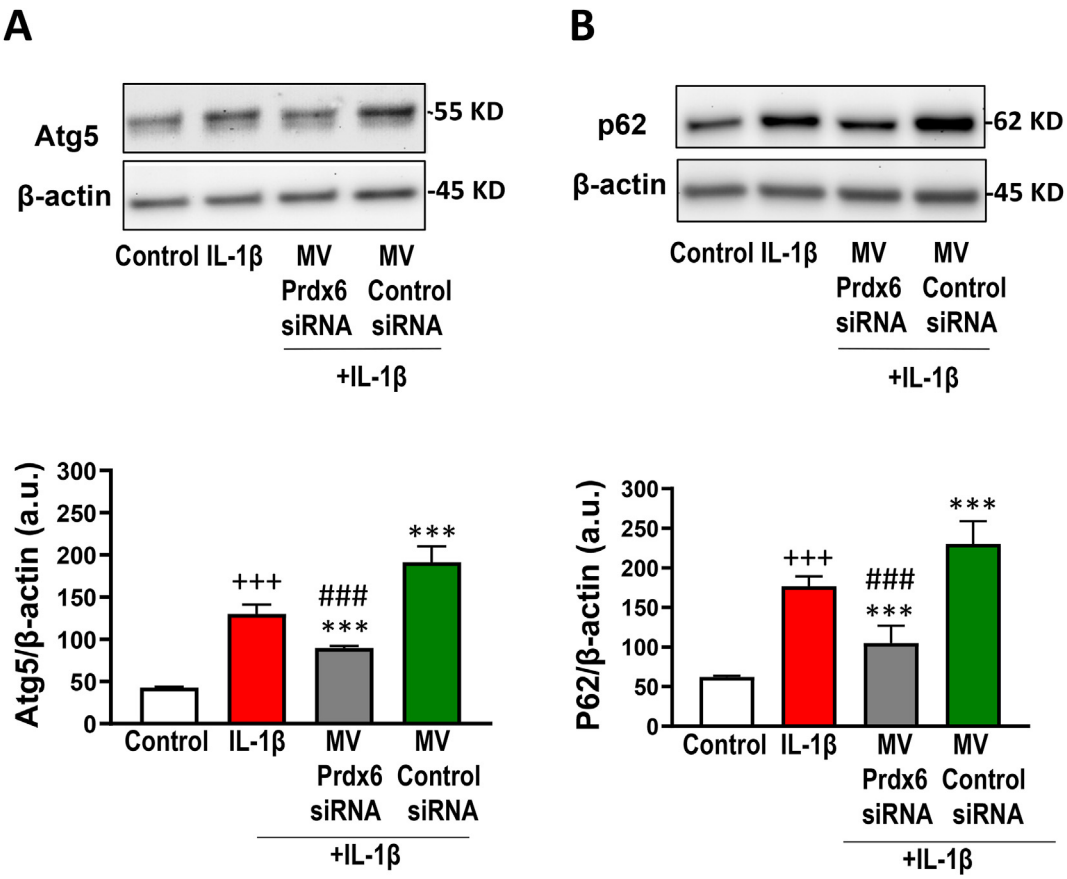

Fig. 6. Contribution of Prdx6 to MV effects on autophagy markers. OA chondrocytes were incubated with MV from AD-MSCs or Prdx6-silenced AD-MSCs in the presence of IL-1β for 24 h. Expression of atg5 (A) and p62 (B) was determined by Western blotting in independent cultures of chondrocytes from six different donors. Data show the mean ± SEM (densitometry, arbitrary units, a.u.) relative to β-actin. $+++p < 0.001$ versus nonstimulated cells (control); $**p < 0.01$, $***p < 0.001$ versus IL-1β; $###p < 0.001$ versus MV from AD-MSCs treated with control siRNA.

degradation.

Credit author contribution statement

I.G., M.J.A., A.S. and M.A.C. were responsible for the conception, design and interpretation of data. M.T. and I.G. performed the experiments and analyzed data. M.J.A. wrote the paper.

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Ethics approval and consent to participate

This study was carried out according to the recommendations of the Declaration of Helsinki of 1975, as revised in 2013. All subjects gave written informed consent. The protocol was approved by the local ethics committees: University of Valencia, Spain, and La Fe Polytechnic University Hospital, Valencia, Spain, H1389967869063 and 2014–0054.

Availability of data and materials

The datasets in this study are available.

Consent for publication

Not applicable.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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