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Short report

Adipose-derived stromal cell secretome reduces TNFα-induced hypertrophy and catabolic markers in primary human articular chondrocytes

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ABSTRACT

Recent clinical trials show the efficacy of Adipose-derived Stromal Cells (ASCs) in contrasting the osteoarthritis scenario. Since it is quite accepted that ASCs act predominantly through a paracrine mechanism, their secretome may represent a valid therapeutic substitute. The aim of this study was to investigate the effects of ASC conditioned medium (ASC-CM) on TNFα-stimulated human primary articular chondrocytes (CHs).

CHs were treated with 10 ng/ml TNFα and/or ASC-CM (1:5 recipient:donor cell ratio). ASC-CM treatment blunted TNFα-induced hypertrophy, reducing the levels of Osteocalcin (−37%), Collagen X (−18%) and MMP-13 activity (−61%). In addition, it decreased MMP-3 activity by 59%. We showed that the reduction of MMP activity correlates to the abundance of TIMPs (Tissue Inhibitors of MMPs) in ASC secretome (with TIMP-1 exceeding 200 ng/ml and TIMP-2/3 in the ng/ml range) rather than to a direct down-modulation of the expression and/or release of these proteases. In addition, ASC secretome contains high levels of other cartilage protecting factors, i.e. OPG and DKK-1.

ASC-CM comprises cartilage-protecting factors and exerts anti-hypertrophic and anti-catabolic effects on TNFα-stimulated CHs in vitro. Our results support a future use of this cell-derived but cell-free product as a therapeutic approach in the management of osteoarthritis.

1. Introduction

Osteoarthritis (OA) is a common age-related condition affecting millions of people worldwide. It is a multifactorial disease whose pathogenesis involves multiple causes, processes and tissues (Martel-Pelletier et al., 2016). This pathology is characterized by the destruction of articular cartilage associated with subchondral bone erosion and inflammation. Cartilage damage seems to be one of the earliest disease-causing events (Berenbaum, 2013). In OA, articular chondrocytes (CHs) undergo a phenotypic change: from quiescent and stable they engage a hypertrophic differentiation, characterized by increased cell proliferation and altered expression and activity of matrix-degrading enzymes (matrix metalloproteinases, MMPs) (Singh et al., 2018). The hypertrophic shift of CHs starts with the abnormal modulation of several signaling molecules and transcription factors that leads to the overexpression of Collagen X and distinct MMPs, such as MMP-1, MMP-9 and MMP-13. Among these enzymes, the latter is considered the main marker of hypertrophy (Singh et al., 2018). In animal models, the down-modulation of hypertrophy-inducing factors enhances the resistance to OA development, suggesting the arrest of chondrocyte hypertrophy as a valid therapeutic approach (Bottini et al., 2016). In addition, several in vitro models of hypertrophic chondrocytes have been developed treating cells with IL-1β or TNFα, the two major players in OA physiopathology (Cecil et al., 2009; Platas et al., 2013). Up to now, most treatments against OA are not curative. Cartilage regeneration does not occur spontaneously and the most common surgical approaches to circumvent the loss of cartilage, e.g. microfracture, subchondral drilling or autologous cartilage implantation, often lead to the formation of low-quality fibrocartilage. In the last years, the use of Mesenchymal Stromal Cells (MSCs) has emerged as a promising tool (Lopa et al., 2018). Its efficacy in contrasting cartilage damage has been shown in vitro (Manferdini et al., 2013; Maumus et al., 2013) and in

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vivo (Lee et al., 2007; Xie et al., 2012). Moreover, > 50 clinical trials have been investigating the safety, feasibility and efficacy of MSC intra-articular injection (clinicaltrials.gov). Since nowadays it is widely recognized that MSC therapeutic action largely depends on paracrine mechanisms, the scientific interest has shifted to their secretome, namely the conditioned medium (CM). MSC-CM has been successfully tested in several preclinical models (e.g. Brini et al., 2017; Kay et al., 2017; Kuljanin et al., 2019), suggesting its promising potential in future clinical applications. Here, we have investigated the anti-hypertrophic and anti-catabolic action of Adipose-derived Stromal Cell conditioned medium (ASC-CM) on an in vitro model of TNFα-stimulated primary human articular chondrocytes.

2. Methods

2.1. Human primary cells

Human ASCs were isolated from the adipose tissue of 8 healthy donors (2 males and 6 females; 46 ± 16 y/o) undergoing aesthetic or prosthetic surgery at IRCCS Galeazzi Orthopaedic Institute, following previously described protocols (Niada et al., 2016). Human CHs were isolated from the articular cartilage of the femoral heads collected from 18 patients (11 males and 7 females; 62 ± 11 y/o) undergoing total hip replacement surgery at the same Clinical Institute (additional information in supplementary methods). All waste tissues were collected following the procedure PQ 7.5.125, version 4, dated 22.01.2015, approved by IRCCS Istituto Ortopedico Galeazzi. Written informed consent was obtained from all the patients.

2.2. Concentrated conditioned media

Conditioned media were collected from about 90–95% confluent ASCs cultured for 72 h in starving conditions (i.e. without FBS) and concentrated through Amicon Ultra-15 Centrifugal Filter Devices with 3 kDa cut-off (Merck Millipore)(Brini et al., 2017; Niada et al., 2018). The product was concentrated about 40–50 folds becoming handy for in vitro treatments (final volume of 50-60 µl~10^6 ASCs).

2.3. Cell viability

CHs were stimulated with 10 ng/ml TNFα and/or ASC-CM (added at a 1:5 recipient to donor cell ratio). Cell viability was assessed by Alamar Blue assay (Thermo Fisher Scientific) (Giannasi et al., 2018) before the first treatment and after 3, 5 and 7 days. After 4 h incubation with Alamar Blue (1:10 dilution), emitted fluorescence was measured using Wallac Victor II plate reader (Perkin Elmer).

2.4. Analyses of gene and protein expression

CHs were seeded at the density of 8×10^3 cells/cm^2 in complete medium with 1% FBS and treated with 10 ng/ml TNFα and/or ASC-CM (1:5 recipient to donor cell ratio). 24, 48 and 72 h later, cells were lysed, and total RNA was extracted with RNaeasy kit (Qiagen). cDNA was synthesized using the High Capacity Reverse-Transcription Kit (Thermo Fisher Scientific). The expression levels of the target genes and of the housekeeping gene *TBP* were quantified by RT-qPCR using TaqMan technology (MMP3: hs00968305_m1; MMP13: hs00233992_m1; TBP: hs00427600_m1). The real-time PCR was conducted on a StepOne Plus Applied Biosystem apparatus (Life Technologies). Data were analysed with the $2^{-\Delta\Delta Ct}$ method.

MMP-3, MMP-13 and Collagen X protein expression was assessed after 1 or 3 days of treatment using western blotting, as described in supplementary methods.

2.5. Luminex multiplex assay

CH culture supernatants were prepared as described in supplementary methods. The analyses were conducted using MILLIPLEX MAP Human Bone Panel (HBNMAG-51 K, Millipore), Human MMP Panel 1 and 2 (HMMP1MAG-55 K and HMMP2MAG-55 K), and Human TIMP Magnetic Luminex Performance Assay (LKTM003, R&D Systems). Technical duplicates were analysed for each condition (25-50 µl/sample) and read through Bio-Plex Multiplex System (Bio-Rad) following standard procedures. Data analysis was performed with MAGPIX xPONENT 4.2 software (Luminex Corporation).

2.6. MMP-3 and MMP-13 activity assay

72-h culture supernatants were analysed to assess the activity of MMP-3 and MMP-13 with SensoLyte 520 Generic MMP Assay Kit (AnaSpec) following the manufacturer's instructions. Pro-MMP activation was achieved after incubating samples with 1 mM 4-aminophenylmercuric acetate (APMA) at 37 °C for 4 h (MMP-3) or 40 min (MMP-13). Fluorescence (490 nm excitation λ, 520 nm emission λ) was read with Wallac Victor II plate reader (Perkin Elmer).

2.7. Statistical analysis

Data are expressed as mean ± standard deviation (SD) of at least 3 independent experiments. Statistical analysis was performed by one-way or two-way ANOVA using Prism 5 software (GraphPad Software Inc). Differences were considered significant at $p \leq .05$.

3. Results

ASC-CM exerted specific effects on OA-related factors, blunting the increase of hypertrophic markers induced by TNFα stimulation. Collagen X expression and Osteocalcin (OC) release were reduced by 18% (day 1) and 37% (day 3) (Fig. 1C and D, Supplementary Fig. 1 A), while MMP-13 activity was lowered by 61% (Fig. 1F). In addition, ASC-CM halved the activity of MMP-3, another OA-related cartilage-degrading enzyme (Fig. 1E). These were most likely specific effects. Indeed, ASC-CM did not induce any alteration in chondrocyte viability and proliferation (Fig. 1A and B), not even when TNFα significantly increased these parameters (+27% and + 31% at day 5 and 7, respectively). Since in the OA context the reduction in MMP-3 and MMP-13 activity represents a promising goal, we explored its possible causes. Initially, we investigated MMP expression and release. At an early time point (24 h after treatments), ASC-CM reduced the expression of *MMP-3* (−49%) and, by a lower extent, *MMP-13* (−31%) in TNFα-treated CHs (Fig. 2A and B). The down-modulation of *MMP-3* was maintained up to day 3 (−38%, data not shown), while *MMP-13* one was lost at later time points (data not shown). Surprisingly, ASC-CM treatment stimulated both the intracellular (Fig. 2C) and the extracellular (Fig. 2E) levels of MMP-3. The intracellular expression was increased in both untreated (+81%) and TNFα-treated CHs (+39%) (Fig. 2C and Supplementary Fig. 1 B) and a similar induction was revealed extracellularly (+245% versus control CHs and + 33% compared to TNFα-treated cells) (Fig. 2E). On the other hand, ASC-CM caused a mild reduction of MMP-13 expression (−12%, Fig. 2D and Supplementary Fig. 1 B) in TNFα-stimulated CHs, in full accordance with its lower release (−20%, Fig. 2F). Taken together these evidences cannot explain the blunting effect of ASC-CM on TNFα-induced MMP activity. Since MMP activities are physiologically modulated by their endogenous inhibitors, we analysed the levels of TIMPs in CH supernatants after 3 days of TNFα stimulation and/or ASC-CM administration. Following CM treatment, TIMP levels were significantly increased in respect to both control and TNFα-stimulated cells (Fig. 3). TIMP-1 was the more abundant (> 200 ng/ml), followed by TIMP-2 (around 80 ng/ml), TIMP-3 (around 5 ng/ml) and the less represented TIMP-4 (Fig. 3A-D

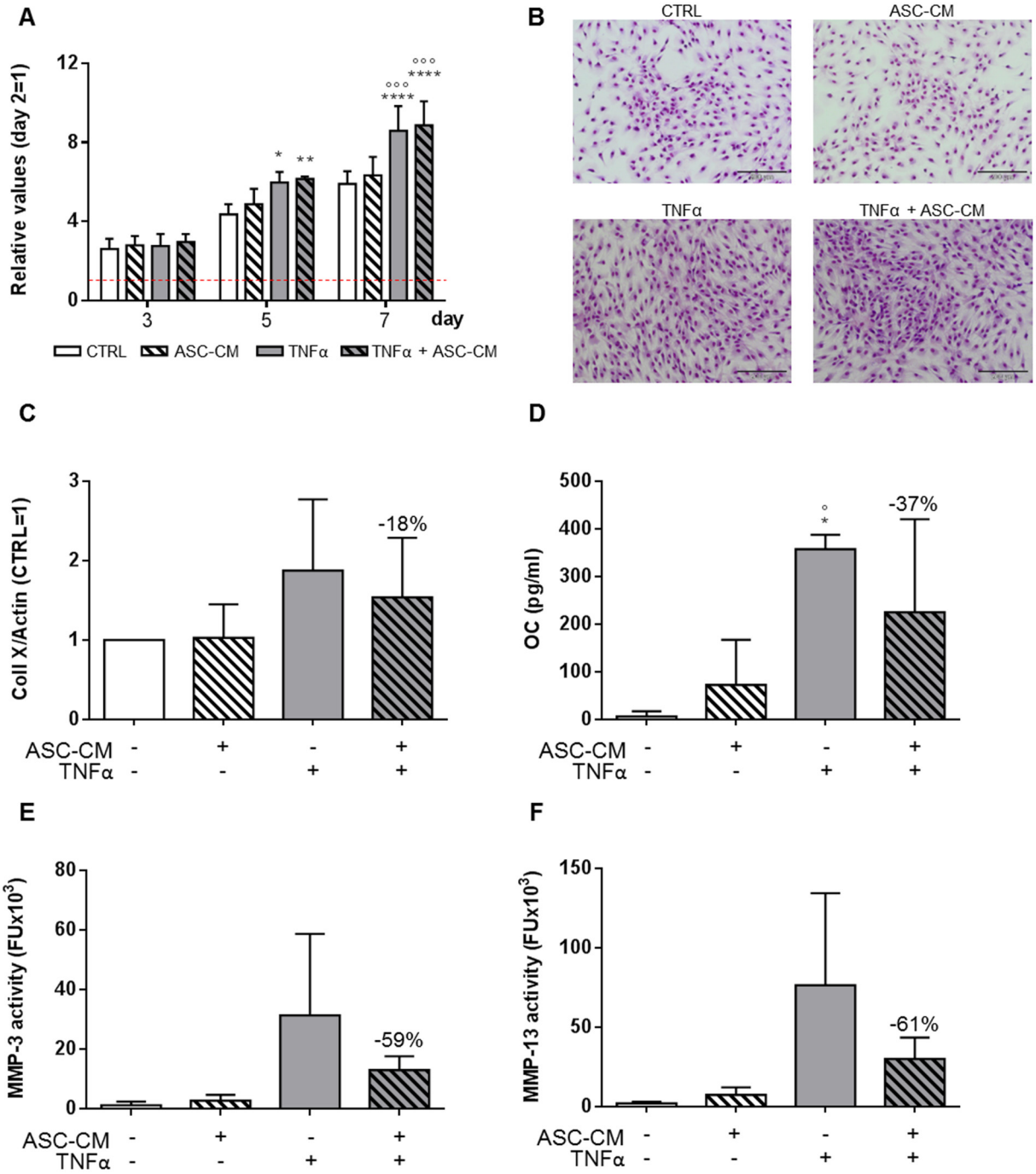

Fig. 1. Reduction of hypertrophic markers and MMP activity by ASC-CM treatment in TNFα-stimulated articular chondrocytes. (A) Cell metabolic activity measured by Alamar Blue assay. Data are shown as mean ± SD ($n = 3$) of relative values calculated as ratios on day 2 (red dashed line). Two-way ANOVA was performed, and significance vs CTRL is shown as $^{*}p < .05$, $^{**}p < .01$ and $^{****}p < .0001$, vs CM as $^{\circ\circ\circ}p < .001$. (B) Cell confluency at the final time point (day 7) displayed by Diff Quick staining (100× magnification; scale bar: 200 μm). (C) Collagen X expression at day 1 by Western Blot. Data ($n = 4$) were normalized on β-Actin and expressed as relative values (CTRL = 1). (D) Osteocalcin (OC) levels in CH culture media (day 3) measured by Luminex Multiplex Assay ($n = 9$ CHs, 3 pools). (*E*-F) Activity of MMP-3 and MMP-13 in chondrocyte culture medium ($n = 10$ CHs, 3 pools and 1 single population) expressed as fluorescence units (FU). Data were analysed by one-way ANOVA followed by Tukey's multiple comparison test. Significance vs CTRL is shown as $^{*}p < .05$, vs CM as $^{\circ}p < .05$.

respectively). Similar levels were already measured after 24 h of treatments (data not shown). This, together with the analysis of the naïve treating medium (Table 1), indicates that the high levels of TIMPs depend on their abundance in ASC secretome rather than to an increased production by treated CHs.

In conclusion, our data suggest that the reduction of MMP activity in TNFα-stimulated-CHs can be ascribed to the presence of TIMPs in ASC secretome rather than to its direct modulation of MMP gene and protein levels. Similar speculations can be made considering other MMPs whose secretion was enhanced by TNFα, such as MMP-1 (reduced by 31% following ASC-CM administration), MMP-9 (−52%) and MMP-10 (−16%) (Supplementary Fig. 2).

Moreover, the analysis of the naïve treating medium revealed the presence of two other factors involved in cartilage protection, namely OPG and DKK-1 (Table 1). MMP-1, MMP-3 and MMP-10 were also detected, even though their activity was probably inactivated by TIMPs

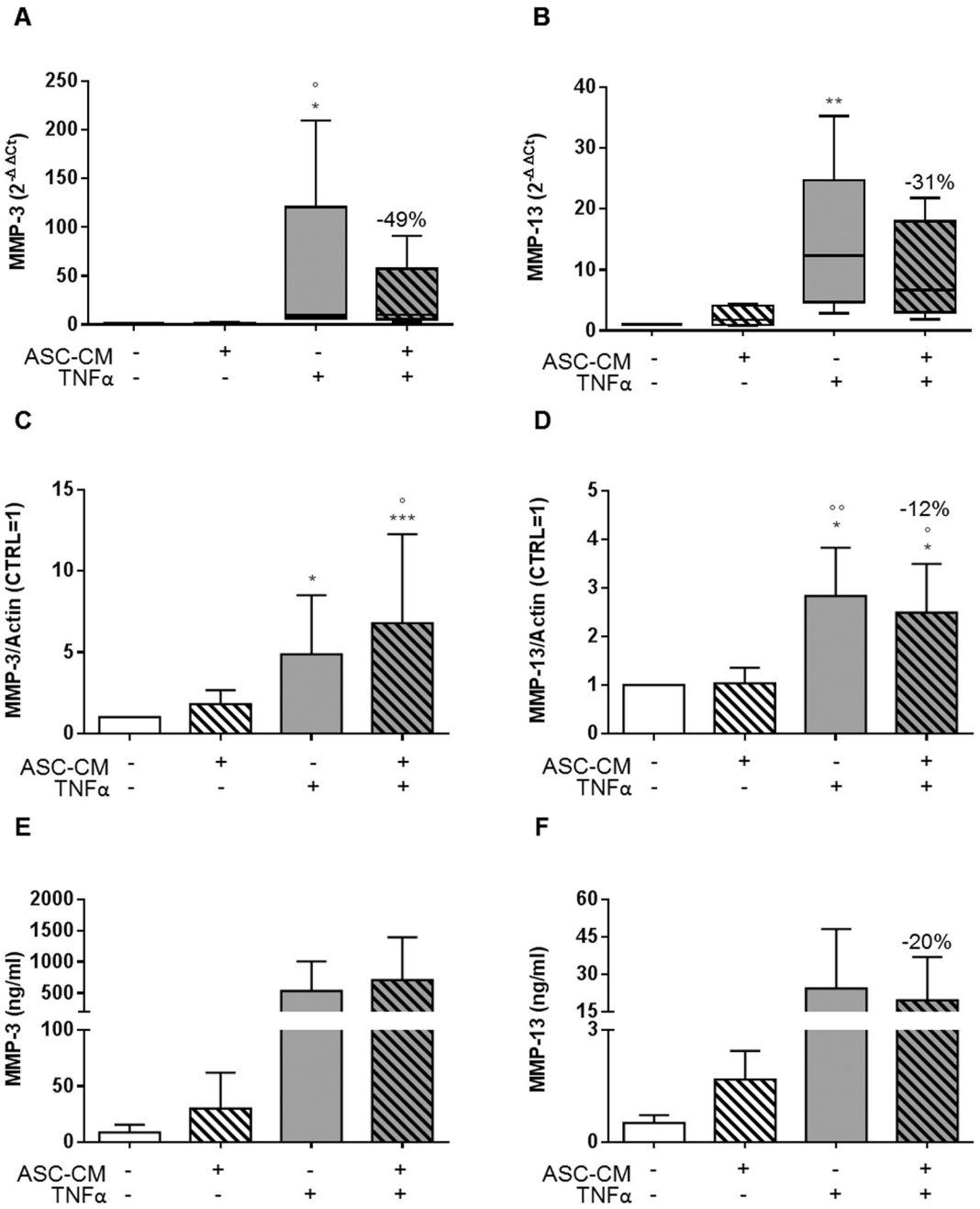

Fig. 2. Gene, protein expression and extracellular levels of MMP-3 and MMP-13 by ASC-CM-treated and/or TNFα-stimulated chondrocytes. (A-B) MMP-3 (A) and MMP-13 (B) mRNA expression at day 1 measured by real-time PCR. Data are expressed as $2^{-\Delta\Delta Ct}$ (TBP was used as housekeeping gene). Data were analysed by Friedman's test followed by Dunn's test. (C-D) MMP-3 (C) and MMP-13 (D) protein expression measured at day 3 by Western Blot. Data ($n = 7$) were normalized on β-Actin, expressed as relative values (CTRL = 1) and analysed using one-way ANOVA followed by Tukey's test. (E-F) MMP-3 (E) and MMP-13 (F) levels measured by Luminex Multiplex Assay (n = 9 CHs, 3 pools). Significance vs CTRL is shown as *p < .05 and ***p < .001, vs CM as °p < .05.

(e.g. MMP-3 in Fig. 1E).

4. Discussion

The complex nature of osteoarthritis might demand a multifactorial treatment. Adipose-derived Stromal Cell secretome is a mixture of soluble (proteins, lipids and nucleic acids) and vesicular elements, representing the entire regenerative milieu of its cell source. However, in the OA context, despite the chondroprotective action of ASCs has been described in vitro (Manferdini et al., 2013; Maumus et al., 2013; Tofino-Vian et al., 2018), in vivo (Choi et al., 2018; Desando et al., 2013) and in clinical trials (Lopa et al., 2018), there is no consensus yet on the efficacy of cell secretome (Manferdini et al., 2015; Platas et al., 2013). The studies of Platas (Platas et al., 2013), Manferdini (Manferdini et al., 2015) and Tofino-Vian (Tofino-Vian et al., 2018) suggest that ASC-CM action depends on the "activated" status of recipient. Our data confirm this hypothesis, as in our hands ASC-CM acts on TNFα-stimulated articular chondrocytes only. Moreover, the reduction of OA-related factors did not correlate with a decrease in cell metabolism nor proliferation, providing further evidence of the specificity of ASC-CM effects. One of the most promising results is the blunting of the TNFα-mediated hypertrophic shift. The reduction in MMP-13 activity is particularly interesting. Indeed, in vivo evidences on MMP-13-deficient or -depleted mice demonstrate that the action of this hypertrophy-

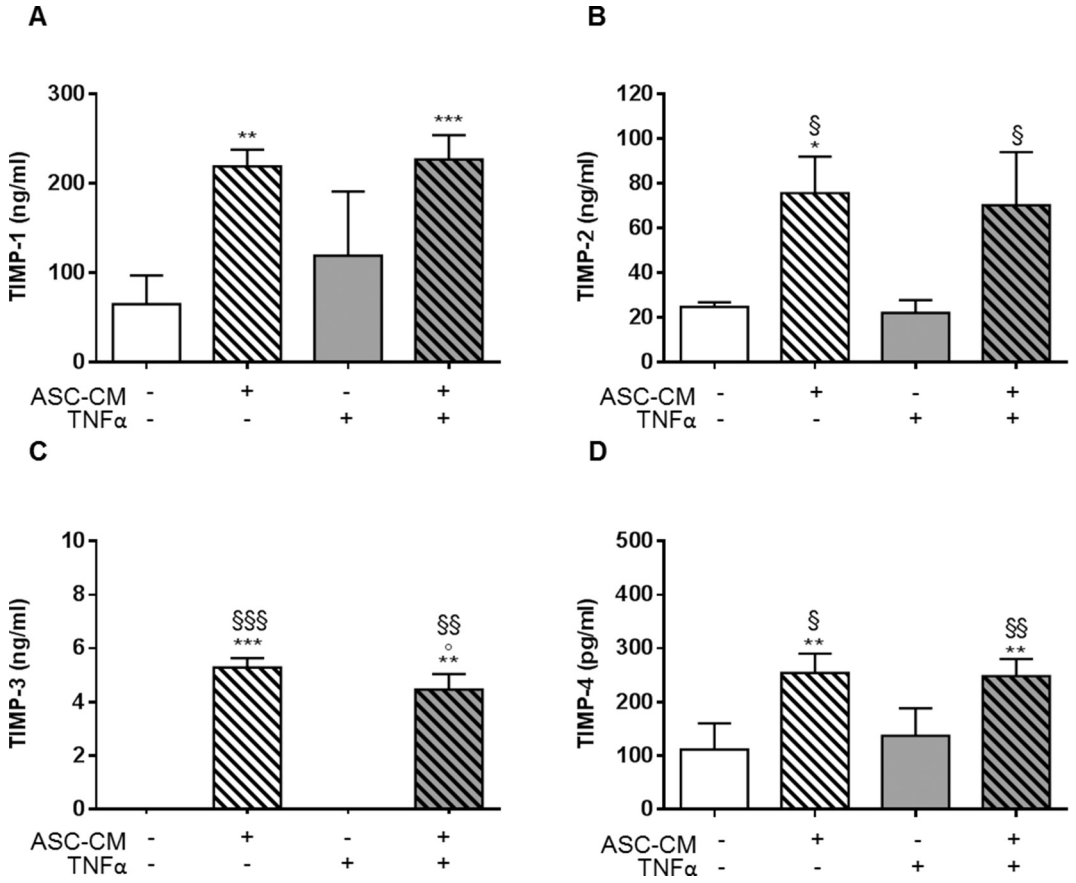

Fig. 3. TIMP levels in ASC-CM-treated and/or TNFα-stimulated chondrocyte culture media. (A-D) TIMP-1 (A), TIMP-2 (B), TIMP-3 (C) and TIMP-4 (D) levels measured by Luminex Multiplex Assay (n = 10 CHs, 3 pools and 1 single population). Data were analysed by one-way ANOVA followed by Tukey's multiple comparison test. Significance vs CTRL is shown as *p < .05, **p < .01 and *** < 0.001, vs CM as °p < .05 and vs TNFα as § p < .05, §§ p < .01 and §§§ p < .001.

Table 1
Concentrations of factors in the treating medium containing ASC-CM. Protein levels were measured by Luminex Multiplex Assay.

	[] of factors in the treating medium containing ASC-CM (pg/ml)
TIMP-1	2.6×10^5
TIMP-2	2.7×10^4
TIMP-3	3.8×10^3
TIMP-4	166
OC	nd
OPG	$1.1–1.9 \times 10^3$
DKK-1	$1.8–2.1 \times 10^3$
MMP-1	$2.1–2.6 \times 10^3$
MMP-3	$1.9–2.1 \times 10^3$
MMP-9	nd
MMP-10	17.7–20.3
MMP-12	0–56
MMP-13	nd

associated metalloprotease plays a key role in cartilage erosion during OA onset (Little et al., 2009; Wang et al., 2013). Here, we show that the ASC-CM-mediated reduction of MMP-13 activity, as well as MMP-3 one, depends only partially by the modulation of their expression. Indeed, the downmodulation of *MMP-3* and *MMP-13* transcription, the latter fully in agreement with what observed by Tofino-Vian (Tofino-Vian et al., 2018), was evident until day 1 only. When we investigated MMP-13 mRNA levels at subsequent time points (48 and 72 h,-data not shown-), no clear-cut regulation emerged. A possible explanation is that the effectors of *MMP-13* downmodulation could be active only in a short time period. As example, several miRNAs are among the major players involved in MMP-13 inhibition (Li et al., 2017). In this perspective, the short-lasting effect of CM could be ascribed to miRNA limited half-life, usually considered < 24 h. Regardless mRNA, also intracellular and secreted MMP-13 levels were decreased to a minor extent compared to the enzymatic activity. Moreover, the influence of ASC secretome on MMP-3 protein expression is not consistent with what observed at mRNA level. Even though this discrepancy was not expected, a lack of correlation between MMP mRNA and protein levels has been described before (Lichtinghagen et al., 2002). We hypothesize that CM reduces both MMP-3 mRNA transcription and its degradation rate, favouring the translation of MMP-3 and increasing the protein levels. These aspects might demand further investigations. However, in our setting, the robust inhibition of MMP-3 and MMP-13 activity is mainly due to the presence of active TIMPs. Besides MMP-3 and MMP-13, ASC-CM reduced also the activity of the collagenase MMP-1, the metallopeptidase MMP-12 and MMP-9/10, both Aggrecan-degrading enzymes (preliminary data not shown). The presence of these inhibitors in the secretome of MSCs is particularly interesting considering the relevance of developing clinical grade MMP inhibitors (Liu and Khalil, 2017). Of note, TIMPs in ASC secretome did not alter the physiologic MMP activity but buffered the TNFα-induced one. In the OA context, it is noteworthy that TIMPs are known to inhibit other metalloproteinases, namely ADAM-10 (a disintegrin and metalloproteinase-10), ADAM-12, ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motif) and ADAMTS-5 (Liu and Khalil, 2017; Yang et al., 2017). Moreover, TIMP-1 has been implicated in the reduction of angiogenesis mediated by MSC secretome (Zanotti et al., 2016). Consequently, ASC-CM could also play a role in re-establishing the anti-angiogenic environment of a healthy cartilage.

Besides TIMPs, other cartilage-protecting factors, namely OPG and DKK-1, were detected in ASC-CM (Table 1). OPG is known to prevent cartilage degradation by inhibiting proteoglycan loss and chondrocyte apoptosis (Feng et al., 2015; Kadri et al., 2008) while DKK-1 acts mainly by preserving CH phenotype, counteracting hypertrophy (Zhong et al., 2016) and inhibiting the expression of catabolic factors (Oh et al., 2012). Of note, we have recently shown that DKK-1 is one of the 34 proteins more abundantly secreted by ASCs compared with dermal fibroblast (Niada et al., 2018).

In the last few years, the first in vivo evidences of the beneficial effect of MSC-secretome administration in pre-clinical OA models have been produced (Cosenza et al., 2017; Khatab et al., 2018; Tao et al., 2017; Toh et al., 2017; Wang et al., 2017; Zhang et al., 2018; Zhu et al., 2017). Even though many of these experimental plans rely on the administration of the purified vesicular component, Khatab et al. showed the effects of the whole secretome (Khatab et al., 2018) on pain reduction and arrest of cartilage damage in a murine collagenase OA model. The rationale of their choice relies on the lack of current knowledge on which component plays a major role. We chose to use a cell product containing both freely dissolved factors and vesicular components (Supplementary Fig. 3) for the same reason. Moreover, in the OA context, the use of selected CM subcomponents may lead to a diminished efficacy of the treatment. In fact, many factors released by ASCs that can be therapeutically exploited are both conveyed in vesicles and released as soluble mediators. TIMPs have been described both in the whole secretome (Niada et al., 2018)(Egashira et al., 2012; Kono et al., 2014; Maffioli et al., 2017) and in the vesicular elements (exosomes or microvesicles) (Haraszti et al., 2016). Similarly, DKK-1, HGF, recognized as a mediator of ASC anti-fibrotic effect (Maumus et al., 2013), and Prostaglandin E2, an immunosuppressive factor acting also on chondrocyte hypertrophic shift (Li et al., 2004; Manferdini et al., 2013), have been identified both in whole ASC-CM (Manferdini et al., 2013; Maumus et al., 2013) and in vesicles (vesiclepedia (Pathan et al., 2019)). Therefore, the use of secretome fractions would subtract effectors at the additional cost of increasing manipulations. This is particularly risky, also considering how different isolation methods can alter EV content (Gualerzi et al., 2019).

In conclusion, ASC-CM might constitute a novel tool to counteract OA development. It inhibits the aberrant activity of MMPs and blunts the hypertrophic changes induced by the inflammatory cytokine TNFα (Graphical Abstract). This complete cell product can be easily obtained, prepared in advance and stored. Therefore it constitutes a ready-to-use product. Both the soluble factors and the extracellular vesicles released by ASCs may be responsible of CM beneficial action, including its well-known anti-inflammatory properties. Further investigations should aim at disclosing all the components of the secretome that are involved in its therapeutic role in the perspective of a future clinical setting.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101463.

Disclosure of potential conflicts of interest

The authors declare that they have no competing interests.

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