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Original Paper

Synergistic Effects of a Mixture of Glycosaminoglycans to Inhibit Adipogenesis and Enhance Chondrocyte Features in Multipotent Cells

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Key Words
Glycosaminoglycans • Adipogenesis • Chondrogenesis • Osteoarthritis • Joint health • Obesity

Background/Aims: Multipotent mesenchymal stem cells affect homeostasis of adipose and joint tissues. Factors influencing their differentiation fate are of interest for both obesity and joint problems. We studied the impact of a mixture of glycosaminoglycans (GAGs) (hyaluronic acid: dermatan sulfate 1:0.25, w/w) used in an oral supplement for joint discomfort (Oralvisc™) on the differentiation fate of multipotent cells. ***Methods:*** Primary mouse embryo fibroblasts (MEFs) were used as a model system. Post-confluent monolayer MEF cultures non-stimulated or hormonally stimulated to adipogenesis were chronically exposed to the GAGs mixture, its individual components or vehicle. The appearance of lipid laden cells, lipid accumulation and expression of selected genes at the mRNA and protein level was assessed. ***Results:*** Exposure to the GAGs mixture synergistically suppressed spontaneous adipogenesis and induced the expression of cartilage extracellular matrix proteins, aggrecan core protein, decorin and cartilage oligomeric matrix protein. Hormonally-induced adipogenesis in the presence of the GAGs mixture resulted in decreased adipogenic differentiation, down-regulation of adipogenic/lipogenic factors and genes for insulin resistance-related adipokines (resistin and retinol binding protein 4), and up-regulation of oxidative metabolism-related genes. Adipogenesis in the presence of dermatan sulfate, the minor component of the mixture, was not impaired but resulted in smaller lipid droplets and the induction of a more complete brown adipocyte-related transcriptional program in the cells in the adipose state. ***Conclusions:*** The Oralvisc™ GAGs mixture can tip the adipogenic/chondrogenic fate balance of multipotent cells away from adipogenesis while favoring chondrocyte related gene expression. The mixture and its dermatan sulfate component also have modulatory effects of interest on hormonally-induced adipogenesis and on metabolic and secretory capabilities of adipose cells.

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Introduction

Obesity and degenerative joint disease (osteoarthritis) are multifactorial pathologies that are becoming major public health issues with the aging of the world population. The relationship between obesity and osteoarthritis, which often appear together, extends beyond biomechanical basis to also include metabolic and developmental links (reviewed in [1]). Dysregulated production of adipokines and inflammatory mediators, hyperlipidemia and increased systemic oxidative stress are, for instance, conditions frequently associated with obesity that may favor joint degeneration [1, 2]. There is interest in agents and interventions capable of simultaneously tackling the two conditions.

Mesenchymal stem cells (MSCs) play a role in the homeostasis and metabolic fate of adipose and joint tissues and provide potential for intervention in both obesity and degenerative joint diseases [3-5]. MSCs are multipotent stromal cells capable of self-replenishment and differentiation into multiple cell types, including chondrocytes, osteoblasts and adipocytes. MSCs are mainly found in the bone marrow, from which they can be mobilized to home to tissues, and in low numbers in almost all adult tissues including joint tissues (synovium, periosteum, perichondrium and cartilage) and adipose tissues. MSCs are viewed as a promising tool for cell therapy of musculoskeletal disorders, including cartilage defects [5]. In white adipose tissue (WAT), MSCs are involved in the dynamics of white and inducible brown (i.e. brite or beige) adipocytes and in hyperplasic expansion under conditions of positive energy balance, making them possible therapeutic targets in obesity and related metabolic derangements [3, 6]. Thus, factors influencing the differentiation fate of MSCs are of interest in regard to joint health and obesity, among other aspects.

Glycosaminoglycans (GAGs) are a special group of carbohydrates with chondroprotective effects [7]. Chemically, GAGs are high molecular weight polymers formed by a repeating disaccharide unit composed of N-acetyl-glucosamine or N-acetyl-galactosamine and an uronic acid. Hyaluronic acid (HA) is an unbranched GAG polymer of N-acetyl-glucosamine and glucuronic acid disaccharide units; it is present in the extracellular matrix (ECM) of practically all tissues and one of the main components of cartilage, the synovial membrane and synovial fluid. HA is used in the treatment of joint dysfunctions, generally by intra-articular route, although beneficial effects of HA and HA-rich mixtures/extracts following oral administration have also been demonstrated [8-11]. Dermatan sulfate (DS) is a sulfated GAG abundant in the skin composed of sulfated N-acetyl-galactosamine and iduronic acid disaccharide units.

Based on previous reports that chondrocyte differentiation can be potentiated in HA-rich three-dimensional microenvironments [12, 13] and that differentiation into different cell lineages is often competitively balanced [14-16], we hypothesized that exposure to GAGs could affect the adipogenic/chondrogenic fate balance of multipotent cells. We tested this hypothesis by assessing the effects of a mixture of HA and DS (1:0.25, w/w) that is used in an oral supplement for the management of joint discomfort [17] (Oralvisc™) on primary mouse embryo fibroblasts (MEFs). MEFs have an even greater pluripotency than MSCs [18]. We show that in this cell model the GAGs mixture (a) prevents spontaneous adipose formation while promoting the expression of typical cartilage-enriched ECM proteins, and (b) regulates adipogenesis and the expression of metabolic and adipokine genes of physiopathological significance.

Materials and Methods

MEFs isolation and culture

MEFs were isolated from mouse embryos derived from dams at day 13.5 of gestation. Briefly, head, liver and other organs were removed and the remaining carcass was minced and incubated at 37°C for one hour with collagenase. After that, fragment embryos were passed over a syringe (20G) in order to obtain single cells. Collagenase was inactivated by addition of Dulbecco's modified Eagle's medium (DMEM)

supplemented with 10% fetal calf serum (FCS) and antibiotics. The outgrowing primary cell population was passaged by trypsinization at a ratio of 1:3 to 1:4 upon confluency. All experiments were carried out using cells between passage 3 and 5. Cell culture reagents were from Invitrogen (Carlsbad, CA, USA). MEFs were routinely cultured in monolayer in AmnioMAX-C100 basal medium supplemented with 7.5% AmnioMAX-C100 supplement, 7.5% FCS, 50 IU/mL penicillin, 50 µg/mL streptomycin, and 2 mmol/L-glutamine (growth medium), in 12-well culture plates of 1 mL capacity/well, essentially as previously described [19]. Two days after reaching confluence (considered day 0 of culture), the cells were exposed to vehicle (water), HA (Sigma-Aldrich, Madrid, Spain), DS (Sigma-Aldrich), or the HA:DS 1:0.25 (w/w) GAGs mixture at the concentrations indicated in the figure legends. In some experiments, cells exposed to human recombinant bone morphogenic protein 2 (BMP2) (Sigma-Aldrich) were included. The culture medium in contact with the cells was changed every two days with fresh growth medium containing the corresponding treatments. Cells were collected on days 3, 8 and 13 of culture, as indicated in the figure legends. Spontaneous adipogenesis was monitored by morphological examination of the cells for lipid accumulation using phase-contrast microscopy.

Adipogenic induction

For hormonally-induced adipocyte differentiation, 2-day post-confluent MEFs were exposed on day 0 of culture to growth medium containing a standard adipogenic cocktail (1 µmol/L dexamethasone, 0.5 mmol/L methylisobutylxanthine, 5 µg/mL insulin and 0.5 µmol/l rosiglitazone) for 48 h [19]. The cells were subsequently refed every other day with fresh medium containing 5 µg/mL insulin and 0.5 µmol/L rosiglitazone (adipogenic medium). From day 6 of culture onwards, cells were deprived of insulin and rosiglitazone. Adipogenic differentiation as described above was assayed in the continuous presence, from day 0 of culture, of vehicle (control cultures) or HA, DS, or the HA+DS mixture, at the concentrations indicated in the figure legends. Cells were harvested at day 8-9, when more than 90% of the cells in control cultures had acquired the adipocyte phenotype, as assessed by phase-contrast microscopy examination.

Oil Red O Staining

Differentiated adipocytes were fixed in 4% paraformaldehyde for 30 min at room temperature, washed with PBS, rinsed with isopropanol and stained with 0.2% Oil red O (Sigma, St. Louis, Mo., USA) in 60% isopropanol for 1 hour. The intracytoplasmatic dye extracted with isopropanol was quantified as described previously [20].

RNA isolation and analysis

Total RNA was extracted from monolayer cultures using Trizol reagent (Invitrogen) E.Z.N.A.® Total RNA Kit I (Omega Bio-Tek, Norcross, GA, USA) or NucleoSpin TriPrep kit (Macherey-Nagel, Dueren, Germany), according to the supplier's instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies Inc Wilmington, DE, USA) and its integrity confirmed using agarose gel electrophoresis. A_{260}/A_{280} ratios were between 1.9 and 2 for all samples, indicating good purity. Retrotranscription followed by real-time polymerase chain reaction (RT-qPCR) was used to measure the mRNA expression levels of: chondrogenesis related genes: aggrecan protein (Acan); cartilage oligomeric matrix protein (Comp); collagen type II, alpha 1 (Col2a1); collagen type X, alpha 1 (Col10a1); delta-like 1 homolog (Dlk1, also known as preadipocyte factor 1, Pref-1); decorin (Dcn); proline arginine-rich end leucine-rich repeat protein (Prelp); Sry-related high-mobility-group box 9 (Sox9); adipogenesis and lipogenesis related genes: CCAAT/enhancer binding protein alpha (Cebpa); fatty acid synthase (Fasn); peroxisome proliferator-activated receptor gamma (Pparg); genes related to oxidative metabolism and thermogenesis: carnitine palmitoyltransferase 1b, muscle (Cpt1b); cytochrome c oxidase II mitochondrial (mt-Co2); cytochrome c oxidase subunit Va (Cox5a); nuclear respiratory factor 1 (Nrf1); peroxisome proliferator-activated receptor alpha (Ppara); PPARγ coactivator 1 alpha (Ppargc1a); PPARγ coactivator 1 beta (Ppargc1b); PR domain containing 16 (Prdm16); uncoupling protein 1 (Ucp1); and adipokines: adiponectin (Adipoq); leptin (Lep); resistin (Retn); retinol binding protein 4 (Rbp4). β-actin was used as internal control. In brief, 0.25 µg of total RNA (in a final volume of 5 µL) was denatured at 65°C for 10 min and then reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystems, Madrid, Spain) at 20°C for 15 min, 42°C for 30 min, with a final step of 5 min at 95°C in an Applied Biosystem 2720 Thermal Cycler. Sense and antisense primers used in the qPCR reactions were designed with specific primer analysis

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Table 1. Genes analysed and primers used in the qPCR reactions

Gene ID	Name	Description	Primer forward (5'-3')	Primer reverse (5'-3')	Product size (bp)
11595	Acan	aggrecan	CACGCTACACCCTGGACTTTG	CCATCTCCTCAGCGAAGCAGT	270
11461	Actb	actin beta	TACAGCTTCACCACCACAGC	TCTCCAGGGAGGAAGAGGAT	120
11450	Adipoq	adiponectin	TCTCACCCTTAGGACCAAG	GCTCAGGATGCTACTGTTG	255
12606	Cebpa	CCAAT/enhancer binding protein alpha	AGGTGCTGGAGTTGACCAGT	CAGCCTAGAGATCCAGCGAC	233
12813	Col10a1	collagen, type X, alpha 1	GCCTCAAATACCCTTTCTGCTG	TGCCTTGTTCTCCTCTTACTGG	176
12824	Col2a1	collagen, type II, alpha 1	GCTCATCGCCGCGGTCCTAC	CTGGCAGTGGCGAGGTCAGC	243
12845	Comp	cartilage oligomeric matrix protein	CCACAGATGCTGCGAGAACT	TCCGCAAGCATCACATTCCA	126
12858	Cox5a	cytochrome c oxidase subunit Va	TTGATGCCTGGGAATTGCGTAAAG	AACAACCTCCAAGATGCGAACAG	143
12895	Cpt1b	carnitine palmitoyltransferase 1b, muscle	AAGGGTAGAGTGGGCAGAGG	GCAGGAGATAAGGGTGAAAGA	223
13179	Dcn	decorin	TCGAGTGGTGCAGTGTTCTG	TAGCAAGGTTGTGTCGGGTG	73
13386	Dlk1	delta-like 1 homolog (Drosophila)	TTCTGCGAAATAGACGTTCGG	TCGTTGTCGCATGGGTTAGG	202
14104	Fasn	fatty acid synthase	TTCGGTGTATCCTGCTGTCC	TGGGCTTGTCCTGCTCTAAC	167
16846	Lep	leptin	AGACCACTCGCATTCCTTTG	TCGCACTTTGGTATTCTTGG	154
17709	mt-Co2	cytochrome c oxidase II mitochondrial	AAGACGCCACATCCCCTATT	CTTCAGTATCATTGGTGCCCT	291
18181	Nrf1	nuclear respiratory factor 1	CGAAAGAGACAGCAGACACG	TTGAAGACAGGGTTGGGTTT	125
19013	Ppara	peroxisome proliferator activated receptor alpha	AGTGCCCTGAACATCGAGTGT	AAGCCCTTACAGCCTTCACATG	89
19016	Pparg	peroxisome proliferator activated receptor gamma	AGACCACTCGCATTCCTTTG	TCGCACTTTGGTATTCTTGG	154
19017	Ppargc1a	peroxisome proliferator activated receptor gamma coactivator 1 alpha	CATTTGATGCACTGACAGATGGA	CCGTCAGGCATGGAGGAA	70
170826	Ppargc1b	peroxisome proliferator activated receptor, gamma coactivator 1 beta	TGAGGTGTTCGGTGAGATTG	CCATAGCTCAGGTGGAAGGA	165
70673	Prdm16	PR domain containing 16	ACTTTGGATGGGAGATGCTG	AGGAACACGCTACACGGATG	159
116847	Prelp	proline arginine-rich end leucine-rich repeat	GAACAGAAGAGTGCCCCAGAG	CACTGAGGCCAAGATGAGGAG	117
19662	Rbp4	retinol binding protein 4	ACTGGGGTGTAGCCTCCTTT	GGTGTCGTAGTCCGTGTCG	71
57264	Retn	resistin	TTCCTTTTCTTCCTTGTCCCTG	CTTTTTCTTCACGAATGTCCC	247
20682	Sox9	SRY (sex determining region Y)-box 9	GAGGCCACGGAACAGACTCA	CAGCGCCTTGAAGATAGCATT	51
22227	Ucp1	uncoupling protein 1	GGCATTCAGAGGCAAATCAG	GCATTGTAGGTCCCCGTGTA	239

software Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA), and their specificity analyzed by the Primer-BLAST databases tool (National Center for Biotechnology Information, Bethesda, MD, USA). The primers were produced by Sigma (Madrid, Spain), and their sequences are shown in Table 1. Each qPCR was performed from diluted (1/20) cDNA template, forward and reverse primers (0.25-1 μM each), and Power SYBER Green PCR Master Mix (Applied Biosystems). Real time PCR was performed using the Applied Biosystems StepOnePlusTM Real-Time PCR Systems (Applied Biosystems) with the following profile: 10 min at 95°C, followed by a total of 40 two- temperature cycles (15 s at 95°C and 1 min at 60°C). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle (Ct) was calculated by the instrument's software (StepOne Software v2.0) and the relative expression of each mRNA was calculated according to Pfaffl [21], using -actin as a reference gene.

Immunoblotting analysis

Total protein was isolated in parallel with DNA and RNA with NucleoSpin TriPrep kit, resuspended in protein solving buffer set PSB/TCEP and denatured as per manufacturer´s (Macherey-Nagel) protocol. Protein concentration was determined with Protein Quantification Assay from the same manufacturer. Protein (15-30 μg /lane) was loaded and separated in precast 4-15% gradient gel (Bio-Rad, Hercules, CA, USA), and transferred onto 0.2 μm nitrocellulose membrane using Trans-Blot Turbo semi-dry transfer apparatus (Bio-Rad). Membranes were blocked for 1 h at room temperature with Odyssey Blocking Buffer (Li-Cor, Lincoln, NE, USA) and incubated overnight at 4°C with gentle shaking with primary antibodies (1:1000 in Tris Buffered Saline-Tween 20, TBS-T). The following were used: anti-C/EBPα (sc-61), anti-retinoblastoma protein (Rb) (sc-74562) and anti-tubulin (sc-8035) antibodies from Santa Cruz Biotechnology (Dallas, TX, USA); and anti-FASN (#318), anti-PPARγ (#2443) and anti–phosphoSer780Rb (# 9307) antibodies from Cell Signaling (Danvers, MA, USA). Membranes were then incubated with the corresponding secondary IRDye antibodies (1:10000 in TBS-T, 1 h at room temperature) and the signal was detected by Odyssey near-infrared scanner (Li-Cor).

Immunofluorescence analysis

MEFs were grown in 12-well plates containing 10 mm glass coverslips (pre-treated with poly-L-lysine solution (Sigma) for 5min and sterilized by UV light) and subjected to treatments as indicated. Following fixation with 4% paraformaldehyde in PBS, cells were permeabilized with 0.2% (v/v) Triton X-100 in PBS for 5 min at room temperature. After washing with PBS, the coverslips were blocked in 5% normal rabbit serum (NRS) in PBS for 1 h at room temperature. Anti-agrecan and anti Col2a1-antibodies (sc-16492 and sc-7764, respectively, from Santa Cruz Biotechnology) were diluted 1:100 in 5% NRS and applied over the coverslips in a humidified chamber at 4°C overnight. The coverslips were washed, incubated with fluorescein

isothiocyanate-conjugated secondary antibody for 1 h at room temperature, washed and mounted in the presence of 1.5 μg/ml DAPI in the mounting media to stain the nuclei. Images were obtained using a Leica TCS SPE Confocal Laser System (Leica Microsystems S.L.U., Barcelona, Spain).

Statistical analysis

Data are expressed as means ± SEM. The statistical differences in mean values were assessed by Student's t test. All experiments were repeated at least twice (in triplicates or quadruplicates per each tested condition) except time-course experiments for chondrogenic induction that were performed once (in triplicates). Threshold of significance was set at $P<0.05$.

Results

The GAGs mixture inhibits spontaneous adipogenesis in MEFs

Serum included in the culture medium can induce spontaneous adipogenesis in multipotent bone marrow-derived MSCs [22]. In fact, we noticed some degree of spontaneous adipogenic differentiation upon microscopically examination of our MEFs cultures. We therefore began by examining the impact of the GAGs mixture and its individual components, HA and DS, used at the same concentration as provided by the mixture, on spontaneous adipogenesis in MEFs not exposed to any adipogenic additives. MEFs in growth medium were chronically exposed from day 2 post-confluence (day 0) to vehicle or treatments, and adipocyte appearance and the expression of adipogenic markers was assessed 8 days later. Phase contrast microscopic examination revealed by day 8 the presence of clusters of cells spontaneously differentiated into adipocytes filled with refringent fat droplets in the control cultures and the cultures exposed to 80 μg/mL (f.c.) HA or 20 μg/mL (f.c.) DS. On the contrary, lipid laden cells were absent in the cultures exposed to 100 μg/mL (f.c.) of the HA:DS (1:0.25) GAGs mixture (Fig. 1A). In good concordance with these morphological observations, at the molecular level gene expression of adipogenic transcription factors (Pparg, Cebpa) and adipocyte markers (Lep, Fasn) was dramatically reduced in the cells treated with the GAGs mixture, but not in the cells treated with the individual components of the mixture alone (Fig. 1B). Similar results were obtained in an independent experiment using a different stock of MEFs: although the degree of spontaneous adipogenic differentiation was lower in this second experiment, spontaneous adipogenesis as assessed by Oil Red staining was completely suppressed at 100 μg/mL of the GAGs mixture and to a lesser extent at 10 μg/mL, indicative of a dose-dependent effect, while the individual components of the mixture showed no or much less anti-adipogenic activity when tested alone (Fig. 1C). Taken together, these results indicate that the mixture of HA and DS synergistically inhibits spontaneous adipogenesis in MEFs.

The GAGs mixture induces chondrocyte related gene expression in MEFs

Because reciprocal control of adipogenesis and osteo-chondrogenesis of MSCs has been described [15] we next studied the impact of the mixture of GAGs on the expression of chondrogenic markers in our cell system. Previous studies have established that chondrogenic differentiation can be induced in post-confluent MEF cultures by cell-cell contact and BMP2 treatment [23, 24]. Therefore, we used BMP2 as a sort of positive control and compared the impact of the mixture of GAGs (100 μg/mL) and BMP2 (100 ng/mL) on the time-course of chondrogenesis related gene expression in post-confluent monolayer MEF cultures. The impact of HA, the major component of the GAGs mixture, at an f.c. of 100 μg/mL was also initially assessed. Gene expression of Sox9 – the "master" chondrogenic transcription factor [25] – was induced between days 3 and 13 of culture in the cells exposed to BMP2, as expected [23], and, to a lesser extent, the GAGs mixture, but not in those exposed to HA or vehicle (Fig. 2A). Gene expression of Acan, coding for aggrecan protein – the core protein of the cartilage-specific proteoglycan that is a main component of the ECM surrounding chondrocytes – was robustly induced in the cells exposed to the GAGs mixture or BMP2 (by

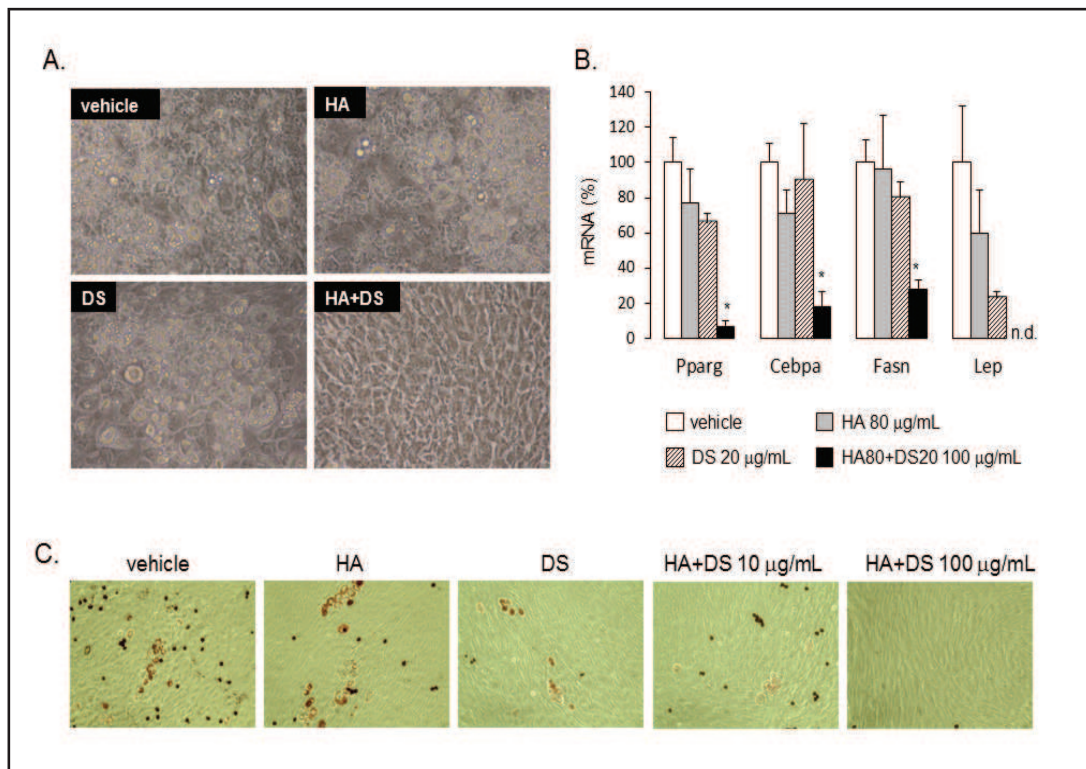

Fig. 1. The hyaluronic acid (HA): dermatan sulfate (DS) mixture (HA80+DS20) inhibits spontaneous adipogenesis in mouse embryonic fibroblasts (MEFs). (A) Phase contrast micrographs of post-confluent MEFs cultured for 8 days in growth medium containing: vehicle (water, Control); HA at a final concentration (f.c.) of 80 µg/mL; DS at an f.c. of 20 µg/mL; or the HA80+DS20 mixture at an f.c. of 100 µg/mL. Lipid-laden cells refringent under the phase contrast microscope were seen in all cultures except those treated with the HA80+DS20 mixture (x 400 magnification). (B) Expression levels of messenger RNAs for the adipogenic transcription factors PPARγ (Pparg) and C/EBPα (Cebpa) and the adipocyte marker genes leptin (Lep) and fatty acid synthase (Fasn) in MEFs cultures grown and treated as in (A). Data are expressed as percentage of the expression level in the control cells treated with vehicle (which was set at 100%), and are the mean+SEM of at least six cultures per tested condition, distributed in two independent experiments. *$P < 0.05$ *vs* vehicle as determined by Student's *t*-test. (C) Representative images of Oil Red stained post-confluent MEFs cultured for 8 days in growth medium supplemented with vehicle (water); HA at f.c. of 80 µg/mL; DS at f.c. of 20 µg/mL; or the HA80+DS20 mixture at f.c. of 10 or 100 µg/mL. (A and C) correspond to independent cultures, and note that the degree of spontaneous adipogenic differentiation was lower in C.

8- and 5-fold at day 13, respectively), but not in those exposed to HA or vehicle (Fig. 2B). Time-course of changes in Dlk1 mRNA levels was also strikingly similar in the cells exposed to the GAGs mixture or BMP2, and very different in these cells than in those exposed to HA or vehicle (Fig. 2C). The time-course found in the cells exposed to the GAGs mixture or to BMP2 – with a peak in Dlk1 expression at day 3 followed by a marked down-regulation by day 8 on, coincident with the up-regulation of aggrecan gene expression – is in keeping with previously reported changes in Dlk1 gene expression in BMP2-stimulated MEFs [24]. Interestingly, the expression of adipogenesis related genes and adipocyte markers (Pparg, Cebpa, Fasn, Lep) was dramatically reduced by days 8 and 13 of culture in the cells exposed to the GAGs mixture or BMP2 (Fig. 2D-G), coincident with the induction of the aggrecan gene and the Sox-9 gene, strongly suggesting that inhibition of spontaneous adipogenesis accompanied chondrogenic gene induction in MEFs. Col2a1 was not induced in this first experiment by any of the treatments applied, including BMP2 (not shown).

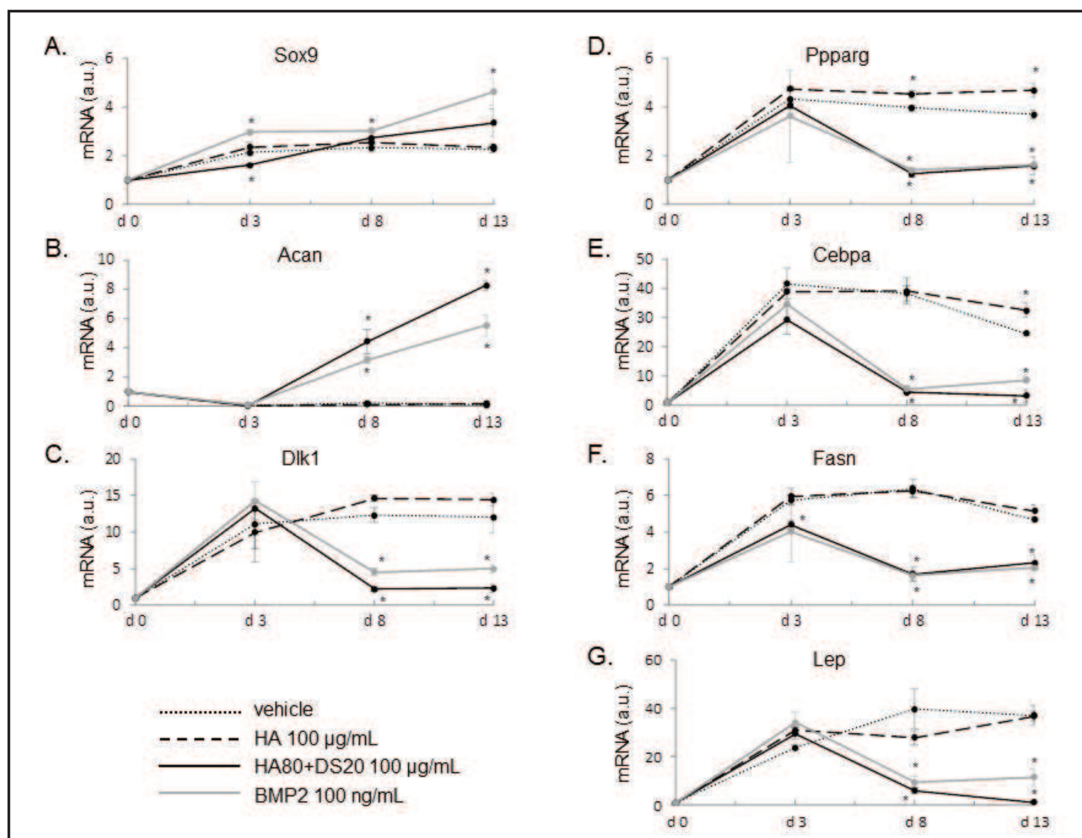

Fig. 2. Temporal changes in the mRNA expression levels of chondrogenesis and adipogenesis related genes in post-confluent mouse embryonic fibroblasts cultured in growth medium supplemented with the hyaluronic acid (HA): dermatan sulfate (DS) mixture (HA80+DS20), HA alone, bone morphogenetic protein 2 (BMP2) at the indicated final concentrations or vehicle (water). Data are the mean+SEM of three cultures per time point and treatment. *P < 0.05 *vs* the corresponding time-point vehicle as determined by Student's *t*-test.

In an independent experiment using a different batch of BMP2 and a different stock of MEFs, induction of aggrecan protein expression by the GAGs mixture (100 µg/mL) and BMP2 (100 ng/mL) was confirmed by both qPCR (Fig. 3A) and immunofluorescence analysis (Fig. 3B), while BMP2 but not the GAGs mixture effectively induced Col2a1 and Col10a1 gene expression already at day 8 (Fig. 3A; induction of Col2a1 by BMP2 was corroborated at the protein level by immunofluorescence analysis, not shown). Interestingly, besides aggrecan protein, treatment with the GAGs mixture induced gene expression by day 8 of Comp, Dcn and Prelp, which code for cartilage-enriched ECM proteins implicated in the enhancement of matrix assembly during chondrogenesis and chondrogenesis itself [26-28] (Fig. 3A). Similar to treatment with the GAGs mixture, treatment with BMP2 also resulted in the up-regulation of Comp and Prelp expression. On the opposite, when individually tested, HA (80 µg/mL) and DS (20 µg/mL) had no effect or down-regulated gene expression of Acan, Comp, Dcn and Prelp in the MEF cultures. The induction of Acan, Comp, Dcn and Prelp expression by the HA+DS GAGs mixture was dose-dependent (Fig. 4). We conclude that the mixture of HA and DS can synergistically and dose-dependently induce gene expression of cartilage related genes in MEFs.

Impact of the GAGs mixture and its individual components on hormonally-induced adipogenesis and metabolic gene expression following adipogenic stimulation of MEFs

The GAGs mixture and its individual components were next compared for their impact on hormonally-induced adipogenesis of MEFs. To this end, MEFs were induced to differentiate

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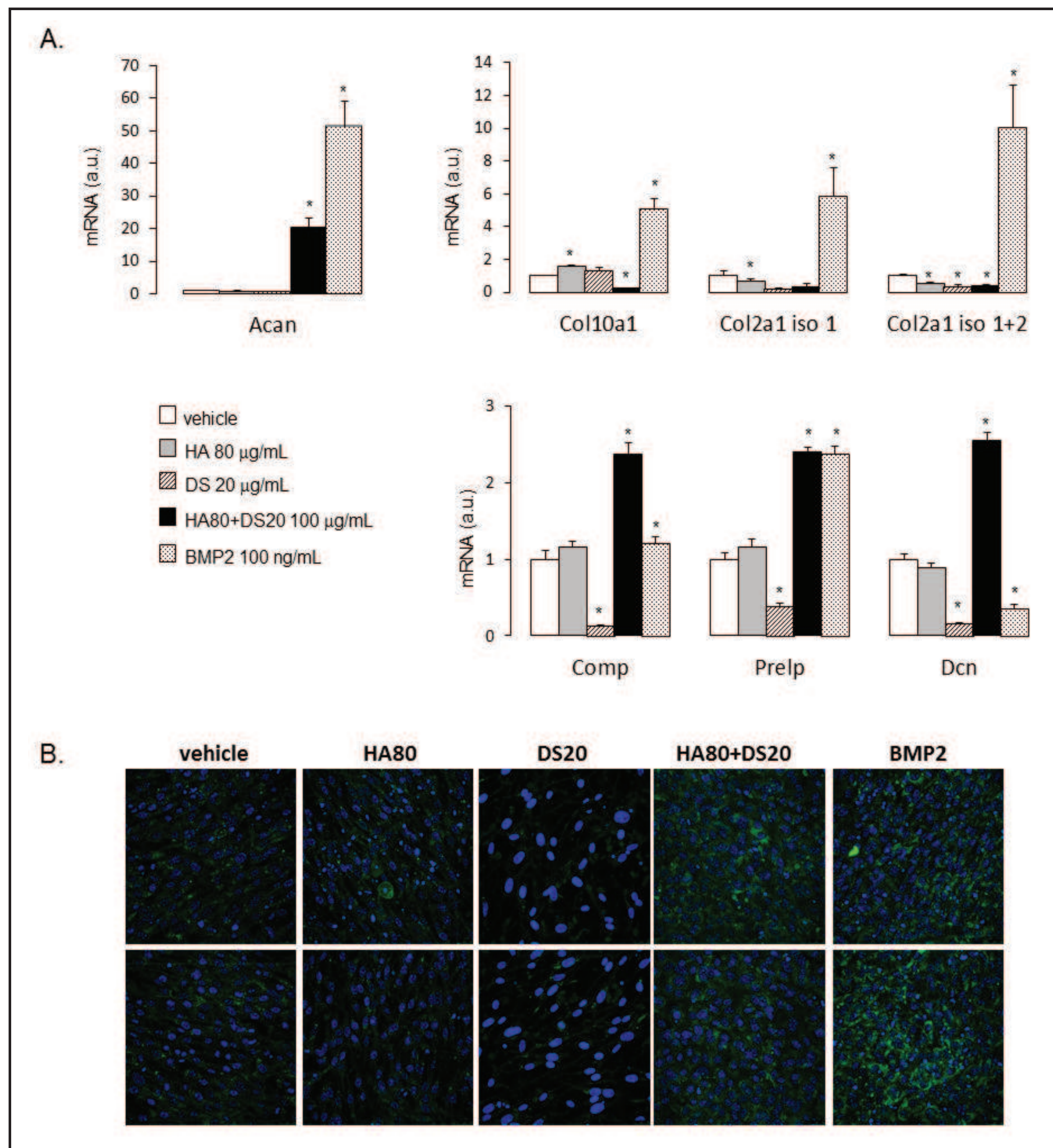

Fig. 3. (A) Gene expression of cartilage extracellular matrix enriched proteins in post-confluent mouse embryonic fibroblasts cultured for 8 days in growth medium supplemented with: vehicle, hyaluronic acid (HA), dermatan sulfate (DS), HA80+DS20 mixture or bone morphogenetic protein 2 (BMP2) at the indicated final concentrations. Data are the mean+SEM of four cultures per treatment. *P < 0.05 *vs* vehicle as determined by Student's *t*-test. (B) Immunofluorescence detection of aggrecan protein in cultures as in A) at day 13. Aggrecan protein fluorescence appears as green. Nuclei were stained with DAPI (blue).

into adipocytes using a standard adipogenic cocktail plus the PPARγ agonist rosiglitazone, in the continuous presence of the GAGs mixture (200 µg/mL), HA (160 µg/mL) or DS (40 µg/mL). Control cultures received the vehicle. After 9 days of differentiation, most (>90%) cells in the control cultures and the cultures exposed to HA or DS had differentiated into lipid-laden cells, even if lipid droplet size appeared smaller in the cultures exposed to DS (Fig. 5A). However, in the cultures exposed to the GAGs mixture lipid-laden cells were less abundant and more undifferentiated cells remained (Fig. 5A). From the quantification of the Oil Red stained material, neutral lipids accumulated per well at day 9 were estimated to be decreased by 20% in the cultures exposed to DS (P = 0.059) and by 42% (P = 0.005)

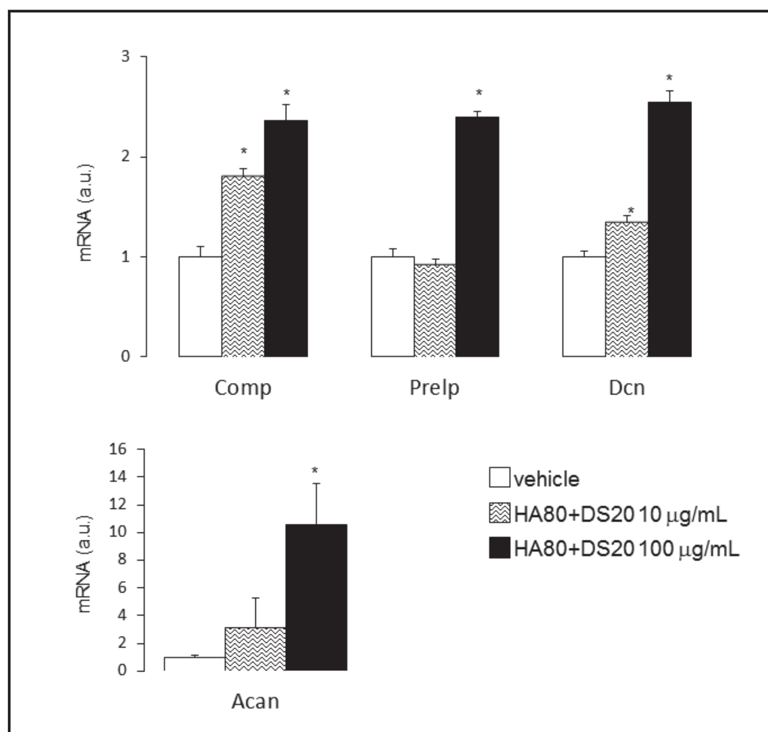

Fig. 4. Gene expression of cartilage extracellular matrix enriched proteins in post-confluent mouse embryonic fibroblasts cultured for 8 days in growth medium supplemented with vehicle (water) or the HA80+DS20 mixture at an f.c. of 10 or 100 μg/mL. Data are the mean ± SEM of four cultures per treatment. *P < 0.05 *vs* vehicle as determined by Student's *t*-test.

in those exposed to the GAGs mixture relative to the control cultures (Fig. 5A, right). Total RNA isolated from cultures at day 9 was used for analysis of genes related to adipogenesis and lipogenesis. Whereas Pparg was similarly expressed independently of treatments, the mRNA levels of Cebpa and Fasn were significantly down-regulated selectively in the cultures exposed to the GAGs mixture (Fig. 5B). Moreover, immunoblotting analysis indicated a concerted down-regulation of PPARγ, C/EBPα and FASN protein levels in the cultures exposed to the GAGs mixture only (Fig. 5B right). In keeping with these results, total levels of Rb – which in adipogenic cultures marks differentiated adipocytes [29, 30] – were lower and the ratio of inactive to active Rb protein (phosphoRb/Rb) higher in the cultures exposed to the GAGs mixture, whereas these two parameters were unchanged in the cultures exposed to HA or DS alone (Fig. 5C).

We looked at the impact of treatments on the expression of genes related to brown adipocytes/oxidative metabolism, since induction of these genes in white adipocytes might be of interest in the context of anti-obesity strategies [31, 32]. Genes of this type analyzed in MEFs following 9 days of adipogenic stimulation included: Prdm16, Ppargc1a, Ppargc1b, Ppara, Nrf1, Cpt1b, Ucp1, mt-Co2 and Cox5a (Fig. 6A). Whereas exposure of the differentiating cells to HA (160 μg/mL) did not affect the expression of any of these genes, exposure to DS (40 μg/mL) resulted in the concerted up-regulation of many of them, namely: Ppargc1b, Cpt1b, Ucp1 and, albeit non-significantly, Ppargc1a (P = 0.067), Ppara (P = 0.098) and Cox5a (P = 0.068). Exposure of the differentiating cells to the GAGs mixture (200 μg/mL) led to a significant up-regulation of the expression of mt-Co2 and a trend towards increased expression of Ppargc1a (P = 0.153), with no noticeable effect on the expression of the other genes in this category assayed.

Adipose differentiation in the presence of the GAGs mixture affects adipokine expression

The many established connections of adipokines with both joint health and obesity-associated metabolic disorders [1] prompted us to analyze the impact of the treatments on the expression of genes coding for relevant adipokines in this context, namely leptin, adiponectin, resistin and RBP4 (Fig. 6B). Adipogenic differentiation in the presence of the GAGs mixture (200 μg/mL) did not affect expression of Lep and Adipoq genes and led

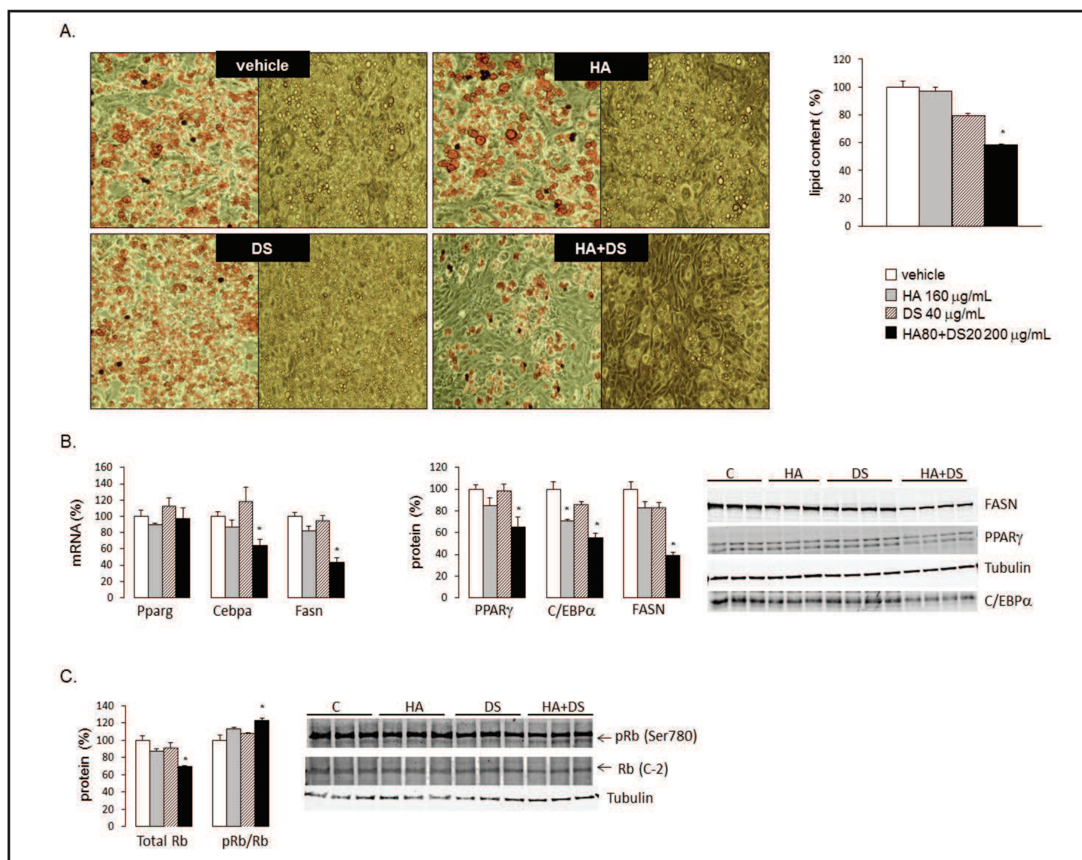

Fig. 5. (A) Representative images of Oil Red stained mouse embryonic fibroblasts following 9 days of hormonally-induced adipogenic differentiation in the continuos presence of vehicle (water), hyaluronic acid (HA), dermatan sulfate (DS) or the HA80+DS20 mixture at the indicated final concentrations. Phase contrast micrographs are also shown (magnification x 400 for both images). The bar diagram corresponds to the quantification of the Oil Red stained lipids eluted per well (n=3 per treatment condition). (B) Expression of mRNA and protein levels of adipogenic/lipogenic genes in the above described cultures. mRNA data are the mean+SEM of at least six cultures per tested condition distributed in two independent experiments. Protein data are the mean+SEM of 3-4 cultures per tested condition. Immunoblots for the adipogenic/lipogenic proteins and tubulin used as internal control are shown on the right (15 µg protein per lane). (C) Total Rb protein and ^{pSer780}Rb-to-total Rb protein ratio in the above described cultures. Data are the mean+SEM of 3 cultures per tested condition. Immunoblots are shown on the right (30 µg protein per lane). In A to C, *P < 0.05 *vs* vehicle, Student's *t*-test.

to reduced expression of Retn and Rbp4. The latter effects were not reproduced by the individual components of the mixture, HA (160 µg/mL) and DS (40 µg/mL), which did not significantly affect the expression of the adipokine genes tested. Resistin and RBP4 have been implicated as insulin resistance and pro-inflammatory factors often elevated in obesity and in the osteoarthritic joint (reviewed in [1, 2]).

Discussion

MEFs have the capacity to differentiate into different cell types [18]. A main finding in this work is the demonstration of the potential of a HA:DS (1:0.25, w/w) GAGs mixture to synergistically inhibit spontaneous adipogenesis while enhancing spontaneous chondrocyte related gene expression in MEFs. The appearance of lipid-laden cells and the expression of

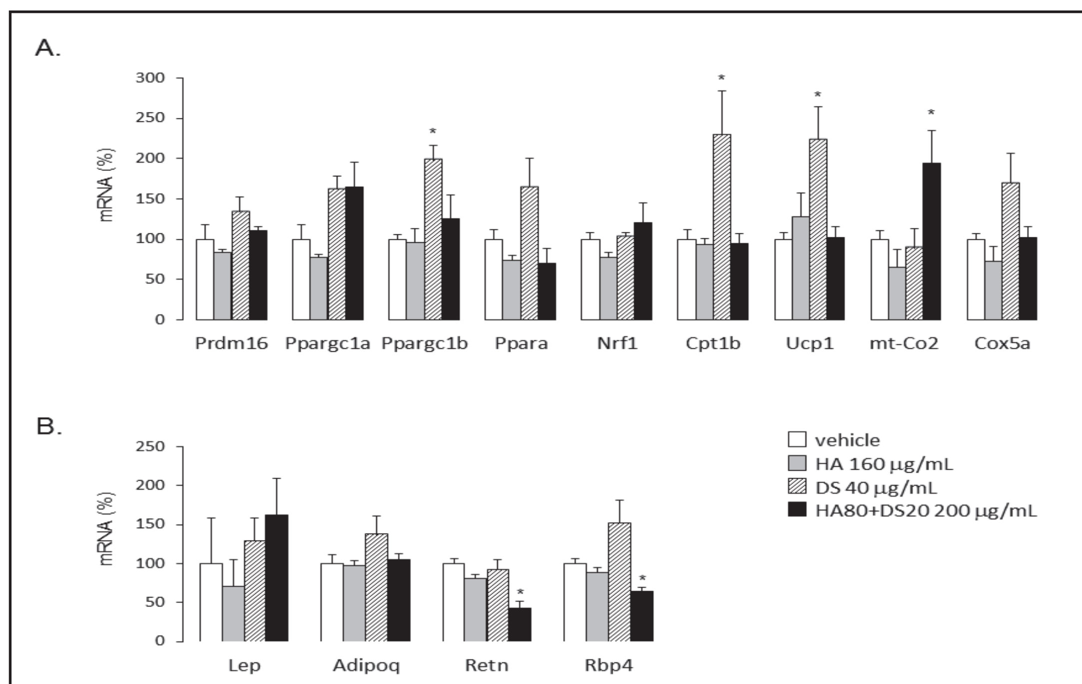

Fig. 6. Expression levels of mRNAs for selected genes related to mitochondrial oxidative metabolism/thermogenesis/brown adipocyte determination (A) and for adipokines (B) in mouse embryonic fibroblasts following 9 days of hormonally-induced adipogenic differentiation in the continuos presence of vehicle (water), hyaluronic acid (HA), dermatan sulfate (DS) or the HA80+DS20 mixture at the indicated final concentrations. Data are expressed as percentage of the expression level in the control cells treated with vehicle and are the mean+SEM of at least six cultures per tested condition, distributed in two independent experiments. *P < 0.05 *vs* vehicle as determined by Student's *t*-test.

typical adipogenic genes (Pparg, Cebpa, Fasn, Lep) were decreased in the cultures exposed to the GAGs mixture at the same time that expression of aggrecan and genes for other cartilage ECM enriched proteins (Comp, Dcn, Prelp) was induced. Moreover, the GAGs mixture triggered changes in gene expression similar to those triggered by BMP2, a known inductor of chondrogenesis in MEFs [23, 24], other multipotent cells [33] and the developing embryo [34]. These changes included an early transient induction of Dlk1 which is in keeping with proposed roles of DLK1 in chondrogenesis, as this protein promotes early commitment of MSCs into the chondrocyte lineage but prevents chondrocyte maturation, so that Dlk1 downregulation is required for later stages in the process, including Acan gene expression [15]. Overall, these results might be in line with the concept that differentiation of multipotent cells is competitively balanced in that mechanisms that promote one cell fate actively suppress mechanisms that induce alternative lineages [14, 15]. Even though only BMP2 effectively induced Sox9 and collagen genes, indicating that the pro-chondrogenic drive provided by the GAGs mixture is at most partial, results herein are remarkable. Aggrecan core protein, COMP and decorin are defectively expressed or fragmented in the altered (osteoarthritic) joint [26, 35, 36]. Evidence indicates that COMP and decorin can facilitate matrix assembly through interaction with major structural cartilage ECM components, such as aggrecan and collagens, and chondrogenesis itself, through interaction with growth factors [26, 27]. Therefore, upregulation of these proteins is of potential interest for joint health.

The GAGs mixture inhibited adipogenic differentiation of MEFs even when the cells were submitted to a strong adipogenic drive (a standard hormonal adipogenic cocktail plus rosiglitazone as a PPARγ activating ligand), as evidenced by decreased abundance of lipid-laden cells and of lipid accumulated per well by day 9 of differentiation. Accordingly, protein expression levels of PPARγ, C/EBPα and FASN were decreased in the adipogenic cultures

exposed to the GAGs mixture. PPARγ is the master regulator of adipogenesis and it is also required to maintain the adipocyte phenotype and for high fat diet-induced adipocyte hypertrophy [37]. C/EBPα is a PPARγ target gene and a late adipogenic transcription factor critically involved in lipogenesis in adipocytes through the transcriptional induction of FASN, which is a key lipogenic enzyme for the de novo fatty acid synthesis [38]. Again, effects of the GAGs mixture on these adipogenesis and lipogenesis related genes were not reproduced by the mixture individual components: a decrease in neutral lipid accumulation in the cultures exposed to DS was independent of PPARγ, C/EBPα or FASN down-regulation and apparently not due to decreased number of lipid-laden cells, but to a reduction in lipid droplet size, while most of these parameters were unaffected in the cultures exposed to HA.

Increasing mitochondrial oxidative metabolism in WAT, per se or as part of a more complete program of WAT browning (i.e., the acquisition of brown adipocyte features in white fat depots) has been suggested might be key to a lean phenotype [31, 32]. Obese and insulin-resistant rodents and humans show decreased mitochondrial density and function in white adipose tissue [39]. Factors including food ingredients and nutrient derivatives targeting mitochondrial activity in white adipocytes or white to brown adipocyte remodeling are of potential interest for obesity management [32, 40-43]. Together with reduced lipogenic capacity, MEFs stimulated for adipogenesis in the presence of the GAGs mixture had by day 9 higher mt-Co2 expression – coding for a mitochondrially encoded component of the respiratory chain – and a trend to increased expression of Ppargc1a – coding for PGC1α, an important transcriptional coactivator for mitochondrial biogenesis and function – suggestive of increased capacity for mitochondrial oxidative metabolism. However other related genes assayed were unaffected and the functional relevance of these changes remains to be established. Remarkably, adipose differentiation in the presence of DS, the minor component of the GAGs mixture, resulted in a robust, concerted induction at the transcriptional level of genes related to mitochondrial biogenesis and function (Ppargc1a, Ppargc1b, Cox5a), fatty acid oxidation (Cpt1b, Ppara) and thermogenesis (Ucp1). Induction of these genes associated with reduced lipid droplet size in the absence of changes in lipogenic capacity, suggesting functional consequences, even if UCP1 could not be detected at the protein level by immunoblotting in the DS-treated cells (data not shown). Defective induction of browning related genes by the GAGs mixture as compared to the DS component alone may relate to the anti-adipogenic effect of the GAGs mixture, since there are molecular players, such as PPARγ that are common to white and brown/beige adipogenesis (see [31, 32]). To our knowledge, modulation of adipogenesis or metabolic capabilities of adipocytes by exogenously added GAGs had not been addressed previously.

Rb has been implicated in the development and maintenance of the mature white adipocyte phenotype [29, 30, 44] and as a negative modulator of brown adipogenesis [45]. We found Rb expression and activity to be decreased in the MEF cultures exposed to the GAGs mixture, in which adipogenesis was impaired, but not in those exposed to DS alone, in which a brown-like transcriptional program was induced, suggesting a preponderant role of Rb in the development and maintenance of the white adipocyte phenotype, at least in our MEF-based model. Thus, even if suppression of Rb favors white-to-brown adipocyte remodeling in cultured adipose cells and *in vivo* [45-47], it would not be a necessary requirement for this process.

How might GAGs affect the differentiation fate of MEFs? Although mechanistic issues have not been addressed in full, several non-mutually exclusive, tentative possibilities can be proposed. One is that GAGs are hydrolyzed extra or intracellularly to render active synergistically interacting building blocks responsible of intracellular effects. In fact, chitosan oligosaccharides (copolymers of glucosamine and N-acetyl-glucosamine), sulfated glucosamine and phosphorylated glucosamine reportedly inhibit adipogenesis in 3T3-L1 pre-adipose cells [48-50]. Another possibility is that GAGs or active fragments act by interacting with dedicated cell surface receptors – such as the hyaluronan receptor CD44, which is expressed in MSCs and also in MEFs [18] – to activate downstream signaling pathways. In fact, stimulation of chondrogenesis from stem cells in HA-enriched three-dimensional

microenvironments (e.g., HA-coated wells) is dependent on HA-CD44 interaction ([13] and references therein). CD44 interacts with sulfated GAGs including DS besides HA [51], and it is conceivably that simultaneous binding of HA and DS synergistically enhances CD44 signaling. A third possibility is that exogenous GAGs influence cell fate by integrating themselves into the cells-derived ECM, where they may interfere with cell-derived or serum-contained signaling factors. In any case, results herein illustrate that individual components in GAGs mixtures can exert both synergic and antagonist biological effects when presented together.

MSCs are involved in the safeguard of adult joint homeostasis [4] and aberrant activity or depletion of these cells associates with the onset of degenerative changes in the joint [5]. Our results suggest that one additional mechanism through which GAGs and particularly the GAGs mixture contained in OralviscTM can contribute to joint health is by modulating the local stem cell niche to suppress adipogenic differentiation while favoring chondrocyte related gene expression. Suppression of adipogenesis in multipotent or pluripotent cells by GAGs mixtures is also of interest for bone health, since this activity, if demonstrated *in vivo*, could help controlling bone-marrow adipogenesis, an aging-related phenomenon that is connected to osteoporosis [52]. Last but not least, the OralviscTM GAGs entail potential applicability in strategies aimed at the management of body fat and metabolic health, on the basis of observed effects on metabolic and secretory capacities of adipocytes. This mixture may therefore be of special interest for the management of joint discomfort in obesity.

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Disclosure Statement

PDP, NG, AP and MLB have no conflict of interest, financial or scientific. DMP and CC are employed by Bioiberica S.A. (Palafolls, Barcelona, Spain), the producer of Oralvisc™. Bioiberica S.A. had no role in the scientific interpretation of the results and did not influence the way these results were reported.

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