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High density micromass cultures of a human chondrocyte cell line: A reliable assay system to reveal the modulatory functions of pharmacological agents

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ABSTRACT

Osteoarthritis is a highly prevalent and disabling disease for which we do not have a cure. The identification of suitable molecular targets is hindered by the lack of standardized, reproducible and convenient screening assays. Following extensive comparisons of a number of chondrocytic cell lines, culture conditions, and readouts, we have optimized an assay utilizing C-28/I2, a chondrocytic cell line cultured in high-density micromasses. Utilizing molecules with known effects on cartilage (e.g. IL-1β, TGFβ1, BMP-2), we have exploited this improved protocol to (i) evoke responses characteristic of primary chondrocytes; (ii) assess the pharmacodynamics of gene over-expression using non-viral expression vectors; (iii) establish the response profiles of known pharmacological treatments; and (iv) investigate their mechanisms of action. These data indicate that we have established a *medium-throughput* methodology for studying chondrocyte-specific cellular and molecular responses (from gene expression to rapid quantitative measurement of sulfated glycosaminoglycans by Alcian blue staining) that may enable the discovery of novel therapeutics for pharmacological modulation of chondrocyte activation in osteoarthritis.

1. Introduction

Osteoarthritis (OA) is a leading cause of disability worldwide, affecting up to 2/3 of the population over 50 years of age. With incidence rates increasing with age and higher life expectancies, OA economic and social burden is ever increasing [1]. To date, a cure for osteoarthritis is not yet available though it is accepted that chondroprotection and cartilage regeneration would represent a successful strategy, if applied early enough, and chondrocytes present an attractive target for therapeutic intervention.

Chondrocytes are responsible for the synthesis and balance of the extracellular matrix (ECM) that confers tensile strength and flexibility to articular surfaces. ECM is formed by several specific macromolecules including collagen type II [2], aggrecan core protein coated with highly sulfated glycosaminoglycans (GAG) and several other smaller structural and non-structural proteins [3]. Chondrocytes are responsible for maintaining the homeostasis of cartilage and of the ECM through a low turnover state of equilibrium between synthetic activity and catabolic remodeling mediated by specific enzymes including aggrecanases (ADAMTS family) and matrix metalloproteinases (MMPs) [4]. A loss of this homeostatic equilibrium results in the destruction of articular cartilage, which is characteristic of OA [5]. With an ever-increasing understanding of the molecular processes involved in cartilage degradation and the availability of large collections of small biologically active compounds and/or suitable macromolecules, the development of high/medium throughput assays for the identification of therapeutic molecules for chondroprotection is a priority in modern medicine. Primary cultures of adult human articular chondrocytes (AHAC) have several shortcomings for screening assays. Firstly, sample availability often represents a problem and, furthermore, there is an issue linked to donor variability that might reflect genetic factors, co-morbidity, lifestyle and more. Secondly, within the same donor there is a large variability deriving from the donor site, and also from OA being a focal disease. Thirdly, culture expansion of primary AHACs is associated with a loss of phenotype and biological behavior introducing, on the one hand, a further element of variability and, on the other, further complexity in obtaining a sufficient number of authentic chondrocytes suitable for screening purposes [6]. Finally,

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primary AHACs are difficult to transfect with sufficient efficiency. The recent availability of immortalized human chondrocytic cell lines [7] represents a potential solution to some of these problems. Following comparison of different cell lines and culture conditions, we establish C-28/I2 cell line in micromass cultures [8] as optimal to test the ability of different modalities of therapeutic intervention (from drugs to gene delivery) to influence chondrocyte metabolism.

2. Material and methods

2.1. Monolayer and micromass cultures of chondrocytic cell lines

The chondrosarcoma cell lines: JJO12 (kindly provided by Dr. Joel Block, Rush Medical College, Rush-Presbyterian-St Luke's Medical Center, Chicago, IL) [9] and H-EM-SS (extraskeletal myeloid chondrosarcoma; European Collection of Animal Cell Cultures; Salisbury, UK); and the immortalized C-28/I2 cell line [8] were selected for study and compared using the same culture conditions.

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1; Gibco-Invitrogen, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS; Gibco-Invitrogen), 100 units/mL penicillin, and 50 μg/mL streptomycin (Omega Scientific, Tarzana, CA) (growth medium) and maintained in the presence of 5% CO_2 in air. The medium was changed every 3 days. Cells were cultured in monolayer ($2 \times 10^4/cm^2$), grown to sub-confluence (around 80% confluent) and passaged at a ratio of 1:8.

For micromass cultures, the protocol described by De Bari et al. [10] using human periosteum-derived cells was employed, with some modifications. Briefly, confluent monolayer cultures of chondrocyte cell lines were released by trypsin-EDTA, tested for viability by trypan blue exclusion, and re-suspended in growth medium at a density of 2.5×10^7 viable cells/mL. Micromasses were obtained by pipetting 20 μL of cell suspension into individual wells of 24-well plates. Following a 3-h attachment period without medium, the growth medium was gently added and cultures left resting for a further 24 h. The medium was then changed to serum-free and phenol red-free medium (Gibco BRL) for 24 h (Supplementary data Fig. 1). Differentiation was promoted by serum starvation and ITS supplementation as described [11,12]. On day 3 of the culture, fresh differentiation medium was added and treatments were performed as described in the results. After 48 h, some of the micromasses were harvested for Alcian blue matrix staining and others for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) gene expression analysis of marker genes for chondrocytes: type II collagen alpha-1 (*COL2A1*), aggrecan (*ACAN*), sex determining region (SRY)-box 9 (*SOX9*), matrix metalloproteinase-1 and -13 (*MMP1*, *MMP13*), and a disintegrin and metalloproteinase with a thrombospondin type 1 motif 5 (*ADAMTS5*), as described in the section 2.6.

2.2. Cell proliferation assay

Cells were counted with a Neubauer hemacytometer and plated at different densities in phenol red- and serum-free medium. The top cell density was 8×10^4, and subsequent serial dilutions (1:2, 1:4 and 1:8 1:16) were made; thus, various concentrations of cells were then seeded in triplicate in a 48-well plate (400 μL $well^{-1}$) and incubated in 5% CO_2 at 37 °C. After an initial 4 h period to allow cell attachment, 40 μL of alamar blue solution (Alamar, Sacramento, CA) was directly added to the medium resulting in a final concentration of 10% and the cell were returned to incubation for 24 h. The absorbance, presented as mean optical density (O.D.), of test and control wells was read after 24 h at 570 and 595 nm with a standard spectrophotometer [Spectronic 2000 spectrophotometer (Bausch & Lomb, Rochester, NY)].

2.3. Cartilage harvest and primary adult human articular chondrocyte (AHAC) isolation

AHACs were obtained with informed consent from patients who underwent joint replacement for knee OA. Cartilage samples were provided by Mr P. Achan (Barts and the London National Health Service Trust, London, UK). All procedures were approved by the East London and The City Research Ethics Committee 3. Cartilage tissue was dissected from preserved areas of the femoral condyles and the patellar groove, as recently reported [13]. Briefly, cartilage was sliced full thickness, excluding the mineralized cartilage and the subchondral bone and initially washed twice in high-glucose DMEM (DMEM/F-12 1:1 plus GlutaMax; Invitrogen, Eggenstein, Germany) containing 10% FBS, 1 mM sodium pyruvate and 2% antibiotic antimycotic solution (Invitrogen, Eggenstein, Germany). Chondrocytes were isolated from the cartilage samples by enzymatic digestion with 1 mg/mL pronase (Roche, Welwyn, UK) for 30 min at 37 °C and then overnight at 37 °C with 1 mg/mL collagenase P (Roche, Welwyn, UK) prepared in complete medium (same composition as above, with 1% antibiotic/antimycotic solution) under agitation. The AHACs recovered from the digestion were then resuspended in complete media, assessed for cell viability using trypan blue, and seeded at a density of 10,000 cells/cm^2. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Only cells obtained from samples with a histological Mankin score <4 for features of OA were used for subsequent experiments [14,15]. All experiments were performed using freshly isolated or confluent P0 cells, cultured either in monolayer or micromasses.

2.4. Alcian blue (AB) staining in vitro

For semi-quantitation of cartilage-specific sulfated glycosaminoglycans, we used a protocol optimized by De Bari et al. [16], based on staining with AB 8GS at pH 0.2, which is highly specific for cartilage ECM [10,16]. Micromasses were rinsed twice with PBS, fixed with 4% glutaraldehyde solution (v/v in distilled water) for 15 min at room temperature (RT), washed with 200 μL of 0.1 N HCl (Sigma, St. Louis, MO, USA) solution in distilled water, and covered with AB dye at pH < 1 (1% Alcian blue 8 GS in 0.1 N HCl; Carl Roth, Karlsruhe, Germany) at RT. Cultures were then washed extensively with distilled water, prior to extraction with 200 μL of 6 M guanidine HCl (Sigma–Aldrich, St. Louis, Mo, USA) overnight at RT. The optical density (OD) of the extracted dye was measured at 630 nm using a Spectronic 2000 spectrophotometer (Bausch & Lomb, Rochester, NY, USA), and OD values were interpolated with an AB standard curve (from 625 to 9.76 μg/mL). Protein content for each sample was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Initially, the amount of extracted AB was normalized with protein content and expressed as AB/protein (μg/μg), such as in the experiments where cell lines and primary AHAC were compared (Supplementary data Fig. 2). In all other assays, GAG accumulation was measured by AB quantification and normalized to DNA content (μg/μg), as described below.

2.5. DNA quantification

DNA content was measured by fluorescence using SYBR Green I dye (Invitrogen, Paisley, UK). For each micromass sample, DNA concentration was assessed against a DNA standard curve [dsDNA for Standard Curve–Lambda DNA (Invitrogen, Paisley, UK)] after preparation in an assay solution [10 mM Tris–HCl, 1 mM EDTA, pH8, containing 1% (v/v) of SYBR Green I dye]. DNA samples were diluted (1:50), homogenized with the assay solution (1:20), measured at 485/535 nm by using a spectrophotometer

(TECAN-M200; Tecan, Männedorf, Switzerland). These values were used to normalize GAG concentrations.

2.6. Aggrecan protein detection by Western blotting

Micromasses were washed and lysed with cold lysis buffer [20 mM Tris–HCl pH 8, 137 mM NaCl, 1% (v/v), Nonidet P-40, 2 mM EDTA] supplemented with protease inhibitors and sodium vanadate (both in a dilution of 1:100). Cell lysates were centrifuged at 15,000 × *g* for 15 min and the supernatants were recovered. Protein samples were subjected to SDS–polyacrylamide gel electrophoresis using a 10% (w/v) gel. Proteins were blotted to polyvinylidene difluoride membranes (PVDF; Millipore, Watford, UK) and blocked for 1 h on a plate rocker in either 5% (w/v) BSA or 5% milk solution (w/v) in Tris-buffered saline (TBS) (150 mM sodium chloride, 2 mM Tris base, pH7.4) containing 0.1% Tween 20 (Sigma) (TBS-T). Aggrecan was detected following overnight incubation at 4 °C, on a plate rocker in TBS-T with primary Ab; a rabbit polyclonal antibody (GeneTex, Inc., USA) diluted 1:1000 in PBS containing 5% (w/v) nonfat dry milk. The following day, membranes were incubated for 1 h at RT with the secondary antibody [donkey anti-rabbit IgG (1:4000; Amersham Biosciences, Amersham, UK)]. Membranes were re-probed with monoclonal mouse anti-human β-actin (dilution 1:10,000 in 5% milk solution, clone AC-15, Sigma). Proteins were detected using the ECL Detection Kit and visualized on Hyperfilm (Amersham Biosciences, Amersham, UK). Densitometry was performed using ImageJ software analysis. The value from each band was subtracted from background values, and the protein band was normalized against β-actin as detected in the same sample.

2.7. Real-time PCR

Total RNA was extracted using a commercially available kit (Qiagen RNeasy Mini Kit; Qiagen, Hilden, Germany), according to the manufacturer's instructions. The concentration and purity of the RNA was analysed using the Nandrop ND-1000 (NanoDrop Technologies, Wilmington, DE). Complementary DNA (cDNA) were obtained by reverse transcription (RT) of 2 μg of total RNA, with the Superscript III reverse transcriptase system (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol and using oligo(dT)$_{15}$ as primer. Real-time PCR was performed with the ABI Prism 7900 Real-time PCR system (Applied Biosystems Inc., CA, USA). The following amplification profile was used: 95 °C for 15 min; 35 cycles- 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s, followed by a melt curve analysis. For each reaction, a total volume of 10 μL was used, which consisted of 2 μL of diluted cDNA (10 ng/μL of RNA), 5.2 μL of 1× Power SYBR Green mastermix (Applied Biosystems, Inc., CA, USA), 1.8 μL of dH_2O, and 1 μL of gene specific primer. Commercially available primers (Qiagen, West Sussex, UK) were used to probe for target mRNA (Hs_*SOX9*, QT00001498; Hs_*COL2A1*, QT00049518; Hs_*ACAN*, QT00001365; Hs_*MMP1*, QT00014581; Hs_*MMP13*, QT00001764; Hs_*ADAMTS5*, QT00011088; Hs_*GAPDH*, QT01192646; and Hs_*BMP2*, QT00012544). mRNA data were normalized relative to *GAPDH* and then used to calculate expression levels. The comparative Ct method was used to measure the gene transcription in samples [17]. Results are expressed as relative units based on calculation of $2^{-\Delta\Delta Ct}$, which gives the relative amount of target gene normalized to endogenous control (*GAPDH*) and to the control (untreated) samples with the expression set as 1. Negative controls were either RT without enzyme or PCR without cDNA template.

2.8. Quantification of PGE_2 levels

The PGE_2 concentrations in cell culture supernatants were measured using a specific enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's recommendation (sensitivity of 9 pg/mL). All assays were performed in triplicate.

2.9. Transfection of C-28/I2 cells

Transient transfection experiments were carried out in C-28/I2 chondrocytes in monolayer using the lipid-based transfection reagent FuGene6 (Transfection Reagent; Roche Molecular Biochemical, Indianapolis, IN, USA). The human BMP-2 plasmid (pMT7T3-BMP2) was a gift from Dr Gerhard Gross (Helmholtz Centre for Infection Research, Braunschweig, Germany). Cell number was determined by hemocytometry. Viability, as determined by trypan blue exclusion, always exceeded 90%. Cells were cultured in monolayers in 6-well plates at 2.5×10^4 cells/cm^2 in medium containing 10% fetal bovine serum; transfection experiments were performed at sub-confluence (density 60–70%). The pMT7T3-BMP2/Fugene or EGFP (control) complexes were added in a dropwise fashion to the cell cultures. Following preliminary experiments, optimal transfection efficiency was obtained with 3 μg DNA and 9 μL of Fugene or with a ratio DNA/Fugene of 3:3 (w/v), using 8 h exposure. After incubation at 37 °C, bright field and fluorescent images were taken of the cultures transfected with the control (EGFP) and the percentage of transfected cells was calculated. The cells were harvested and resuspended in a 2.5×10^7 cells/mL to establish micromass cultures. Using GFP-vector, a transfection efficiency of 30 ± 3% with 95% viability could be calculated.

2.10. Statistical analysis

All data are reported as mean ± SD of n observations, and were performed in duplicate and repeated at least three times. Statistical evaluation was performed using one-way analysis of variance (ANOVA) (Prism4 GraphPad Software, San Diego, USA) followed by Bonferroni test for post hoc analyses. When two experimental groups were compared, a Student's *t*-test was used. In all cases, a probability p value ≤ 0.05 was taken as significant.

3. Results

3.1. Selection of C-28/I2 cells for micromass cultures

One aim of this study was to establish a rapid and reproducible screening assay for chondroprotective molecules and anti-inflammatory drugs. We began by comparing several human chondrocytic cell lines for their growth rate and differentiation markers. H-EMC-SS displayed a gene expression profile of a chondrocytic cell line in monolayer (*SOX9* mRNA expression is almost 50% increased when compared to C-28/I2) (Fig. 1A), yet these cells had slow proliferation rates when compared to JJO12 and C-28/I2 lines (Fig. 1B). The latter two cell lines showed similar proliferation rates (Fig. 1B); however our choice fell on the C-28/I2 human chondrocyte cell line because of the combination of a suitable growth rate and a gene profile of phenotypic markers which resembles more closely that of primary adult human articular chondrocytes (AHACs) (Fig. 1A).

When compared to primary AHACs, the C-28/I2 cells displayed an overall lower expression of phenotypic marker genes (*SOX9*, *COL2A1*, *ACAN*, *MMP1*, *MMP13* and *ADAMTS5*) upon culture in monolayer (Fig. 1A), associated with lower accumulation of highly sulfated glycosaminoglycans as measured by Alcian blue staining and spectrophotometric quantification (Supplementary Fig. 2). However, when compared to the other cell lines tested in this study, C-28/I2 cells in micromass displayed enhanced expression of chondrocyte marker genes (with significant up-regulation in

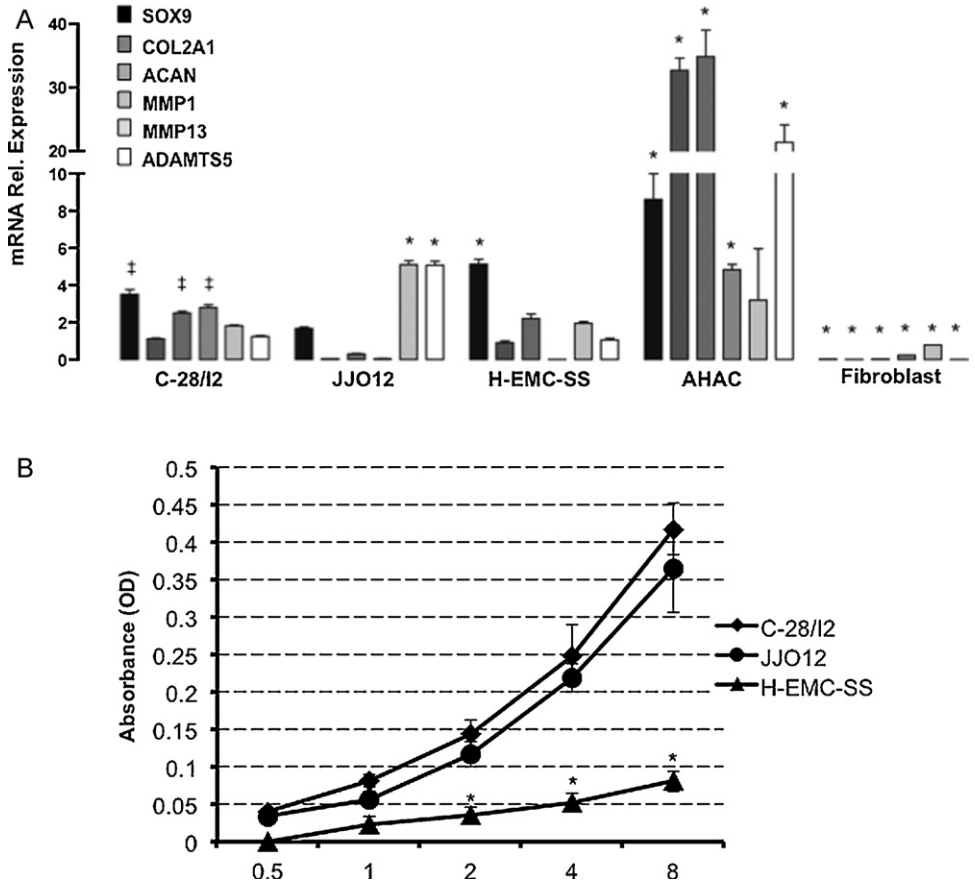

Fig. 1. Molecular characterization and growth rate of different chondrocyte cell lines and primary adult human articular chondrocytes (AHACs). (A) C-28/I2, JJ012, and H-EMC-SS cells, and primary AHAC were cultured in monolayers and analysed for expression of anabolic (*SOX9*, *COL2A1*, *ACAN*) and catabolic (*MMP1*, *MMP13* and *ADAMTS5*) chondrocyte marker genes. Cells underwent chondrocytic stimulation by culturing in serum free conditions, with control (unstimulated) cells kept at 10% serum. The relative units are based on the calculation of $2^{-\Delta\Delta Ct}$, which gives the relative amount of target gene normalized to endogenous control (*GAPDH*) and to the control value set at 1. Fibroblasts were used as negative control. Results are mean ± SD of 3 independent experiments performed in duplicate; $^{\ddagger}p < 0.05$ *vs.* C-28/I2 10% FCS and $^{*}p < 0.05$ *vs.* C-28/I2 0% FCS. (B) C-28/I2, JJO12 and H-EMC-SS cells were cultured in monolayer (serum-free) at different plating densities (8, 4, 2, 1 and 0.5×10^4) and assayed for their growth rate using Alamar blue assay. The absorbance (OD; optical density) was read after 24 h, at 570 and 595 nm. Data are mean ± SD of 3 different experiments performed in triplicate; $^{*}p < 0.05$ *vs.* C-28/I2.

SOX9, *COL2A1* and *ACAN* mRNA; Supplemental Table 1) and slightly increased accumulation of GAG-rich proteins (Supplemental Fig. 2). Thus, the C-28/I2 cell line cultured in high-density micromass was selected for further analyses.

3.2. C-28/I2 cells in micromass respond to anabolic and catabolic stimuli

Treatment of micromasses with TGFβ1 induced significant up-regulation of cartilage phenotypic markers (*SOX9*, *COL2A1*, *ACAN*), with down-regulation of the catabolic (*MMP13* and *ADAMTS5*) gene products (Fig. 2E). This was mirrored by an augmented accumulation of GAG-rich extracellular matrix *in vitro* (Fig. 2A) reflected by higher values for AB extraction (Fig. 2B). These ECM-related responses were paralleled by TGFβ1-enhanced aggrecan protein expression as assessed by Western blot (Fig. 2C and D).

In contrast, treatment of these micromasses with catabolic signal IL-1β led to a significant ∼50% reduction in GAG content (Fig. 2A and B) and aggrecan protein blotting (Fig. 2C and D). Gene expression responses to the anabolic and catabolic stimuli of C-28/I2 cells cultured in micromasses were qualitatively similar (Fig. 2E), though not so marked in their degree, to that measured with primary AHACs (Fig. 2F).

Next, we tested whether C-28/I2 cells could be amenable to gene transfection studies. Using the Fugene®6 protocol, an efficiency of ∼30% could be determined, as evaluated by counting fluorescent cells following transfection with control EGFP plasmid. The cells remained EGFP-positive after 7 days of culture (some transfected cells were kept up to 10 days for verification purposes). Transfection efficiency did not result from any significant change in cell viability, which remained well over 95% (Table 1). When the pMT7T3-BMP2 vector was transfected, C-28/I2 cells responded with a marked accumulation of sulfated GAGs (Fig. 3A and Table 1) which was, again, associated with the expected changes in the anabolic gene markers *SOX9*, *COL2A1* and *ACAN*, with ∼3-fold, >12-fold and ∼6-fold increase, respectively (Fig. 3B). Of note was the up-regulation (∼4-fold increase) observed on BMP-2 mRNA after BMP-2 transfection (Fig. 3B).

Effect of prototypical anti-inflammatory drugs on C-28/I2 micromass responses. Finally we determined whether this cell culture assay, could be applied to reveal chondroprotective properties of pharmacological molecules and to do so we tested naproxen (NAP), prototype of cyclo-oxygenase inhibitors, and prednisolone (PRED), prototype of clinically relevant glucocorticoids. In resting C-28/I2 micromass cultures, treatment with PRED (10–1000 nM) and NAP (1–100 μM) for 48 h did not alter the accumulation of GAG (Fig. 4A), nor was there any change observed in mRNA levels of anabolic genes following treatment with PRED (Table 2). However, NAP reduced *COL2A1* and *ACAN* mRNA levels by approximately 30% (Table 2).

In the presence of catabolic stimulation by IL-1β, NAP inhibited the accumulation of GAG-rich proteins without returning values

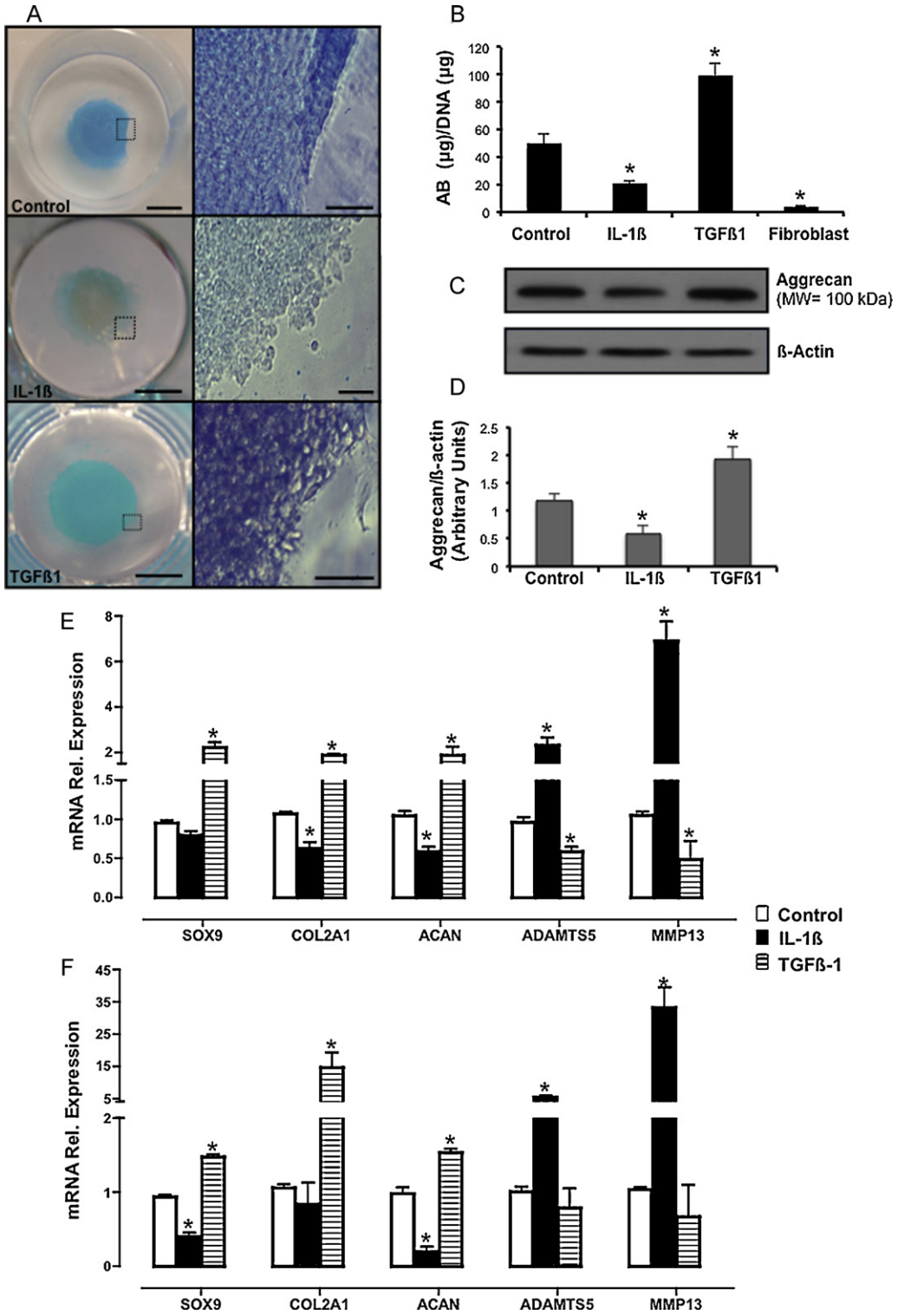

Fig. 2. Effect of anabolic and catabolic stimuli on C-28/I2 cells in micromasses (MM). Images in the left panel show MM stained with Alcian blue (AB) to detected glycosaminoglycan (GAG) accumulation after 48 h incubation with TGFβ1 (10 ng/mL; anabolic stimulus) or with IL-1β (20 ng/mL; catabolic stimulus) *vs.* control (scale bars = 3 mm). Right panel shows a higher-magnification of the boxed areas (scale bars = 100 μm). (B) Accumulation of GAG-rich extracellular matrix *in vitro* was measured by AB staining and normalized to the DNA content (μg/μg). Fibroblasts were used as negative control. (C) Western blotting for total aggrecan after treatment of MM with either IL-1β or TGFβ1 and (D) quantification of pixel density (ratio aggrecan/β-actin). mRNA relative quantification of the chondrocyte phenotypic marker genes (*SOX9*, *COL2A1*, *ACAN*, *MMP13* and *ADAMTS5*) was assessed by quantitative PCR in (E) C-28/I2 cell line and (F) human primary chondrocytes (AHACs), both in MM incubated with TGFβ1 or IL-1β. Data (mean ± SD of 5 experiments in duplicate) are expressed as relative units ($2^{-\Delta\Delta Ct}$). $^{*}p < 0.05$ *vs.* untreated control.

back to basal levels (Fig. 4A). Nevertheless, NAP produced a marked attenuation of IL-1β-induced *MMP1* and *MMP13* mRNA expression (e.g., >60% inhibition on *MMP13*) (Fig. 4C). No beneficial effect on anabolic gene expression could be detected. Additionally, no modulation of *ADAMTS5* was observed following treatment with this cyclooxygenase inhibitor.

The glucocorticoid PRED prevented the catabolic effect of IL-1β on GAG content, with significantly enhanced responses through all

Table 1
Parameters of the EGFP and BMP-2 transfection.

Parameter	C-28/I2 transfection	
	EGFP	BMP-2
Cell viability	94 ± 3	95 ± 2
Transfection efficiency (%)	28.3 ± 3.1	ND
GAG content (μg AB/μg DNA)	50.2 ± 4.9	125 ± 21*

Summary of data acquired upon C-28/I2 cells transfection, showing cell viability, transfection efficiency (%), and GAG accumulation measured by Alcian blue (AB) quantification normalized to DNA content (μg/μg). Cells were transfected either with a plasmid encoding bone morphogenetic protein (BMP)-2 (pMT7T3-BMP2) or EGFP. Each data point it the mean ± SD of 5 different experiments performed in duplicate. BMP-2, bone morfogenetic protein-2; EGFP, enhanced green florescent protein. ND, not determinate.
* $p < 0.05$ *vs.* respective EGFP value

tested concentrations (Fig. 4A). The anti-catabolic effect of PRED was underpinned by strong inhibition of all three proteolytic enzyme genes: *MMP1*, *MMP13* and *ADAMTS5* mRNA levels were decreased by over 60% (Fig. 4B). Moreover, *COL2A1* mRNA expression was also up-regulated.

To gain further notion on the modulation of cell responses by NAP and PRED, we measured the production of PGE_2 in the supernatants of micromasses. NAP afforded significant reductions at all concentrations tested, in the presence or absence of IL-1β (Table 3). In contrast to NAP, PRED did not significantly modulate PGE_2 production (Table 3), leaving open the possibility of other chondroprotective mechanisms, which need to be further investigated. Based on these results, we selected 10 μM NAP and 0.1 μM PRED for testing on primary chondrocytes to challenge the predictive value of the C-28/I2 micromass-culture assay.

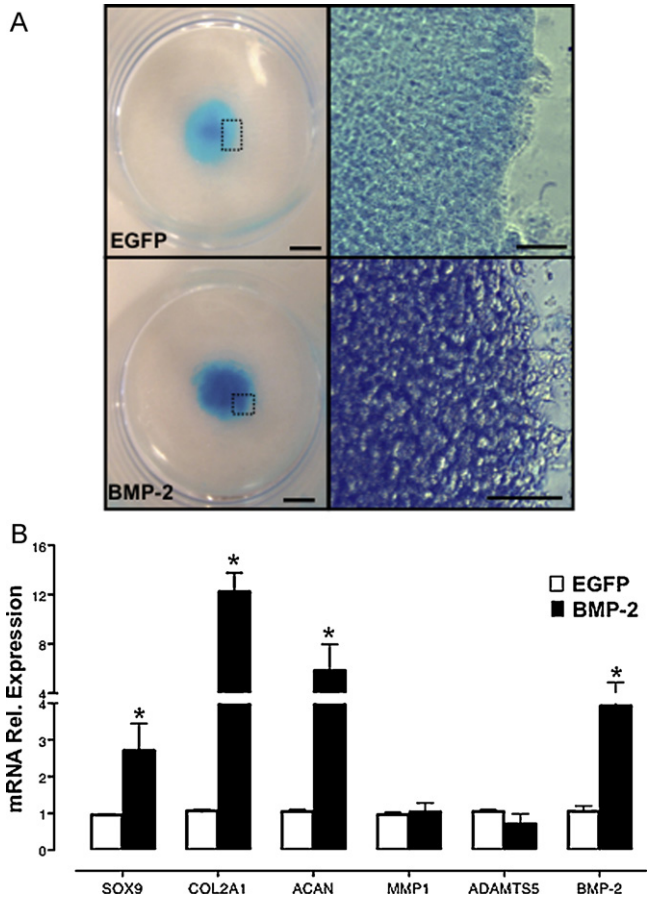

Fig. 3. Transfectability of C-28/I2 cells and effects of BMP-2 overexpression on gene expression and matrix formation. (A) C-28/I2 cells in monolayer were transfected with a plasmid encoding bone morphogenetic protein (BMP)-2 (pMT7T3-BMP2) or EGFP (control) and then cultured in micromass (MM). Left-hand side: images of C-28/I2 cells in MM stained with Alcian blue (AB) after transfection (bar = 3 mm). Right-hand side: higher-magnification views of the boxed areas (bar = 100 μm). (B) Relative quantification of the anabolic (*SOX9*, *COL2A1*, *ACAN*) and catabolic (*MMP1* and *ADAMTS5*) marker gene products, as well as BMP-2 mRNA upon transfection with either EGFP or BMP-2 plasmids. Data (mean ± SD of 5 independent experiments performed in duplicate) are expressed as relative units ($2^{-\Delta\Delta Ct}$). *$p < 0.05$ *vs.* EGFP transfected (control).

Primary AHAC in micromass were stimulated with IL-1β in the presence or absence of the two pharmacological tools for 48 h. As shown in Fig. 5, both NAP and PRED abrogated the effects of IL-1β on the catabolic enzyme gene expression profile, e.g. *MMP13* and *ADAMTS5*, but only PRED displayed modest effects on anabolic genes, as indicated by the up-regulation of *SOX9* (Fig. 5A) and *ACAN* (Fig. 5C) mRNA expression.

Altogether the results with primary chondrocytes were, at least qualitatively, similar to those obtained with C-28/I2 micromass cultures, depicting NAP and PRED modulation of cell reactivity to a similar degree, though not necessarily to a similar extent.

4. Discussion

In this study, we describe a reliable culture assay using a chondrocytic cell line, providing strong evidence for its utility to reveal anabolic and catabolic responses, including those induced by gene transfection, as well as to investigate the properties of chondroprotective and anti-inflammatory drugs. Use of primary chondrocytes afforded strong proof-of-concept for the validity and relevance of a chondrocyte cell line that we chose for characterization in the micromass culture system.

Primary adult human articular chondrocytes (AHACs) cultures constitute an attractive tool for investigating intracellular and molecular features of chondrocyte differentiation and activation [18]. However, during assessments of phenotype and responsiveness to stimuli [7], marked variability between cells from different donors or even between different areas of the same joint can produce artifacts that bias results and lead to limited experimental analysis. Hence, better strategies for using chondrocyte cell lines are remarkably required [4,19].

We began this study by comparing the growth rates and gene expression profiles of three well-known human chondrocytic cell lines in different culture conditions. We selected the chondrosarcoma cell lines JJO12 [20,21] and H-EMC-SS [22], and the immortalized chondrocytes, C-28/I2 [7,8] and compared their behavior in monolayer and micromass cultures. Selected readouts were expression profiles of genes involved in matrix production (*COL2A1* and *ACAN*) [3], and *SOX9*, a pivotal transcriptional regulator that is essential for chondrocyte differentiation [23]. As catabolic markers, we selected: *MMP13*, the major type II collagen-degrading collagenase, known to be regulated by stress-, inflammation-, and differentiation-induced signals [24], *MMP1* [25], and the main aggrecanase involved in matrix degradation, *ADAMTS5* [26,27].

In monolayer, all analysed cell lines showed only very limited similarities in gene expression of cartilage differentiation markers compared to primary AHAC. Nevertheless, we selected the C-28/I2 cells for their rapid growth rate combined with a relatively better chondrocyte-like phenotype, compared to other cell lines, in micromass culture. The results obtained in this study are in line with the known positive effects of high-density cultures on the phenotypic profile of primary cells [11,28–32]. In accordance with the literature, this protocol yielded high *SOX9* gene expression, the principle phenotypic marker of chondrocytes [33], and associated changes in *COL2A1* and *ACAN*, genes involved with cartilage-specific ECM.

Interestingly, C-28/I2 in micromasses expressed higher levels of genes associated with matrix degradation (*MMP1* and *ADAMTS5*),

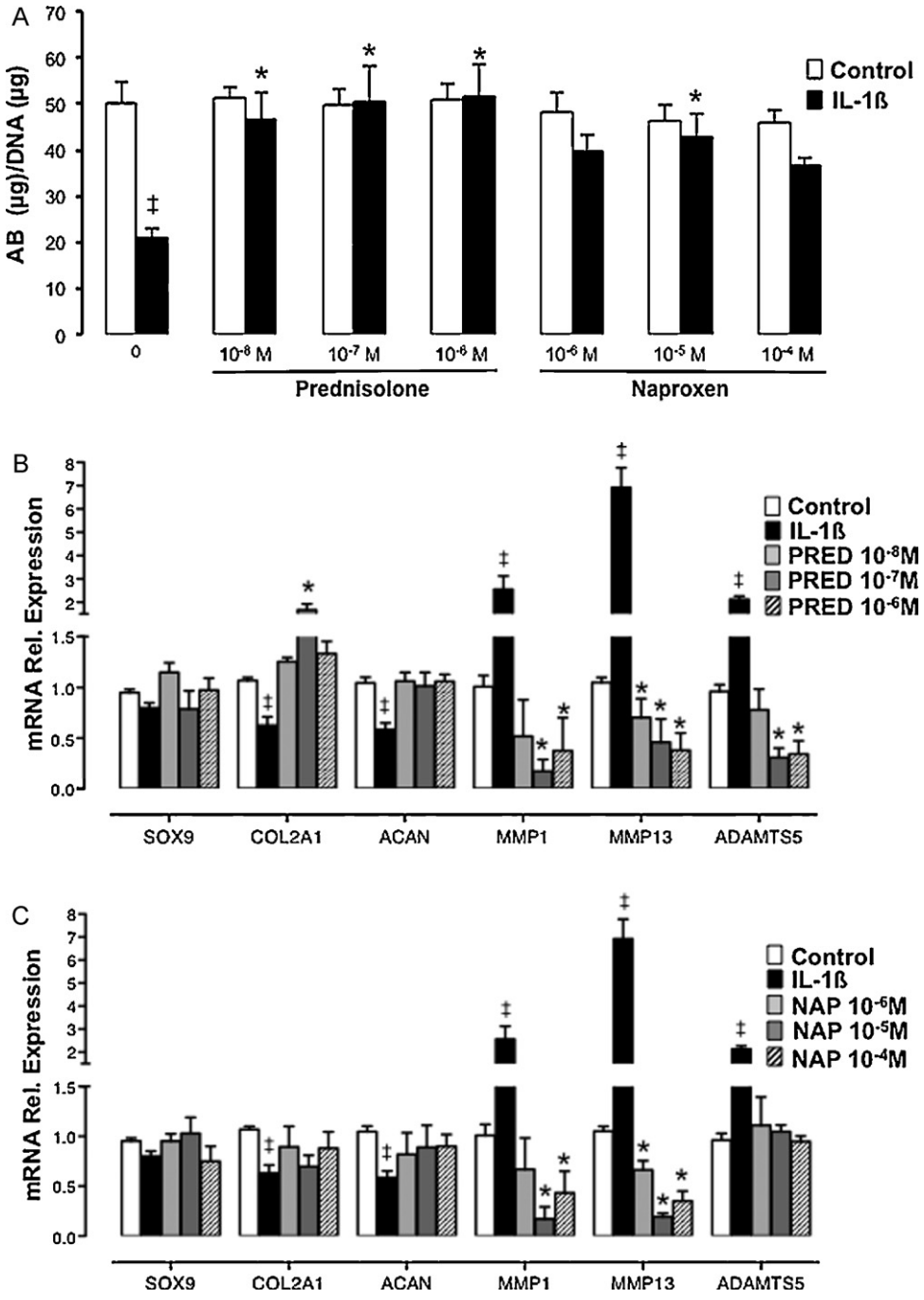

Fig. 4. Modulation of C-28/I2 in micromasses (MM) by prednisolone and naproxen. C-28/I2 cells in MM were incubated for 48 h with prednisolone (PRED; 10^{-8}–10^{-6} M) or naproxen (NAP; 10^{-6}–10^{-4} M) in the absence or presence of IL-1β (20 ng/mL; catabolic stimulus), and analysed by (A) Alcian blue (AB) staining (values reported as µg AB per µg DNA). Relative expression of anabolic (*SOX9*, *COL2A1* and *ACAN*) and catabolic genes (*MMP1*, *MMP13* and *ADAMTS5*) in presence of IL-1β (20 ng/mL) are shown after (B) PRED and (C) NAP treatments. Data (mean ± SD of 5 independent experiments performed in duplicate) for gene expression are calculated as relative units ($2^{-\Delta\Delta Ct}$). $^{\ddagger}p < 0.05$ *vs.* untreated C-28/I2 (control) and $^{*}p < 0.05$ *vs.* IL-1β treated MM.

which has also been detected in primary AHAC in high-density cultures [28]. This suggests that this cell line might be a good option to investigate not only matrix anabolism but also catabolism in chondrocytic cells [7]. Other advantages of the micromass system using cell lines include its rapidity and simplicity, allowing rapid screenings to measure accumulation of GAG-rich extracellular matrix by Alcian blue staining. AB binds to anionic groups on highly sulfated GAG [34,35] and, at low pH values, selectively stains cartilage-specific matrix [16].

One important aspect of this study is the retrospective validation of the detection of bioactive agents that have been proven to be active *in vivo*. In spite of its relatively simplicity, this system proved to be sensitive to agents that are known to be either catabolic or anabolic for chondrocytes *in vivo*, such as IL-1β [36,37] and TGFβ1 [38,39], respectively. Application of these two chondrocyte stimuli yielded the expected modulation of C-28/I2 responsiveness, which was strictly reflected in the amount of AB staining. The patterns of relative mRNA expression of chondrocyte marker genes in C-28/I2 cells upon TGFβ1 and IL-1β stimulation were also clearly comparable with the results produced with primary AHAC, giving confidence for this protocol as an alternative to assays based upon primary cells.

Table 2
Effect of anti-inflammatory drugs prednisolone and naproxen on C-28/I2 micromasses in resting conditions.

	PRED (M)			NAP (M)		
	10^{-8}	10^{-7}	10^{-6}	10^{-6}	10^{-5}	10^{-4}
SOX9	1.18 ± 0.1	0.72 ± 0.01	0.71 ± 0.01	0.92 ± 0.04	1.10 ± 0.18	0.98 ± 0.07
COL2A1	1.12 ± 0.2	1.03 ± 0.08	1.13 ± 0.15	1.04 ± 0.08	0.71 ± 0.03*	0.7 ± 0.02*
ACAN	1.22 ± 0.2	1.24 ± 0.12	1.10 ± 0.07	0.76 ± 0.01	0.60 ± 0.01*	0.62 ± 0.01*
MMP13	0.84 ± 0.02	0.86 ± 0.03	0.90 ± 0.05	1.07 ± 0.03	0.8 ± 0.02	0.76 ± 0.01
ADAMTS5	0.78 ± 0.01	0.95 ± 0.01	0.74 ± 0.01	0.87 ± 0.02	0.95 ± 0.15	1.06 ± 0.3

C-28/I2 cells were grown in micromasses and incubated for 48 h with prednisolone (PRED; 10^{-8}–10^{-6} M) and naproxen (NAP; 10^{-6}–10^{-4} M). Relative expression of anabolic (*SOX9*, *COL2A1* and *ACAN*) and catabolic genes (*MMP13* and *ADAMTS5*) was measured by real time PCR. *GAPDH* was used as endogenous control. Data are expressed as relative units based on calculation of $2^{-\Delta\Delta Ct}$, which gives the relative amount of target gene normalized to endogenous control (*GAPDH*) and to the control (non-stimulated), set at 1. Data are expressed as means ± SD of 5 independent experiments performed in duplicate.
* $p < 0.05$ *vs.* untreated C-28/I2 (control).

Table 3
Prostaglandin E_2 (PGE_2) levels in micromass cultures.

	Control	PRED (M)			NAP (M)		
		10^{-8}	10^{-7}	10^{-6}	10^{-6}	10^{-5}	10^{-4}
Basal	120 ± 16	111 ± 7	102 ± 9	112 ± 18	65 ± 20*	56 ± 5*	46 ± 4*
IL-1β	191 ± 20‡	128 ± 23	120 ± 18	121 ± 21	89 ± 5*	88 ± 10*	82 ± 8*

Cell supernatants (serum- and phenol-red free) generated as in Fig. 4 were assayed for PGE_2 concentrations (pg/mL) using a specific enzyme immunoassay (EIA). PRED = prednisolone; NAP = naproxen. Each data point is the mean ± SD of 5 different experiments performed in triplicate.
* $p < 0.05$ *vs.* respective control value.
‡ $p < 0.05$ *vs.* control/basal value.

Finally, we postulated another advantage of this cell line over the primary cells, which is its potential use for gene transfection studies, as primary AHAC are resistant to most transfection methods and consequently not amenable to *high-throughput* mechanistic studies to screen the consequences of gene over-expression [40]. To this end, we used a plasmid vector to overexpress BMP-2, a pivotal anabolic molecule that can increase *COL2A1* and ACAN gene expression and is induced by cytokines in articular chondrocytes [41,42].

C-28/I2 cells could be transfected to approximately 30% and yet produce viable micromasses, as evident following transfection with the control EGFP-positive plasmid. Transfection with human pMT7T3-BMP2 plasmid yielded an up-regulation of BMP-2 mRNA, as well as a selective anabolic response chiefly assessed both by AB staining and augmented SOX-9, *COL2A1* and *ACAN* gene expression. This response is congruent with studies showing that BMP-2 is a critical molecule in cartilage development [43] with a dramatic improvement of the chondrogenic phenotype of human articular chondrocytes [44].

Inflammation is the main pathogenic mechanism for cartilage destruction in rheumatoid arthritis and is also a major risk factor in OA [45,46]. Marked differences exist among anti-inflammatory drugs with respect to their ability to modulate inflammation in the joint; their capacity to modulate proteases and inhibitors is unproven, suggesting a requirement for new approaches to study their actions on chondrocytes [47–49].

We have therefore assessed the effects of two compounds that are known to counteract inflammation: the glucocorticoid prednisolone (PRED), and naproxen (NAP), a non-selective cyclo-oxygenase inhibitor [50,51]. In line with published data produced using primary chondrocytes or human explants [52–55], addition of NAP decreased the IL-1β-induced catabolic activity on chondrocytes, however, no further effect on ECM protein accumulation was observed [56]. The more evident effect of NAP in counteracting IL-1β-elicited catabolic responses was in down-regulating matrix metalloproteinases (*MMP)-1* and *13* mRNA. It has been reported in the literature that NAP decreases MMPs and this effect may be in part addressed to its positive effect on tissue inhibitors of metalloproteinases, which form inhibitory complexes with MMPs in a 1:1 stoichiometry [48,55].

Our results using NAP are at variance with those using PRED; addition of this drug to micromasses did not elicit detectable responses on its own, but profoundly affected IL-1β-elicited responses in terms of AB staining, which could be correlated with marked down-regulation of expression of the most potent matrix degradative protease *ADAMTS5*, as well as both *MMP1* and *MMP13*. At high concentrations, PRED could fully overcome the suppression of anabolism by IL-1β with higher expression of *COL2A1* and augmented AB staining.

It is interesting to note how NAP and PRED effects on C-28/I2 cell reactivity to IL-1β was replicated with primary chondrocytes, satisfying the notion that this high-density culture system using this chondrocytic cell line can be predictive of chondrocyte biological responses and their modulation by therapeutic treatments. We propose that this assay can be run as a medium-throughput screening system for anti-inflammatory and chondroprotective drugs.

Mechanistically, we showed that the effects of NAP were related to the inhibition of PGE_2 synthesis and release. Although the biologic activities of this prostaglandin in articular cartilage have sparked controversy [57–59], it appears to be protective in chondrocytes [60,61], increasing anabolic activity in OA cartilage [62,63]. At this level, cytokine-induced synthesis of PGE_2 is part of a circuit that positively regulates *COL2A1* transcription [64]. The protective role of PGE_2 on chondrocytes could be indirectly confirmed by this study, as NAP reduced expression of genes involved in matrix production (*COL2A1* and *ACAN*) in resting conditions, which in part may be due to a reduction of PGE_2 production. Moreover at higher doses of NAP, both PGE_2 and anabolic gene expression were reduced, highlighting the impact of NAP as a suppressive compound in chondrocyte biology.

Our data also indicate a profound inhibition of PGE_2 levels by NAP upon IL-1β stimulation, which has been reported previously [65]. We confirmed and also demonstrated that this effect on PGE_2 correlates with no change in the phenotypic genes related to ECM synthesis, since a similar degree of expression was observed

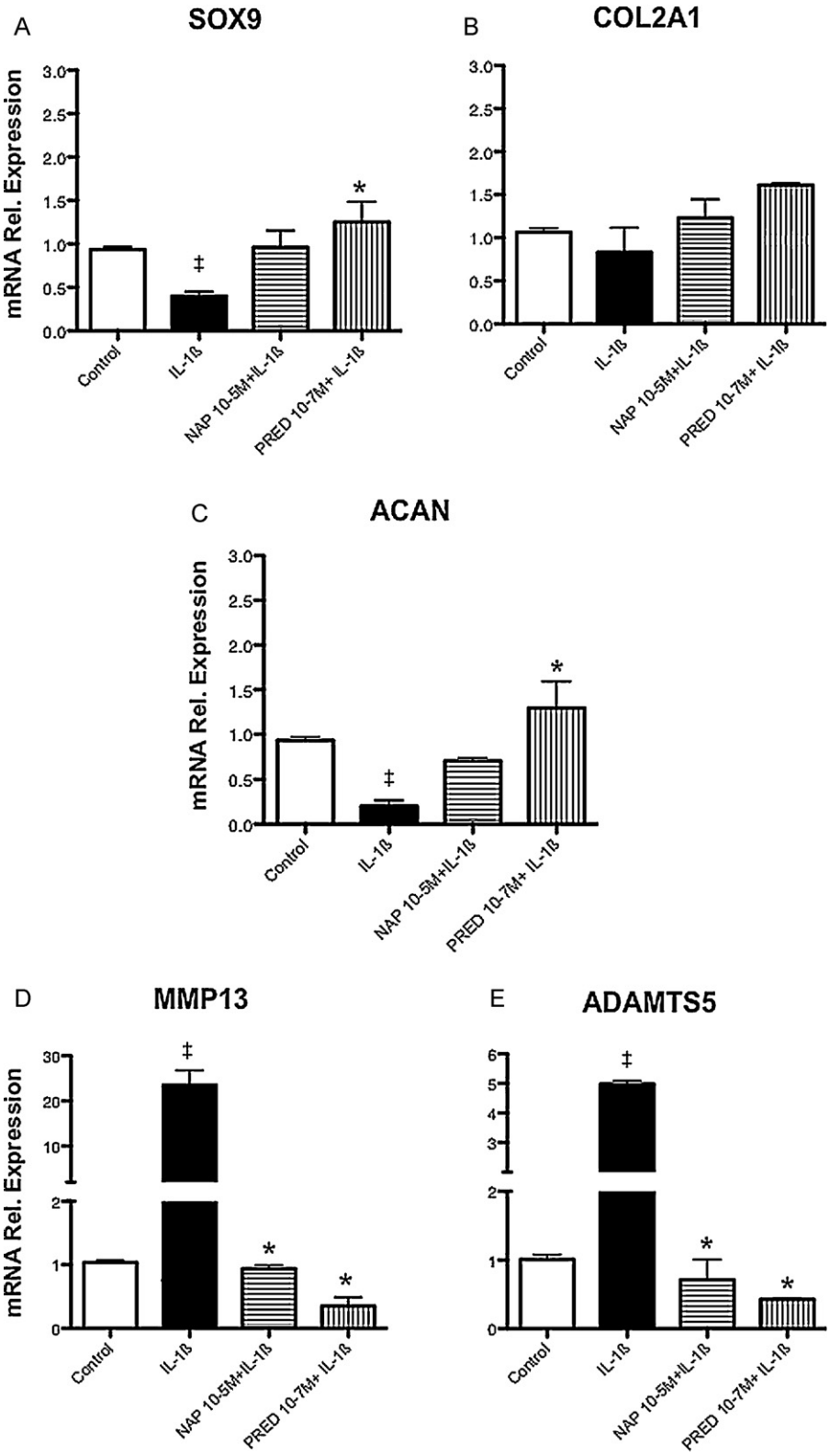

Fig. 5. Effect of prednisolone and naproxen on primary adult human chondrocytes (AHACs). AHACs were treated for 48 h with either prednisolone (PRED 10^{-7} M) or naproxen (NAP 10^{-5} M) in presence of IL-1β (20 ng/ml), and were subjected to gene expression analysis by quantitative PCR. PRED and NAP suppressed the up-regulation of (D) *MMP13* and (E) *ADAMTS5* genes. PRED also augmented (A) *SOX9* and (C) *ACAN* mRNA expression. Data (mean ± SD of 3 independent experiments performed in duplicate) are expressed as relative units ($2^{-\Delta\Delta Ct}$). $^{\dagger}p < 0.05$ *vs.* control AHAC and $^{*}p < 0.05$ *vs.* AHACs + IL-1β.

following stimulation with IL-1β or NAP treatment. The inhibitory effect on chondrocyte metabolism exerted by NAP was also highlighted in this study by using primary chondrocytes. Although this observation has been demonstrated only in short-term *in vitro* studies, it may be clinically relevant, as nonsteroidal anti-inflammatory drugs have been used often to relieve symptoms of OA patients; however, they may also potentially affect cartilage repair in these circumstances [66,67].

Controversially, the potential mechanism(s) behind the beneficial effect of PRED on chondrocytes are clearly independent from any effect on PGE_2 levels, opening the possibility that other downstream regulators of glucocorticoids could be operative in these settings, including Annexin A1, dual-specificity phosphatase-1 or macrophage-inflammatory factor [68,69]. In initial analyses we have observed high Annexin A1 gene expression in C-28/I2 cells (Greco and Dalli, not shown), however its potential involvement in the observed actions of PRED is still to be verified.

In summary, high-density micromass cultures of a human chondrocytic cell line represent a reliable experimental tool to test strategies that might interfere with matrix synthesis and degradation. The biological effects of BMP-2 overexpression, and pharmacological application of NAP and PRED allow us to propose the potential for this system to unveil novel chondroprotective molecules; in addition, such human chondrocyte micromasses could also be used to study the impact of pathogenic circuits with the potential of shedding light on mechanism relevant to OA, hence suitable for testing novel therapeutic approaches.

Conflict of interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.09.009.

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