An improved diagnostic tool to predict cartilage formation in an osteoarthritic joint environment

Tissue Engineering

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Abstract

Osteoarthritis (OA) is characterized by progressive articular cartilage loss. Due to the chondrogenic potential of human mesenchymal stromal cells (MSCs), MSC-based therapies are promising treatment strategies for cartilage loss. However, the local joint microenvironment has a great impact on the success of cartilage formation by MSCs. There are great inter-patient differences in this local joint environment and therefore the result of MSC therapies is uncertain. We previously developed gene promoter-based reporter assays as a novel tool to predict the effect of a patient's OA joint microenvironment on the success of MSC-based cartilage formation. Here we describe an improved version of this molecular tool with increased prediction accuracy. For this, we generated fourteen stable cell lines using transcription factor (TF) binding elements (AP1, ARE, CRE, GRE, ISRE, NFAT5, NFκB, PPRE, SBE, SIE, SOX9, SRE, SRF, TCF/LEF) to drive luciferase reporter gene expression, and evaluated the cell lines for their responsiveness to an osteoarthritic microenvironment by stimulation with OA synovium-conditioned medium (OAs-cm; n=31). To determine the effect of this OA microenvironment on MSC-based cartilage formation, MSCs were stimulated with OAs-cm while cultured in a three-dimensional pellet culture model.Pellets were assessed histologically and sulfated glycosaminoglycan (sGAG) production was quantified as a measure of cartilage formation. Six TF reporters correlated significantly with the effect of OAs-cm on cartilage formation. We validated the predictive value of these TF reporters with an independent cohort of OAs-cm (n=22) and compared the prediction accuracy between our previous and the current new tool. Furthermore, we investigated which combination of reporters could predict the effect of the OA microenvironment on cartilage repair with the highest accuracy. A combination between the TF (NFκB) and the promoter-based (IL6) reporter proved to reach a more accurate prediction compared to the tools separately. These developments are an important step towards a diagnostic tool that can be used for personalized cartilage repair strategies for OA patients.

Impact statement

We demonstrate the improvement of a novel diagnostic tool to predict if an OA joint microenvironment is permissive for cartilage repair or not. The enhanced prediction

accuracy is of great importance for the development of a diagnostic tool that can determine the success of MSC-based cartilage repair strategies.

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Introduction

Progressive articular cartilage degeneration is the main hallmark of osteoarthritis (OA). It causes symptoms such as severe pain, stiffness and loss of joint function [1, 2]. Unfortunately, articular cartilage has limited healing capacity due to its poor vascularity and the limited capacity of chondrocytes to proliferate [3, 4]. Because of the frequent occurrence of OA [5], effective cartilage repair strategies are highly needed in the clinic. Mesenchymal stromal cells (MSCs) can be used for this because of their ability to differentiate into chondrocytesand to repair damaged areas of cartilage [6, 7]. Current treatment outcomes of MSC-based cartilage repair therapies are variable and not always satisfactory [8-10], as the amount and quality of *de novo* formed cartilage is not adequate [11-13].

Various factors that can influence the results of MSC-based cartilage repair strategies have been identified. Especially, the local joint microenvironment, which is mainly determined by the synovial fluid, can (negatively) influence the outcome of MSC-based cartilage repair strategies [14-16]. It has been shown that high concentrations of inflammatory factors, such as IL1β and TNFα in this environment suppress MSC chondrogenesis and expression of SOX9, an essential transcription factor involved in chondrogenesis and cartilage extracellular matrix production [17-19]. Both IL1β and TNFα are known to be elevated in OA synovial fluid [20] and can counteract the MSC therapy outcome. Besides being elevated in OA, it is also reported that levels of these inflammatory cytokines is variable between OA patients [21-23]. OA is known to be a very heterogenous disease containing multiple endotypes, i.e. underlying disease mechanisms, and differences in joint microenvironment between patients could explain why MSC-based cartilage repair therapies report variable responses. We thus hypothesize that the composition of the patient-dependent joint microenvironment determines the results of MSC-based cartilage repair strategies. Therefore, personalized treatment options could be of great benefit. However, no diagnostic tools are available yet that can determine the effect of a patient's joint microenvironment on cartilage formation.

Our approach to develop a diagnostic tool capable of testing the effect the microenvironment has on cartilage formation, is to use a luciferase bioassay. A luciferase-based bioassay is a model system which makes use of an artificial promoter to drive the

expression of a luciferase reporter gene [24]. Expression of luciferase can subsequently be easily measured using a luminometer. Such a luciferase bioassay can be used to analyse the complexity of the joint microenvironment [25, 26]. Using gene-derived promoters to drive luciferase reporter gene expression, we previously have developed such a bioassay that is able to predict the effect of the joint microenvironment on MSC-based *in vitro* cartilage formation with 87.5% accuracy [26]. In this study, we investigated if we could augment the prediction accuracy by changing the complex gene promoters to transcription factor binding elements or to use a combination of both.

Materials and Methods

Chondrocyte H11 cell line culture [27]

Healthy human adult articular chondrocytes derived from femoral head cartilage of a 57 years old Caucasian male (Tebu-Bio, Cat#402-05a) were transduced with a retroviral construct encoding a temperature-dependent SV40 large T oncogene and neomycin resistance. The next day, cells were transduced with a retroviral construct encoding human telomerase reverse transcriptase and hygromycin B resistance (Both retroviral constructs were a gift from Dr. Michael J. O'Hare, Ludwig Institute For Cancer Research). Transduced cells were selected for resistance to both antibiotics at 32 °C. Thereafter, cells were cultured for 3 days at 37 °C to stop proliferation and subsequently sorted by fluorescence-activated cell sorting, based on the amount of aggrecan expressed on their cell surface. Subsequently, H11 cells were cloned by limiting dilution in Mesenchymal Stem Cell Growth Medium (MSCGM™; Lonza, Basel, Switzerland) supplemented with 1% penicillin-streptomycin-glutamine (Gibco, Carlsbad, CA) at 32 °C with 5% CO_2 and 95% humidity.

Promoter reporter construction, transient transfection and stimulation H11 cells

Promoter reporters were cloned in the pNL1.2 backbone as described previously (Promega, Madison, WI, Cat# N1011)[26]. Transient transfection of H11 cells was performed as described in [26]. In short, H11 cells (85.000 cells/cm^2) were seeded and after overnight attachment of the cells, promoter reporters were introduced by transient transfection with plasmid DNA (3.7 pg. plasmid/cell) using Lipofectamine 2000 (Thermo Scientific) according to manufacturer's instructions. After five hours, transfection was stopped and

cells were trypsinized (Trypsin; Thermo Scientific) and re-seeded into 96-well plates (Greiner Bio-One) at a cell density of 62.500 cells/cm^2.Transient transfected cells were serum-starved for sixteen hours in DMEM/F12 supplemented with 0.5% FCS and 1% penicillin-streptomycin prior to stimulation. Serum-starved cells were stimulated with 5% OA synovium conditioned medium (OAs-cm) for 24 hours. OAs-cm was made using synovium obtained from joint capsule biopsies of OA patients undergoing total knee replacement. The synovium was divided into pieces and cultured in a six-well plate in DMEM supplemented with 0.1% BSA (0.3 g tissue/mL). Before weighing the tissue, tissue was dabbed on gauze two times to remove excessive fluid. The supernatant was collected after 24 hours. Debris was removed by 15 minutes centrifugation at 300 x *g* and medium was stored in aliquots at -20 °C until further use. OAs-cm was characterized by measuring levels of 10 different cytokines by Luminex (IL-1β, IL-1, IL-6, IL-8, IL-10, IL-17, GM-CSF, IFN-γ, MCP-1 and TNF-α; Supplemental Table 1).

Construction transcription factor reporter plasmids and virus production

Binding sequences (Table 1) specific for 14 transcription factors were extended with a minimal promoter and were synthesized by Genecust (Boynes, France). Binding sequences were directionally cloned into the pNL1.2 vector (Promega, Madison, WI). Subsequently, lentiviral constructs were generated by re-cloning pNL1.2 reporters with the In-Fusing Cloning method (TakaraBio, Kusatsu, Shiga, Japan) into the ClaI site of the pLVX-EF1α-IRES-Puro producer vector (TakaraBio). The 4th generation lentiviral production system (TakaraBio) was used to generate viral supernatants in Lenti-X HEK 293T cells (TakaraBio). 1 mg/mL polyethylenimine (PEI; Polysciences, Warrington, USA) was used to transfect viral constructs into Lenti-X 293T cells. Viral supernatant collected after 48 and 72 hours post-transfection were concentrated using ultracentrifugation (2 hours, 25000 x*g*, 4 °C). Lentiviral titer was determined by p24 ELISA (Fujirebio, Gent, Belgium).

SW1353 cell culture, stable cell line generation and stimulation

SW1353 cells (ATCC, HTB-94) were cultured in growth medium consisting of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12; ThermoFisher, Carlsbad, CA, USA) supplemented with 10% Fetal Calf Serum (FCS; Sigma, Saint Louis, Missouri, USA) and

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1% penicillin-streptomycin-glutamine (Gibco, Carlsbad, CA) in a humidified atmosphere containing 5% CO_2 at 37 °C. SW1353 reporter cell lines were generated using lentiviral transduction (250 ng virus particles/62.500 cells) with 8 µg/mL hexadimethrine bromide (Sigma) for eight hours. Two days post-transduction, cells were selected using 1 µg/mL puromycin (Sigma) and expanded for three passages before cryopreservation and stored in liquid nitrogen till further use. All 14 reporter cell lines were functionally validated with known positive stimuli (Supplementary Figure 1). SW1353 reporter cell lines were defrosted and expanded for another 2 passages before the start of the experiment. Cells were trypsinized and re-seeded (95.250 cells/cm^2) into white polystyrene 384-well plates (Greiner Bio-One, Alpen aan den Rijn, the Netherlands) and serum-starved overnight in 0.5% FCS supplemented DMEM/F12. Serum-starved cells were stimulated with 5% OAs-cm for six hours.

Reporter gene assay

After stimulation cells were lysed using either 30 µL or 15 µL ultrapure H_2O for 96-well or 384-well plates respectively. Nano-Glo (Promega) was added at 1:1 ratio to the cell lysate to measure luminescence. All luminescent measurement were performed with the CLARIOstar (BMG Labtech) at room temperature.

hMSCs cell culture, chondrogenic differentiation and incubation with OAs-cm

Human fetal bone-marrow derived MSCs (n=1, sex unknown) were purchased from ScienCell Research Laboratories (Carlsbad, CA). Passage 2 stem cells were expanded for 12 days in Mesenchymal Stem Cell Growth Medium (MSCGM™; Lonza, Basel, Switzerland) supplemented with 1% penicillin-streptomycin-glutamine (Gibco, Carlsbad, CA). Cells were cultured at 37 °C with 5% CO_2 and 95% humidity. After four passages, the MSCs were stored in liquid nitrogen. Per experiment, cells were defrosted and expanded in MSCGM for another 2 passages before start of the pellet experiment. MSCs were cultured in high cell density pellets by centrifugation of 200.000 cells at 300 x *g* for 8 minutes in polystyrene V-bottom tubes (Greiner Bio-One, Alphen a/d Rijn, Netherlands). Pellets were cultured for 14 days in 0.5 mL of serum-free chondrogenic medium, consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 1% penicillin-streptomycin (Gibco), insulin (6.25 µg/mL), transferrin (6.25 µg/mL) sodium selenite (6.25

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ng/mL), proline (0.4 mg/mL), sodium pyruvate (1 mg/mL), linoleic acid (5.35 µg/mL), ascorbic acid (50 µg/mL) and bovine serum albumin (1 mg/mL; all from Sigma-Aldrich). The growth factors transforming growth factor beta 1 (TGFβ1; 10 ng/mL) and bone morphogenetic protein 2 (BMP2; 20 ng/mL; both R&D Systems, Minneapolis, MN) were added during each medium refreshment (thrice a week)[26]. After 10 days of MSC differentiation, 2% OAs-cm was added from different donors (number of donors is specified per experiment; in total n=52). 2% OAs-cm was used as this induced at least 50% reduction in sGAG content of MSC pellets in previous experiments[28]. The media of stem cells pellets was refreshed at day 12 and for each medium refreshment a different OAs-cm aliquot was used. At day 14, pellets were harvested for measurement of sGAG content and/or histology.

Glycosaminoglycan measurement

Each individual pellet was digested overnight with 0.1% (w/v) papain (Sigma-Aldrich) in 100 µL papain digestion buffer (200 mM $NaPO_4$, 100 mM NaOAc, 10 mM cysteine-HCl, 50 mM EDTA; pH 6.0) at 60 °C in a 0.5 mL Eppendorf tube. Samples were diluted in ultrapure water depending on amount of sGAG content (1:4 or 1:6) to measure sulfated GAG content using the Farndale assay [29]. Hereby, 200 µL of dimethylmethylene blue (DMB) solution (0.05 mM DMB, 41 mM NaCl, 45 mM glycine; pH 3.0) was added to 40 µL papain-digested sample. The absorbance was measured at λ=595 nm using a spectrophotometer (iMark Reader; Bio-Rad). Chondroitin sulfate sodium salt from shark cartilage (Sigma-Aldrich) was used for generation of a standard curve.

Histology

Pellets were fixed in formalin (4%) overnight, dehydrated, and embedded in paraffin. Tissue sections of 5 µm were prepared. To visualize sGAGs, sections were stained with 0.1% aqueous safranin-O and counterstained with 0.1% aqueous fast green (both Brunschwig Chemie, Amsterdam, the Netherlands).

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Data analysis

Luciferase reporter measurement were investigated in quadruple and MSC chondrogenic differentiation experiments were performed in triplicate. Fold change data from reporter measurements were calculated by consecutively subtracting the background signal and normalizing to the mean of the unstimulated negative control conditions. In Figure 3A, data are normalized to percentage of positive control. To quantify the degree to which two variables were related (reporters and reduction of sGAG content) correlations were calculated and p-values were corrected for multiple testing using the Bonferroni multiple comparison test, which were considered statistically significant when $p \leq 0.003125$ (GraphPad Prism 5.03). All data were presented as mean of three replicates ± SD.

To test the association between the two continuous variables sGAG inhibition and fold change of promoter reporters the Pearson's correlation coefficient was determined (Two-tailed). The 95% prediction band of the best-fit line is represented in the graphs. Statistical differences were considered as significant if $p \leq 0.05$.

To determine which reporters would contribute substantially to predicting the effect of OAs-cm on cartilage repair we performed Multiple Regression analysis using the Forward method (IBM SPSS Statistics 25).

Results

Effect OAs-cm on MSC cartilage formation correlates with the effect of OAs-cm on transcription factor reporters

We previously developed a bioassay that can predict the effect of the OA joint microenvironment on MSC-based cartilage formation with an accuracy of 87.5% [26]. Here, we investigated if we could increase prediction accuracy by changing from the complex gene promoter reporters used previously to transcription factor binding site-based reporters. First, we validated the TF reporters with known positive stimuli (Supplementary Figure 1)[30-34]. All fourteen TF reporters were significantly upregulated by known positive stimuli. Next, we examined if the effect of OAs-cm (n=31) on TF reporter activity (fold change compared to control condition) correlated with their effect on MSC-based *in vitro* cartilage formation (reduction in sGAG content compared to control) (Figure 1A). Six out of fourteen TF reporters revealed significant (strong) correlations between the two

parameters (AP1, ARE, ISRE, NFAT5, NFκB and SRE; Figure 1B). Of these six reporters that did correlate we continued without ARE, because the response window of this reporter was very low (max. fold change of 1.3). The other eight TF reporters showed no significant correlations (CRE, GRE, PPRE, SBE, SIE, SOX9, SRF and TCF/LEF; Supplementary Figure 2).

Transcription factor reporter responses can predict the effect of individual OAs-cm on MSC in vitro cartilage formation

To investigate the predictive value of the five selected TF reporters, we tested a second independent cohort of OAs-cm samples (n=22) on the TF reporters. All five TF reporters revealed variation in response between individual OAs-cm (Figure 2A). For example, the ISRE reporter showed a range between fold change 1.1 and 4.7 (SD ± 1.1). Based on the correlations in Figure 1, we formulated an expectation of the effect of the various OAs-cm on MSC-based *in vitro* cartilage formation via linear approximation (Figure 2B). To investigate if these predictions were accurate, we differentiated MSCs to chondrocytes in a 3D pellet model and incubated these pellets with the same OAs-cm. The sGAG content differed from 28 µg/pellet in the control condition to 6 µg/pellet when incubated with certain OAs-cm (Figure 2C). In addition, on histological level the difference in sGAG could also be clearly observed (Figure 2D). Pellets with lower sGAG content were smaller and had a less intense red staining. A reliable prediction was defined as follows: the outcome of the tested OAs-cm should be within the 95% prediction interval of the linear approximation that was constructed from the first cohort of OAs-cm. The AP1 and NFκB TF reporters correctly predicted 100% of the data points (Figure 3A; red dots; n=22). The SRE and ISRE TF reporters predicted respectively 86% and 82% of the data points correctly, while the NFAT5 TF reporter reached 73% prediction accuracy. Another method to determine if the model bestows a reliable prediction, is the correlation coefficient between the predicted values and the actual measured values (blue squares versus red dots; Figure 3A). There was a significant correlation between the predicted values and the actual measured values for AP1 (R square=0.78), NFκB (R square=0.84) and ISRE (R square=0.82), whereas there was no significant correlation for the SRE reporter (R square=0.16; Figure 3B). The NFAT5 did not have a linear relation between the predicted values and the actual measured values and therefore was left out of further analysis.

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Bioassay consisting of a combination of transcription factor binding element reporter and gene promoter reporter is the best predictive model for the effect of OAs-cm on MSC in vitro cartilage formation

To compare the TF reporters with the gene promoter reporters, we also investigated how accurate the gene promoter reporters could predict the effect of the OAs-cm on MSC-based cartilage formation (Figure 3C). Both the hWISP1 and the hIL6 gene promoter reporter predicted the effect of OAs-cm on MSC-based cartilage formation with an accuracy of 95% (Figure 3C). To establish the most reliable bioassay for predicting the effect of individual OAs-cm on MSC *in vitro* cartilage repair, we investigated which reporters contribute substantially to the ability of the assay to predict the effect of OAs-cm on cartilage formation. For this, we used multiple regression analysis in which reporters are added one-by-one to the regression calculation (starting with the largest significant predictor of the dependent variable). This resulted in a total of 6 different possible models (Table 2). Measuring the NFκB reporter alone results in an Adjusted R Square of 0.742 with an standard deviation error of the estimate with 10.51. Adding the IL6 reporter significantly increased the Adjusted R Square to 0.795 and reduced the standard deviation error of the estimate to 9.36. Further expanding the model with other reporters did not have a significant improving effect on the Adjusted R Square and thus does not contribute to a more accurate prediction model. In summary, the combination of the NFκB TF reporter together with the IL6 gene promoter reporter is the best predictive luciferase reporter model for the effect of OAs-cm on MSC-based *in vitro* cartilage formation, as the prediction accuracy is 100% and together they have the highest Adjusted R Square.

Discussion

MSCs could be used for the repair of articular cartilage defects, however the outcome of treatment strategies that use MSCs is unsatisfactory and variable between patients. The joint microenvironment is a major contributor to the success of cartilage repair strategies [14, 17, 19, 35]. Here, we aimed to improve a previously developed molecular tool that can be used to determine the effect of the OA joint microenvironment on MSC-based cartilage repair. We evaluated the use of TF reporters instead of gene promoter reporters for our diagnostic tool. From the fourteen TF reporters evaluated in this study, six correlated

significantly with the effect of OAs-cm on *in vitro* cartilage formation. Based on the TF reporter response of a new cohort of OAs-cm, predictions about the effect of the OAs-cm on cartilage repair were constructed. Next, the accuracy of these predictions were experimentally evaluated and the NFκB, AP1 and ISRE TF reporters exhibited the highest prediction accuracy. To determine if the current tool was better than the previous tool we compared the two, however only a very small difference could be observed between the prediction accuracy between the tools [26]. Subsequently, we established that a combination of the TF reporter together with a gene promoter reporter resulted in the highest prediction certainty. In summary, we have improved our molecular tool and are therefore a step closer to a diagnostic tool that can be used to subdivide patients with OA based on responsiveness to MSC-based treatment strategies.

Here, we investigated if we could augment the prediction accuracy of our molecular tool by changing the response elements to transcription factor binding elements instead of gene promoters [26]. Gene promoter reporters are complex, consisting of multiple transcription factor binding elements together, which can either activate or repress transcription [36]. Furthermore, gene promoters can also be regulated by cis- and trans-acting elements hundreds to thousands base pairs from the transcription start site by e.g. enhancers or silencers [36, 37]. Only a limited part of the native gene promoter is used in our gene promoter reporters and therefore these can miss the genomic context. An advantage of using TF reporters is their simplicity. A transcription factor is the endpoint of a signalling pathway and thus individual pathways can be identified that are activated upon stimulation with OAs-cm [38].

The local joint microenvironment is a major determinant for the success of MSC-based cartilage repair strategies [14]. It is known that inflammatory factors, like IL1β, can inhibit MSC chondrogenesis [35]. Therefore, cartilage repair by MSCs can be compromised in inflamed joints. OA is a very heterogenous disease and the local joint microenvironment can differ enormously within OA knee joint patients [39]. In multiple animal studies, MSC injection resulted in cartilage repair [40]. However, limited studies have been performed in humans and the results are not consistent [13, 41-43]. Our diagnostic tool could help to predict patients as responders or non-responders. Adjusting the joint microenvironment to be permissive for cartilage repair, by co-injection of MSCs with certain growth factors or

cytokines, could also be a possible option. A cell-based bioassay has a main advantage due to its ability to integrate multiple signals, which is similar to the *in vivo* situation. Our reporter assay receives all signals that are present in the OAs-cm and integrates these signals to a single output. It therefore gives a more complete picture of the different processes that play a role/are activated in an inflamed joint compared to a single cytokine. Future work using the developed TF reporters could shed light on the molecular mechanisms behind the variable effect of the OA microenvironment on MSC cartilage formation and could help define which cofactors are needed for successful MSC-based cartilage repair. Besides, a large effort has recently been made on identifying OA endotypes, meaning stratifying OA patients based on the underlying pathophysiological mechanisms that trigger the disease [44]. Patient stratifications based on endotyping has been successful in other diseases, such as atopic dermatitis, asthma, COPD and allergic diseases [45-47]. The use of TF reporters could aid the search for OA endotypes as this can help identify different intracellular signalling pathways activated during the disease. However, SW1353 cells have been shown to respond differently to IL1β than primary human articular chondrocytes, therefore results should be validated in model systems closer to the *in vivo* situation [48].

A combination of the NFκB TF reporter and the IL6 gene promoter reporter proved to reach the best prediction accuracy (up to 100%) and the highest Adjusted R Square for the effect of OAs-cm on MSC-based *in vitro* cartilage formation. This prediction model analysis assumes a linear distribution, which was observed for most of the reporters (NFAT5 excluded). For a diagnostic tool in the clinic, an on/off readout would be preferred as this circumvents misjudgment of the results. However, such an on/off readout is not clearly present in our current tool and sucha cutoff point has to be determined clically.. This will require studies where patients will be tested with our bioassay before receiving MSC-based treatment (for cartilage repair) and prediction accuracy *in vivo* can then be determined.

To create an OA microenvironment, we incubated end-stage knee OA synovium for 24 hours in medium to create OA synovium conditioned medium. This OAs-cm is a complex mixture of cytokines, chemokines and growth factors that are secreted by the synovium derived from OA patients. The synovium is an important contributor to the OA

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microenvironment, as it is known to secrete catabolic and inflammatory mediators into the synovial fluid [49]. Most studies have only investigated the effect of a single cytokine on chondrogenesis and the OAs-cm we used in this study is a complex mixture of factors, such as IL6, IL8, TNFα and IL1β [21, 50]. These factors are also present in the synovial fluid of OA patients [20, 51]. However, despite the similarities between OAs-cm and synovial fluid, the composition of synovial fluid is determined by more than the synovium alone. The composition of the synovial fluid is also determined by other adjacent joint tissues, such as the cartilage, the infrapatellar fat pad and meniscus [49]. Furthermore, synovial fluid is also formed by ultrafiltration of plasma [52]. Future work should aim at the further development of this tool in combination with synovial fluid from OA patients as this does not require a synovium biopsy. We have previously shown that OA synovial fluid can be used in combination with our bioassay [25 53], however we have not investigated the correlation between MSC cartilage formation and synovial fluid reporter responses. To evaluate if our bioassay can also predict the effect of MSC-based therapies for individual patients, joint fluids should be collected before treatment and bioassay results coupled to cartilage repair outcome.

Conclusion

To conclude,we improved our diagnostic tool that can be used to predict the effect of the joint microenvironment on MSC-based cartilage formation by using TF reporters. A combination of the NFκB TF reporter and the IL6 gene promoter reporter is the best predictive model. The improvement of this tool is an important step towards a diagnostic tool that can be used for personalized cartilage repair strategies for OA patients.

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Author contributions

Conception and design: M.N., B.A.C.H., G.G.H.A., T.J.M.W., A.P.M.V.C. and P.M.V.D.K. Collection and acquisition of data: M.N., N.G.M.T., H.M.V.B, E.L.V and B.A.C.H. Analysis and interpretation of data: M.N., N.G.M.T., B.A.C.H., G.G.H.A., T.J.M.W., A.P.M.V.C. and P.M.V.D.K. Drafting of the article: M.N. Critical revision: M.N., B.A.C.H., G.G.H.A., T.J.M.W., A.P.M.V.C. and P.M.V.D.K. Final approval of the article: M.N., B.A.C.H., N.G.M.T., H.M.V.B, E.L.V, G.G.H.A., T.J.M.W., A.P.M.V.C. and P.M.V.D.K.

Author disclosure statement

The authors declare no competing interest.

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Table 1. Transcription factor reporter element sequences. Underlined is the minimal promoter including a TATA-box, which is the same for every sequence.

Transcription factor element	Sequence
AP1	TGAGTCAGTGACTCAGTGAGTCAGTGACTCAGTGAGTCAGTGACTCAGCTCGAGGATATCAAGATCTGGCCTCGGCGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAG
ARE	TAGCTTGGAAATGACATTGCTAATGGTGACAAAGCAACTTTTAGCTTGGAAATGACATTGCTAATGGTGACAAAGCAACTTTCTCGAGGATATCAAGATCTGGCCTCGGCGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAG
CRE	TGACGTCAGCTGCCAGATCCCATGGCCGTCATACTGTGACGTCTTTCAGACACCCCATTGACGTCAATGGGAGAACAGATCTGGCCTCGGCGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAG
GRE	AGAACATTTTGTCCGAGAACATTTTGTCCGAGAACATTTTGTCCGAGAACATTTTGTCCGAGAACATTTTGTCCGCTCGAGGATATCAAGATCTGGCCTCGGCGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAG
ISRE	TAGTTTCACTTTCCCTAGTTTCACTTTCCCTAGTTTCACTTTCCCTAGTTTCACTTTCCCTATTTCACTTTCCCCTCGAGGATATCAAGATCTGGCCTCGGCGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAG
NFAT5	TGGAAAAGTCCATGGAAAAGTCCATGGAAAAGTCCATGGAAAAGTCCATGGAAAAGTCCATGGAAAAGTCCATGGAAAAGTCCACTCGAGGATATCAAGATCTGGCCTCGGCGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAG
NFκB	GGGAATTTCCGGGGACTTTCCGGGAATTTCCGGGGACTTTCCGGGAATTTCCAGATCTGGCCTCGGCGGCCTAGATGAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAG
PPRE	GTCGACAGGGGACCAGGACAAAGGTCACGTTCGGGAGTCGACAGGGGACCAGGACAAAGGTCACGTTCGGGAGTCGACAGGGGACCAGGACAAAGGTCACGTTCGGGAGTCGACCTCGAGGATATCAAGATCTGGCCTCGGCGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAG

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SBE	AGTATGTCTAGACTGAAGTATGTCTAGACTGAAGTATGTCTAGACTGACTCGAGGATATCAAGATCTGGCCTCGGCGGCCTAGATGAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAG
SIE	AGCTTCATTTCCCGTAAATCGTCGAAGCTTCATTTCCCGTAAATCGTCGAAGCTTCATTTCCCGTAAATCGTCGAAGCTTCATTTCCCGTAAATCGTCGAAGCTTCATTTCCCGTAAATCGTCGACTCGAGGATATCAAGATCTGGCCTCGGCGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAG
SOX9	AGAACAATGGAGAACAATGGAGAACAATGGAGAACAATGGAGAACAATGGAGAACAATGGAGAACAATGGCTCGAGGATATCAAGATCTGGCCTCGGCGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAG
SRE	AGGATGTCCATATTAGGACATCTAGGATGTCCATATTAGGACATCTAGGATGTCCATATTAGGACATCTAGGATGTCCATATTAGGACATCTAGATCTGGCCTCGGCGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAG
SRF	AGTATGTCCATATTAGGACATCTACCATGTCCATATTAGGACATCTACTATGTCCATATTAGGACATCTTGTATGTCCATATTAGGACATCTAAAATGTCCATATTAGGACATCTAGATCTGGCCTCGGCGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAG
TCF/LEF	AGATCAAAGGGTTTAAGATCAAAGGGCTTAAGATCAAAGGGTATAAGATCAAAGGGCCTAAGATCAAAGGGACTAAGATCAAAGGGTTTAAGATCAAAGGGCTTAAGATCAAAGGGCCTACTCGAGGATATCAAGATCTGGCCTCGGCGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAG

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Table 2. Results Multiple Linear Regression analysis. The multiple linear regression analysis allows you to determine the overall fit of the model and the relative contribution of each of the predictors to the total variance explained. Model #1: NFκB. model #2: NFκB+IL6. model #3: NFκB+IL6+AP1. model #4: NFκB+IL6+AP1+SRE. model #5: NFκB+IL6+ AP1+SRE+WISP1. model #6: NFκB+IL6+ AP1+SRE+WISP1+ISRE.

Model	R Square	Adjusted R Square	Std. Error of the Estimate	Sig. F Change
1	0.747	0.742	10.51	7,63E-17
2	0.803	0.795	9.36	0.000432
3	0.808	0.796	9.33	0.265
4	0.811	0.795	9.38	0.448
5	0.812	0.792	9.44	0.534
6	0.812	0.788	9.53	0.768

Figure legends

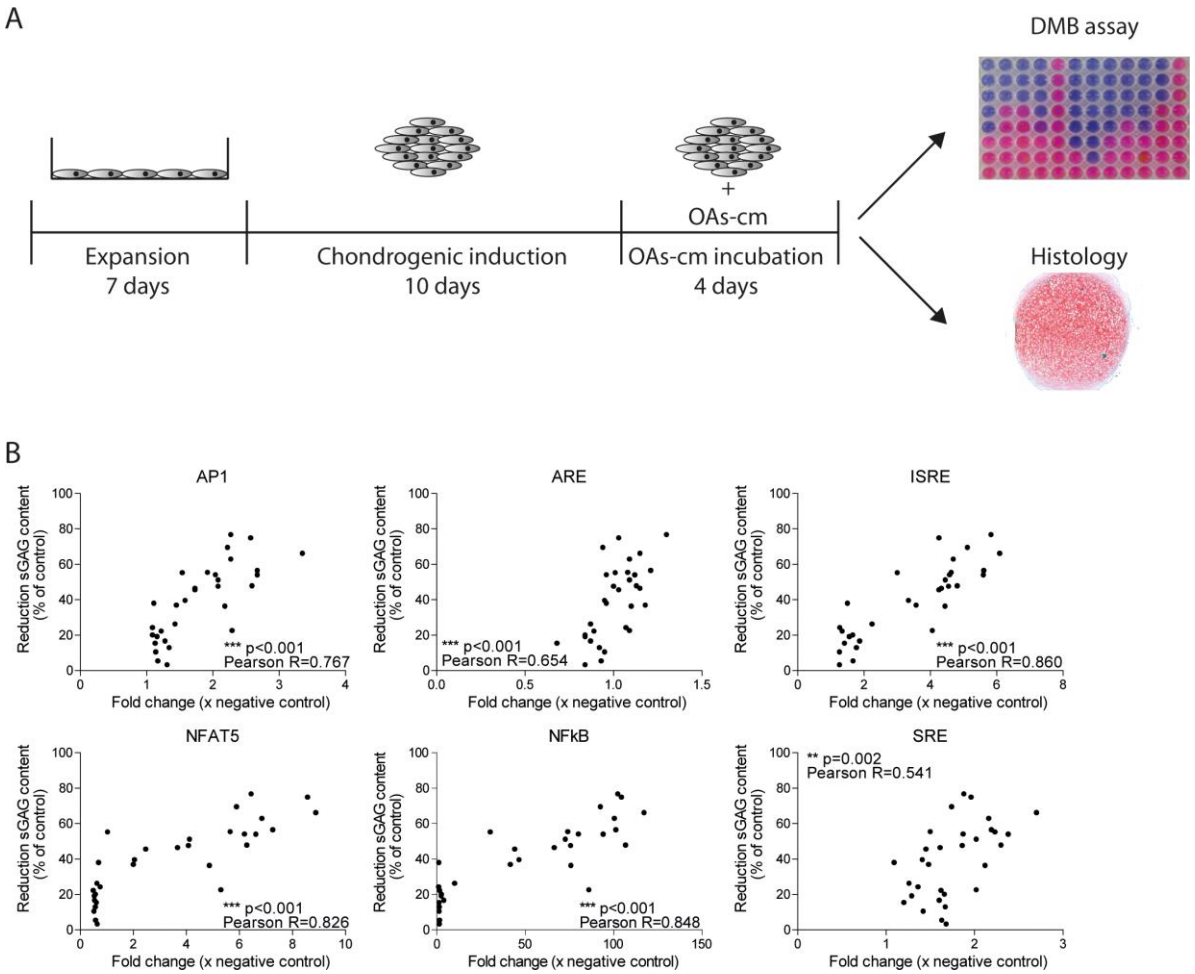

Figure 1. Fold change transcription factor reporters correlates with reduction of sGAG content. (A) Schematic depiction of experimental design 3D pellet culture exposed to OAs-cm from individual donors. (B) Fold change of TF reporters (stable SW1353 cell lines) after 6 hour stimulation with OAs-cm (5%; n=31) significantly correlated (AP1, ARE, ISRE, NFAT5, NFκB and SRE) to the reduction in sGAG content of MSCs stimulated with the same OAs-cm (2%). sGAG content in unstimulated control was set at 100% and reduction in sGAG content was calculated compared to this. To test the association between the two continuous variables reduction in sGAG production and fold change of TF reporters the Pearson's correlation coefficient was determined (Two-tailed). ** p. value ≤0.01, *** p. value ≤0.001. sGAG, sulfated glycosaminoglycan; MSC, mesenchymal stromal cell; OAs-cm, osteoarthritis synovium-conditioned medium.

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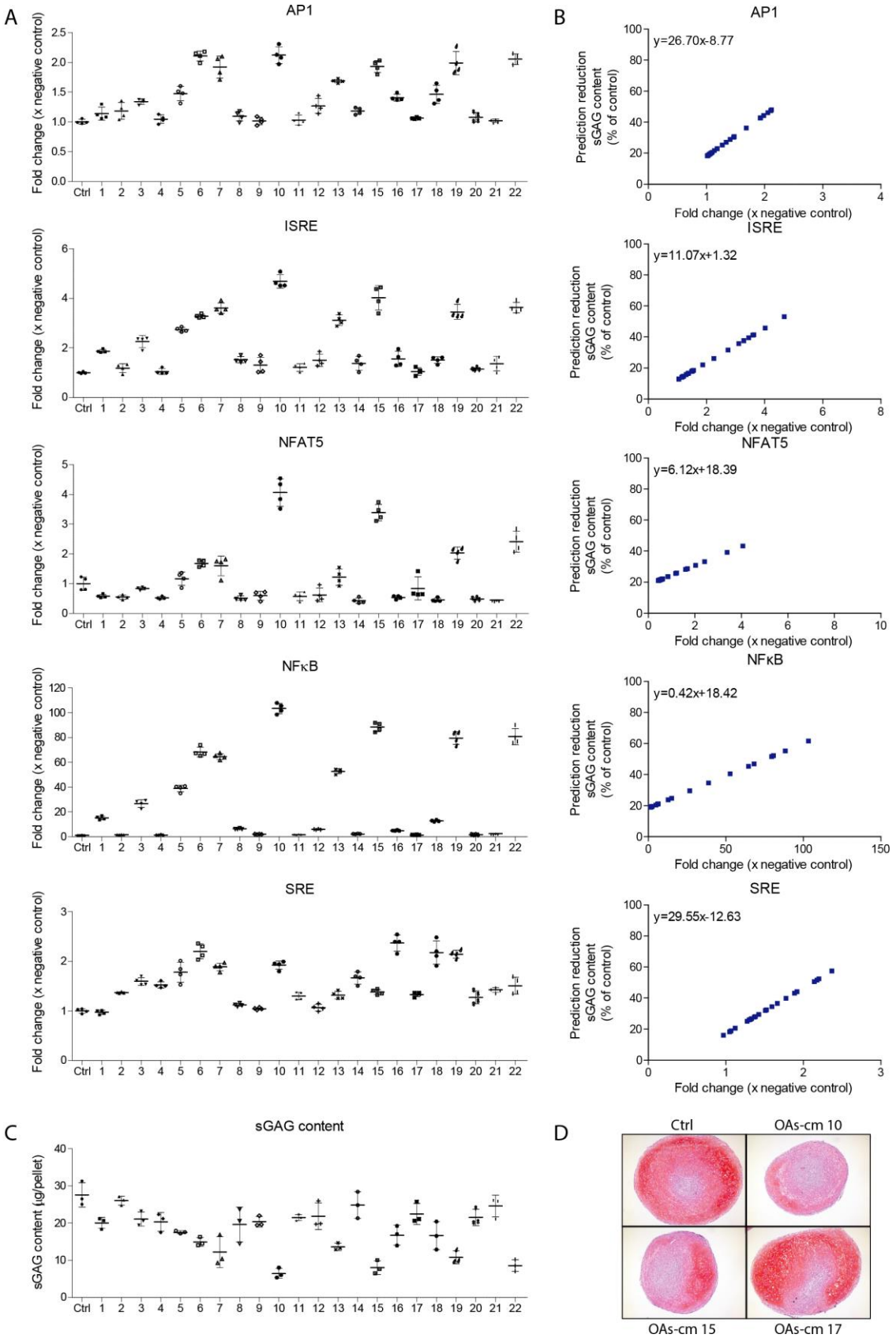

Figure 2. Prediction of the effect of OAs-cm on MSC cartilage formation. (A) A second, independent cohort of OAs-cm donors (n=22) was tested on the TF reporters (stable

SW1353 cell lines) that showed a significant correlation in Figure 1 (AP1, ISRE, NFAT5, NFκB and SRE). TF reporter cell lines were stimulated with OAs-cm of individual donors (5%) for 6 hours. (B) The fold change of the reporters was used to make a prediction on the effect of the same OAs-cm on the reduction of sGAG content. This prediction was based on the significant correlations plotted in Figure 1. (C) Quantification of sGAG content of MSC pellets at day 14 of differentiation after stimulation with the same OAs-cm donors (2%). (D) Histological examination of sGAG production, by safranin-O/fast green staining, of differentiated MSC pellets at day 14 after stimulation with OAs-cm (2%) for 4 days (100x magnification). Representative pictures are presented. sGAG, sulfated glycosaminoglycan; MSC, mesenchymal stromal cell; OAs-cm, osteoarthritis synovium-conditioned medium.

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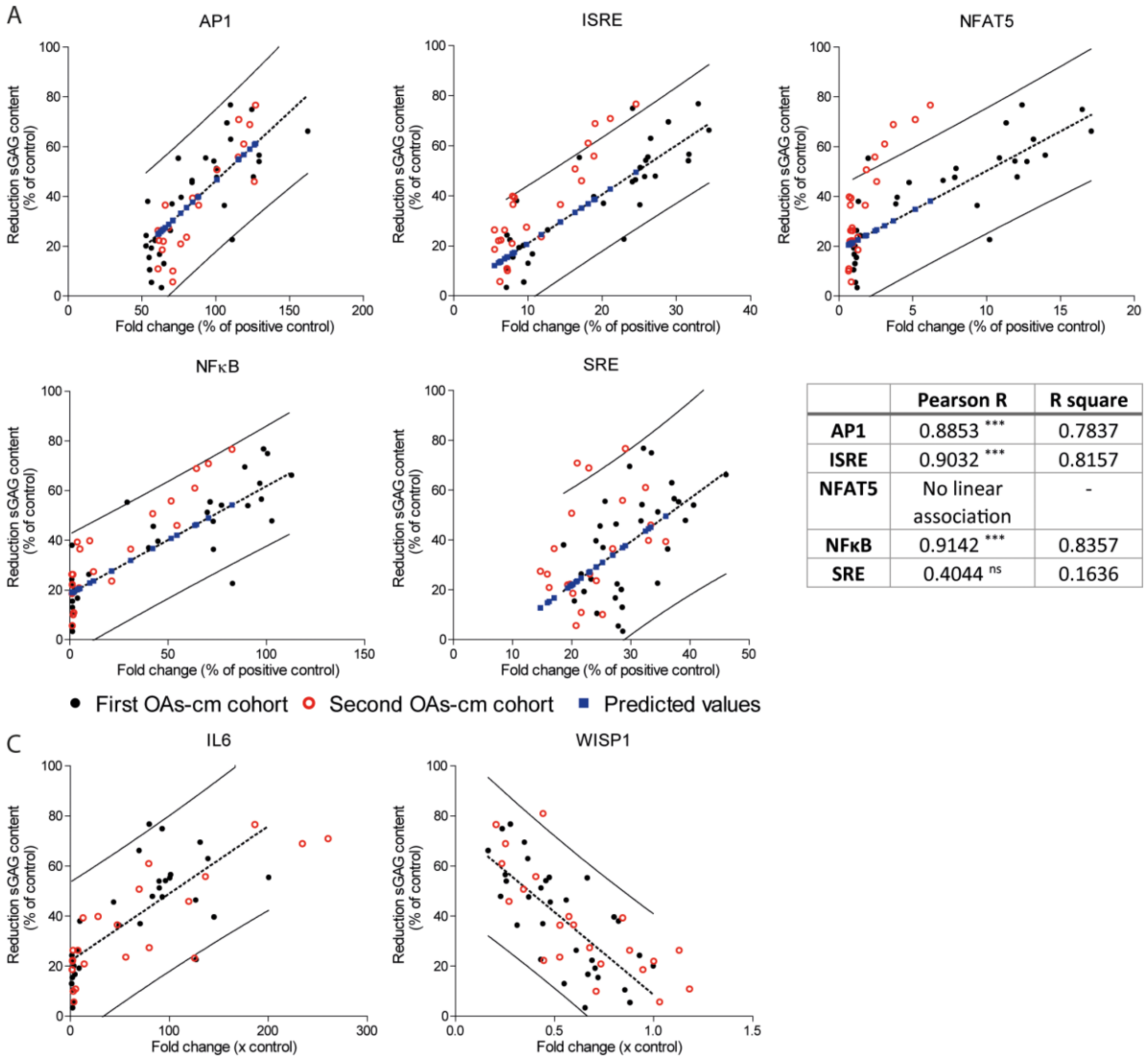

	Pearson R	R square
AP1	0.8853 ***	0.7837
ISRE	0.9032 ***	0.8157
NFAT5	No linear association	-
NFκB	0.9142 ***	0.8357
SRE	0.4044 ns	0.1636

Figure 3. Validation of the predictive capacity of our bioassay. (A) Fold change of the TF reporters (AP1, ISRE, NFAT5, NFκB and SRE) after 6 hour stimulation with the second independent cohort of OAs-cm (n=22; 5%; red dots) was correlated to the reduction in sGAG production of MSCs stimulated with the same OAs-cm (2%). Fold change was determined as percentage of positive control as this figure comprises data of different experiments (n=3). Data was plotted in the corresponding correlation graphs constructed with the first cohort of OAs-cm (black dots). Each dot represents the average of three biological replicates in both the fold change as well as the sGAG experiment. The predicted outcomes (blue squares) were also plotted in the graphs. (B) Pearson R and R square of the correlation between the predicted values (blue squares) and the actual measured values (red dots). (C) Fold change of the promoter reporters hIL6 and hWISP1 after 24 hour stimulation with the second independent cohort of OAs-cm (n=22; 5%; red dots) in H11

chondrocytes was correlated to the reduction in sGAG production of MSCs stimulated with the same OAs-cm (2%). Data was plotted in the corresponding correlation graphs constructed with the first cohort of OAs-cm (black dots). Each dot represents the average of three biological replicates in both the fold change as well as the sGAG experiment (black dots n=3, red dots n=1). *** p. value ≤0.001, ns=not significant. sGAG, sulfated glycosaminoglycan; MSC, mesenchymal stromal cell; OAs-cm, osteoarthritis synovium-conditioned medium.

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