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SODIUM HYALURONATE-SUPPLEMENTED CULTURE MEDIUM COMBINED WITH JOINT-SIMULATING MECHANICAL LOADING IMPROVES CHONDROGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS

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Abstract

In vitro models aim to recapitulate the *in vivo* situation. To more closely mimic the knee joint environment, current *in vitro* models need improvements to reflect the complexity of the native tissue. High molecular weight hyaluronan (hMwt HA) is one of the most abundant bioactive macromolecules in healthy synovial fluid, while shear and dynamic compression are two joint-relevant mechanical forces.

The present study aimed at investigating the concomitant effect of joint-simulating mechanical loading (JSML) and hMwt HA-supplemented culture medium on the chondrogenic differentiation of primary human bone-marrow-derived mesenchymal stem cells (hBM-MSCs). hBM-MSC chondrogenesis was investigated over 28 d at the gene expression level and total DNA, sulphated glycosaminoglycan, TGF-β1 production and safranin O staining were evaluated.

The concomitant effect of hMwt HA culture medium and JSML significantly increased cartilage-like matrix deposition and sulphated glycosaminoglycan synthesis, especially during early chondrogenesis. A stabilisation of the hBM-MSC-derived chondrocyte phenotype was observed through the reduced upregulation of the hypertrophic marker collagen X and an increase in the chondrogenic collagen type II/X ratio.

A combination of JSML and hMwt HA medium better reflects the complexity of the *in vivo* synovial joint environment. Thus, JSML and hMwt HA medium will be two important features for joint-related culture models to more accurately predict the *in vivo* outcome, therefore reducing the need for animal studies. Reducing *in vitro* artefacts would enable a more reliable prescreening of potential cartilage repair therapies.

Keywords: Hyaluronic acid, mesenchymal stem cells, chondrogenic differentiation, articular cartilage, hypertrophy, joint simulating bioreactor, mechanical loading, *in vitro* model, culture medium, TGF-β1.

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List of Abbreviations

ACAN	aggrecan
ADAMTS	ADAM metallopeptidase with thrombospondin type 1 motif
ALP	alkaline phosphatase
ANOVA	analysis of variance
bFGF	basic fibroblast growth factor
CD	cluster of differentiation
COL2A1	collagen type 2
COL10A1	collagen type 10
COMP	cartilage oligomeric matrix protein
DMMB	dimethylmethylene blue
ECM	extracellular matrix
ELISA	enzyme-linked immunosorben assay
EMA	European Medicines Agency
FBS	foetal bovine serum
FDA	Food and Drug Administration
HA	hyaluronic acid
hBM-MSCs	human bone marrow-derived mesenchymal stem cells
hMwt HA	high molecular weight HA
MMP	matrix metallopeptidase
hMSCs	human mesenchymal stem cells
PRG4	proteoglycan 4
RHAMM	hyaluronan-mediated motility receptor

RPLP0	ribosomal protein large P0 housekeeping gene
RUNX2	runt-related transcription factor 2
SD	standard deviation
sGAG	sulphated glycosaminoglycans
SOX9	SRY-box 9 cartilage transcription factor
TGF	transforming growth factor
αMEM	alpha minimum essential medium

Introduction

Trauma, osteoarthritis and osteochondritis are the most common causes of cartilage damage, leading to pain, swelling and impaired movement of the joint (Madry *et al.*, 2011). The demand for effective treatment strategies for cartilage lesions is continually increasing. However, current therapies have considerable limitations, prompting the development of novel cartilage tissue-engineering approaches.

Autologous chondrocytes implantation and matrix-assisted autologous chondrocytes implantation have been approved by the FDA and by the EMA as advanced-therapy medicinal products (Brittberg, 2010; Brittberg *et al.*, 1994; Makris *et al.*, 2015). However, for both treatments, donor site morbidity due to the required cartilage biopsy remains an issue, as does the small size of harvestable cartilage that is associated with a low chondrocyte yield and limited *in vitro* expansion potential (Brittberg, 2010; Brittberg *et al.*, 1994; Erggelet *et al.*, 2003; Knecht *et al.*, 2007). In addition, the need to expand chondrocytes in monolayer increases the risk of dedifferentiation into fibroblastic cells (Benya and Shaffer, 1982; Hegewald *et al.*, 2004).

Due to the limited supply of autologous chondrocytes for transplantation procedures, much attention has focused on MSCs, which are also the cells involved in the regeneration processes when microfracture, a leading surgical technique for healing chondral defect, is used (Kang *et al.*, 2008; Oussedik *et al.*, 2015; Steadman *et al.*, 2001). Particularly, hBM-MSCs represent an attractive alternative cell source to autologous chondrocytes since they can be easily isolated from bone marrow aspirates with limited donor site morbidity and, following expansion, they continue to maintain multilineage potential (Gardner *et al.*, 2013; Hegewald *et al.*, 2004). Indeed, hBM-MSCs are well investigated in the clinical setting, are the best characterised and can be used for subchondral bone and overlying articular cartilage repair (de Vries-van Melle *et al.*, 2014; Nejadnik *et al.*, 2010; Parekkadan and Milwid, 2010; Wakitani *et al.*, 1994; Wakitani *et al.*, 2002). However, one of the main challenges of *in vitro* hBM-MSC chondrogenic differentiation is the lack of a suitable culture environment that reproduces the *in vivo* physiological conditions and, in so doing, prevents or reduces the progression of MSC-derived chondrocytes through hypertrophic differentiation, which causes the neo-formed cartilage to undergo endochondral ossification (Johnstone *et al.*, 1998).

Due to the existing discrepancies, current *in vitro* models need to be improved to reflect the complexity of the joint environment found *in vivo*, thus aiming to reduce the gap between *in vitro* and *in vivo* results. More accurate *in vitro* models will be crucial to prevent *in vitro* artefacts and to produce more reliable results, enabling more accurate *in vitro* prescreening of potential cartilage repair therapies.

To improve the current *in vitro* models, characteristic features that would help mimic the native tissue need to be introduced into the culture system. For this purpose, it is necessary to consider that articular cartilage or hyaline cartilage, due to its unique molecular composition and structure, plays an essential role in joint lubrication and impact absorption lining the articulating surface of bones (Hosseini *et al.*, 2014; Responte *et al.*, 2007; Sophia Fox *et al.*, 2009; Wu and Ferguson, 2017). During joint articulation, mechanical cues profoundly affect cell and tissue responses influencing the homeostasis of healthy tissue or leading to degeneration of articular cartilage (Bader *et al.*, 2011; Grodzinsky *et al.*, 2000; Sophia Fox *et al.*, 2009). Therefore, several studies aim to clarify the mechanisms involved in cell response under loading conditions.

hBM-MSCs have been utilised in multiple mechanical loading studies (Schatti *et al.*, 2011; Schumann *et al.*, 2006). Indeed, complex multiaxial load *in vitro*, by mimicking the mechanical motion of an articulating joint, induces gene expression and protein production of endogenous TGF-β1 and TGF-β3, key molecules that address the chondrogenic differentiation of hBM-MSCs (Li *et al.*, 2010b). In addition, mechanical loading not only induces an increased TGF-β expression but also directly leads to the activation of latent endogenous TGF-β (Li *et al.*, 2010). Therefore, mechanical loading enhances MSC chondrogenic differentiation by resembling the *in vivo* conditions and inducing a more physiological production and activation of TGF-β by hBM-MSCs.

hMwt HA (10^5-10^7 Da), one of the main components of a healthy synovial fluid and cartilage ECM, plays an important role when mechanical loading is applied, mainly by protecting the opposing articular cartilage surfaces, improving joint lubrication and acting as a shock absorber (Hegewald *et al.*, 2004). On the other hand, HA that resides in the synovial fluid is also involved in nutrient and waste transport towards and from the cartilage tissue and is able to maintain the water homeostasis inside the joint due to its excellent osmotic buffering property (Laurent *et al.*, 1996; Lynch *et al.*, 1998; McDonald and Levick, 1995). HA, as well as synovial fluid, can induce *in vitro* chondrogenic differentiation in chicken limb bud bioassays (Kujawa *et al.*, 1986; Maleski and Knudson, 1996; Rodrigo *et al.*, 1995). In addition, during microfracture treatment, MSCs that reside in the bone marrow cavity of the subchondral bone are

exposed to HA contained within the synovial fluid (Kang *et al.*, 2008; Oussedik *et al.*, 2015; Steadman *et al.*, 2001). Therefore, HA has been extensively used as target molecule for scaffold or hydrogel manufacturing, alone or in combination with MSCs, to reproduce engineered cartilage with native structure or to enhance the repair of osteochondral defects (Gallo *et al.*, 2019; Huerta-Ángeles *et al.*, 2018; Li *et al.*, 2018; Radice *et al.*, 2000; Solchaga *et al.*, 2000). However, few studies have described the use of HA as an exogenous medium supplement to promote the chondrogenic differentiation of MSCs (Hegewald *et al.*, 2004; Monaco *et al.*, 2020).

The present study hypothesises a potential beneficial synergistic effect on chondrogenic differentiation of hBM-MSCs during joint-simulating mechanical loading in the presence of hMwt HA -supplemented culture media. The purpose of the present study was to determine if a physiological concentration (2 mg/mL) of exogenous hMwt HA 1.8 MDa administered as a medium supplement in chondropermissive culture media, combined with joint-simulating mechanical loading, would allow a more physiologically driven chondrogenic differentiation of MSCs seeded within polyurethane-fibrin-based construct (Fig. 1). By including these factors in the *in vitro* culture model, a culture system was created that better reflected the complexity of the *in vivo* joint environment. The newly developed culture system, by better approximating the *in vivo* situation, would also offer more reliable results for the screening of potential cartilage repair therapies.

Materials and Methods

Poly(ester-urethane) scaffolds preparation

Poly(ester-urethane) porous sponges were prepared using hexamethylene diisocyanate, poly (1-caprolactone) diol and isosorbide diol (1,4: 3,6-dianhydro-D-sorbitol) through a salt leaching-phase inverse technique (Gorna and Gogolewski, 2002). With this procedure, an interconnected macroporosity ranging from 90 to 300 μm was uniformly achieved within the sponge. The poly(ester-urethane) porous sponge was cut by water-jet (CUTEC AG, Basel, Switzerland) producing cylindrical scaffolds (8 mm dimeter, 4 mm height), sterilised in a cold cycle at 37 °C using ethylene oxide and degassed under vacuum for 6 d before use.

Isolation of hBM-MSCs

Bone marrow was obtained with full ethical approval from Cantonal Ethics Commission, University of Zurich, Zurich (KEK-ZH-NR: 2010–0444/0) and the written consent from patients undergoing routine operations (Table 1).

MSCs were isolated from three different marrow aspirates (two male 50 and 65 year old, one female 62 years old) using Ficoll 400 density separation (Sigma-Aldrich). Mononuclear cells were collected from the interphase and the adherent cell fraction was seeded at a density of 50,000 cells/cm^2 and left to attach for 96 h in αMEM (Gibco), 10 % MSC-tested FBS (Pan Biotech, Aidenbach, Germany), 5 ng/mL bFGF (Peprotech) and 1 % penicillin/streptomycin

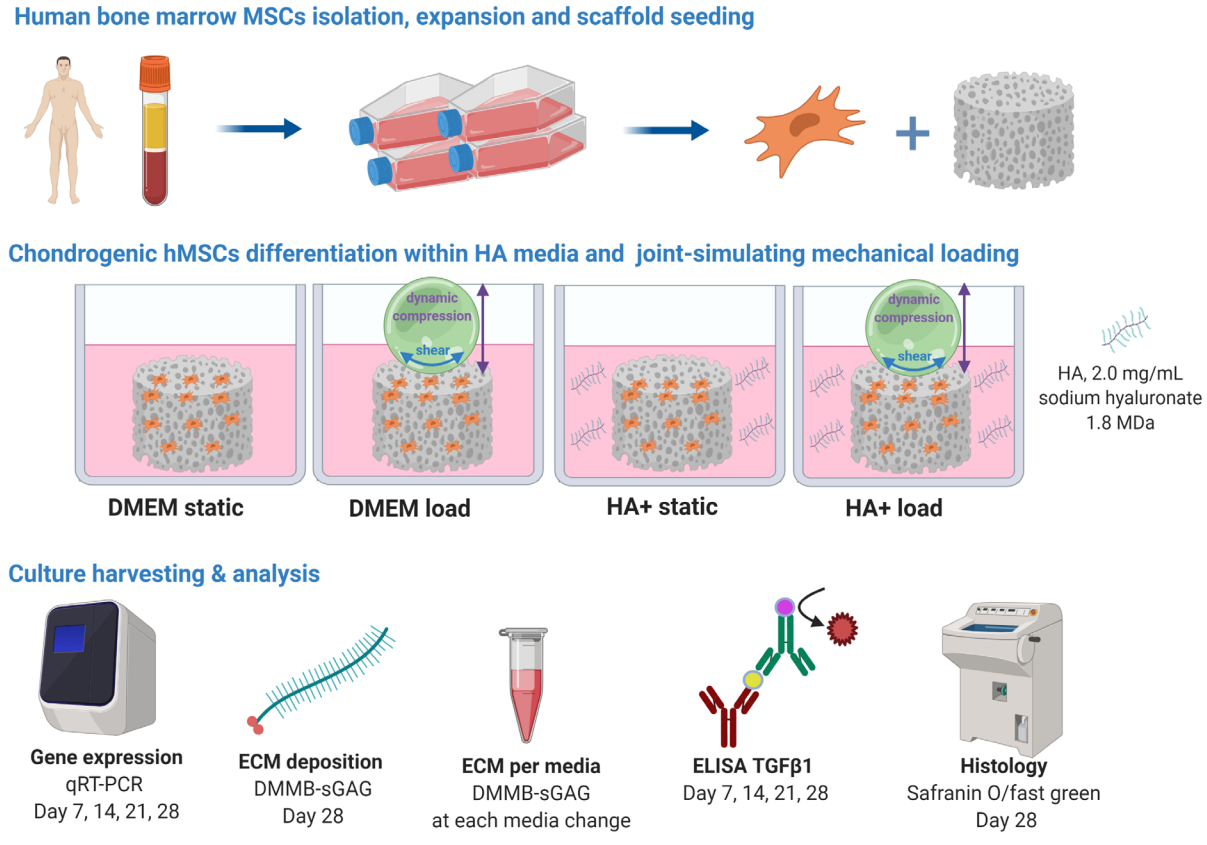

Fig. 1. Representative schema of the study experimental steps and design.

Table 1. Details of the investigated donors.

Donor	Birthdate	Age in 2020	Sex	Cell source
1	1951	69	Male	Vertebra
2	1954	66	Female	Vertebra
3	1967	53	Male	Vertebra

Table 2. Human oligonucleotide primers and probes used for qRT-PCR.

Gene	Primer forward (5′-3′)	Primer reverse (5′-3′)	Probe (5′ FAM- 3′ TAMRA)
COL2A1	5′-GGC AAT AGC AGG TTC ACG TAC A-3′	5′-GAT AAC AGT CTT GCC CCA CTT ACC-3′	5′-CCT GAA GGA TGG CTG CAC GAA ACA TAC-3′
COL10A1	5′-ACG CTG AAC GAT ACC AAA TG-3′	5′-TGC TAT ACC TTT ACT CTT TAT GGT GTA-3′	5′-ACT ACC CAA CAC CAA GAC ACA GTT CTT CAT TCC-3′
ACAN	5′-AGT CCT CAA GCC TCC TGT ACT CA-3′	5′-CGG GAA GTG GCG GTA ACA-3′	5′-CCG GAA TGG AAA CGT GAA TCA GAA TCA ACT-3′
RUNX2	5′-AGC AAG GTT CAA CGA TCT GAG AT-3′	5′-TTT GTG AAG ACG GTT ATG GTC AA-3′	5′-TGA AAC TCT TGC CTC GTC CAC TCC G-3′
RPLP0	5′-TGG GCA AGA ACA CCA TGA TG-3′	5′-CGG ATA TGA GGC AGC AGT TTC-3′	5′-AGG GCA CCT GGA AAA CAA CCC AGC-3′

Table 3. Assays on demand used for qRT-PCR.

Gene	Assays on demand
Sox9	Hs00165814_m1
ALP	Hs00758162_m1
CD44	Hs01075861_m1
RHAMM	Hs00234864_m1
PRG4	Hs00981633_m1
COMP	Hs00164359_m1
MMP3	Hs00968305_m1
ADAMTS4	Hs00192708_m1
ADAMTS5	Hs01095518_m1

(Gibco). When most of the colonies were confluent, the cells were passaged and seeded into new flasks at a cell density of 3,000 cells/cm^2. The chondrogenic potential of each donor was confirmed using standard techniques. hBM-MSCs isolated from each donor were used separately in three independent experiments.

Scaffold seeding and chondrogenic differentiation

hBM-MSCs at passage 3 were trypsinised at 80 % confluence, suspended in a 150 µL fibrinogen-thrombin-solution and evenly seeded at a cell density of 5 × 10^6 cells/150 µL in cylindrical (8 mm × 4 mm) macroporous polyurethane scaffolds. Constructs were fed with two different media for 28 d. Control medium was serum-free basal medium containing DMEM high-glucose, supplemented with 1 % ITS+, 1 % penicillin/streptomycin, 1 % non-essential amino acid, 50 µg/mL ascorbate-2-phosphate, 5 µmol/L ε-amino-caproic acid, 10^{-7} mol/L dexamethasone (DMEM). This medium was further supplemented with 0.2 % 1.8 MDa HA (HA+) (Stanford Chemicals) to simulate the synovial fluid concentration under normal conditions (2.3 mg/mL) (Fam *et al.*, 2007). The culture medium was refreshed every second day and conditioned medium was collected for analysis.

Mechanical loading

Control constructs for both culture media (DMEM and HA+ groups) were kept under static culture for the entire culture period (4 weeks). A custom-made joint-simulating bioreactor based on tribological principals was used to exert the multi-axial loading on the surface of the experimental constructs (Wimmer *et al.*, 2004). Loaded constructs were exposed to 20 cycles of 10 % compression superimposed on top of a 10 % pre-strain and shear loading (± 25°) at 1 Hz for 1 h a day, five times a week. The application of multi-axial mechanical load to fibrin-poly(ester-urethane) constructs in this system has been described previously (Zahedmanesh *et al.*, 2014).

Gene expression analysis: RNA isolation, cDNA synthesis, Real Time qPCR

After 0, 7, 14, 21 and 28 d of chondrogenic culture, constructs were harvested and total RNA was isolated

using TRI Reagent (MRC, Cincinnati, OH, USA). Then, TaqMan reverse transcription was performed using 1 μg of total RNA sample, random hexamer primers and TaqMan reverse transcription reagents (Applied Biosystems).

Real-time qPCR was performed using the QuantStudio 6 Flex real-time PCR system (Applied Biosystems). A panel of human genes associated with chondrogenic markers (*COL2A1*, *ACAN*, *SOX9*), the hypertrophic marker *COL10A1*, osteogenic markers (*RUNX2*, *ALP*), hyaluronan receptors (*CD44*, RHAMM) and mechanically responsive genes (*PRG4*, *COMP*) were investigated.

Primers for *RPLP0*, *COL2A1*, *COL10A1*, *ACAN* and *RUNX2* were synthesised by Microsynth AG (Balgach, Switzerland) (Table 2). Primers for *SOX9*, *ALP*, *CD44*, *RHAMM*, *PRG4*, *COMP*, *MMP3*, *ADATS4* and *ADAMTS5* were purchased from Applied Biosystems (Table 3).

Relative quantification of target mRNA was determined according to the comparative Ct method, with *RPLP0* as an endogenous control. In addition, the level of gene expression for each gene was determined relative to day 0 monolayer through a ΔΔCt comparison (Table 2 and 3).

sGAG and DNA quantification

After 28 d of culture, constructs were digested for 16 h at 56 °C with 1 mL proteinase K (0.5 mg/mL). Total DNA content was measured spectrofluorometrically following reaction with Bisbenzimide Hoechst 33258 dye (Polysciences Inc., Warrington, PA, USA), with purified calf thymus DNA as standard (Lubio Science, Luzern, Switzerland) (Labarca and Paigen, 1980).

Amount of sGAG retained within the scaffolds was determined by a direct spectrophotometric microassay, according to the DMMB dye method (Sigma-Aldrich) at pH 1.5, using bovine chondroitin 4-sulphate sodium salt from bovine trachea (Fluka) (Farndale *et al.*, 1986). Total GAG content of the culture media was also measured to assess the release of matrix molecules from the constructs. All samples containing hyaluronan were blanked with media containing 0.2 % hyaluronan and DMMB at pH 1.5 was used to eliminate background due to the residual interaction between DMMB and hyaluronan.

ELISA TGF-β1 quantification

Both the amount of total TGF-β1 and active TGF-β1 in collected culture media was quantified using the human TGF-β1 DuoSet ELISA (R&D systems). To measure the total amount of TGF-β1 in each sample, an acidic activation step was performed as per the manufacturer's instructions. Analyses of the samples without this activation step provided the amount of active TGF-β1 within the sample.

Histology and staining

After 28 d of culture, the constructs were fixed in 70 % methanol and 10 μm-thick specimen sections were cut using a cryostat (CryoStar NX70, Thermo Fisher Scientific), stained with safranin O and counterstained with fast green to detect proteoglycan presence and proteoglycan-depleted collagen-rich areas.

Statistical analysis

Data were produced from three individual experiments, each carried out using MSCs from a different donor. All experiments were performed in triplicate or quadruplicate for each group at different timepoints to reduce methodological variability. Each measurement was performed in duplicate. Analyses were performed between the appropriate control group and treatment groups as well as between different treatment groups, using one-way or two-ways ANOVA with Tukey's or Sidak's *post-hoc* testing as required. A significance level of $p < 0.05$ was applied and data are presented as mean and SD. Analyses were carried out using the GraphPadPrism 8.1.0 software (GraphPad Software Inc.).

Results

Gene expression analysis

A panel of genes associated with chondrogenic differentiation (*COL2A1*, *ACAN*, *SOX9*), hypertrophy-associated *COL10A1* as well as genes associated with osteogenic differentiation (*RUNX2*, *ALP*) and mechanical loading (*PRG4*, *COMP*) were investigated (Fig. 2). To gain further understanding of the underlying mechanism, the hyaluronan receptors (*CD44*, *RHAMM*) and genes associated with matrix breakdown (*MMP3*, *ADAMTS4* and *ADAMTS5*) were also investigated. All the donors investigated displayed similar trends in the levels of gene expression, with a varying degree of magnitude.

Concomitant loading and HA-supplemented medium led to a significant *COL2A1* upregulation at day 14 and 21 compared with DMEM (* $p < 0.05$) and at day 14 compared with HA medium (* $p < 0.05$) under static conditions (Fig. 2). No significant differences were detected between the two loaded groups, however, the HA+ medium-loaded group showed a slight increase in the average value of *COL2A1* expression at day 14 and 21 for all three donors investigated compared with load alone. Load plus HA showed a significant upregulation of *ACAN* expression when compared with static DMEM for all the timepoints investigated (* $p < 0.05$ and ** $p < 0.01$), while loaded DMEM showed a significant *ACAN* upregulation only at day 28 (* $p < 0.05$). Therefore, HA+ under static and to a greater extent under loading conditions contributed to an overall increase in *ACAN* expression. *SOX9* was upregulated due to mechanical loading in both DMEM at day 14 and 21 (* $p < 0.05$ and ** $p < 0.01$) and HA+ medium at day 14 (** $p < 0.01$). No significant effect can be attributed to the culture medium. *RUNX2* expression was neither affected by the mechanical loading nor by the culture medium (Fig. 2).

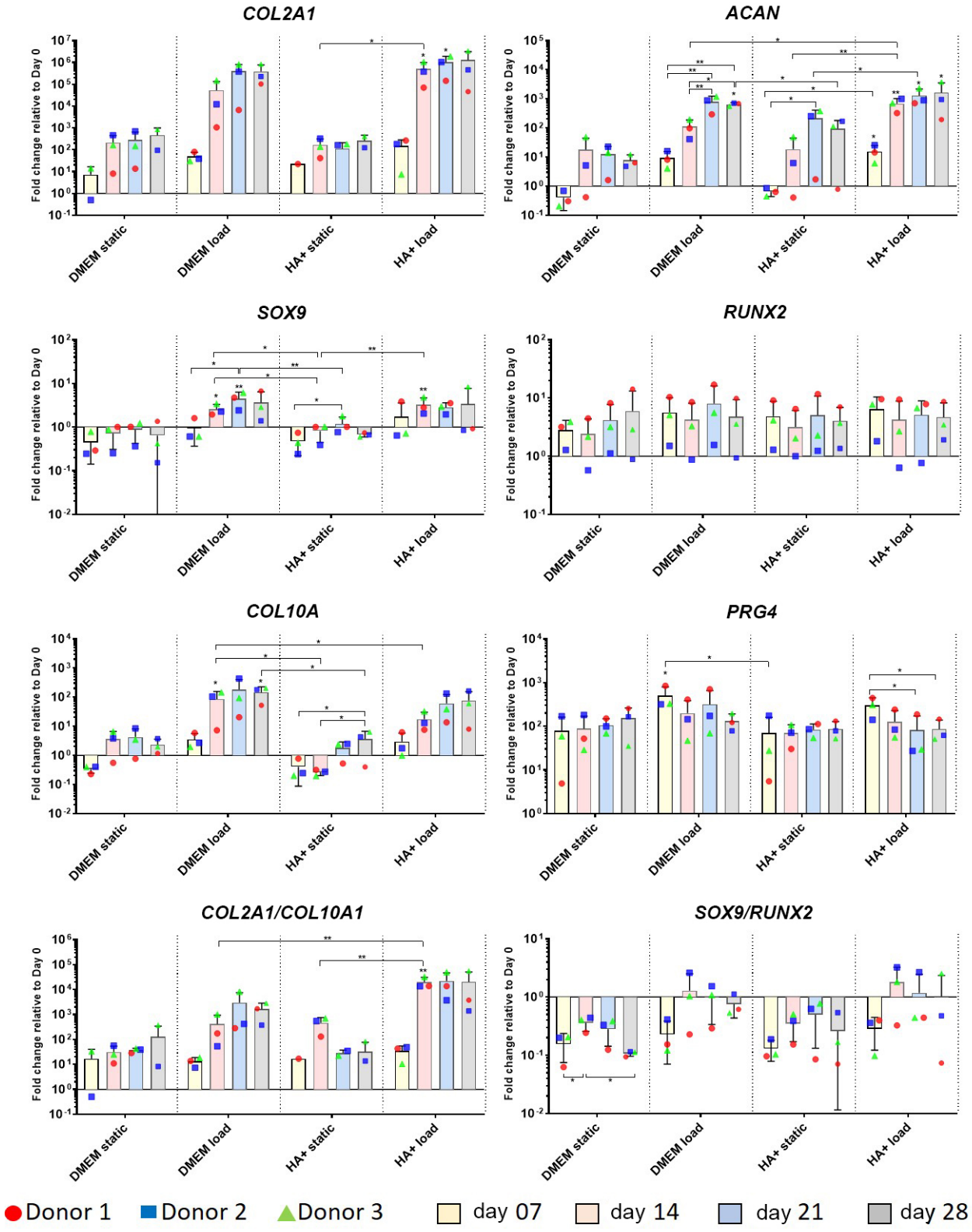

Fig. 2. Gene expression. Gene expression measured by qRT-PCR of chondrogenically differentiating hBM-MSCs-based constructs fed with i) standard chondropermissive medium under static (DMEM static) or under mechanical loading (DMEM load) and ii) standard chondropermissive medium supplemented with 0.2 % 1.8 MDa hMwt HA under static (HA+ static) or under mechanical loading (HA+ load). Samples were harvested at day 7, 14, 21 and 28. Under loading, HA-supplemented medium supported the chondrogenic gene expression of *COL2A1* and *ACAN*, reduced the upregulation of the hypertrophic cartilage marker *COL10A1* and increased *COL2A1*/*COL10A1* ratio. Relative quantification of target mRNA was performed according to the comparative Ct method. Values represent mean ± SD of three independent hBM-MSC donors in experimental triplicate. Statistical significance was defined as * $p < 0.05$ and ** $p < 0.01$.

COL10A1 was upregulated under mechanical load compared with static conditions (Fig. 2). However, exogenous HA reduced *COL10A1* upregulation when concomitant load was applied. The reduction in *COL10A1* expression in HA+ medium under load was significant at day 14 compared with the DMEM loaded group (* $p < 0.05$). In addition, *COL10A1* expression in the DMEM-loaded group was significantly higher than DMEM static at day 14 and 28 (* $p < 0.05$) and HA+ static at day 28 (* $p < 0.05$). A significant increase in *COL10A1* was observed at the later stages of culture for the HA+ static group. This increase was significant because *COL10A1* was initially downregulated at day 7 and 14, therefore when compared with day 21 and 28, the slight upregulation became significant (* $p < 0.05$). No significant differences were detected between DMEM static and HA+ static. In addition, DMEM static showed upregulation of *COL10A1* already at day 14, when *COL10A1* was still downregulated for HA+ medium static group. Therefore, HA+ medium delayed and overall reduced the upregulation of *COL10A1* both under static and mechanical loading conditions.

The static groups did not show any changes in lubricin (*PRG4*) expression over time. A significant *PRG4* upregulation was observed at day 7 in the DMEM loaded compared with the DMEM static (* $p < 0.05$) and HA+ static (* $p < 0.05$) groups. However, *PRG4* expression under mechanical shear and dynamic compression was significantly reduced over time in HA+ medium loaded between day 7-21 (* $p < 0.05$) and day 7-28 (* $p < 0.05$). This reduction was not observed for the DMEM loaded group. In addition, the average value of *PRG4* expression in the HA+ loaded group was never significantly different from the static groups (Fig. 2).

To gain further understanding of the impact of HA+ medium on hMSC chondrogenic differentiation, the *COL2A1*/*COL10A1* ratio was investigated. HA+ medium combined with loading showed an improved ratio compared with DMEM loaded and this was consistent among all donors investigated. Particularly at day 14, HA+ medium loaded significantly increased the *COL2A1*/*COL10A1* ratio compared with all other groups and particularly compared with the corresponding DMEM loaded group (** $p < 0.01$), demonstrating the positive effect of HA and loading in reducing the hypertrophic hMSC phenotype in favour of a more stable chondrogenesis. The *COL2A1*/*COL10A1* ratio for the DMEM loaded group was upregulated at day 14, 21 and 28 compared with DMEM static and at day 21 and 28 compared with HA+ static. The expression of *MMP3* and *ADAMTS4* was upregulated during loaded conditions, but the presence or absence of HA had no effect (Fig. 3). The expression of *ADAMTS5* was unaffected by load or HA addition (Fig. 3). The *SOX9*/*RUNX2* chondrogenic ratio appeared to be slightly increased by mechanical load compared with static conditions but the culture medium did not affect the ratio level. Cartilage oligomeric matrix protein (*COMP*), the hyaluronan receptors (*CD44* and *RHAMM*) and the osteogenic marker *ALP* expressions were not affected by the mechanical loading nor by the culture media investigated in the present study (data not shown).

sGAG and DNA quantification

After 28 d of hMSC chondrogenic differentiation, no significant differences were observed in DNA content among the groups (Fig. 4**a**). An overall increase in sGAG production and deposition was observed under loading conditions when the medium was supplemented with HA. sGAG deposited in the scaffold after 28 d of culture (Fig. 4**b**) were significantly more in the HA+ medium loaded group compared with HA+ medium (* $p < 0.05$) and DMEM under static conditions (* $p < 0.05$). Particularly, donor 2 showed a notable increase in sGAG deposition under load and HA supplementation increased this further. The concurrent effect of HA+ medium and loading significantly increased GAG deposition and this result was consistent among the three different hBM-MSCs donors.

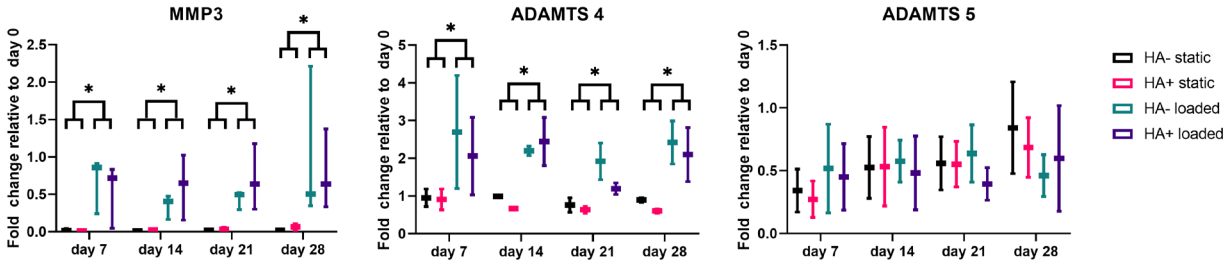

Fig. 3. Metalloproteinase gene expression. Gene expression measured by qRT-PCR of chondrogenically differentiating hBM-BSCs-based constructs fed with i) standard chondropermissive medium under static (DMEM static) or under mechanical load (DMEM load) and ii) standard chondropermissive medium supplemented with 0.2 % 1.8 MDa hMwt HA under static (HA+ static) or under mechanical loading (HA+ load). Samples were harvested at day 7, 14, 21 and 28. Load induced the expression of *MMP3* and *ADAMTS4*, while no differences in *ADAMTS5* expression were seen. HA presence or absence had no effect. Relative quantification of target mRNA was performed according to the comparative Ct method. Values represent mean ± SD of three independent hBM-MSC donors in experimental triplicate. Statistical significance was defined as * $p < 0.05$.

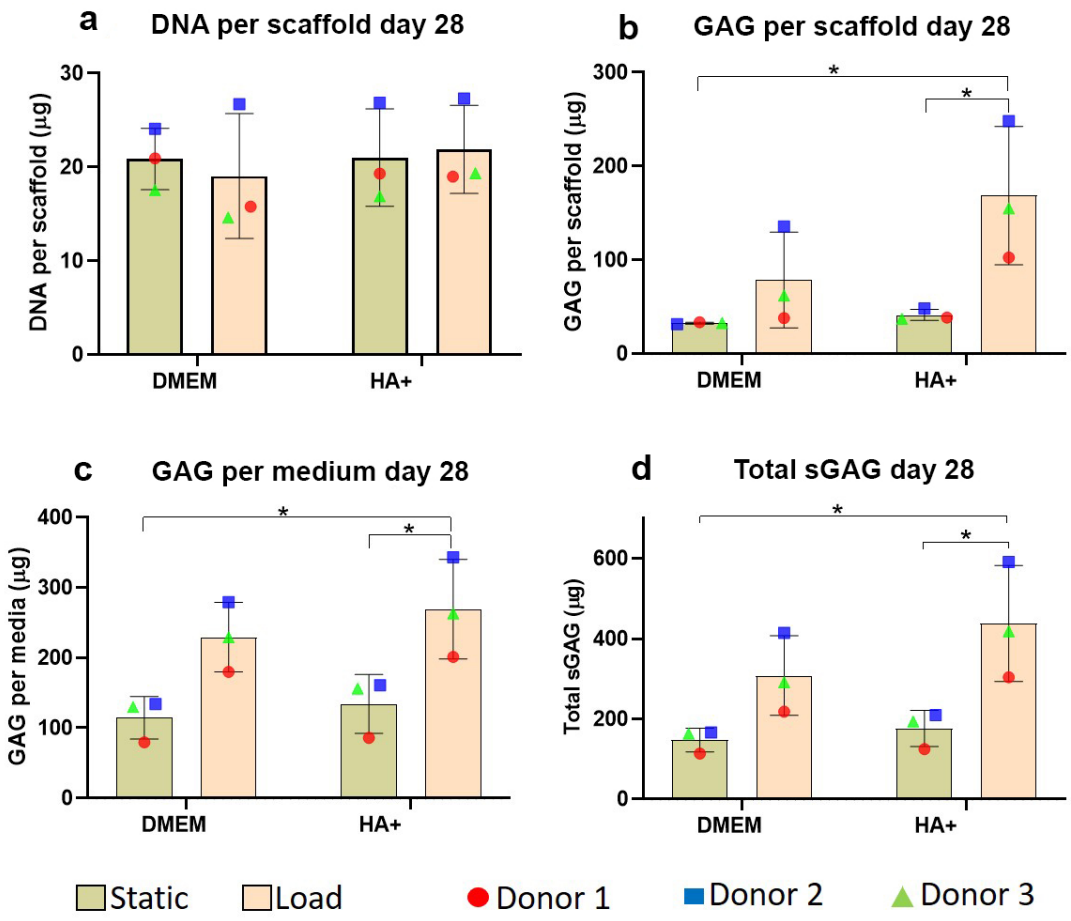

Fig. 4. GAG analysis. Biochemical analysis of chondrogenically differentiated constructs after 28 d in culture showed significantly more sGAG when 0.2 % HA was supplemented in culture medium under mechanical load (HA+ load). (**a**) Bisbenzimide Höchst 33528 dye was used to quantify the DNA in proteinase K digests of scaffolds. DMMB at pH 1.5 was used to determine sGAG produced by MSCs present in each group from (**b**) the proteinase K construct digests, (**c**) the collected culture medium and (**d**) both. All culture medium samples containing HA were blanked with medium containing HA. Values represent mean ± SD of three independent hBM-MSC donors in experimental triplicate or quadruplicate. Statistical significance was defined as * $p < 0.05$.

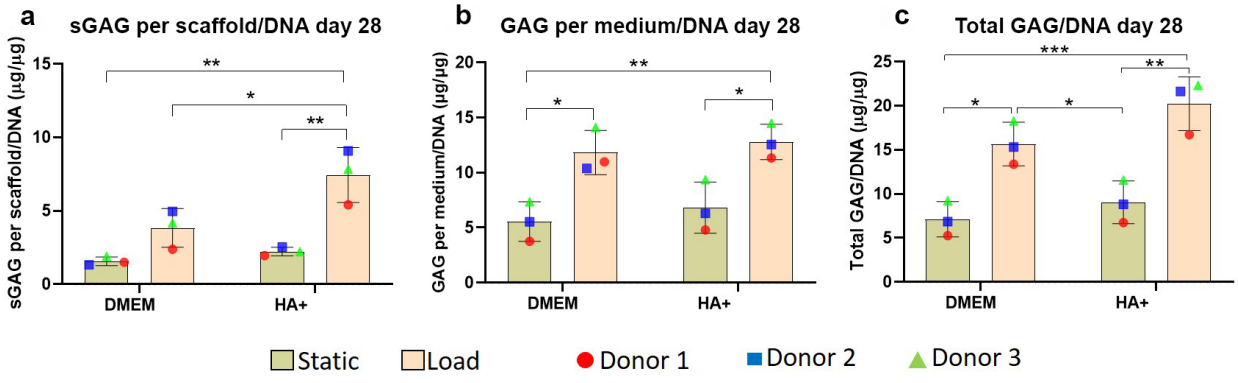

Fig. 5. GAG normalised to DNA. Biochemical analysis of chondrogenically differentiated constructs after 28 d in culture showed a significant increase in sGAG/DNA ratio when 0.2 % HA was supplemented in culture medium under mechanical load (HA+ load). DMMB at pH 1.5 was used to determine sGAG produced by MSCs. GAG/DNA ratio was calculated from total DNA and GAG values to show the production of GAG relative to the MSCs present in (**a**) scaffolds, (**b**) collected culture medium and (**c**) both. All culture medium samples containing HA were blanked with medium containing HA. Values represent the mean ± SD of three independent hBM-MSC donors in experimental triplicate or quadruplicate. Statistical significance was defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

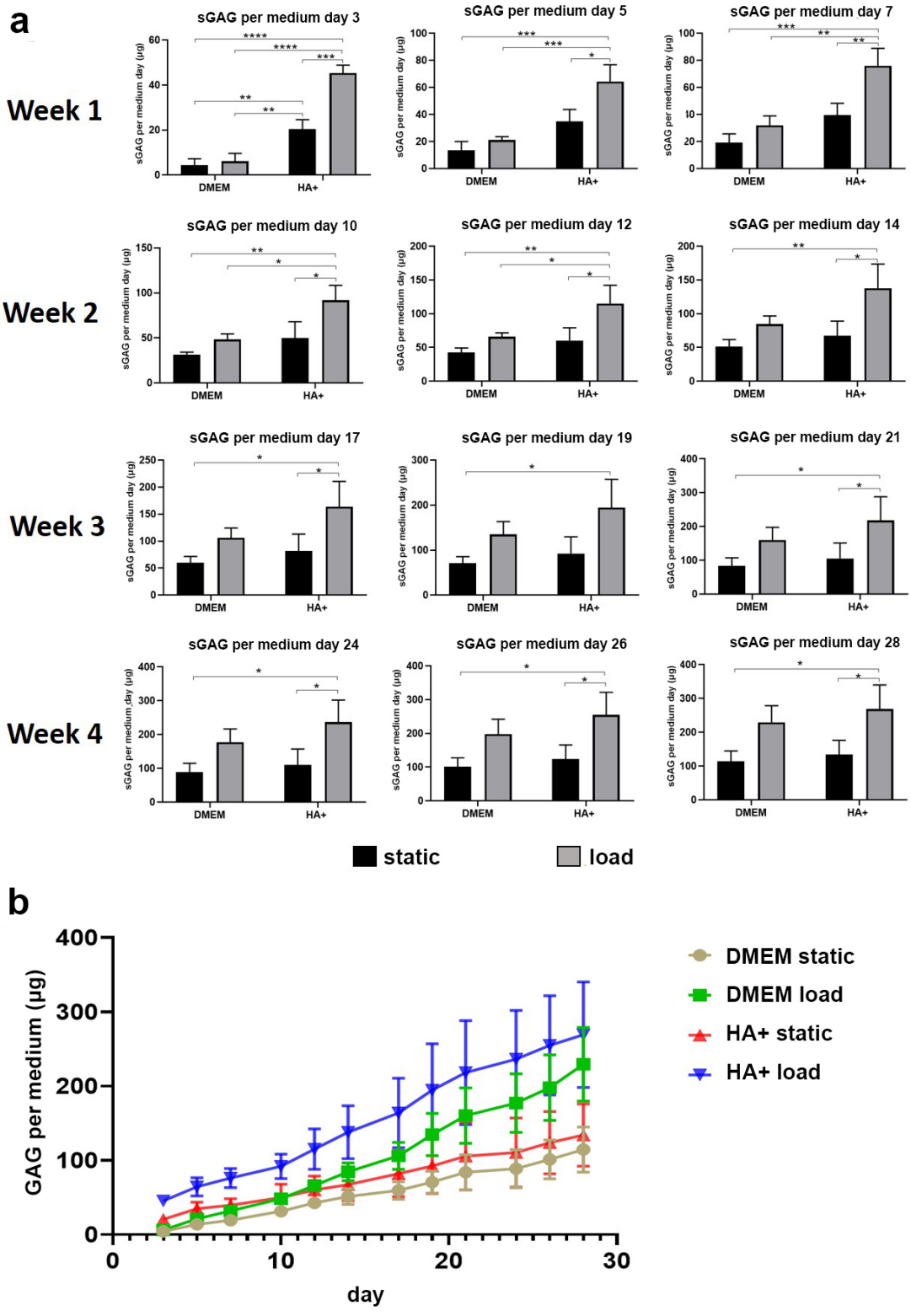

Fig. 6. sGAG level produced and released into culture medium from chondrogenically differentiated hBM-MSC-based constructs over 28 d in culture. (**a**) Chondropermissive medium supplemented with 0.2 % HA under static (HA+ static) and, with a greater extent, under mechanical loading (HA+ load) significantly increased sGAG level in culture medium in the early days of the hBM-MSC chondrogenesis. sGAG content in culture medium was determined spectrophotometrically following reaction with DMMB pH 1.5. All samples containing HA were blanked with medium containing HA. Values represent mean ± SD of three independent hBM-MSC donors in experimental triplicate or quadruplicate. Statistical significance was defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. (**b**) Cumulative sGAG produced and released into culture medium from chondrogenically differentiated hBM-MSC-based constructs along 28 d in culture.

GAG per medium (Fig. 4**c**, Fig. 5) was also monitored and analysed at each medium change to investigate the trend of GAG release over the 28 d of hBM-MSC chondrogenic differentiation. An important sGAG release within the first week of hMSC chondrogenesis was already observed for HA+ medium static compared with DMEM static (** $p < 0.01$) and DMEM loaded (** $p < 0.01$) at day 3 (Fig. 5). However, the GAG release observed with HA+ medium was further enhanced when mechanical loading was applied. Indeed, the HA+ loaded group at day 3 showed significantly more GAG per medium not only compared with DMEM static (**** $p < 0.0001$) and HA+ static (*** $p < 0.001$) groups but also when compared with DMEM loaded (**** $p < 0.0001$) group. HA+ loaded continued to show significantly more GAG per medium compared with all the other groups until day 12 (* $p < 0.05$ and ** $p < 0.01$). Then, from day 14 to day 28, HA+ loaded continued to show significantly more GAG per medium with respect to the static groups (* $p < 0.05$) but no longer towards the DMEM loaded group. Overall, DMEM load showed a constant release of GAG into the medium over time while HA+ loaded promoted an early GAG release, as well as a progressive release during hBM-MSC chondrogenic differentiation. Compared with the static condition, loading increased total GAG (Fig. 4**d**) over 4 weeks of chondrogenic differentiation. However, exogenous HA combined with loading significantly increased GAG production compared with both HA+ medium (* $p < 0.05$) and DMEM under static condition (* $p < 0.05$).

GAG per scaffold/DNA ration at day 28 (Fig. 6**a**), as an indicator of hMSC chondrogenic differentiation, was significantly higher in the HA+ loaded group compared with DMEM static (** $p < 0.01$) and HA+ static (** $p < 0.01$) but also compared with DMEM load (* $p < 0.05$), demonstrating a clear synergistic effect of HA+ medium and loading in inducing a notable enhancement of the cartilage-like matrix production and deposition by hBM-MSCs within poly(ester-urethane)-fibrin scaffolds. Mechanical loading significantly increased GAG per medium/DNA ratio at day 28 independent of the culture medium used (Fig. 6**b**). However, HA+ medium combined with load showed a significantly higher GAG per medium/DNA ratio compared with static conditions (* $p < 0.05$ and ** $p < 0.01$). On the other hand, DMEM loaded showed a significantly higher GAG media/DNA ratio only when compared with the corresponding DMEM static condition (* $p < 0.05$). However, no significant differences were observed between DMEM loaded and HA+ loaded.

Total GAG/DNA ratio at day 28 (Fig. 6**c**) demonstrated the beneficial effect of HA and loading in increasing GAG production and matrix deposition.

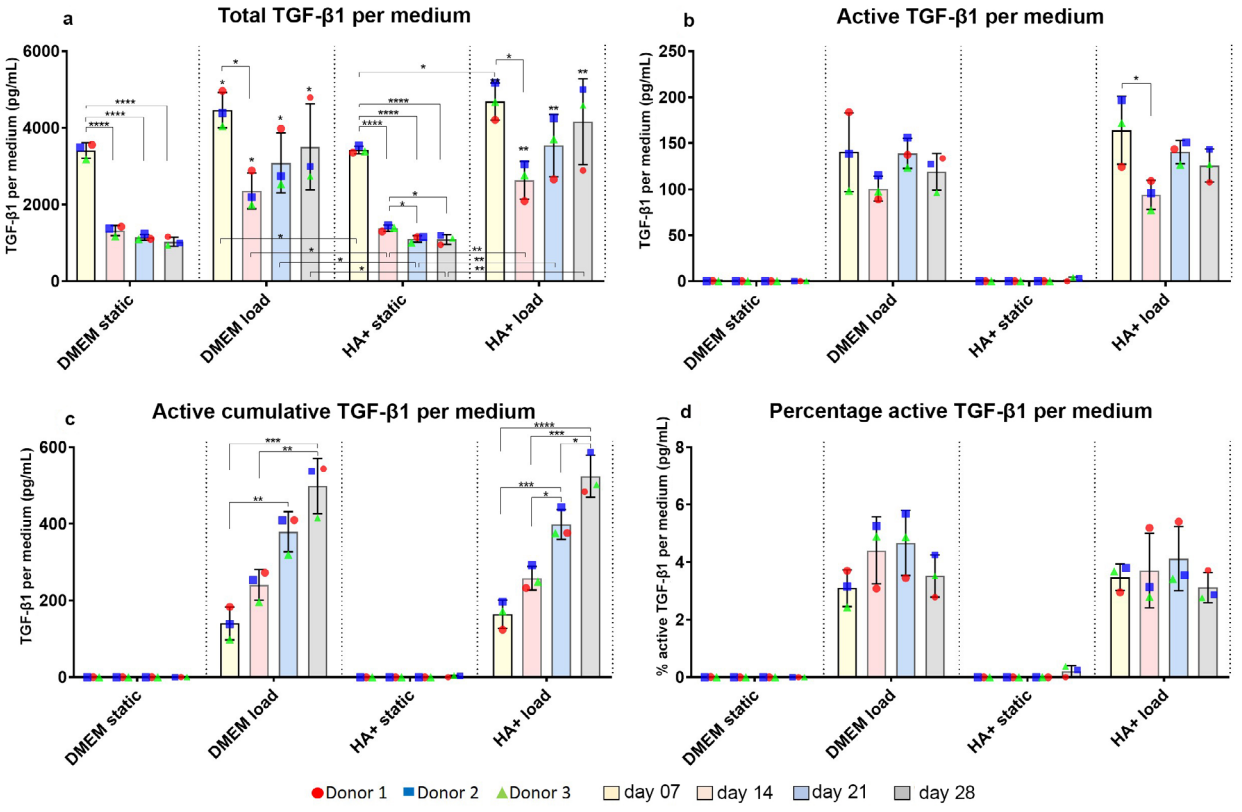

Fig. 7. ELISA data on endogenous TGF-β1 produced by hBM-MSCs-based constructs from week 1 to week 4. (**a**) Total endogenous TGF-β1, (**b**) active TGF-β1, (**c**) active cumulative TGF-β1, (**d**) active TGF-β1 as a percentage of total TGF-β1. Values represent mean ± SD of three independent hBM-MSC donors in experimental triplicate or quadruplicate. Statistical significance was defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ and determined using one-way ANOVA and Tukey's multiple comparison.

A significant increase in total GAG/DNA was observed in both loaded groups compared with both static conditions. While no significant differences were detected between the two loaded groups, total GAG/DNA ratio in the HA+ medium loaded group reached a higher level of significance when compared with the static groups (** $p < 0.01$, *** $p < 0.001$) than DMEM loaded (* $p < 0.05$). Indeed, the highest total GAG/DNA ratio was observed for the HA+ loaded group. Thus, HA+ medium combined with load led to a more consistent GAG production and ECM development compared with DMEM plus load in chondrogenically differentiating MSCs.

Over 28 d of hBM-MSC chondrogenesis under loading conditions, HA+ medium exerted a beneficial effect facilitating the matrix deposition inside the constructs. Overall, GAG analysis demonstrated a consistent effect of HA and loading in increasing GAG production and deposition. Although the magnitude of sGAG production was donor-dependent, the trend observed in the present study was reproducible and robust.

ELISA TGF-β1

Total TGF-β1, active TGF-β1, active cumulative TGF-β1 and percentage of active TGF-β1 were analysed to assess whether HA+ medium under mechanical loading affected TGF-β1 expression and activation during hBM-MSC chondrogenic differentiation.

Under static conditions, significantly more TGF-β1 production was observed for both culture media at day 7 when compared with all the other timepoints investigated (day 14, 21 and 28) (Fig. 7**a**). Similarly, loaded samples had a peak of TGF-β1 production at day 7. A lack of mechanical load in the static conditions led to a significant decrease in TGF-β1 production, which decreases over time (**** $p < 0.0001$), independent of culture media. A similar but less intense drop from day 7 to day 14 was also observed in the loaded groups in both DMEM and HA+ media (* $p < 0.05$). However, when mechanical load was applied, a recovery from day 14 to day 28 was observed. Overall, mechanical load, compared with static conditions, induced significantly more TGFβ1 production over 28 d of MSC chondrogenic differentiation. The average total TGFβ1 was significantly more for HA+ load (** $p < 0.01$) and DMEM load (* $p < 0.05$) groups at all timepoints investigated when compared with both static conditions. Active TGF-β1 was detected only in the loaded groups (Fig. 7**b**), clearly demonstrating the importance of mechanical load for TGF-β1 activation. No effect related to the culture medium supplementation was observed. Cumulative active TGF-β1 showed a significant increase over time under load between day 7 and 21 (** $p < 0.01$) and day 7 and 28 (*** $p < 0.001$) but also between day 14 and 28 (** $p < 0.01$) for the DMEM loaded group (Fig. 7**c**). The HA+ loaded group promoted a slightly more

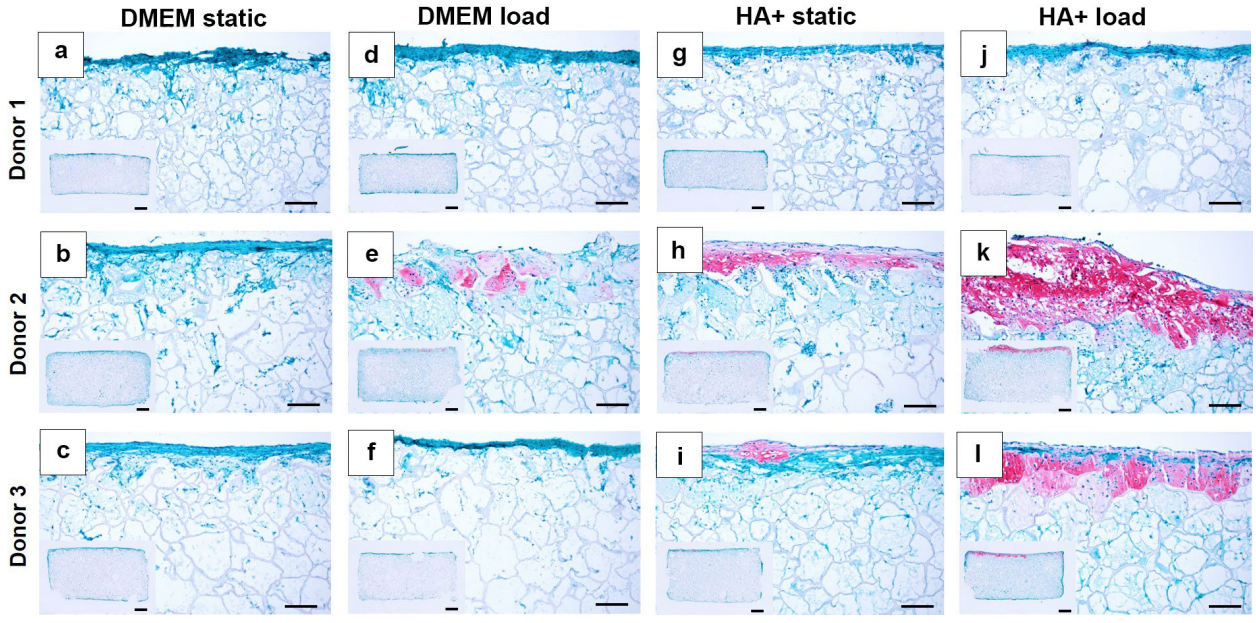

Fig. 8. Differentiated constructs stained with safranin O and counterstained with fast green after 28 d of chondrogenic culture. Images showing higher magnification of the middle upper side and the full section of the constructs cut longitudinally for three independent hBM-MSC donors. The HA-supplemented medium under loading group (HA+ load) showed the best matrix deposition. Scale bar full section: 200 μm; scale bar upper middle magnification: 2 mm. (**a**-**c**) Control group fed with chondropermissive medium under static conditions (DMEM static). (**d**-**f**) Constructs fed with chondropermissive medium under mechanical loading (DMEM load). (**g**-**i**) Constructs fed with chondropermissive medium supplemented with HA under static conditions (HA+ static). (**j**-**l**) Constructs fed with chondropermissive medium supplemented with HA under mechanical loading (HA+ load).

marked increase in TGF-β1 activation over time since significant differences were detected between day 7 and 21 (*** $p < 0.001$), day 7 and 28 (**** $p < 0.0001$), day 14 and 21 (* $p < 0.05$), day 14 and 28 (*** $p < 0.001$), day 21 and 28 (* $p < 0.05$). The percentage of active TGF-β1 was calculated to estimate the fraction of active protein produced by hBM-MSCs (Fig. 7**d**). No culture-medium-induced differences were observed in the percentage of active TGF-β1.

Taken together, mechanical load promoted both the production and the activation of TGF-β1, independently of the medium and timepoint investigated.

Histology and safranin O/fast green staining

To show the deposition of sGAG, differentiated constructs were stained with safranin O and counterstained with fast green after 28 d of chondrogenesis (Fig. 8**a-l**). DMEM culture medium under static conditions showed no ECM deposition with any donor. Addition of HA under static conditions improved ECM deposition in donor 2 (Fig. 8**h**) and partly also in donor 3 (Fig. 8**i**). Mechanical loading alone increased ECM deposition and safranin O staining along the upper surface of the poly(ester-urethane)-fibrin scaffolds fed with DMEM only in donor 2. The concomitant effect of HA+ culture medium and mechanical loading notably increased ECM deposition and safranin O staining along the upper surface, in 2 donors out of 3 (donor 2 and 3).

Discussion

The simultaneous investigation of bioengineered culture medium and load, in the absence of exogenous growth factors, can provide more physiological conditions to improve the understanding of hBM-MSCs chondrogenesis and functional cartilage-like tissue development. *In vivo*, the forces that most cartilages within joints are exposed to during day-to-day physiological movements are a combination of compression – caused by gravitational or muscular loading – and shear stress – generated by the movement of the two articular surfaces against each other and the movement of synovial fluid across the surface of the tissue. Therefore, mechanical loading and particularly multiaxial shear and compression are important cues addressing MSC fate and have been shown to induce chondrogenesis in the absence of any exogenous growth factors, *e.g.* TGF-β1 (Kupcsik *et al.*, 2010; Li *et al.*, 2010; Li *et al.*, 2010b; Neumann *et al.*, 2013). On the other hand, the formulation of a suitable culture medium to develop *in vitro* tissue-tailored constructs has been attempted in several ways (Hegewald *et al.*, 2004; Monaco *et al.*, 2020). Previous findings have demonstrated that supplementing culture medium with exogenous HA enhances the chondrogenic differentiation of equine MSCs in pellet culture and hBM-MSCs in 3D tissue engineered constructs, with increased ECM production and reduced hypertrophy (Hegewald *et al.*, 2004; Monaco *et al.*, 2020). An increased expression of *SOX9* and *ACAN* has been demonstrated when supplementing culture medium with TGF-β3 and using HA as a culture plate coating (Bhang *et al.*, 2011). The improved chondrogenic differentiation, by combining TGF-β3 and a bioactive molecule such as HA, was shown to be additive (Bhang *et al.*, 2011). Previous studies have also demonstrated that hMwt HA administered at a physiological concentration (2 mg/mL) as a medium supplement in chondropermissive culture medium exerts a beneficial effect on the expression of *SOX9* and *ACAN* but does not induce a significant increase in *COL2A1* expression under static conditions (Monaco *et al.*, 2020). Furthermore, addition of HA to the medium or as part of tissue-engineered constructs leads to a reduced *COL10A1* expression that is also dose-dependent (Amann *et al.*, 2017; Hegewald *et al.*, 2004; Monaco *et al.*, 2020).

The present study aimed at investigating the synergistic effects of exogenous hMwt HA and joint-simulating mechanical load on the chondrogenic differentiation of hBM-MSCs over 28 d. The physiological concentration of 2 mg/mL and hMwt HA (1.8 MDa) were selected since they have been shown to enhance the chondrocyte function of hBM-MSCs and cartilage tissue development under static and mechanical load, respectively, in a model based on chondrocytes isolated from fetlock joints (Monaco *et al.*, 2020; Wu *et al.*, 2017). Exogenous hyaluronan (MW 500-730 kDa) administered by intraarticular delivery, in combination with anti-inflammatory signals, acts as a disease modifying drug showing anti-hypertrophic and pro-chondrogenic effects (Prasadam *et al.*, 2013). In another study, intraarticular injection of allogeneic MSCs in combination with HA in rabbits led to a reduction in peri-chondrocyte *COL10A1* (Chiang *et al.*, 2016). Therefore, previous studies have demonstrated that mechanical load and hMwt HA, when added to the *in vitro* culture systems or when HA was intraarticularly delivered *in vivo*, beneficially affected hBM-MSC chondrogenesis and cartilage homeostasis (Chiang *et al.*, 2016; Hegewald *et al.*, 2004; Monaco *et al.*, 2020; Prasadam *et al.*, 2013; Wu *et al.*, 2017). In the present study, hMwt HA supplemented into the culture medium and mechanical loading were concurrently applied, with an increase in *COL2A1* and *ACAN* expression. Combined with the reduced *COL10A1* expression, particularly within the first 14 d of chondrogenesis, a more chondrogenic *COL2A1*/*COL10A1* ratio was observed at all timepoints investigated. The higher *COL2A1*/*COL10A1* ratio observed in HA+ medium loaded when compared with DMEM loaded, demonstrated the beneficial effect of hMwt HA in reducing the hypertrophic hMSC differentiation in favour of a more stable chondrogenesis.

In addition to collagen molecules and chondrogenic markers, lubricin gene expression was also investigated. *PRG4* expression did not show

any variation within the static groups, while under mechanical shear and dynamic compression a slight increase in expression was observed especially at earlier timepoints. *PRG4* expression was significantly upregulated under DMEM loading conditions at day 7 compared with the static groups. However, when HA+ medium was combined with loading, a significant reduction in *PRG4* expression was observed over time, resulting in no differences when compared with the static groups. The reduced *PRG4* expression observed in the present study differed from previous findings (Wu *et al.*, 2017). However, Wu *et al.* (2017) used bovine chondrocytes and only exposed the cells to HA for 1 h/d. It is well known that the main function of lubricin is to provide lubrication to articulating regions and to prevent cell and protein adhesion (Jay and Waller, 2014). In addition, surface motion was previously shown to be specifically responsible for lubricin levels (Grad *et al.*, 2005). Therefore, in addition to the different cell type (chondrocytes *vs.* hBM-MSCs), it is also possible that HA, as a natural lubrication molecule, would reduce friction generated during mechanical loading.

Matrix accumulation is a combination of synthesis and degradation. In the present study, the mRNA expression of the matrix degrading enzymes *MMP3* and *ADAMTS4* was increased in response to mechanical load, while *ADAMTS5* expression was unaffected. As the differences were similar, it did not explain the enhanced safranin O staining obtained with HA addition. To gain further understanding of potential interaction between hMwt HA and hBM-MSCs, the expression of hyaluronan receptor CD44 and receptor for HA-mediated motility, RHAMM, was investigated. The expression of both receptors was downregulated for all the groups investigated. In a previous study, it has been observed that naïve MSCs from bone marrow naturally lack CD44 expression (Qian *et al.*, 2012). However, *in vitro* cultivation results in acquisition of CD44 expression, especially during monolayer expansion (Qian *et al.*, 2012). Therefore, the lack of CD44 upregulation upon HA stimulation may be the result of an artificially high expression generated during cell expansion.

hMwt HA+ medium and joint-simulating mechanical loading significantly increases sGAG/DNA deposition and early sGAG synthesis. Several studies have shown enhanced matrix synthesis due to medium supplementation with exogenous HA. An improved sGAG synthesis and chondrocyte proliferation after medium supplementation with 0.1 mg/mL 800 KDa HA (Kawasaki *et al.*, 1999) has been shown, while 0.1-1 mg/mL has been proposed as the best concentration range to be used within culture media to promote chondrocyte metabolic activity and sGAG deposition within cartilage tissue (Akmal *et al.*, 2005). Adipose MSCs seeded in a collagen gel supplemented with different concentrations of HA showed the highest sGAG/DNA ratio with 1 % 1.5-1.8 MDa HA (Amann *et al.*, 2017). In the present study, no significant differences were observed in DNA content among the four groups investigated and no increase in DNA content was observed from day 0 to day 28.

Over 28 d of hBM-MSC chondrogenesis, an increase in production, deposition as well as release of sGAG into the culture medium was observed under mechanical load alone and this was further enhanced by the addition of hMwt HA. sGAG per scaffold/DNA not only confirmed that HA+ medium loaded was the group with the largest sGAG deposition, but also showed that the concomitant effect of HA+ medium and mechanical loading was significantly beneficial compared with all other groups. Differently, sGAG per medium/DNA ratio was significantly higher in both loaded groups, regardless of the addition of HA. Total sGAG/DNA, which considered both sGAG per scaffold/DNA and sGAG per medium/DNA, at day 28 was also evaluated. Although both loaded groups were significantly superior in total sGAG/DNA ratio compared with the static groups and no significant differences were detected between the two loaded groups, HA+ medium loaded group showed a higher average level of total sGAG/DNA in comparison with the DMEM load group.

Both mechanical load and hMwt HA+ medium increased safranin O ECM staining at day 28. However, the concomitant application of hMwt HA+ medium and mechanical load notably increased ECM deposition. The localisation of matrix deposition to the upper border of the scaffolds was mainly due to the proximity of this area to the region where the load was applied (Zahedmanesh *et al.*, 2014), with load leading to an increased matrix deposition and deposition depth. The enhanced matrix deposition within the HA+ loaded group could also be due to an enhancement in hMwt HA transport from the culture medium into the developing construct caused by mechanical load. Indeed, load can facilitate large molecular weight solute transport into the constructs (Mauck *et al.*, 2003). Therefore, it is possible that the amount of hMwt HA transported into the mechanically active regions of the construct exposed more cells to the stimulus. The matrix production and deposition was donor dependent, with donor 1 in particular being weak in histological staining. This could be related to the donor age, as older donors were used, or to the monolayer expansion/selection.

hMwt HA+ medium under mechanical loading promoted TGF-β1 synthesis by hBM-MSCs

Previous studies have identified the role of TGF-β1 in the chondrogenic differentiation of hBM-MSCs (Johnstone *et al.*, 1998). hBM-MSC chondrogenesis can be induced by mechanical stimulation, even in the absence of exogenous TGF-β (Li *et al.*, 2010; Li *et al.*, 2010b). Therefore, in the present study no exogenous TGF-β1 was used. TGF-β1 is secreted by hBM-MSCs in an inactive form, since the active TGF-β1 peptide is bound to the latency-associated peptide and latent TGF-β1 binding peptide (Robertson and Rifkin, 2013). Shear and compressive load

mimicking the motions of a diarthrodial joint have been observed as chondrogenic inducers leading to mechanical activation of TGF-β1 (Gardner *et al.*, 2016; Li *et al.*, 2010). TGF-β1 activation also occurred in fluid environments following fluid shear stress or stirring forces (Ahamed *et al.*, 2008; Albro *et al.*, 2012). Mechanical forces have been shown to activate the latent form of TGF-β1, residing within the ECM, due to cell traction after integrin binding (Annes *et al.*, 2004; Madej *et al.*, 2016; Wipff *et al.*, 2007). Taken together, this would suggest that at early stages of tissue development, where pericellular matrix is limited, direct mechanical activation of TGF-β1 by shear is the dominant activator. As the ECM develops, the matrix-assisted activation would increase in importance.

The present study confirmed previous findings on the effect of mechanical load on the production and activation of TGF-β1 by hBM-MSCs. Total TGF-β1 production peaked at day 7 and this was particularly pronounced under static conditions. Over time, the static groups showed a strong decrease in total TGF-β1 from day 7 to the end of the experiment. Differently, the loaded groups, after an initial decrease from day 7 to day 14, showed a progressive recovery over time, indicating that mechanical stimulation was crucial for tissue homeostasis. It is interesting to observe that, despite the high TGF-β1 production at day 7 under static conditions, no active TGF-β1 was detected. This result further confirmed the importance of mechanical load in the activation of TGF-β1.

The addition of hMwt HA to the culture medium slightly increased the average total TGF-β1 at all timepoints investigated in comparison with DMEM load. However, due to the donor variation in the absolute TGF-β1 levels, no significant differences were detected between the two loaded groups. In any case, the TGF-β1 levels observed in HA+ medium loaded did not appear significant enough to justify the increases in sGAG deposition, overall matrix production and positive safranin O staining. It is also possible that, due to the increased matrix production in HA+ load group, the newly produced matrix might act as a net to entrap more efficiently TGF-β1, thus reducing its release into the culture medium. To confirm this hypothesis, further studies need to be performed to quantify the TGF-β1 retained by the matrix.

Clinical significance and potential applications

To make *in vitro* culture systems more clinically relevant, it is necessary to increase their complexity to better recapitulate the *in vivo* situation. Joint-simulating mechanical load, which consists of combined dynamic compression and shear, will help to create an *in vitro* environment more representative of the *in vivo* physiological motion. As TGF-β is not approved for clinical use, the absence of exogenous TGF-β1 enables this system to be used to investigate therapies in a more clinically relevant context. In addition, the present study demonstrated that a more complex culture medium including hMwt HA, an important synovial fluid component, helps to improve matrix production and deposition, while stabilising the hBM-MSC-derived chondrocyte phenotype through the reduction in hypertrophic differentiation. The clinical outcome after microfracture is reported to be type I collagen fibrocartilage, while the most often reported *in vitro* outcome is reported to be type X collagen and hypertrophy. Thus, more work is needed to understand this discrepancy. The concomitant effect of joint-simulating mechanical load and hMwt HA medium better reflects the complexity of the *in vivo* synovial joint environment. In so doing, it reduces the need for animal studies, which is beneficial in view of animal welfare and 3Rs principles.

A limitation of the current study was the application of load for 1 h/d. In part, this was due to limitations within the bioreactor system itself. A high throughput bioreactor system is currently under development to enable the study of loading duration on the differentiation process.

The present system also included hMSCs and a fibrin gel (a material used in clinical practice) making the whole culture system more clinically relevant i) to be used as a more reliable *in vitro* preclinical testing system for the screening of potential cell-based cartilage repair therapies, new biomaterials developed for tissue engineering and clinical application, new drug candidates for cartilage regeneration and osteochondral repair; ii) to increase the clinical effectiveness of *de novo* functional cartilage engineered constructs developed *in vitro* in a more synovial joint-like environment; iii) to improve rehabilitation protocols for patients receiving intra-articular injections of hMwt HA or hMwt HA combined with hBM-MSCs.

Conclusion

The results of the study demonstrated that culture medium supplementation with 12 mg/mL 8 MDa hMwt HA, with the concomitant application of joint-simulating mechanical load, notably improved hBM-MSC chondrogenesis with increased matrix production. The present study also demonstrated that a combination of hMwt HA+ medium and mechanical loading contributed to an enhanced expression of the chondrogenic markers *COL2A1* and *ACAN* and, most importantly, to a reduced expression of the hypertrophic marker *COL10A1*. This was also reflected by a notable increase in the *COL2A1*/*COL10A1* ratio over time. Therefore, the development of a more physiological synovial-like bioengineered culture medium, associated with a joint-mimicking mechanical load, contributed to a better preservation of a more stable hBM-MSC-derived chondrocyte phenotype.

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Discussion with Reviewer

Reviewer: The literature suggests that *in vitro* application of multi-axial mechanical loads to MSCs promotes chondrogenesis. However, rehabilitation protocols that include early weight bearing and aggressive strengthening following a procedure such as microfracture are more likely to produce fibrocartilage. Please comment on how we can better translate these important *in vitro* findings to improve the understanding of suboptimal *in vivo* outcomes for patients.

Authors: This is a clear paradox in the field. Currently, we do not have a definite answer. However, our working hypothesis is based on the localised activation of TGF-β. We showed this under complex load and this potentially couple a local mechanical strain to a local biological response. It would also explain why the concentration of TGF in the mechanically stimulated samples was so low and yet a chondrogenic response was observed. Data also showed that the material properties needed to reach a threshold of stiffness, below which this activation could not occur. Our hypothesis regarding microfracture is that the initial marrow clot is too soft to allow for localised mechanically induced TGF-β activation. It could also offer some insight into why a microfracture becomes less effective the larger the defect, as the soft surface area increase, the localised TGF-β activation decreases. We believe adding a stiffer macroporous support, a role played by polyurethane in the present study, would increase TGF-β activation and potentially improve outcomes.

Editor's note: The Scientific Editor responsible for this paper was Chris Evans.