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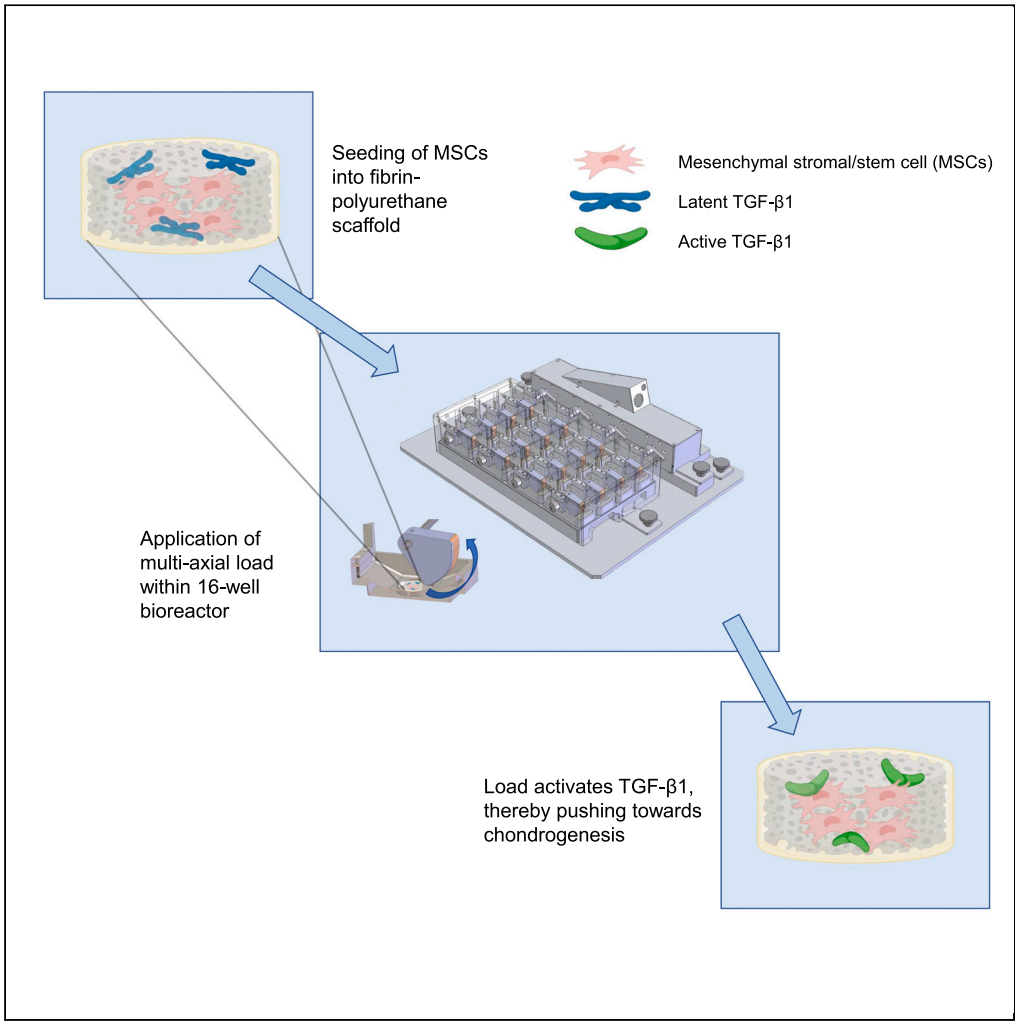

Yann D. Ladner, Hermann Kasper, Angela R. Armiento, Martin J. Stoddart

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Highlights

We present a simple 16-sample multiaxial load bioreactor for chondrogenesis

Human MSCs are exposed to joint-mimicking mechanical load

Mechanical stimulation activates TGF-β1, a key regulator of chondrogenesis

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A multi-well bioreactor for cartilage tissue engineering experiments

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SUMMARY

Cartilage tissue engineering necessitates the right mechanical cues to regenerate impaired tissue. For this reason, bioreactors can be employed to induce joint-relevant mechanical loading, such as compression and shear. However, current articulating joint bioreactor designs are lacking in terms of sample size and usability. In this paper, we describe a new, simple-to-build and operate, multi-well kinematic load bioreactor and investigate its effect on the chondrogenic differentiation of human bone marrow-derived stem cells (MSCs). We seeded MSCs into a fibrin-polyurethane scaffold and subsequently exposed the samples to a combination of compression and shear for 25 days. The mechanical loading activates transforming growth factor beta 1, upregulates chondrogenic genes, and increases sulfated glycosaminoglycan retention within the scaffolds.
Such a higher-throughput bioreactor could be operated in most cell culture laboratories, dramatically accelerating and improving the testing of cells, new biomaterials, and tissue-engineered constructs.

INTRODUCTION

Hyaline articular cartilage is the connective tissue lining the surface of opposing bones within diarthrodial joints. Due to its weight-bearing location, the tissue is constantly exposed to compressive and shear stresses. While cartilage is a resilient tissue, the lack of vascularization and consequently low metabolic activity of its resident cells can severely impede self-healing after tissue injury.[1] In fact, traumatic articular cartilage injuries can lead to advanced cartilage degeneration and increase the risk of post-traumatic osteoarthritis, which increases the burden on human health and health-care cost in general.[2,3] Current cartilage repair strategies remain inadequate, which is why hope is placed upon tissue engineering (TE) strategies that could restore longer-term tissue function. Nevertheless, the success of TE depends on understanding how cells differentiate and/or regulate the repair process.

Bioreactors provide the unique opportunity to investigate the behavior of cells under a controlled physiological environment, in contrast to simple monolayer or 3D cultures using unphysiological static conditions. For instance, bioreactors can be used to expose cells to various stimuli, including, but not limited to, hydrostatic pressure, electromagnetic fields, tension, thermal changes, and shear and compressive forces.[4–9] This is especially useful for cartilage cells, or their progenitors, that reside in a highly organized extracellular matrix where they are exposed to mechanical stimulation.[1] On the path towards clinical translation, pre-clinical *in vivo* experiments offer an alternative to *in vitro* testing. However, in accordance with the 3R principles (replacement, reduction, and refinement),[10,11] one should strive to improve *in vitro* experiments with the goal to relieve the burden on animals. In our lab, we previously developed a 4-station bioreactor that can be used to expose TE constructs to a joint-mimicking multi-axial load.[5] The multi-axial mechanical loading consists of a combination of compression and shear, thereby mimicking the kinematics within the knee joint. In fact, when seeding mesenchymal stem/stromal cells (MSCs) in this system, the mechanical loading has been shown to lead to human MSC chondrogenesis[12] and does so in part by activating endogenous transforming growth factor beta 1 (TGF-β1).[13] Studying cell behavior under kinematic load allows more representative loading that better mimics the clinical situation, providing the opportunity to screen more potential therapeutics prior to animal tests. However, the aforementioned bioreactor has two main limitations: (1) only 4 samples can be processed at the same time and (2) each sample has to be individually transferred to the bioreactor after each run. To overcome these limitations, we developed a new multi-well bioreactor (MWB—Figure 1A) with space for a maximum of 16 samples that are run in parallel. Similar to our 4-station bioreactor, the MWB can be placed into a cell culture incubator

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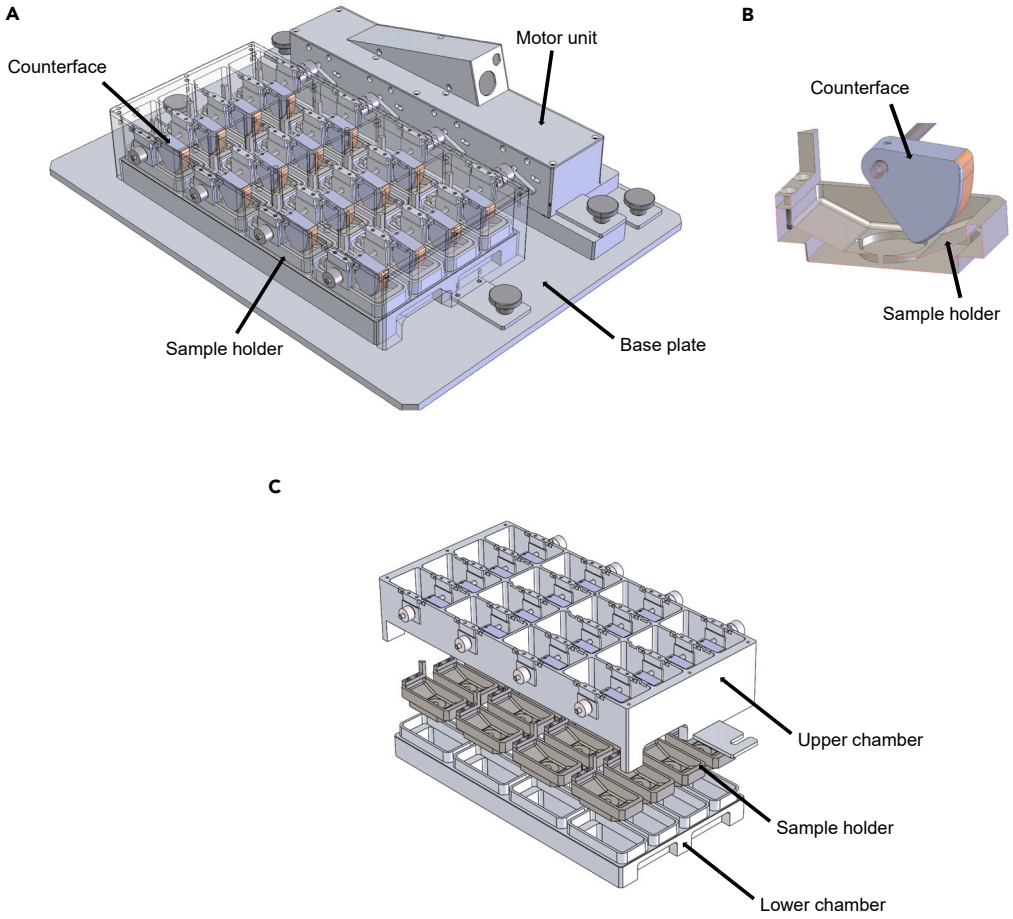

Figure 1. Bioreactor design

(A and B) Scheme of the MWB and (B) enlarged picture of the counterface and holder.

(C) Shows the chamber that can be transferred to the incubator. See also Figure S1.

for a controlled environment. In contrast to the 4-station bioreactor, samples within the MWB are kept in the bioreactor during the whole experiment apart from media change, where the entire holder plate is transferred to a laminar flow hood.

The MWB was developed to allow the application of multi-axial mechanical stimulation to several tissue-engineered constructs in parallel. In the bioreactor, the lower chamber (Figure 1C) consists of a box with 16 positions where samples can be placed, with each sample contained in individual holders. Therefore, various samples can be loaded at the same time in different media. The sample holders (Figure 1B) are made of polyether ether ketone (PEEK), a bioinert material with high mechanical stiffness that is autoclavable.[14] In the center of the holder, there is a hole where different PEEK adaptor rings can be placed, allowing the sample size to be varied. The counterfaces (Figure 1B) are attached to the upper chamber (Figure 1C) and transmit the load to the samples made of MACOR, a machinable glass ceramic. By the teardrop shape of the counterfaces, both compression and shear are applied simultaneously (refer to the movie in the supplementary information). The counterfaces are mounted to the lid of the bioreactor, with four counterfaces connected to one metal rod. Each of the four metal rods is motorized using a stepper motor, which is controlled by a generic soft motion controller. A raspberry pi allows for remote access of the bioreactor using a graphical user interface (GUI—as seen in Figure S1, the LabView code can be found in the supplementary information). During the TE experiment, the MWB is placed remains in a standard incubator, while for medium change, the entire chamber (Figure 1C) is transferred to a laminar flow hood. Lastly, the bioreactor can be programmed to run during scheduled time intervals.

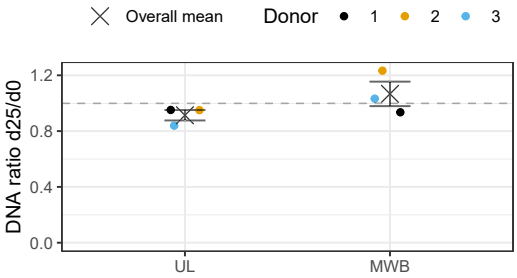

Figure 2. DNA ratio (day 25 vs. day 0) for different conditions

Dashed line indicates a ratio of 1, which constitutes no change in DNA content between day 25 and day 0. UL: unloaded controls; MWB: multi-well bioreactor. Error bars show SEM. Estimated marginal means are adjusted using the Tukey method.

The present work introduces the MWB for higher throughput and investigates its potential in terms of mechano-induced chondrogenesis. The design of the bioreactor focuses on simplicity, thus enabling it to be adopted in as many research laboratories as possible, including those with limited engineering support. The aim of this study was to validate the bioreactor; we, therefore, used protocols and methods previously shown to be successful with our complex 4-station bioreactor. We applied mechanical stimulation to MSCs under growth factor-free conditions as this provides the clearest signal-to-noise ratio, *i.e.* in the absence of load, no chondrogenesis occurs. The newly developed higher throughput bioreactor is a user-friendly tool that could be used to rapidly screen different TE constructs, produced under varying conditions, in a swift manner, thereby saving significant time.

RESULTS

Loading in the MWB does not negatively affect DNA content after 25 days

The DNA ratio between samples harvested on day 25 vs. day 0 remained stable around a value of 1.0, meaning that there was no significant change in DNA content over the course of the experiment when comparing MWB vs. unloaded controls (UL) (Figure 2). Additionally, no statistical difference was observed in the DNA ratio (day 25/day 0) across the different conditions. However, a trend toward a higher DNA ratio was detected for the MWB samples.

MWB activates latent TGF-β1

Total produced TGF-β1, which consists of both latent and active TGF-β1, was significantly increased in MWB compared to UL (UL: 1958.53 μg/μg ± 251.80; MWB: 3359.29 μg/μg ± 320.73—Figure 3A). Active TGF-β1 was only detected in MWB (MWB: 136.27 pg/μg ± 34.69—Figure 3B). Consequently, the percentage of active vs. produced TGF-β1 was the highest for MWB (Figure 3C).

Load increases chondrogenic gene expression

While only statistically significant on day 14, the mRNA expression levels of *SOX9* were higher for MWB compared to UL at all time points (Figure 4). This was also the case for *RUNX2*, albeit no significance was reached. Additionally, MWB samples expressed the highest levels of *SOX9* during earlier time points (days 7 and 14). A significant difference in the *SOX9/RUNX2* ratio was detected between UL and MWB on day 14.

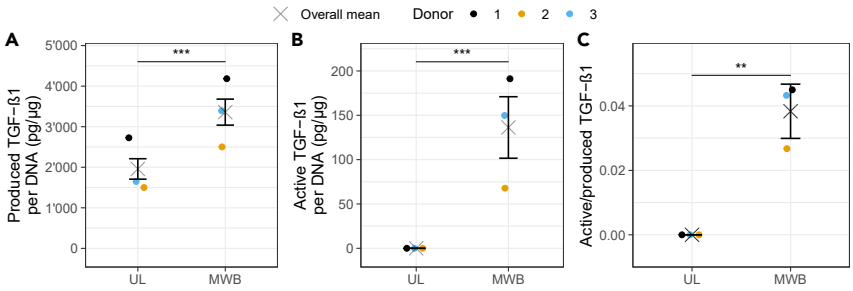

Figure 3. TGF-β1 detected in the conditioned medium after 25 days

(A–C) Produced and (B) active TGF-β1, and (C) the ratio between active and produced TGF-β1. All values are normalized to the DNA content of the respective scaffolds. UL: unloaded controls; MWB: multi-well bioreactor. Error bars show SEM. **p < 0.01, ***p < 0.001, ****p < 0.0001. Estimated marginal means are adjusted using the Tukey method.

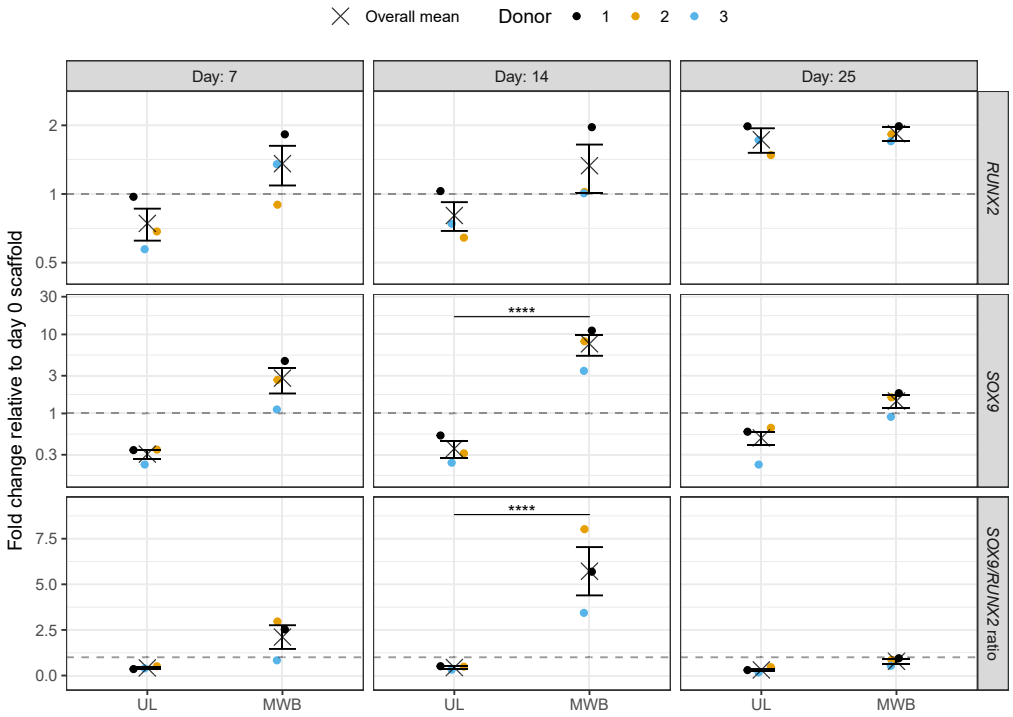

Figure 4. mRNA expression of chondrogenic and osteogenic transcription factors

mRNA expression of *RUNX2* and *SOX9* and their ratio (*SOX9/RUNX2*) on days 7, 14, and 25. UL: unloaded controls; MWB: multi-well bioreactor. Error bars show SEM. ****p < 0.0001. Estimated marginal means are adjusted using the Tukey method.

Loaded conditions demonstrated a higher expression of chondrogenic-related genes *ACAN*, *COL2A1*, and *PRG4* compared to UL (Figure 5). While not statistically significant (apart from day 25 for *ACAN* and day 7 for *PRG4*), the highest levels on each time point were found for the MWB group. For *ACAN*, *COL2A1*, and *PRG4*, mRNA expression was only not always detected in different donors at various time points, which complicates statistical testing despite the clear upregulation of *ACAN* and *COL2A1* in MWB.

Both *COL1A1* and *COL10A1* were detected in all donors at all time points and exhibited significantly higher mRNA levels for MWB compared to UL (apart from *COL10A1* day 7 — Figure 6).

While not statistically significant, mechanical stimulation increased insulin like growth factor 1 (IGF1) gene expression on day 7 and 14 (Figure 7).

No IGF-1 protein detected in conditioned media

No insulin like growth factor 1 (IGF-1) protein was detected above the lowest standard of the ELISA kit.

MWB increases nitrite secretion

While nitrite was detected in both loaded and unloaded samples, the nitrite content was significantly higher in scaffolds loaded with the MWB than in UL (UL: 2.12 μM/μg±0.20; MWB: 4.10 μM/μg±0.61—Figure 8).

Loading in the MWB leads to higher sGAG retention

Significantly more sGAGs were retained within scaffolds loaded with the MWB compared to UL (UL: 3.94 μg/μg±0.30; MWB: 6.14 μg/μg±0.65—Figure 9A). On the other hand, the sGAG per μg of DNA in the conditioned medium was significantly increased in UL samples compared to MWB (UL: 84.34 μg/μg±4.91; MWB: 48.24 μg/μg±4.56—Figure 9B). Since the sGAG in the conditioned medium accounts

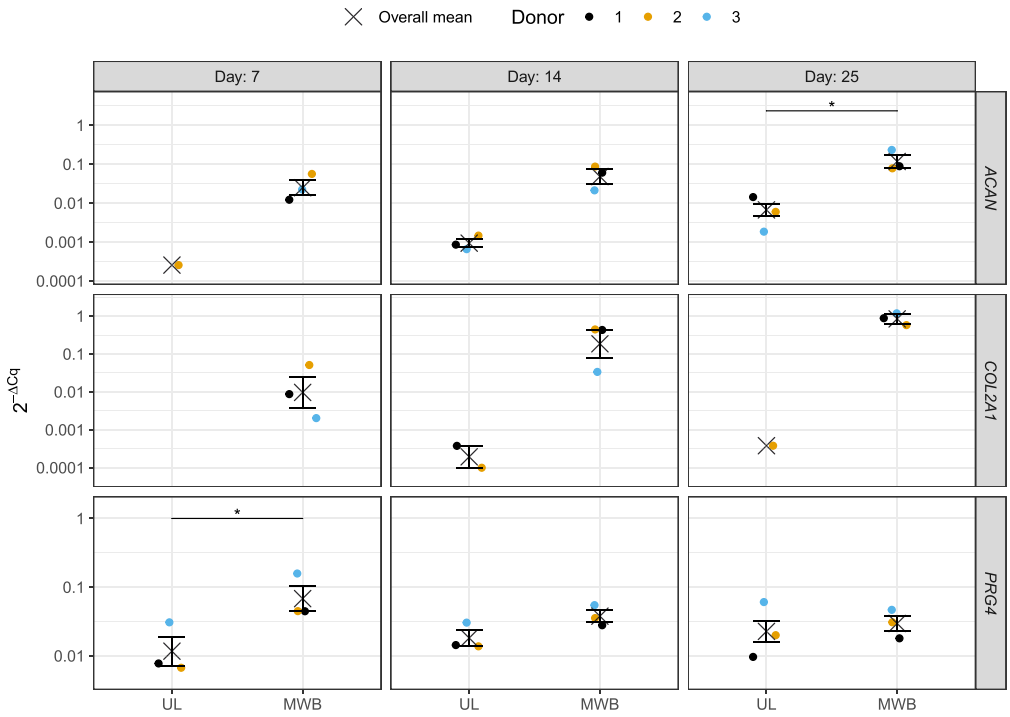

Figure 5. mRNA expression of chondrogenic genes

mRNA expression of *ACAN*, *COL2A1*, and *PRG4* on days 7, 14, and 25. UL: unloaded controls; MWB: multi-well bioreactor. Error bars show SEM. *p < 0.05. Estimated marginal means are adjusted using the Tukey method.

for a greater percentage of the total sGAG than what is retained in the sample, the total sGAG shows the same trend as the conditioned medium (UL: 88.28 μg/μg±5.20; MWB: 54.38 μg/μg±5.1—Figure 9C).

Histology

In agreement with the sGAG retention data, only MWB load scaffolds stained positive for Safranin O, while no positive staining could be observed for UL (Figure 10).

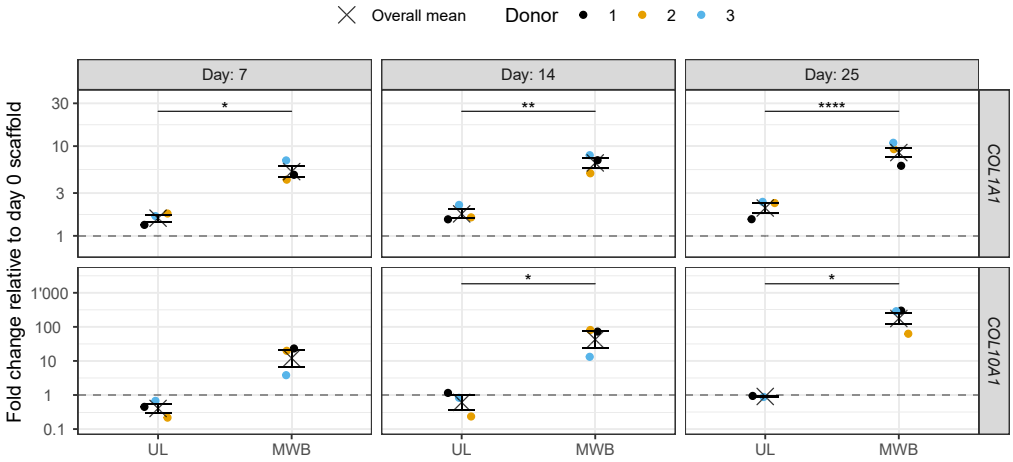

Figure 6. mRNA expression of osteogenic and hypertrophic genes

mRNA expression of *COL1A1* and *COL10A1* on days 7, 14, and 25. UL: unloaded controls; MWB: multi-well bioreactor. Error bars show SEM. *p < 0.05, **p < 0.01, ****p < 0.0001. Estimated marginal means are adjusted using the Tukey method.

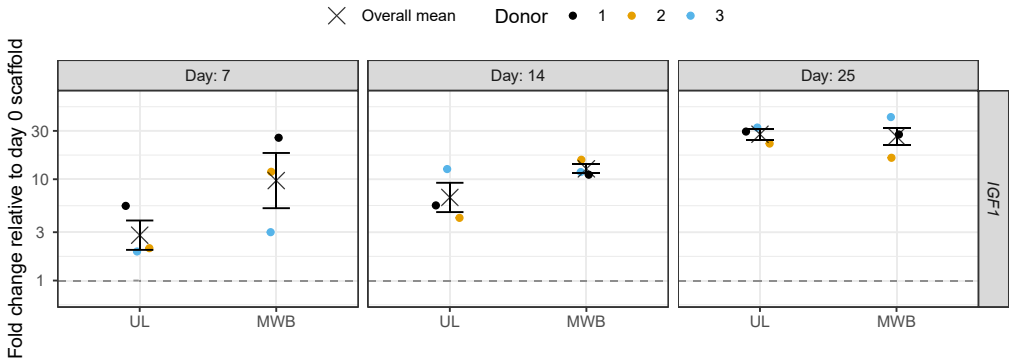

Figure 7. *IGF1* gene expression

mRNA expression of *IGF1* on days 7, 14, and 25. UL: unloaded controls; MWB: multi-well bioreactor. Error bars show SEM. Estimated marginal means are adjusted using the Tukey method.

DISCUSSION

TE is a field that requires the cells to be in a suitable environment. Unsurprisingly, TE for a tissue that is exposed to mechanical forces, such as articular cartilage, entails the use of a bioreactor that allows for mechanical stimulation. The sophisticated kinematics within the knee joint, with the simultaneous application of compressive and shear forces, are difficult to replicate. Thus, bioreactors used for cartilage TE are bulky, difficult to maintain, and often inadequate for experiments with larger sample sizes. For this reason, we developed a new MWB that is easy to handle and can be used for higher throughput experiments. Clearly, higher throughput in TE is of utmost importance, since the plethora of factors that could improve regeneration remains unknown or poorly understood. This study compares the MWB to UL in terms of mechano-induced chondrogenesis. Human MSCs were seeded into fibrin-PU scaffolds and subsequently subjected to joint-mimicking multi-axial load, which has been shown to drive chondrogenesis.[12]

The vast majority of mechanoregulation studies utilize a preculture period with a chondrogenic stimulus, *e.g.* exogenous TGF-β, to establish a pericellular matrix.[15] The load is then applied to further mature the *de novo* tissue. Our research interests have focused on the initial chondrogenesis of MSCs, which is a crucial event during the rehabilitation of patients after the most commonly used clinical treatment of microfracture.[16] We have previously shown that the initial chondrogenic trigger is connected to the mechanical activation of TGF-β protein by the complex load applied.[13] In the absence of load, no chondrogenesis occurs, while complex load leads to TGF-β activation and subsequent chondrogenesis. Mechanical adaptation of precultured cells is possible with the multi-well device described here, but as the load applied modifies the already occurring differentiation, it is more complicated to use this approach for the initial validation of the bioreactor. By using naive cells, and mechanically inducing chondrogenesis, we can show a robust binary response to load (differentiation versus no response) that is comparable to previous results obtained in our laboratory. We have, therefore, validated the functionality of the device, which can now be used for various approaches. Maintaining a comparable experimental setup to previous studies enabled us to establish that the MWB behaves as expected.

Compared to our 4-station bioreactor, which takes up the whole space of a standard incubator, the MWB is much smaller with the potential for two per incubator. Furthermore, the MWB features higher throughput, remote control, simplified maintenance, and ease of handling with improved sterility. Higher throughput is especially important for TE experiments, where the high donor variability stemming from the cells forces the experimenter to use biological repeats and technical replicates. Being able to control the bioreactor remotely and programming the run times frees up the available time of the researcher that can be used for other tasks. During unloaded periods, the samples remain in the MWB and with the use of scheduled runs, the researcher only needs to move the MWB sample chamber (Figure 1C) for medium or sample change. For medium change, the MWB can be transferred to the laminar flow hood and the lid with the counterfaces is easily removed from the sample holder plate. Compared to our previous bioreactor, this process takes much less time and also offers the advantage that all the samples can be processed simultaneously. At the end of the experiment, the counterface lid and the sample holder can be cleaned using a

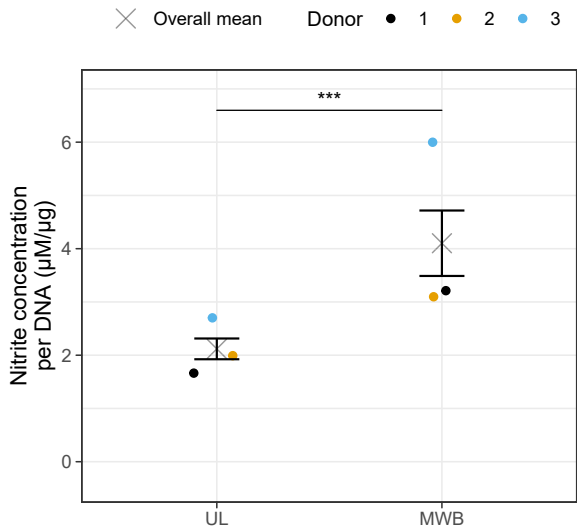

Figure 8. Nitrite content

Nitrite content within the conditioned medium after 25 days. All values are normalized to the DNA content of the respective scaffolds. UL: unloaded controls; MWB: multi-well bioreactor. Error bars show SEM. ***p < 0.001. Estimated marginal means are adjusted using the Tukey method.

conventional laboratory dishwasher and then placed in a sterilization box for heat sterilization. The already-mounted bioreactor can then remain in the sterilization box until further use. Within the MWB, the tear-drop-shaped counterface has a specific geometry that allows for the simultaneous introduction of shear and compression. Compared to other existing bioreactors, where both the compression and the shear motions are decoupled,[17,18] the MWB has the advantage of introducing both motions using a single motor, better mimicking the natural articulation and resolving any timing problems between two motors. It also provides a simpler system to enable easier implementation in laboratories with limited engineering support.

The mechanical loading did not appear to influence the DNA content after 25 days of culture. Consistent with previous findings, where increased compressive strain on loaded samples increases nitrite production,[19] a higher nitrite content was found in loaded samples. Mechanical loading of the MSCs could potentially lead to larger stress on the cells leading to increased nitrite.

Mechanical stimulation increased the expression of *SOX9*, an important transcription factor for chondrogenesis. *SOX9* has been shown to directly regulate *COL2A1*, the main collagen type in articular cartilage, *in vivo*.[20] Similarly, *COL2A1* also increased due to mechanical loading, and while not statistically significant (due to non-detected gene expression in UL), the highest gene expression values were reproducibly found in samples loaded within the MWB. Moreover, as shown within our previous 4-station bioreactor,[12,21] mechanical stimulation increased *PRG4* expression, a mechano-regulated protein that contains sGAGs and plays an important role in the lubrication of articular joints.[22]

Both the osteogenic marker *RUNX2* and the chondrogenic *SOX9* showed a trend toward upregulation in the MWB, especially on day 14. As the inverse of the *SOX9/RUNX2* ratio has been shown to be predictive of early osteogenicity, we deem the *SOX9/RUNX2* ratio to indicate a favorable chondrogenic phenotype at earlier time points.[23]

MSCs cultivated *in vitro* express higher levels of *COL10A1* when exposed to TGF-β1.[24] Indeed, loading increased *COL10A1* gene expression, with a peak after 25 days and the highest levels were found in loaded scaffolds, where the highest amount of active TGF-β1 was detected. The role of TGF-β1 in the fibrotic response has been well studied.[25–27] Unsurprisingly, higher TGF-β1 levels also correlated with higher *Col1A1* gene expression levels.

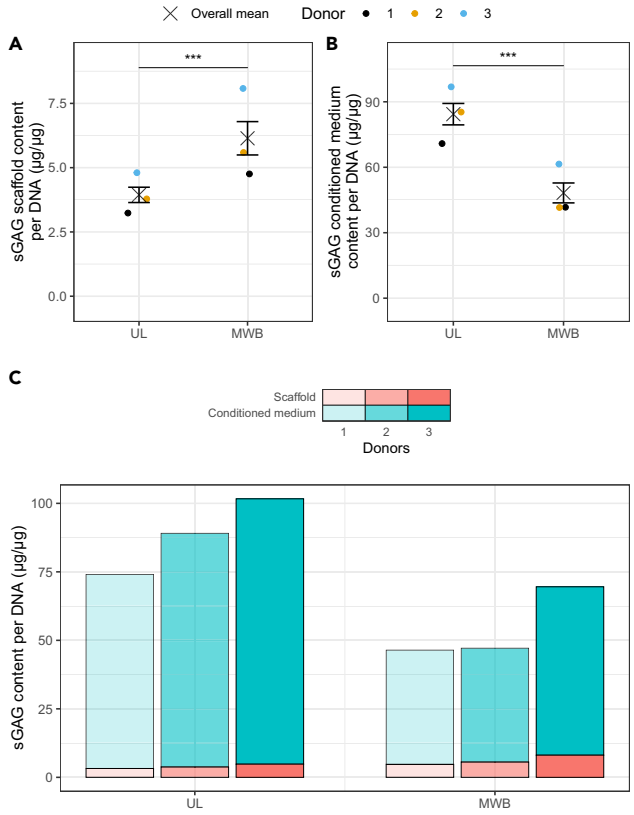

Figure 9. sGAG content

(A–C) sGAG detected in (A) the scaffolds, (B) the conditioned medium, and (C) in total (conditioned medium + scaffold) after 25 days. Red color indicates sGAG found in the scaffolds and blue color indicates what was found in the conditioned medium. The different transparencies indicate different donors. All values are normalized to the DNA content of the respective scaffolds. UL: unloaded controls; MWB: multi-well bioreactor. For (A) and (B), error bars show SEM. ***$p < 0.001$. Estimated marginal means are adjusted using the Tukey method.

In agreement with previous data, mechanical loading in the MWB increased the production of TGF-β1 by the MSCs and also activated the latent TGF-β1.[12,19,28] The mechanical activation of TGF-β is a key advantage of this system. TGF-β1, in its active form, is a key regulator of chondrogenesis. Interestingly, in a previous experiment, we showed that a larger counterface surface applying mechanical load onto the scaffold resulted in a higher TGF-β1 activation.[19] In this study, the counterface surface used in the MWB is also larger than in the 4-station bioreactor, thereby confirming this finding.

Increased active TGF-β1 correlates with total sGAG content (in samples and supernatant)[19] and the majority of sGAG was secreted into the medium, which has also been confirmed in previous studies using this material combination.[12,29] We hypothesize that the fibrin is not able to retain the sGAG as well. In fact, internal, unpublished data from another bioreactor experiment confirms that a material such as gelatin methacryloyl can better retain the sGAG.

In this study, more sGAG was detected retained within loaded samples compared to UL, while more total sGAG—within both supernatant and sample construct—was detected in UL compared to the MWB. Similarly, the Safranin O/Fast Green staining only exhibited sGAG matrix deposition in samples exposed to mechanical loading. There is potentially a negative feedback loop that impedes sGAG production if there is already enough sGAG within the pericellular matrix.

Despite the increase in *IGF1* expression in MWB samples, IGF-1 protein in the media, while detected, was below the threshold of the lowest standard in the ELISA standard curve. Previously, van Osch *et al.* showed that IGF-1 increases proteoglycan deposition within the cell-associated matrix compared to

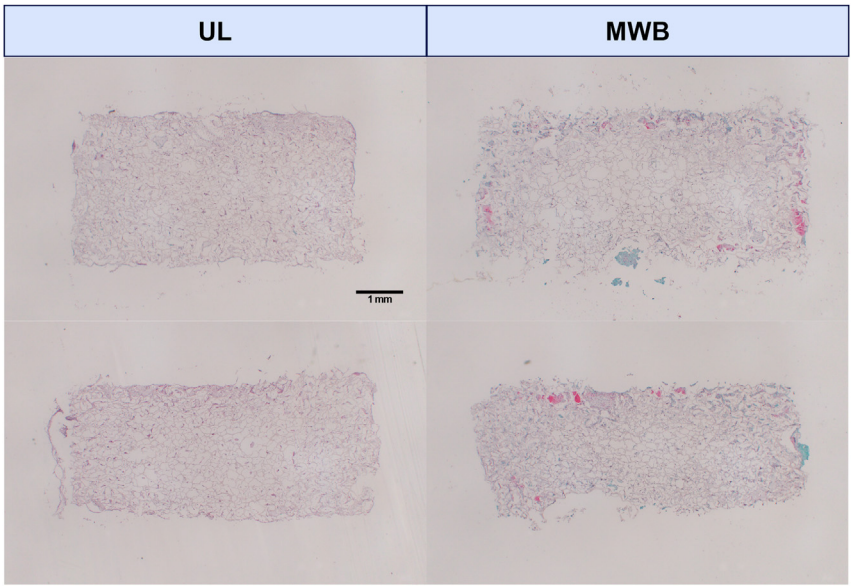

Figure 10. Histology
Safranin O/Fast Green staining of mechanically loaded and unloaded constructs. Scale bar: 1 mm. UL: unloaded controls; MWB: multi-well bioreactor.

further-removed matrix that would not be retained in the scaffold.[30] We hypothesize that there is an increase in endogenous IGF-1 expression which cannot be detected in the conditioned medium, since the low levels of endogenous growth factor have been internalized by the cells. This could have resulted in higher sGAG retention within the scaffolds. Methods to establish pericellular versus bulk media concentrations would be needed to confirm this.

In summary, an MWB for cartilage TE was developed that is able to drive MSCs toward chondrogenesis. This can be combined with exogenous growth factors and used for classical TE studies or alternatively can be used to investigate pure mechanically induced chondrogenesis in the context of regenerative medicine and rehabilitation protocols. The ease of maintenance and handling, and the higher throughput provide a great opportunity for its use in the testing of TE constructs.

Limitations of the study

Currently, this bioreactor can only run one defined loading protocol in terms of compressive strain. However, for future prototypes, we intend to implement a modular counterface system with different geometries that enable different loading protocols.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
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  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Culture of human mesenchymal stromal cells
- METHOD DETAILS
  - Seeding of cells into scaffolds
  - Mechanical loading in bioreactors
  - Sample and medium collection
  - Biochemical assays

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107092.

ACKNOWLEDGMENTS

Should researchers working in the field of cartilage TE be interested in manufacturing such a bioreactor themselves, they can contact the last author of this paper for any information, such as schematics. We can provide sufficient details to any laboratory wishing to reproduce the device.

A special thank you goes to Thomas Krüger for the CAD images and movie of the MWB.

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AUTHOR CONTRIBUTIONS

Conceptualization: A.R.A. and M.J.S.; Methodology: H.K. and Y.L.; Formal analysis: Y.L., A.R.A., and M.J.S.; Investigation: A.R.A. and Y.L.; Resources: M.J.S. and Y.L.; Writing—original draft preparation: Y.L. and A.R.A.; Writing—review and editing: Y.L., A.R.A., H.K., and M.J.S.; Supervision: A.R.A. and M.J.S.; Project administration: M.J.S.; Funding acquisition: M.J.S. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human bone marrow derived mesenchymal stem/stromal cells	Bone marrow donors (Freiburg, EK-326/08)	N/A
Chemicals, peptides, and recombinant proteins		
Proteinase K	Sigma-Aldrich	SKU: 3115879001 (Roche)
Histopaque®-1077	Sigma-Aldrich	SKU: 10771-500ML
Minimum Essential Medium alpha (αMEM)	Thermo Fisher	Cat#: 12000063 (Gibco)
Fetal bovine serum (Sera Plus)	Pan Biotech	P30-3702
Penicillin-Streptomycin	Thermo Fisher	Cat#: 15140122 (Gibco)
FGF basic protein	fisher scientific	Cat#: 50-187-0612 (Fitzgerald Industries International)
Fibrinogen from human plasma	Sigma-Aldrich	SKU: F4883
Thrombin from human plasma	Sigma-Aldrich	SKU: T4393
Dulbecco's modified Eagle medium	Thermo Fisher	Cat#: 52100039 (Gibco)
Sodium pyruvate	Sigma-Aldrich	SKU: P5280
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate	Sigma-Aldrich	SKU: A8960
Dexamethasone	Sigma-Aldrich	SKU: D2915
6-Aminocaproic acid	Sigma-Aldrich	SKU: A7824
Non-Essential Amino Acids Solution (100X)	Thermo Fisher	Cat#: 11140050
ITS+ Premix Universal Culture Supplement	Corning	Prod#: 354352
Hoechst dye (bisBenzimide H 33258)	Sigma-Aldrich	SKU: 14530
1,9-Dimethyl-Methylene Blue zinc chloride double salt (DMMB dye)	Sigma-Aldrich	SKU: 341088
TRI Reagent®	Molecular Research Center Inc.	TR 118
Polyacryl Carrier	Molecular Research Center Inc.	PC 152
Critical commercial assays		
Human TGF-beta 1 DuoSet ELISA	R&D Systems	Cat#: DY240
Human IGF-I/IGF-1 DuoSet ELISA	R&D Systems	Cat#: DY291
MultiScribe™ Reverse Transcriptase	Invitrogen	Cat#: 4311235
Software and algorithms		
R (version 4.2.1)	R Core Team	https://www.r-project.org/
RStudio (2022.7.1.554)	Posit Software, PBC	https://posit.co/download/rstudio-desktop/
LabView	National Instruments	https://www.ni.com/it-it/shop/labview.html
LabView code for device	This paper	Methods S1
Other		
Infinite® 200 PRO	Tecan	N/A
TissueLyser II	Qiagen	N/A
QuantStudio™ 6 Pro Real-Time PCR System	Thermo Fisher	N/A
Metal beads	MARTIN & C	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Martin Stoddart (martin.stoddart@aofoundation.org).

Materials availability

This study did not generate nor use any new unique reagents.

Data and code availability

- All data produced in this study are included in the published article and its supplemental information, or are available from the lead contact upon request.
- All original code has been added as supplementary information.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Culture of human mesenchymal stromal cells

Human MSCs were isolated with full ethical approval (Freiburg, EK-326/08) and written informed donor consent from bone marrow aspirates of three donors (two females aged 57 and 68; one male aged 55) using Ficoll (Histopaque-1077, Sigma-Aldrich) density gradient and plastic adhesion.[31]

The MSCs were expanded in Minimum Essential Medium alpha (αMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (Sera Plus, Pan Biotech), 1% (v/v) Penicillin-Streptomycin (P/S, 100 U/mL penicillin and 100 μg/mL streptomycin, Gibco) and 5 ng/mL recombinant human fibroblast growth factor basic protein (FGF-b, Fitzgerald Industries International) until passage 4.

METHOD DETAILS

Seeding of cells into scaffolds

4.5×10^6 cells were seeded into a cylindrical fibrin-poly(ester-urethane) (fibrin-PU) scaffold (average salt leached pore size between 150 and 300 μm) with a thickness of 4 mm and a diameter of 8 mm according to previous protocols.[32,33] Briefly, the cells were resuspended in 50 mg/mL fibrinogen (Sigma-Aldrich) and added to the sterile lid of a 1.5-mL Eppendorf tube. An equal volume of a 4 U/mL thrombin solution (Sigma-Aldrich) was then mixed with the fibrinogen-cell solution and the porous scaffold was pressed into the lid. Repeated compression of the scaffold using tweezers allowed for the influx of the cell suspension into the pores and even cell distribution as previously shown.[34] Afterward, the scaffolds were transferred to a cell culture incubator (37°C, 5% CO_2 and 95% rH) for 40 min. Another 0.5×10^6 cells were suspended in Dulbecco's modified Eagle medium 4.5 g/L glucose (DMEM, Gibco) and seeded on top of the scaffolds with subsequent incubation for 1 h, as originally described by Gardner *et al.*[33]

Thereafter, the scaffolds were placed into PEEK sample holders that allowed for mechanical stimulation and then into the bioreactor within a cell culture incubator. The scaffold samples were cultured in chondro-permissive medium (CpM) consisting of 4.5 g/L glucose DMEM, 0.11 g/L sodium pyruvate (Sigma-Aldrich), 50 μg/mL L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma-Aldrich), 100 nM dexamethasone (Sigma-Aldrich), Corning ITS+ Premix (6.25 μg/mL human recombinant insulin, 6.25 μg/mL human natural transferrin, 6.25 ng/mL selenious acid, 1.25 mg/mL bovine serum albumin, 5.35 μg/mL linoleic acid, Thermo Fisher), 1% non-essential amino acids (Gibco), 1% (v/v) P/S and 5 μM 6-aminocaproic acid (Sigma-Aldrich). The culture conditions were kept constant at 37°C, 5% CO_2 and 95% rH.

Mechanical loading in bioreactors

Sample constructs were transferred to the MWB and subjected to a loading protocol based on a standard protocol.[12,35] A compressive strain of 10–30% was chosen due to studies indicating improved chondrogenesis with increased amplitude.[19,29] The scaffolds were loaded 20 times for 1 h per day within a total duration of 25 days. Unloaded controls were kept in unloaded (UL) conditions within a cell culture incubator.

Sample and medium collection

Conditioned media from each sample was collected and replaced every second day and stored at −20°C for later biochemical analysis. In total, there were 12 samples of collected conditioned medium (day 0 conditioned medium sample included, which is 1 day after seeding and before the first loading) for each scaffold. At the end of the culture period, scaffold samples were cut in halves and either prepared for biochemical analysis, RNA isolation or histology.

Biochemical assays

For biochemical analysis, the scaffold halves were digested in 1 mL 0.5 mg/mL proteinase K (pK, Roche) at 56°C for 16 h. The pK reaction was inactivated at 96°C for 10 min and the samples were stored at −20°C. DNA content in pK digested scaffold halves was measured using Hoechst 33258 dye (Sigma) and the fluorescence measured at an excitation at 355 nm and emission at 460 nm. The 1,9-dimethylmethylene blue (DMMB) assay was used to quantify the sulfated glycosaminoglycan (sGAG) content in the conditioned medium and pK digested samples. Absorbance was measured at 530 nm and the highest standard in the wells contained 1.25 μg, since at higher contents, the standard curve could leave its linear range.[36] sGAG/DNA was calculated as total sGAG produced during the culture period (scaffold and medium) divided by the DNA content of the scaffold.

DuoSet ELISA kits (R&D Systems) were used to quantify both secreted IGF1 (IGF-1) and TGF-β1 protein in the conditioned medium, according to manufacturer's instructions. Absorbances were measured 450 nm and 560 nm and the values obtained at 560 nm were subtracted from the values obtained at 450 nm and the concentrations were calculated by fitting a four-parameter logistic curve. Total produced TGF-β1 was measured by activating it through acidification and neutralization of the samples, before adding the sample to the plate. Therefore, total produced TGF-β1 consists of already active and activated TGF-β1. The different biochemical assays were measured using an Infinite 200 PRO (Tecan) plate reader."

Gene expression analysis

For RNA analysis, the scaffold halves were transferred to 1 mL TRI reagent +5 μL Polyacryl Carrier (both Molecular Research Center Inc., Cincinnati, OH, USA) and 5 mm metal balls (MARTIN & C) within 2-mL Eppendorf Safe-Lock Tubes (Eppendorf). The samples were then homogenized in the TissueLyser II (Qiagen) for 2 × 3 min at 30 Hz. All the steps were conducted at room temperature. After homogenization, the supernatant was transferred to a new 2-mL Eppendorf tube for subsequent RNA isolation according to Sigma's TRI Reagent Protocol.[37] After isolation, the RNA was reverse transcribed using the MultiScribe™ Reverse Transcriptase (Invitrogen™). qRT-PCR within the QuantStudio 6 Pro Real-Time PCR System (ThermoFisher) was used to quantify the gene expression levels of collagens type I *(COL1A1)*, type II *(COL2A1)*, type X *(COL10A1)*, aggrecan *(ACAN)*, runt-related transcription factor 2 *(RUNX2)*, proteoglycan 4 *(PRG4)*, IGF1, SRY-box transcription factor *(SOX9)*, and ribosomal protein lateral stalk subunit P0 *(RPLP0)*. Primers and probes for qRT-PCR are listed in the Table below. The gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method[38] with *RPLP0* as housekeeping gene and relative to day 0 (1 day after seeding and before the first loading). For *ACAN*, *COL2A1*, and *PRG4*, no mRNA was detected for day 0 for some donors. Therefore, the $2^{-\Delta CT}$ is plotted instead.

Primers and probe sequences for qRT-PCR

Gene	Forward primer	Reverse primer	Probe
RPLP0	5′-TGGGCAAGAACA CCATGATG-3′	5′-CGGATATGAGGCA GCAGTTTC-3′	5′-AGGGCACCTGGAAA ACAACCCAGC-3′
COL1A1	5′-CCCTGGAAAGAA TGGAGATGAT-3′	5′-ACTGAAACCTCTGT GTCCCTTCA-3′	5′-CGGGCAATCCTCGAG CACCCT-3′
COL2A1	5′-GGCAATAGCAGGT TCACGTACA-3′	5′-GATAACAGTCTTGC CCCACTTACC-3′	5′-CCTGAAGGATGGCTG CACGAAACATAC-3′
COL10A1	5′-ACGCTGAACGATA CCAAATG-3′	5′-TGCTATACCTTTACT CTTTATGGTGTA-3′	5′-ACTACCCAACACCAAG ACACAGTTCTTCATTCC-3′
ACAN	5′-AGTCCTCAAGCCT CCTGTACTCA-3′	5′-CGGGAAGTGGCGGT AACA-3′	5′-CCGGAATGGAAACGTG AATCAGAATCAACT-3′

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Gene	Forward primer	Reverse primer	Probe
RUNX2	5′-AGCAAGGTTCAAC GATCTGAGAT-3′	5′-TTTGTGAAGACGGTT ATGGTCAA-3′	5′-TGAAACTCTTGCCTCGTC CACTCCG-3′
PRG4	Assay ID[a]: Hs00981633_m1		
IGF1	Assay ID[a]: Hs01547656_m1		
SOX9	Assay ID[a]: Hs00165814_m1		

RPLP0: ribosomal protein lateral stalk subunit P0; *COL1A1*: collagen type I; *COL2A1*: collagen type II; *COL10A1*: collagen type X; *ACAN*: aggrecan; *RUNX2*: runt-related transcription factor 2; *PRG4*: proteoglycan 4; *IGF1*: insulin like growth factor 1; *SOX9*: SRY-box transcription factor.
[a]TaqMan™ Gene Expression Assay.

Histology

For histology, the halves were fixed with 10% neutral buffered Formalin solution (Sigma-Aldrich) at room temperature for 24 h, then transferred to 70% ethanol at 4°C, and subsequently embedded in paraffin.

Histological samples were sectioned at a thickness of 5 μm, then deparaffinized using xylene and subsequently hydrated. Following sectioning, proteoglycans were visualized using the Safranin O/Fast Green (both Sigma) staining and counterstained with Weigert's haematoxylin (Merck).

QUANTIFICATION AND STATISTICAL ANALYSIS

The experiment was performed with three biological repeats (n = 3). Each time point had 2 technical repeats for PCR and sGAG/DNA. Statistical analysis was performed using R (version 4.2.1 (2022-06-23))[39] within the integrated development environment RStudio (2022.7.1.554).[40] A linear mixed model with the interaction between loading condition and the donor and time point (where applicable) as a random effect was employed. Estimated marginal means were adjusted using the Tukey method. For the linear mixed model, the R packages *lmer* and *lmerTest* were used and for the estimated marginal means, the R package *emmeans* was used. Data are shown as mean ± SEM of three independent experiments with cells from three individual donors.

Statistical significance was set at $p < 0.05$. In the graphs, statistical significance is denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.