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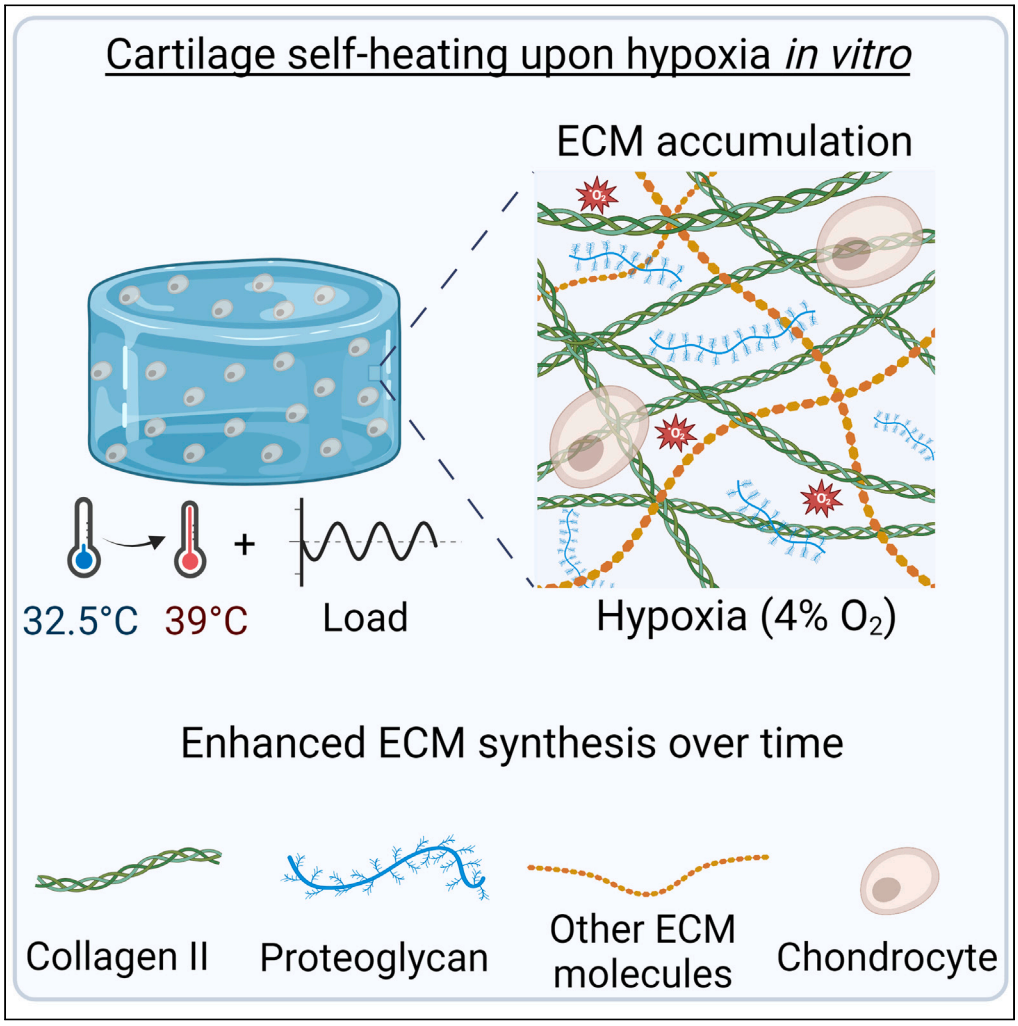

Theofanis Stampoultzis, Yanheng Guo, Naser Nasrollahzadeh, Vijay Kumar Rana, Peyman Karami, Dominique P. Pioletti

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Highlights

Hypoxia and thermomechanical cues synergistically affect human chondrocytes

Hypoxia alters chondrocyte sensitivity to isolated thermal or mechanical signals

Combined stressors induce maximum collagen synthesis through SOX9 factor

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Low-oxygen tension augments chondrocyte sensitivity to biomimetic thermomechanical cues in cartilage-engineered constructs

Theofanis Stampoultzis,[1,2] Yanheng Guo,[1,2] Naser Nasrollahzadeh,[1] Vijay Kumar Rana,[1] Peyman Karami,[1] and Dominique P. Pioletti[1,3,*]

SUMMARY

Chondrocytes respond to various biophysical cues, including oxygen tension, transient thermal signals, and mechanical stimuli. However, understanding how these factors interact to establish a unique regulatory microenvironment for chondrocyte function remains unclear. Herein, we explore these interactions using a joint-simulating bioreactor that independently controls the culture's oxygen concentration, evolution of temperature, and mechanical loading. Our analysis revealed significant coupling between these signals, resulting in a remarkable ~14-fold increase in collagen type II (COL2a) and aggrecan (ACAN) mRNA expression. Furthermore, dynamic thermomechanical stimulation enhanced glycosaminoglycan and COL2a protein synthesis, with the magnitude of the biosynthetic changes being oxygen dependent. Additionally, our mechanistic study highlighted the crucial role of SRY-box transcription factor 9 (SOX9) as a major regulator of chondrogenic response, specifically expressed in response to combined biophysical signals. These findings illuminate the integration of various mechanobiological cues by chondrocytes and provide valuable insights for improving the extracellular matrix content in cartilage-engineered constructs.

INTRODUCTION

Articular cartilage lacks the ability for self-renewal, and therefore lesions in the tissue do not heal.[1] At the same time, traditional treatments for cartilage repair do not achieve long term functionality and rarely return the tissue to its native normal state, leading to insufficient outcomes.[2] To this end, tissue engineering is considered a puissant tool for the treatment of such injuries. One of the key tenets of the cartilage tissue engineering paradigm is the integration of exogenous biomechanical stimuli that accurately mimic the joint microenvironment, ultimately accelerating and/or improving extracellular matrix production.[3] Indeed, articular cartilage is a mechanically dynamic and dissipative environment where fast and adaptive responses guide the interactions between chondrocytes and their surroundings.[4] Biophysical and/or biomechanical cues are key characteristic features of the tissue and significant previous work has provided understanding into how certain individual factors may modulate chondrocyte physiology.[5]

Indeed, in light of the essential role of the biophysical interactions between chondrocytes and their microenvironment in tissue formation and homeostasis, mechanobiology has encouraged the integration of various forms of stimuli into current cartilage-engineering strategies.[6] Within the knee joint, cyclic loading, for instance, constitutes an integral aspect of normal mechanical stimulation. The compression of cartilage, in turn, results in changes within the tissue primarily including matrix/cell deformation and subsequent heat accumulation within the joint.[7] Such effects, in turn, are detected by specialized receptors on the cell surface, initiating intracellular signaling cascades that help maintain tissue homeostasis.[8] With this respect, both dynamic compression and heat have been harnessed *in vitro* to enhance the maturation of cartilage-engineered constructs. Previous investigations have demonstrated that dynamic compression bolsters the accumulation of ^{35}S-sulfate and ^{3}H-proline, surrogates of proteoglycan and collagen synthesis, by up to 35%.[9] Meanwhile, periodic heat exposure has been shown to stimulate glycosaminoglycan accumulation within engineered constructs by 150%, compared to untreated controls.[10]

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Low-oxygen tension (hypoxia) has also been shown to be beneficial for engineering cartilage neo-tissues.[11] Articular cartilage develops in a hypoxic milieu, and therefore adaptations to such conditions do not only involve cell survival responses but also enhancement of its specific function.[12] In engineering articular cartilage *in vitro*, hypoxia has been found to elevate the expression of SOX9, as well as the accumulation of major structural proteins, such as aggrecan.[13] More recently, low-oxygen tension culture was also established as a reliable strategy for fostering collagen cross-linking in tissue-engineered cartilage, thus resulting in improved biomechanical performance.[14]

Nonetheless, despite the significant effects that such individual biophysical inputs can yield during chondrocyte culture, the vast majority of previous studies have assessed a single type of stimulus at a time and neglected to consider potential interactions between them. These studies have been confined to assessing a single type of stimulus at a time, failing to explore the intricate interplay and possible synergies that may exist between them. It is only by integrating diverse stimulation modalities concurrently within a single culture period that we can unlock the remarkable prospects of synergetic enhancements. Indeed, inspired by the self-heating capacity of cartilage tissue following joint loading, we have recently demonstrated that the beneficial effects of dynamic mechanical loading (dynamic compression) on the transcriptome response of human chondroprogenitor cells and on the biosynthetic rate of primary human chondrocytes, can be significantly enhanced when dynamic mechanical stimuli act concomitantly with a biomimetic temperature evolution regime.[15,16] Building upon this work, and since low-oxygen tension has also been recognized as an indispensable element of the chondrocyte microenvironment, it is consequently of interest to explore whether hypoxia could act in tandem with dynamic thermomechanical signals to more closely resemble cartilage *in vivo* environment, thus amplifying chondrocyte performance.

Accordingly, the overall purpose of the current study was to address the unique biophysical interplay of joint-level dynamic mechanical loading, oxygen level, and evolution of temperature. The principal aim of our study was to identify the most effective modality of stimulation, whether administered in isolation or in combination, to maximize the biosynthetic content of cartilage-engineered constructs. Prompted by the absence of investigations exploring the relative effects of multimodal physiological cues on adult chondrocytes, we embarked on a comprehensive series of studies to: (i) capture transcriptional changes corresponding to the sensing of different signals/cues in the human chondrocytes microenvironment, (ii) investigate hypothesis pertaining to the coupling of these signals, and (iii) explore how these cues mechanistically could be used to improve cartilage tissue engineering. Toward achieving these objectives, we encapsulated primary human chondrocytes in gelatin methacrylamide (GelMA) hydrogels. Next, we investigated cells chondrogenic differentiation under the application of isolated or combined forms of stimulation in a bioreactor culture. We demonstrated that chondrocytes respond more positively to a physiological thermomechanical-loading regime under hypoxia (compared to isolated forms of stimulation and conventional culture conditions at 37°C), in terms of biological cell response and subsequent collagen type II and proteoglycan formation. Overall, this study represents a significant first step toward addressing cartilage thermo-mechanobiology under low-oxygen tension and reinforces the notion that chondrogenesis can be greatly enhanced by incorporating biomimetic cues into engineered cell-hydrogel constructs.

RESULTS

Transcriptomic comparison of the effects of isolated and combined biophysical cues on chondrocyte-laden GelMA hydrogels

Toward addressing the uniqueness of the transcriptional effects of different biophysical cues on chondrocyte function, chondrocyte-laden constructs were placed in a bioreactor designed to simulate thermomechanical cues as experienced in the knee joint, up to 16 days (Figure 1A). The expression of chondrocyte phenotype-associated markers, such as aggrecan (ACAN), collagen type II (COL2a), and cartilage oligomeric matrix protein (COMP), as well as the de-differentiation marker twist protein 1 (TWIST1), were studied. In agreement with previous studies,[11] expression of the two major structural components in cartilage tissue, ACAN and COL2a, displayed a pronounced surge in response to hypoxia (~12-fold). Similarly, expression of cartilage oligomeric matrix protein (COMP), a pentameric protein found in the territorial matrix engulfing chondrocytes, was more than 250% upregulated upon low-oxygen tension (hypoxia) treatment, whereas TWIST1 was not affected by the level of oxygen concentration.

Notably, the effects of oxygen tension on chondrocyte response to isolated biophysical stimuli were found to be substantial. Our results indicated that the application of mechanical or thermal cues had minimal

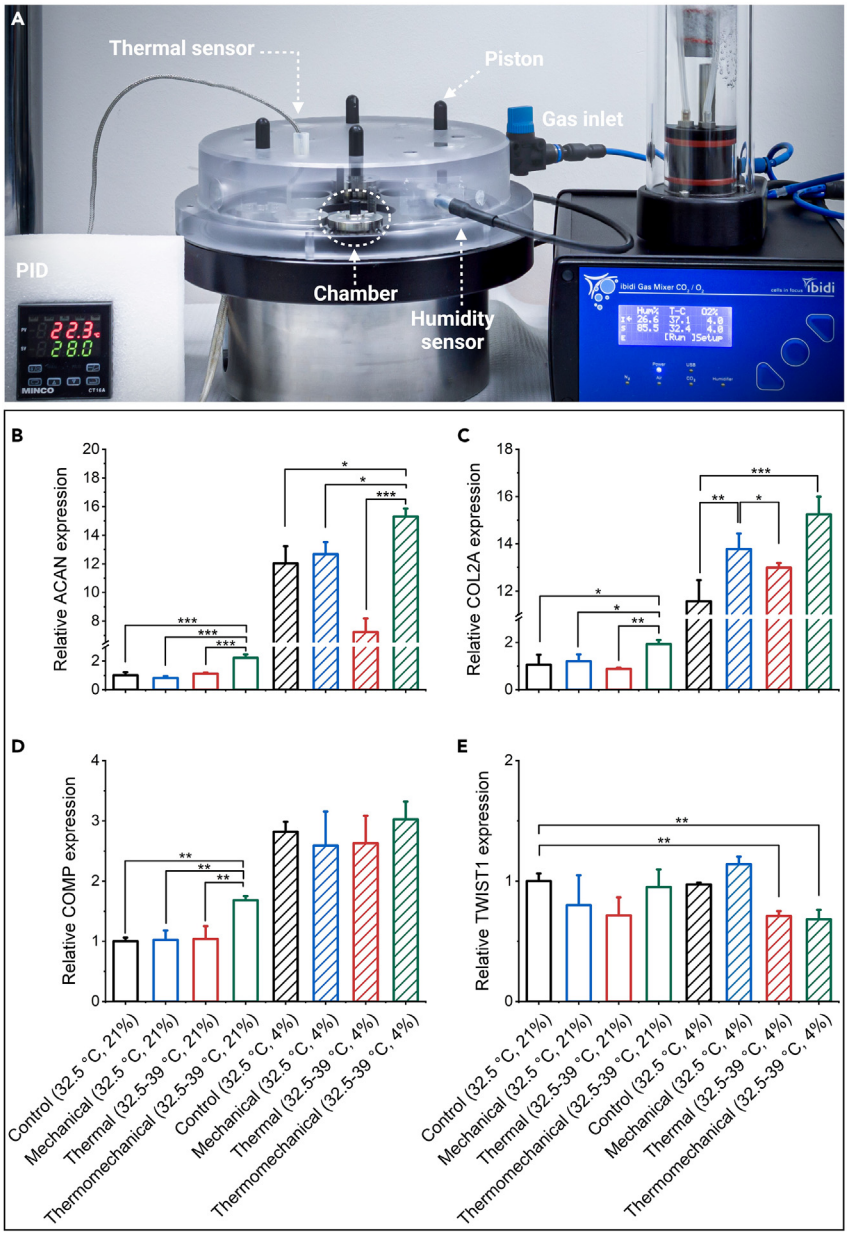

Figure 1. Loading-induced cartilage self-heating *in vitro*

(A–E) (A) Bioreactor apparatus consisting of three modules. A PID controller regulates the temperature profile applied in the engineered constructs through a thermal sensor. A gas mixer ensures desired CO_2 and O_2 concentration to achieve either normoxia or hypoxia conditions. The bioreactor apparatus is designed to fit an Instron E3000 machine to apply compressive loads on samples. Comparison of the relative expressions of genes of interest: (B) ACAN, (C) COL2A, (D) COMP, and (E) TWIST1 are shown for the different forms of stimulation normalized to the normoxia free-swelling control groups. RPL13a was used as the housekeeping gene. Samples for mRNA analysis were collected immediately after the last loading cycle was ceased. Control groups were treated similarly.

Error bars represent mean+standard deviation.

impact on the mRNA levels of major chondrogenic markers (ACAN, COL2a, and COMP) when constructs were cultured under normoxia conditions. However, a noteworthy shift in chondrocyte transcriptional sensitivity to isolated mechanical or thermal signals was observed upon reducing the oxygen concentration to physiological levels (4% v/v).

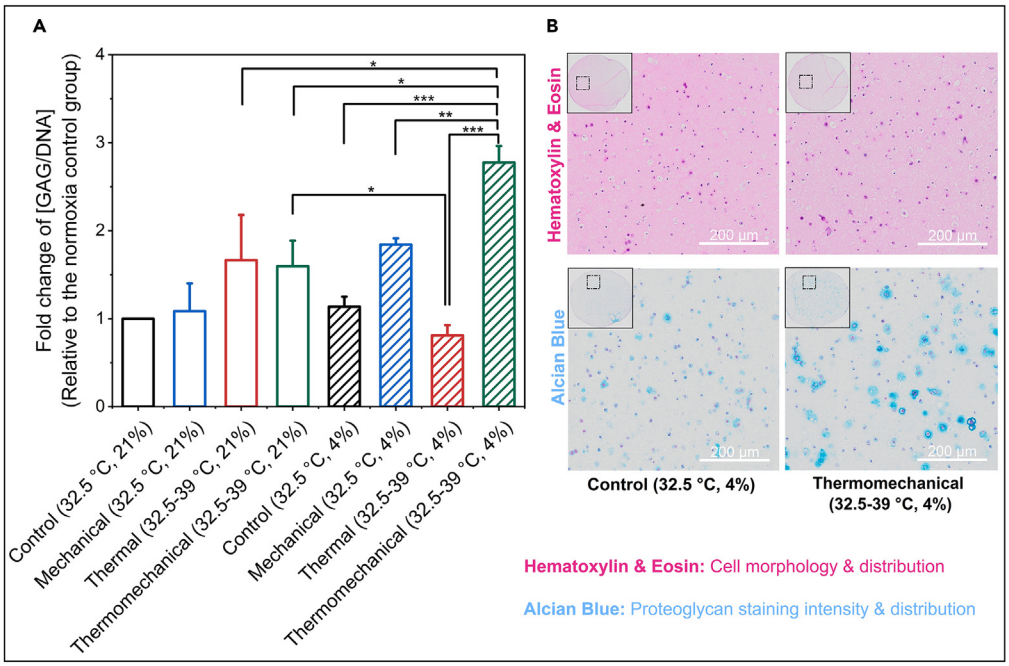

Figure 2. Calorimetric analysis of protein accumulation in engineered constructs under various biomimetic stimulations

(A) Fold change of [Glycosaminoglycan/DNA] over the culture period of 16 days for the different forms of stimulation normalized the normoxia free-swelling control groups.

(B) H&E and Alcian blue staining for the realistic physiologically relevant conditions.

Error bars represent mean+standard deviation.

Ultimately, our gene expression findings demonstrated that the simultaneous application of thermal stimulation and compression positively improves cell chondro-induction and outperforms mechanical or thermal stimulus alone. A 200% increase in ACAN and COL2a can be seen in Figures 1B and 1C when dynamic thermal stimulation acted in tandem with dynamic compression. Such coupled improvements were further enhanced under hypoxia conditions, leading to a remarkable ~15-fold increase in ACAN and COL2a expression and a ~3-fold increase in COMP expression over the unstimulated normoxia controls. In parallel, expression of TWIST1, a critical chondrogenic inhibitor, was notably more than 50% decreased when thermomechanical cues acted under low oxygen concentration.

Biomimetic thermomechanical stimulation under low oxygen tension led to increased GAGs content in the engineered hydrogels

To confirm, visualize, and assess potential enhancements in extracellular matrix-level arising from combined biomimetic stimulation, we first assessed deposition of glycosaminoglycans (GAGs) via calorimetric DMMB assay. In line with the gene expression data, glycosaminoglycan synthesis trended highest when chondrocytes perceived thermomechanical signals upon a hypoxic milieu, as illustrated in Figure 2A. This trend was further supported by Alcian blue staining as illustrated in Figure 2B.

The morphology and location of chondrocytes in the GelMA constructs were also assessed 16 days after encapsulation and were not affected by the application of different stimuli as indicated by the similar staining intensities under H&E. Collectively, these findings demonstrate how combined physiologically relevant cues contextualize each other to enhance cell chondro-inductivity.

A combination of thermomechanical stimulation and hypoxiagenerated enhancements in COL2A protein expression

Functional collagenous matrix content also reflected the trends seen in gene expression (Figures 3A–3C). The collagen content of the engineered constructs was assessed using histology and immunofluorescence

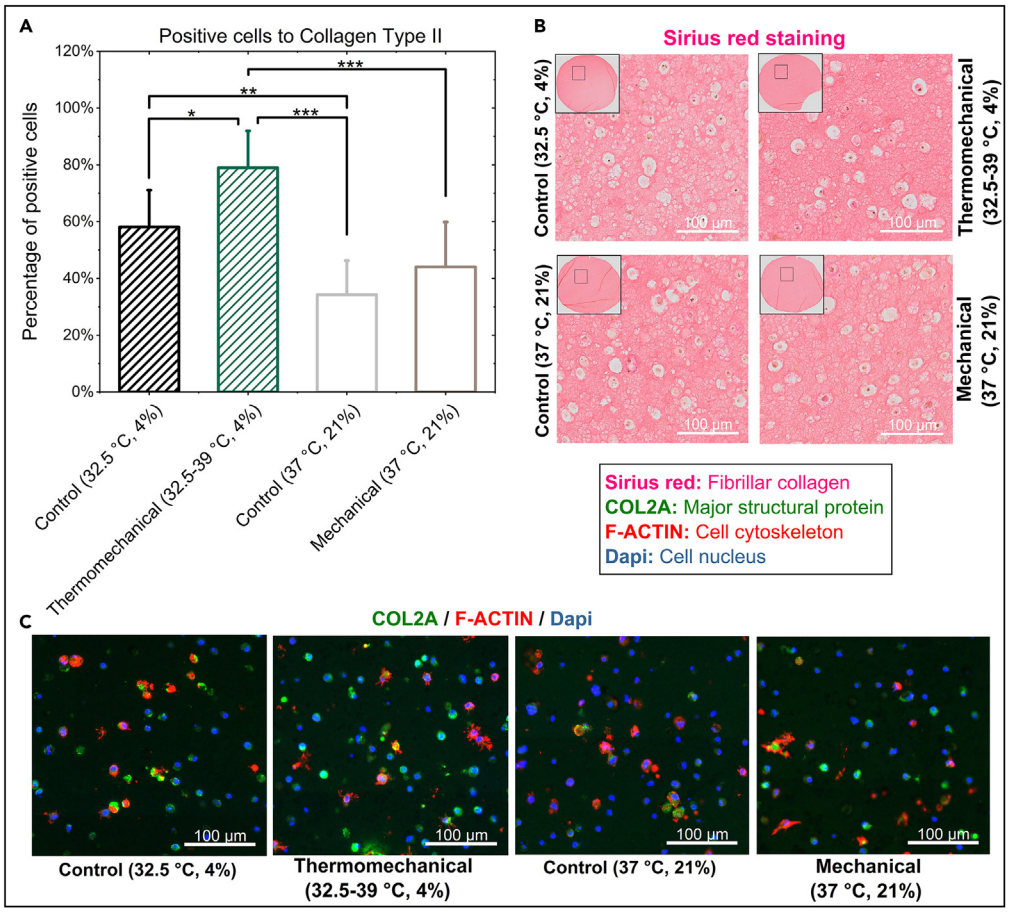

Figure 3. Histological and immunofluorescence staining for collagen type II protein

(A) Percentage of positive COL2A-cells for the different experimental groups.

(B) Sirius red staining for total collagen visualization.

(C) Representative fluorescent images of human articular chondrocytes seeded in GelMA-based hydrogels channels after 16 days of culture.

Error bars represent mean+standard deviation.

staining. In this series of experiments, we also included experimental groups mimicking the standard, however, unrealistic culture temperatures that have been so far used in cartilage mechanobiological studies. Inter-group comparison was conducted by calculating the percentage of COL2A-positive cells with respect to different stimuli. Implementation of a synergistic regime involving dynamic thermomechanical stress and low-oxygen tension resulted in a discernible upward trend in collagen levels within the engineered constructs. In terms of the number of COL2A-positive cells, constructs that received combined treatments (heat + load + hypoxia) trended higher (~80%), significantly different from free-swelling controls under hypoxia conditions (Figure 3A). Interestingly, conventional, unrealistic culture conditions at 37°C brought a stark decrease in COL2A accumulation within the engineered constructs, irrespective of whether or not mechanical loading was applied (~41% and ~32% respectively). Deposition of total collagen was also monitored by sirius red staining (Figure 3B). No significant differences were detected between the experimental groups denoting that the [COL2A/total collagen] ratio is higher when samples receive a combination of three biomimetic signals.

Thermomechanical stimulation in conjunction with hypoxia yields the largest SOX9 transcriptional improvements over control groups potentially through HIF1a expression

After observing a synergistic effect on GAG and COL2A protein deposition following combined biomimetic cues, we then sought to elucidate potential mechanisms behind such improvements. SOX9, a crucial transcription factor for embryonic chondrogenesis, has been demonstrated to counteract the

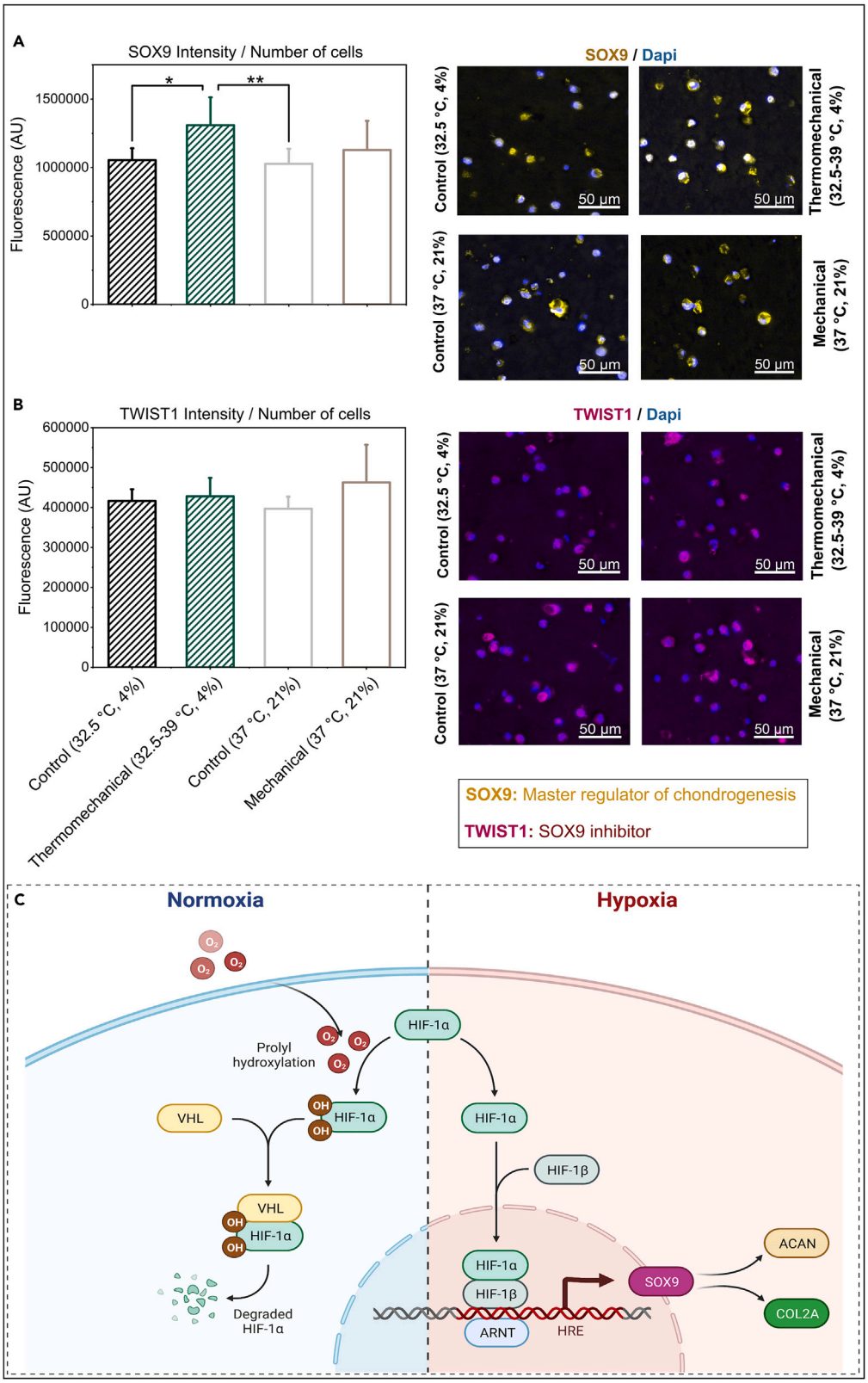
A
SOX9 Intensity / Number of cells
Fluorescence (AU)
1500000
1000000
500000
0
*
**
SOX9 / Dapi
Control (32.5 °C, 4%)
Thermomechanical (32.5-39 °C, 4%)
Control (37 °C, 21%)
Mechanical (37 °C, 21%)
50 µm
B
TWIST1 Intensity / Number of cells
Fluorescence (AU)
600000
500000
400000
300000
200000
100000
0
Control (32.5 °C, 4%)
Thermomechanical (32.5-39 °C, 4%)
Control (37 °C, 21%)
Mechanical (37 °C, 21%)
TWIST1 / Dapi
SOX9: Master regulator of chondrogenesis
TWIST1: SOX9 inhibitor
C
Normoxia
Hypoxia
O2
HIF-1α
Prolyl hydroxylation
OH
VHL
HIF-1β
Degraded HIF-1α
ARNT
HRE
SOX9
ACAN
COL2A

Figure 4. Proposed mechanism of thermo-mechanotransduction process under low-oxygen tension condition
(A) Integrated fluorescent intensities for SOX9 with representative confocal images of human articular chondrocytes seeded in GelMA-based hydrogels channels after 16 days of culture.
(B) Integrated fluorescent intensities for TWIST1 with representative confocal images of human articular chondrocytes seeded in GelMA-based hydrogels channels after 16 days of culture.
(C) Proposed mechanism of ACAN and COL2A synthesis upon thermomechanical loading through HIF1a signaling pathway. Schematic was created with BioRender.com.
Error bars represent mean+standard deviation.

de-differentiation process of mature cartilage.[17] Our immunofluorescence staining findings revealed that this chondrogenic marker is significantly enhanced when chondrocytes are subjected to a combination of three physiologically relevant stressors (over the free-swelling control groups). Our findings further demonstrated that expression of SOX9 protein is significantly lower during conventional/standard culture conditions (free-swelling condition at 37°C) that may be improved by mechanical compression; nonetheless, the increase was less pronounced (Figure 4A).

Expression of TWIST1, a pleiotropic factor with dominant anti-chondrogenic activity,[18] was further assessed with respect to different stimuli. Chondrocytes were stained positive irrespective of the form of stimulation, and TWIST1 expression appeared unaffected by either treatment (Figure 4B). Chondrocytes possess a highly regulated response mechanism to address the challenge of hypoxia, and the hypoxia-inducible factor (HIFa) serves as a pivotal player in driving the expression of target genes. Through our investigation, we have discovered that the HIF1a gene displays analogous response patterns to the SOX9 gene, which, in turn, exhibits similar patterns to the expression of the aggrecan (ACAN) gene (See Figure S2).

Furthermore, our experimental data demonstrate that both the SOX9 and ACAN factors reach their maximum protein levels upon exposure to thermomechanical stimulation. This compelling evidence leads us to postulate that the HIF1a signaling pathway potentially plays a vital role in governing these intricate cellular responses to such stimuli. Figure 4C summarizes the potential role of HIF1a signaling in regulating the expression of SOX9, which in turn results in higher ACAN and COL2A synthesis. Overall, these data denote that the concomitant application of 3 stressors can significantly improve the expression of pivotal transcription factors that may ultimately result in neocartilage with enhanced glycosaminoglycan and collagen type II content.

DISCUSSION

Historically, a biomechanics-inspired strategy was deemed highly attractive during functional cartilage tissue engineering.[19] Indeed, given its avascular and aneural character, articular cartilage possesses, primarily, a biomechanical function.[20] With this respect, many research groups, including ours, have convincingly illustrated the beneficial effects of mechanical stimulation on chondro-induction either in tissue explants or in cell-hydrogel constructs.[21–23] Nonetheless, despite its significance, previous mechanobiological studies thus far normally consider a static culture temperature (37°C) and a constant oxygen concentration of ~21% v/v (normoxia conditions) that are far from reflecting the physiological *in vivo* scenario within the human knee joint. For the first time, we explored cartilage thermo-mechanobiology under low-oxygen tension conditions and demonstrated the additive and/or synergistic effects of loading, heat and hypoxia stimuli at the transcriptional, as well as protein level of chondrogenesis (Figure 5). By analyzing these interrelated factors, this study underscores the critical significance of considering their combined impact in future research endeavors aimed at refining and optimizing cartilage tissue engineering strategies. Collectively, this study carries immense practical significance as it has the potential to revolutionize strategies for cartilage repair and regeneration. By unraveling the intricate effects of hypoxia and seamlessly integrating thermal and mechanical stimulation, the current research aims to create *in vitro* models that faithfully replicate the physiological environment. This approach holds great promise in advancing tissue engineering methods and optimizing culture conditions specifically tailored to the complexities of cartilage engineering.

We have recently gained an understanding on how biomimetic thermomechanical cues can alter chondrocyte biological responses through activation of calcium (TRPV4) and potassium (TREK1) ion channels to facilitate matrix biosynthesis in short-term studies.[16] Building upon previous work and in an attempt to expand our knowledge on how low-oxygen tension (hypoxia) might add to these improvements, we first

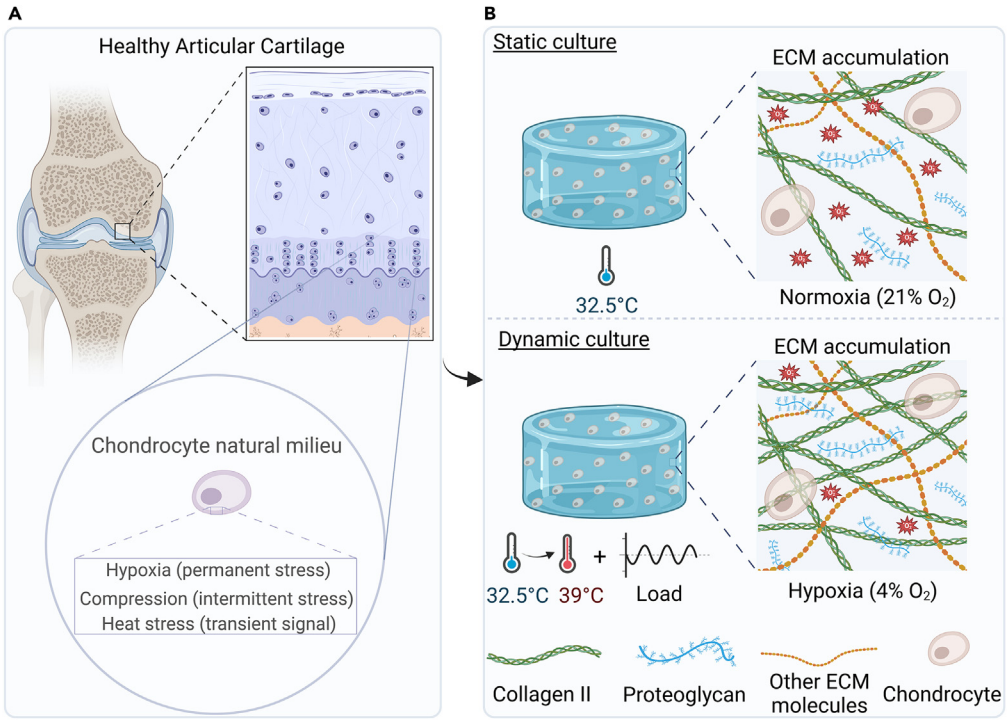

Figure 5. Summary of research work

(A) Chondrocytes in cartilage tissue develop under hypoxia conditions, where intermittent mechanical or transient thermal cues can occur (left).

(B) By re-enacting these biophysical cues *in vitro* (right) tissue maturation is accelerated through enhanced biosynthetic content. Schematic was created with BioRender.com.

compared gene expression data with respect to isolated or combined forms of stimulation. Overall, the obtained findings for ACAN, COL2a, COMP, and TWIST1 clearly indicate that physiologically relevant thermomechanical cues are more effective when applied under low-oxygen tension, positively enhancing chondrogenic expression of cells. With regards to isolated types of stimuli, we consistently observed that mechanical or thermal cues could alter the expression of major chondrogenic genes only when oxygen concentration was reduced to physiological levels. Regarding the effect of isolated mechanical stimulation, previous studies conducted under normoxic conditions have yielded mixed results when employing similar loading magnitudes as those utilized in our study, highlighting the influence of variables such as pre-culture period and duration. Notably, Visser et al. demonstrated that subjecting human chondrocytes in 3D culture to compressive regimes similar to our study (~15% strain, 1 Hz sinusoidal loading) but for a duration of 3 h per day instead of 1.5 h, led to a significant augmentation in ACAN transcription.[24] In another study, Meinert and colleagues showed that mRNA transcription of major chondrogenic markers such as ACAN, COL2A1, and PRG4, could only be significantly higher at different strain levels compared to free-floating samples only when the preculture period was extended from 7 to 14 days before mechanical stimulation.[25]

Consequently, it seems that the duration of mechanical loading and the culture system of chondrocytes can yield significant effects on cells biosynthetic and phenotypic capacity with regards to dynamic compression; and changing any of these conditions can produce completely different results. This latter, could somewhat explain why we didn't observe significant differences among the control groups and isolated mechanical or thermal cues for the constructs in normoxia conditions, as we precultured constructs for only 7 days prior application of corresponding stimuli.

The biomimetic temperature evolution signal led to a significant reduction in aggrecan transcription and subsequent protein synthesis when applied in a low-oxygen environment. Surprisingly, such adverse effects were reversed when mechanical compression was introduced. This observation can be considered as the most provocative finding of the current research work. So far, there is no available information in

the literature pertaining to the mode of action of combined stressors on chondrocytes biological responses. However, previous research conducted in fish suggests that heat and hypoxia stressors are very likely to interact.[26,27] A mechanism that is proposed to explain the observed effects stems from the interplay between temperature and low-oxygen tension, and their impact on oxidative metabolism. It has been postulated that, as temperature increases, the metabolic rate and associated oxygen demand also rise, while hypoxia limits oxygen supply. Therefore, these two stressors are likely to interact in an antagonistic manner, which in turn may yield adverse effects for the expression of a variety of genes and/or proteins. Such adverse effects are diminished when mechanical load acts in tandem with heat and oxygen stimuli. The fact that constructs subjected to a combination of the 3 stressors, simultaneously, showed maximum expression of ACAN, leads us to posit that the application of thermomechanical stress under hypoxic conditions may play a role in shaping the extracellular matrix of neocartilage in ways that were not investigated in this study. For instance, a great corpus of evidence suggests that externally applied mechanical load alters the regional distribution of oxygen and glucose within cells.[28] Oxygen and glucose, in turn, guide the process of anaerobic glycolysis, which is the main mechanism of energy production during hypoxia conditions and essentially modulates extracellular matrix synthesis.[29] Another possible explanation could be that chondrocytes are able to develop mechanisms that improve their cross-tolerance in an attempt to maintain homeostasis. Cells cross-tolerance in turn occurs when exposure to one stressor conveys enhanced tolerance to a subsequent and distinct stressor. In other words, cells pre-induction to one stress could significantly alter the physiological responses during subsequent exposure to a second stress, ultimately harnessing the benefits of each individual signal and conferring improved biosynthetic performance. Gene expression data illustrate that oxygen tension is a dominant cue. We hypothesize that an isolated mechanical load can optimize oxygen distribution inside the cells boosting the effect of oxygen tension. However, the isolated temperature signal requires more oxygen to show its contribution, while there is a limited amount of oxygen in the static hypoxia condition, which leads to a sub-optimal/conflicting condition.

Last but not least, our investigation at the gene level has provided compelling evidence that hypoxia-inducible factor 1a (HIF1a) exhibits remarkably similar response patterns to both SOX9 and ACAN genes when exposed to various forms of stimuli. These findings strongly suggest the potential involvement of the HIF signaling pathway in this intricate cellular process. However, in order to gain a more comprehensive understanding of the underlying mechanisms, we recognize the need for additional experiments, such as utilizing RNA sequencing. Such an approach will provide a more in-depth and precise assessment, enabling us to obtain a more definitive answer regarding the role of the HIF signaling pathway in regulating the responses of SOX9 and ACAN genes to different stimuli.

The present study aligns with previous investigations indicating that chondro-inductive potential can be augmented by conditions that more closely approximate the native *in vivo* cartilage milieu. For example, the application of multiple loading modalities, including compression, sliding, and shear, has been shown to yield greater results compared to a single mode of loading.[30] In addition, previous studies also showed that by combining hypoxia culture with multi-directional loading showed more potent effects than normoxia.[31] In line with the GAG expression trend, the concomitant application of hypoxia, heat, and load resulted in changes on chondrocyte sensibility, which translates to an improvement in collagen type 2 synthesis. Collagen type II is the predominant collagen type in hyaline cartilage, accounting for approximately 90% of its total collagen content, and is strongly associated with tensile stiffness and strength, as well as articular cartilage compression.[32] Interestingly, collagen type II in articular cartilage has negligible turnover, which may contribute to the low-regenerative capacity of cartilage by impairing the ability to repair and replace damaged collagen.[32] The findings of the current study revealed that by reproducing and combining *in vitro* the different types of stresses as experienced *in vivo* by cartilage, allowed chondrocytes to produce copious amounts of collagen type 2 and outperformed any other form of stimulation strategy.

In an endeavor to understand the greater extracellular matrix accumulation following combined biomimetic cues over the unstimulated hypoxia controls, we found that SOX9 protein accumulation in the latter constructs is significantly higher compared to the untreated controls. In essence, the trends observed in SOX9 protein staining, as illustrated in Figure 4A, align with the quantitative metrics characterizing glycosaminoglycan and collagen type II content, as depicted in Figures 2A and 3A, urging us to believe that higher SOX9 expression may be responsible for higher GAG and COL2a content accumulation. SOX9 is

a transcription factor that is important in both developing and adult cartilage.[33] It is necessary for chondrogenesis because it guarantees chondrocyte lineage commitment, fosters chondrocyte survival and activates elements unique to cartilage. We further showed that the expression of this protein was far lower when constructs cultured under normal culture conditions (21%, 37°). Additionally, expression of TWIST1 that is known to directly inhibit SOX9 was not altered with respect to different culture conditions, indicating that a combination of load, heat, and oxygen can have more profound effects on chondrogenesis compared to unrealistic standard culture conditions.

In summary, various aspects of cartilage thermo-mechanobiology under hypoxia were investigated using a novel customized *in vitro* model in relatively long-term studies. Our findings show that combined biomimetic cues have a greater effect on chondrogenesis than isolated forms, regardless of the oxygen concentration. Externally applied cues that emulate *in vitro* the signals found *in vivo*, such as hypoxia or thermomechanical stress, contribute to the generation of tissue-engineered hyaline-like tissues.[15,16] In this work, we demonstrate that when these stressors co-exist simultaneously during one culture period, can significantly improve the biochemical content (ACAN, COL2A) of engineered cartilage constructs. SOX9 transcriptional factor is hypothesized to be involved as a key element in the thermo-mechanotransduction process. Overall, the findings of this study provide compelling evidence to suggest that the early response to thermomechanical loading in a reduced oxygen milieu engenders significant perturbations in intracellular signaling cascades, notably those involving SOX9. These dynamic changes ultimately give rise to the development of novel cartilaginous tissue characterized by enhanced biosynthetic capacity, as evidenced by the conspicuous upregulation of ACAN and COL2A.

Limitations of the study

1. Knowing the variability of human chondrocytes in response to environmental signals, key investigations may be repeated using cells from other donors during future studies to conclusively identify the precise mechanism of action. However, this was beyond the scope of the current research work, and herein we report the results based on cells derived from one primary human chondrocyte source. To ensure the robustness and generalizability of our results, we have previously seeded human chondroprogenitor cells as well as human chondrocytes from the same donor as in this study in various hydroxyethyl-based hydrogels and observed similar responsive patterns to the thermomechanical stimulation applied in this work.[15,16] Although we acknowledge that testing primary chondrocytes from additional donors would have strengthened the conclusions drawn, the observed consistency in our results across different cell sources and different hydrogel environments underscores the validity and reproducibility of our findings.

2. Our findings strongly suggest the potential involvement of the HIF signaling pathway in this intricate cellular process. However, in order to gain a more comprehensive understanding of the underlying mechanisms, we recognize the need for additional experiments, such as utilizing RNA sequencing. Such an approach will provide a more in-depth and precise assessment, enabling us to obtain a more professional and definitive answer regarding the role of the HIF signaling pathway in regulating the responses of SOX9 and ACAN genes to different stimuli.

STAR★METHODS

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

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- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107491.

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AUTHOR CONTRIBUTIONS

Conceptualization: T.S., Y.G., N.N., V.K.R., P.K., and D.P.P.; Methodology: T.S. and Y.G.; Validation: T.S., Y.G., and N.N.; Formal Analysis: T.S., Y.G., and N.N.; Investigation: T.S., Y.G., and N.N.; Writing—Original Draft: T.S., Writing—Review & Editing: T.S., Y.G., N.N., V.K.R., P.K., and D.P.P.; Supervision: D.P.P.; Funding Acquisition: D.P.P.

DECLARATION OF INTERESTS

The authors declare that they have 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
Antibodies		
Mouse anti-SOX9	Abcam	ab76997 RRID: AB_2194156
Mouse anti-COL2A	Invitrogen	MA5-12789 RRID: AB_10986605
Alexa Fluor 568 phalloidin	Invitrogen	A12380
Mouse anti-TWIST1	Invitrogen	MA5-17195 RRID: AB_2538666
Alexa Fluor 488	Invitrogen	A11029 RRID: AB_2534088
Chemicals, peptides, and recombinant proteins		
Methacrylic anhydride	Sigma Aldrich	276685
Gelatin from porcine skin	Sigma Aldrich	G2500
Experimental models: Cell lines		
Healthy primary human chondrocytes, male donor	INNOPROT, Spain	#cat 0018996 P10970
Oligonucleotides		
Primers for PCR	This paper, Microsynth (Balgach, Switzerland)	See supplemental information
Software and algorithms		
Origin pro 2021	OriginLab	https://www.originlab.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dominique Pioletti (dominique.pioletti@epfl.ch).

Materials availability

This study did not generate unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- 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

Cell lines

Human articular chondrocytes (P10970, Innoprot, 22-year-old male donor) were expanded in T-75 tissue culture flasks inside chemically defined chondrogenic medium consisting of alpha minimum essential medium (a-MEM), with 1% L-glutamine, 1% non-essential amino acids (NEAA), 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1% penicillin, 1% streptomycin, supplemented with 10% fetal bovine serum (FBS), 5 ng/mL fibroblast growth factor (FGF, Sigma) and 1 ng/mL transforming growth factor β 1 (TGF-β1)).

METHOD DETAILS

Macromer synthesis

Gelatin methacryloyl hydrogel (GelMA) was prepared by reaction of type A gelatin (porcine skin, ref. G2500, Sigma Aldrich) with methacrylic anhydride (ref. 276685, Sigma Aldrich) at 57°C for 3 h, as previously

described.[34] Briefly, gelatin was dissolved at a 10% w/v in Dulbecco's phosphate buffered saline (DPBS, Gibco) after which 0.6 g methacrylic anhydride (per gram of gelatin) was added dropwise to achieve an ~80% degree of modification. Removal of any non-reacted molecule was achieved through dialysis of the functionalized polymer against distilled water at 53°C for 1 week. Water bath was changed every day. Solution was filtered, freeze-dried and subsequently stored at 4°C before use. The degree of functionalization was determined via ^{1}H NMR spectroscopy. The spectra were collected with a 400 MHz Bruker Avance NEO. Briefly, 18 mg of the samples were dissolved in 500 L of D2O. After that, the solution was transferred to an NMR tube for analysis. The control samples were treated in the same way.

Human chondrocyte expansion and hydrogel encapsulation

Human articular chondrocytes (P10970, Innoprot, 22-year-old male donor) were expanded in T-75 tissue culture flasks inside chemically defined chondrogenic medium consisting of alpha minimum essential medium (a-MEM), with 1% L-glutamine, 1% non-essential amino acids (NEAA), 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1% penicillin, 1% streptomycin, supplemented with 10% fetal bovine serum (FBS), 5 ng/mL fibroblast growth factor (FGF, Sigma) and 1 ng/mL transforming growth factor β 1 (TGF-β1)).

Hydrogel precursor solution of 10% w/v GelMA were prepared inside phosphate buffered saline (PBS) containing LAP photoinitiator (Lithium phenyl-2,4,6-trimethylbenzoylphosphinate, 0.1 mg/mL). Passage 4 chondrocytes, at a seeding density of 1×10^{7} cells per mL, were carefully resuspended in hydrogel precursor and pipetted in a custom-designed mold (Teflon, mm × mm × mm). Chondrocyte/hydrogel suspension was crosslinked under illumination of 405 nm wavelength for 2 min. Constructs were cultured inside differentiation medium composed of Dulbecco's modified Eagle's medium (DMEM) with 1% L-glutamine, 1% NEAA, 1% HEPES, 1% penicillin/streptomycin antimycotic cocktail, supplemented with 10% ITS-VI (Life Technologies), 10% L-ascorbic acid (Sigma) and 10 ng/mL TGF-β1. Medium was refreshed every other day.

Mechanobiological study

Chondrocyte-seeded hydrogels were divided into 2 categories each one consisting of 4 different groups: 1) cell-laden hydrogels that were stimulated with cyclic compression at 32.5°C (normal intra-articular knee temperature), 2) cell-laden hydrogels that were subjected to dynamic thermal stimulation, 3) cell-laden hydrogels that were stimulated with a combination of dynamic compression and dynamic thermal stimulation and 4) cell-laden hydrogels that remained in free-swelling condition at 32.5°C (control groups). The compressive stimulation protocol was designed to mimic a normal physical activity, by applying a cyclic 15% unconfined compressive strain (5% pre-strain followed by 10% amplitude strain in a sinusoidal waveform), once per second (1 Hz frequency), for 1.5 h. Similarly, the dynamic thermal stimulus was meant to simulate the natural temperature increase inside the knee joint, over a period of 1.5 h.[35] Stimulation was performed either in normoxia (21% v/v oxygen concentration) or hypoxia conditions (4% v/v oxygen concentration). Temperature evolution over time was monitored via a PID controller (Minco-CT16A, Minnesota, USA). The humidity, as well as the CO_2/O_2 concentration inside the bioreactor were controlled via an external gas mixer (ibidi-Gas Incubation System, Martinsried, Germany). Constructs were cultured for a total of 16 days. After stimulation, engineered constructs receiving hypoxia were incubated separately in 4% (v/v) O_2 using a hypoxic incubator (Vitaris AG) that generates hypoxic conditions through appropriate nitrogen regulation, keeping CO_2 concentration constant. A detailed schematic illustration of the mechanobiological study followed in the current work can be seen in supplemental information (Figure S1). On day 0, chondrocytes are seeded on scaffolds and remain at free swelling condition up to Day 7. On Day 7, fetal bovine serum is removed from the medium, while vitamin C and Insulin transferrin selenium are introduced. All constructs were subjected to dynamic stimulation on Days 8, 10, 12, 14 and 16. Constructs were then collected for gene expression and biochemical analysis.

Gene expression analysis

Constructs were homogenized in 300 μL of TRIzol reagent and RNA was extracted using Nucleospin RNA XS kit (Macherey-Nagel, Düren, Germany) following manufacturer's instructions and as previously described.[16,21] Briefly, the hydrogels were blended in Trizol using a polytron (Kinematica, Switzerland) while keeping the tube cold on dried ice. Afterward, chloroform (100 μL) was introduced, and the samples were centrifuged for 10 min at 12000 rpm at 4°C. The watery phase was meticulously transferred to 1.5 mL Eppendorf tubes, and the extraction process was finalized by adding 6.5 μL of RNA carrier and following

the XS kit protocol. The RNA concentration was quantified by spectrophotometry and RNA samples were reversed transcribed into cDNA using Taqman Reverse Transcription Reagents (Applied Biosystems). For each PCR reaction, Fast SYBR Green Master Mix (Applied Biosystems) was added, in a final reaction volume of 20 μL, containing 10 ng of synthesized cDNA. Primers were selected to capture mRNA transcripts pertaining to hyaline cartilage. The relative expression levels of each gene were analyzed using the comparative ΔΔCt method after normalization to RPL13a endogenous control. Primers and probe sequences for qRT-PCR can be found in supplemental information (Table S1).

Biochemical quantification

Constructs dedicated for biochemical analysis were digested inside a 6 μL/mL papain solution, containing 100 mM Na_2HPO_4, 10 mM L-cysteine, 10 mM EDTA at pH = 6.5, overnight at 65°C. Total sulfated glycosaminoglycan content was measured via 1,9-dimethylmethylene blue (DMMB) assay, at pH 1.5. Chondroitin sulfate was selected as the standard and absorbance was measured at 530 and 590 nm. GAG measurements for each construct were normalized to each DNA content. Total DNA content in turn, was measured via Hoechst 33258 DNA intercalating dye method. For the preparation of the standard curve, purified Calf Thymus DNA was used.

Sample preparation for histology and immunohistochemistry

For histological and immunohistochemical staining, the chondrocyte-laden constructs were taken out of the media and subsequently fixed with 4% paraformaldehyde overnight at room temperature. Next day, engineered tissues were incubated sequentially in 15% and 30% sucrose solution for 3 and 2 h respectively. The samples were then embedded in optical cutting temperature compound (OCT) and frozen in liquid nitrogen. Chondrocyte-laden hydrogels were cryosectioned to a thickness of 7 μm using a Leica CM 1950 cryostat and stained with Hematoxylin and Eosin, Serious red, and Alcian blue. Samples were also analyzed using immunofluorescense staining. The primary and secondary antibodies used in this study are listed in the supplemental information (Page S3). Fluorescent and non-fluorescent images of various specimens were obtained utilizing a 20× magnification tile-scan technique with an Olympus VS120 whole-slide scanner. Uniform laser intensity and exposure duration were maintained during imaging for all specimens. The resultant tile-scan images were imported into QuPath v0.3.2 software for analysis and stitching. Subsequently, a representative region of interest (ROI) was meticulously chosen for each specimen, and the DAPI channel was utilized to identify and quantify the number of cell nuclei. For signaling proteins (SOX9, TWIST1), the total intensity of the corresponding channel (FITC) within each ROI was quantified and normalized by the number of detected cells. For functional markers (COL2A), a FITC intensity threshold was set, and the number of positive cells was calculated and compared among the different groups.

QUANTIFICATION AND STATISTICAL ANALYSIS

The statistical analysis for multiple group comparisons was performed using analysis of variance with Tukey's post hoc tests, and the data are presented as mean ± standard deviation. Statistical significance is indicated by (*) for $p \leq 0.05$, (**) for $p \leq 0.01$, and (***) for $p \leq 0.001$. Origin Pro 2021 software was utilized for the statistical analysis. All experiments were carried out with at least three biological replicates. To confirm the results of gene expression (PCR) and biochemical analysis (GAG and immunofluorescence), three independent experiments were conducted, each with a minimum of three biological replicates for each experimental group.