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Enhancing Chondrocyte Biosynthetic Activities: Harnessing the Power of Loading-Induced Evolved Temperature

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Abstract

Chondrocytes within articular cartilage possess the capacity to perceive and react to signals generated by the typical load-bearing actions of daily life, such as walking or running. Articular cartilage is predominantly oriented toward compression-based functionality, and thus the application of such stimuli dominates the field of cartilage regeneration. Nonetheless, despite its potential significance, physiological variations in temperature within the cartilage, associated with mechanical deformation of this tissue, and their biological consequences are still poorly studied. This thesis aims to comprehensively investigate the impact of a transient temperature increase on chondrocyte functionality in knee cartilage. Drawing on an original *in vitro* investigation of cartilage *thermo-mechanobiology*, this thesis unearths the hidden influence of temperature increase on this exquisite tissue, shedding new light on the complex interplay between mechanical and thermal stimuli on chondrocyte function.

Employing a biomimetic approach, we commence by probing into the cellular effects of temperature increase during cyclic loading, unmasking its profound significance in the realm of cartilage mechanobiology. The research findings provide robust evidence substantiating that the synergy between mechanical signals and a biomimetic thermal increase elicits a remarkable amplification in the accumulation of major chondrogenic markers. Furthermore, this distinctive thermo-mechanical combination engenders a significant upregulation of temperature-gated (TREK1) and mechano-gated ion channels (TRPV4), further elucidating underlying thermo-mechanotransduction mechanisms.

Expanding on these insights, we next shift our focus to the intricate relationship between loading-induced evolved temperature and hypoxia, another pivotal factor known to significantly influence chondrocyte function. Utilizing a state-of-the-art bioreactor and chondrocyte-laden hydrogels (specifically, single network covalent-based hydrogels) under controlled conditions, we further elucidate this complex relationship. Our findings challenge the traditional paradigm of solely focusing on mechanical attributes in cartilage, highlighting the influential role of multiple, co-existing stressors on chondrocyte behavior.

Beyond investigations using conventional, covalently-crosslinked hydrogels, we extend our endeavors to the field of biomaterials by developing double network supramolecular hydrogels. These hydrogels use dynamic host-guest links to mimic hyaline cartilage's natural dynamism, fostering a favorable environment for cell encapsulation, as illustrated by enhanced mRNA

transcription and synthesis of cartilage-related proteins. Next, through external application of load, heat, and hypoxia, we observe synergistic enhancements in chondrocyte biosynthetic activity (predominantly in collagen and aggrecan expression), surpassing the effects observed in conventional (single network covalent-based) hydrogels subjected to the same conditions. Our novel approach presents a unique and compelling perspective on the inextricable interplay between biomimetic temperature evolution, hypoxia and cartilage mechanobiology, propelling the significance of thermo-mechanical cues and matrix dynamism to the forefront of bio-engineering strategies for cartilage repair.

Keywords: Mechanotransduction, Cartilage thermo-mechanobiology, Cartilage self-heating, Bioreactors, Hypoxia

Résumé

Les chondrocytes au sein du cartilage articulaire possèdent la capacité de percevoir et de réagir aux signaux générés par des charges mécaniques de la vie quotidienne, telles que la marche ou la course à pied. Le cartilage articulaire est principalement soumis à des contraintes mécaniques en compression. L'application de ce type de stimulus est par conséquent généralement utilisé dans le domaine de l'ingénierie tissulaire du cartilage. Les variations physiologiques de la température dans le cartilage associées à des déformations mécaniques de ce tissu et leurs conséquences biologiques sont encore très peu étudiées. Cette thèse vise donc à étudier de manière approfondie l'impact d'une augmentation transitoire de la température sur la fonctionnalité des chondrocytes dans le cartilage du genou. S'appuyant sur des études *in vitro* originales de la *thermo-mécanobiologie* du cartilage, cette thèse met en évidence l'influence méconnue de l'augmentation de la température de ce tissu sur les chondrocytes. Les résultats obtenus, apportent un éclairage nouveau de l'interaction complexe entre les stimuli mécaniques et thermiques sur la fonction des chondrocytes.

En adoptant une approche biomimétique, nous commençons par explorer les effets sur les chondrocytes de l'augmentation de la température pendant des charges mécaniques cycliques. Les résultats obtenus fournissent des preuves solides qui étayent le fait que la synergie entre les signaux mécaniques et une augmentation physiologique de la température induit une amplification marquée de l'accumulation des principaux marqueurs chondrogéniques. De plus, cette combinaison de stimuli thermo-mécanique entraîne une régulation significative des canaux ioniques activés par la température (TREK1) et des canaux ioniques activés par la mécanique (TRPV4), mettant en lumière les mécanismes sous-jacents de thermo-mécanotransduction.

Se basant sur les résultats obtenus, nous portons ensuite notre attention sur la relation complexe entre l'évolution de la température due à la charge mécanique et l'influence de l'hypoxie, un autre facteur essentiel connu pour influencer la fonction des chondrocytes. En utilisant un bioréacteur innovant et des chondrocytes encapsulées dans des hydrogels (hydrogels covalents à réseau unique), dans un environnement contrôlé, nous approfondissons davantage cette interrelation entre la température résultant de la charge mécanique et l'hypoxie. Nos résultats remettent en question les paradigmes classiques de la mécanobiologie du cartilage, traditionnellement basés uniquement sur les aspects mécaniques. Ils, mettent en évidence le

rôle influent sur le comportement des chondrocytes, de plusieurs facteurs de stress agissant en synergie.

Au-delà des travaux de recherche utilisant des hydrogels conventionnels réticulés chimiquement, nous étendons nos études au domaine des biomatériaux en développant des hydrogels supramoléculaires à double réseau. Ces hydrogels utilisent des liaisons hôte-invité dynamiques pour imiter la dynamique naturelle du cartilage hyalin, induisant un environnement favorable aux cellules encapsulées dans ce type de matériau. Ceci est confirmé par, une transcription accrue de l'ARN messager et par la synthèse de protéines liées au cartilage par les chondrocytes ensemencées dans nos d'hydrogels supramoléculaires à double réseau. Ensuite, grâce à l'application de charges mécaniques externe, ainsi que de chaleur sous un régime d'hypoxie, nous observons des augmentations synergiques de l'activité biosynthétique des chondrocytes (principalement dans l'expression de collagène et d'agrécane), dépassant les effets observés dans les hydrogels conventionnels (à réseau simple réticulé chimiquement). Notre approche novatrice présente une perspective originale sur l'interaction inextricable entre l'évolution physiologique de la température et la mécanobiologie du cartilage. Nos travaux démontrent l'importance de la combinaison des stimuli thermo-mécaniques, de l'hypoxie et de la dynamique de la matrice pour des développements futurs en bio-ingénierie dans la réparation du cartilage.

Mots-clés: Mécanotransduction, Thermomécanobiologie du cartilage, Auto-échauffement du cartilage, Bioréacteurs, Hypoxie

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Chapter 1 - Setting the Scene: Contextualizing the Research Problem and Identifying Knowledge Gaps*

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1.1 Introduction

Hyaline cartilage, a remarkable tissue that provides smooth articulation and load-bearing capacity to joints, has long been a subject of intensive research in the field of mechanobiology. While the mechanical aspects of cartilage have been extensively investigated, one key factor has been largely overlooked: temperature. Unlike other tissues in the body, cartilage experiences dynamic fluctuations in temperature during physiological activities, such as joint loading during daily activities like jogging. *In vivo* studies have shown that the normal intra-articular knee temperature can reach up to ~39 ºC following cyclic loading,[1] suggesting a plausible role for temperature in influencing aspects of cartilage health and function.

In this thesis, we delve into the realm of cartilage *thermo-mechanobiology* to uncover the hidden role of temperature in chondrocyte function. Through a biomimetic approach, we first investigate the cellular effects of transient temperature increase as a byproduct of load and demonstrate its significance in cartilage mechanobiology. Our findings upend the traditional paradigm of reducing cartilage biology to solely mechanical attributes, and instead highlight the critical interplay between temperature variation and cellular behavior (Chapter 2).

Expanding upon such foundational insights, we further explore the significance of temperature variation in knee cartilage by investigating how it interacts with another critical factor: hypoxia (Chapter 3). Phenomenological studies have hinted at the profound influence of oxygen concentration, transient thermal signals, and mechanical cues on chondrocyte function. However, the way in which these factors intricately interact and potentially amplify each other's

effects on the regulatory microenvironment of chondrocytes remains a realm yet to be thoroughly explored. Delving deeper into these subtle interactions holds profound significance, as it promises a more faithful emulation of the native cartilage environment and a clearer grasp of how chondrocytes function within it. In the third chapter of this thesis, we employ a multi-modal bioreactor system to independently manipulate culture oxygen concentration, evolution of temperature, and applied mechanical loading, thereby enabling us to gain comprehensive insights into the transcriptional and biosynthetic profiles of chondrocytes under well-defined combinations of biophysical parameters. In the final chapter (Chapter 4), we advance our investigations into the realm of biomaterials by transitioning from purely conventional covalently-bonded hydrogels to innovative double network supramolecular hydrogels that offer a dynamic system for cell encapsulation, even in free swelling conditions. These supramolecular hydrogels are engineered to closely replicate the dominant non-covalent interactions present in soft tissues like cartilage. This enables the potential for synergistic effects and interactions among various factors, including load, heat, hypoxia, and the dynamic nature of crosslinks. Through meticulous manipulation of these parameters, we observe significant enhancements in cellular behavior compared to conventional (single network covalent-based) hydrogel systems. Our approach provides a unique perspective on the role of multi-modal biophysical cues in cartilage mechanobiology and underscores the importance of considering the thermo-mechanical cues, as well as the dynamic nature of crosslinks, in the design of biomaterials for cartilage repair.

The main body of this thesis comprises a collection of original research (Chapters 2-4), including previously published works, that collectively contribute to our understanding of cartilage *thermo-mechanobiology*. Chapter 1 sets the stage by showcasing previously published works that highlight a gap in our current understanding of this complex field. With a firm foundation established, the ensuing chapters delve into original and groundbreaking research that promises to advance our knowledge of the *thermo-mechanobiology* of cartilage, offering exciting prospects for future scientific inquiry and clinical translation. The outcomes of this work are discussed in the Conclusions section (Chapter 5), and future research directions are proposed to further advance our understanding of this fascinating field.

1.2 Brief Insights into Articular Cartilage Function and Biology

Articular cartilage (hyaline cartilage) is the soft layer of deformable material that covers the ends of long bones that articulate in diarthrodial joints. The principal function of this tissue is

to provide a smooth surface for articulation and to facilitate load transmission with minimum friction.[2] Such essential biomechanical functions require a unique structural tissue architecture and composition.[3] Indeed, hyaline cartilage is principally composed of a dense collagen network enmeshed in a concentrated proteoglycan solution, that give to the tissue the ability to perform under a rigorous mechanical environment.[4]

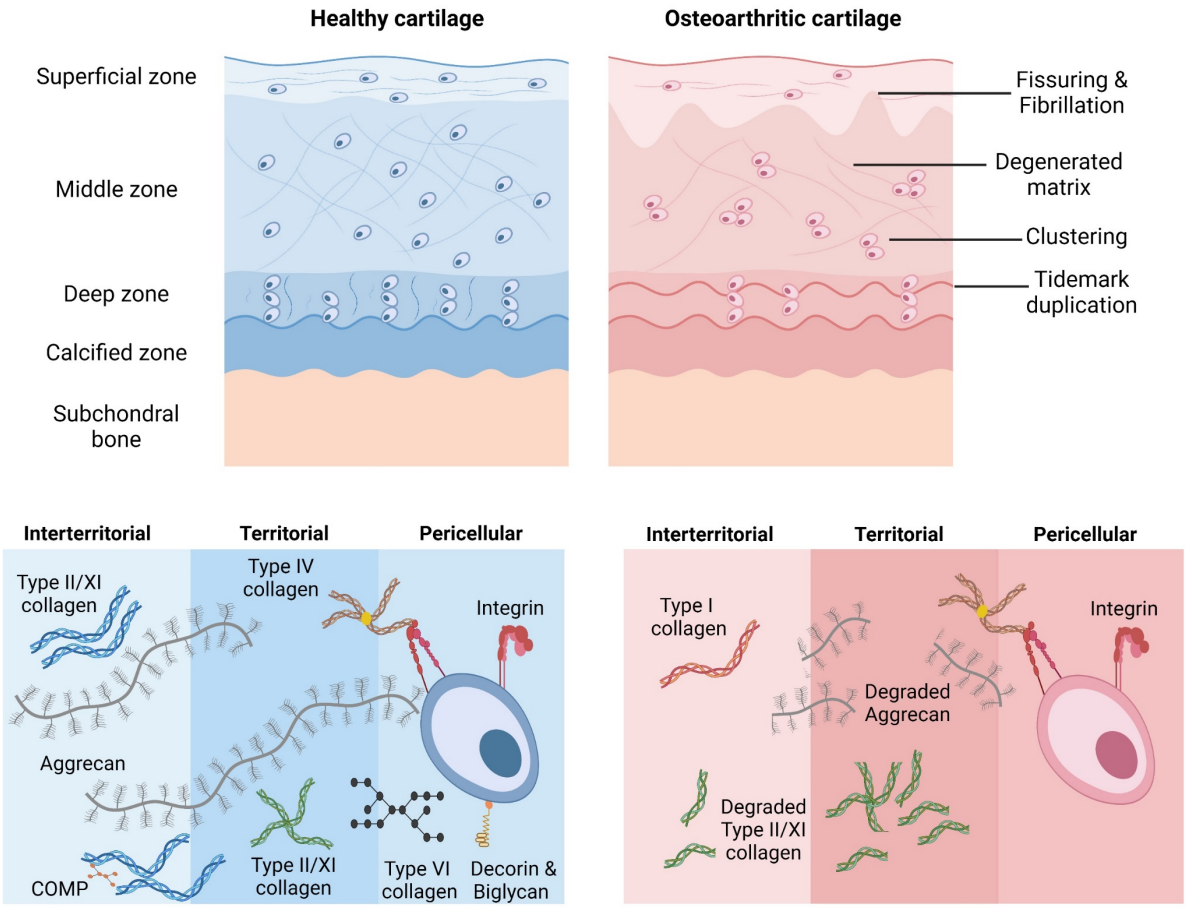

Figure 1.1 - The arrangement of articular cartilage can be categorized into three distinct zones as you move from the joint surface towards the bone: the superficial, middle, and deep zones, followed by the calcified cartilage. Each of these zones has a distinct composition and structure of the extracellular matrix (ECM), which mirrors the encountered forces. In the superficial zone, chondrocytes and type II collagen fibers are aligned horizontally. This arrangement helps distribute shear forces during joint movement. The presence of lubricin in this zone further aids in lubricating the joint. In the middle zone, the random arrangement of type II collagen fibers enables resistance against compressive and shear forces from various directions. In contrast, the deep zone contains thick collagen fibers that are oriented vertically to the joint surface. This alignment helps the cartilage withstand compressive loads. The high

concentration of proteoglycan in this zone supports water retention. The composition and structure of the ECM surrounding chondrocytes vary across three regions moving outward from the chondrocyte: the pericellular matrix (PCM), territorial matrix, and interterritorial matrix. The PCM, which envelops the chondrocyte, includes type VI collagen and affects how chondrocytes respond to mechanical stimuli. Additionally, important elements like hyaluronan, biglycan, and fibronectin are present in the PCM. This integrates with the territorial matrix, characterized by densely packed fibrillar collagen and proteoglycan. The outermost layer, termed the interterritorial matrix, constitutes the majority of the tissue ECM. Although the overall integrity of the ECM plays a role in transmitting forces within the tissue, each specific zone determines the type of load experienced by a chondrocyte, thus affecting cellular responses. Osteoarthritis leads to the breakdown of the functional ECM, loss of tissue hydration, and the generation of incorrect fibrous ECM components. This condition is accompanied by fibrillation, fissuring, altered chondrocyte behavior, chondrocyte clustering, senescence, and inflammation. Characteristic histological features of osteoarthritis include the thickening of calcified cartilage and the infiltration of blood vessels, contributing to tidemark duplication. COMP, cartilage oligomeric matrix protein. The idea for drawing the schematic was inspired from.[8]

The collagen content (predominantly type II) ranges from approximately 10% to 30% (by wet weight), while the proteoglycan content from 3% to 10% (by wet weight); the remaining 60% to 87% is mainly fluid (interstitial water and electrolytes).[5,6] Glycosaminoglycans and collagens, are exquisitely ordered in a three-dimensional network, that constantly interact with a charged fluid environment, ultimately conferring unique mechanical functionality to the tissue.[6] Interestingly, the shape and arrangement of such structural components significantly vary along the tissue's thickness, giving cartilage a layered character. Morphologically, four distinct regions have been identified: the superficial, the transitional/middle, the deep and the calcified zone.[7] Each zone can be depicted in Figure 1.1.[8]

Compared to other tissues, hyaline cartilage is sparsely populated by cells which are called chondrocytes. All chondrocytes in the tissue share similar traits with regards to surface markers and protein level expression. However, some differences do exist in the mechanical and morphological characteristics of the cells with respect to their zone of origin within the tissue.[9,10] Chondrocytes possess a vital role in maintaining the tissue's architecture through a balanced cascade of anabolic and catabolic activities.[11] Surprisingly, such activities take place under low oxygen conditions.[12] Skeletally mature articular cartilage is avascular.[13]

Consequently, the oxygen concentration within the tissue is significantly decreased compared to other vascularized tissues. Measured oxygen levels in articular cartilage range from around 7% (53 mm Hg) in the superficial zone to less than 1.5% (7.6 mm Hg) in the deep zone.[14]

1.3 Repairing Cartilage Damage: The Quest for Effective Solutions

Even though the presence of hyaline articular cartilage results in exceptionally durable and resilient diarthrodial joints, injurious impact and repeated loading, imbalances in biological processes and overuse, can all lead to progressive cartilage tissue loss. Unlike other self-repairing tissues, articular cartilage possesses a poor intrinsic capacity for repair.[15] As a result, once injured, it is very difficult to self-heal. This inability causes even minor lesions to cascade into larger defects that can progressively result in osteoarthritis, the most common degenerative joint disease.[6] Traditional treatments, such as microfracture, osteochondral allograft transplantation or autologous chondrocyte implantation have long been considered as potential treatment options for cartilage repair. However, in all cases these surgeries are inadequate due to their inability to produce a mechanically robust tissue, their failure to integrate with the existing native cartilage or even fill the entire defect.[16] Consequently, the need for alternative solutions is crucial.

The rapid advancements in the field of biomedical engineering has led to the establishment of new pioneering strategies, such as tissue engineering, aiming at healing and restoring even a whole human tissue through a specific process.[17] The ultimate goal of this strategy is the reconstitution of native tissues through the development of artificial scaffolds, by combining them with cells and/or different biomolecules, that will stimulate the process of natural tissue regeneration.[18] At its core, tissue engineering can be summarized into three stages: cells are usually cultured in a scaffold, which may consist of different types of materials, a tissue grows *in vitro*, and the newly formed construct is implanted back to the desired anatomical position.[19]

This promptly flourishing multidisciplinary field has long been considered as a promising alternative for cartilage regeneration.[20] This novel strategy for regeneration of cartilage lesions involves cell-seeded biomaterials in concordance with appropriate biomechanical stimuli.[21] Biomaterial, as a proper microenvironment provides mechanical support for the cells to proliferate and produce their matrix, while biomechanical stimuli (e.g., cyclic compression) facilitates nutrients diffusion as well as inherent transient signals, thus providing the necessary means for chondrocyte homeostasis.[22]

Each of the three ‘’components’’ of the cartilage engineering paradigm will be discussed in the following sections. More specifically, the criteria for the proper biomaterial selection for cartilage research, as well as the different cell sources that have been investigated over the years, will be analyzed in the sub-sections 1.3.1 and 1.3.2 respectively. The role of biomechanical stimulation in cartilage engineering, where this thesis will contribute, will be discussed separately in the context of mechanobiology (Section 1.3)

1.3.1 State-of-the-art biomaterials as *in vitro* models for cartilage research and engineering

The overarching principle for effectively choosing the desired material in any tissue engineering-related research is having meticulously defined design criteria, that are determined, predominantly, by the final use.[23] More precisely, in case of cartilage regeneration, biomaterial’s composition and interactions with cells need to be screened and subsequently enhanced based on the interactions with the native cartilage tissue, its inherent repair mechanisms, as well as its ability to be fabricated at a clinically relevant scale. Crucial prerequisites of any biomaterial to be used, present its biocompatibility and degradation properties. The three-dimensional biomaterial must be able to support and enhance cell growth and maintenance, while align degradation rate with its distinct healing traits (low turnover).[24]

Additionally, in case of cartilage regeneration, the ideal biomaterial should also allow the recreation of tissue’s composition by means of the ratio between the liquid and solid phase of the soft tissue, it should reproduce its zonal architecture, and facilitate a suitable environment for the successful and efficacious integration of the newly-developed tissue with the adjoining native one.[25] Ultimately, functional regeneration can only be reached via distinctive hierarchical structures, suitable cells that will initiate matrix deposition and a well-orchestrated interplay of mechanical properties. This latter concept, has initiated a quest among the scientific community for the most effective way to substitute for damaged cartilage and to produce the optimal *in vitro* cartilage replacement material.

Biomimetic scaffolds with tissue-like architectures and on-demand properties have long been developed to guide specific cellular responses. Scaffold-based culturing systems overall include hydrogels, porous scaffolds or fibrous networks. Hydrogels are three-dimensional hydrophilic networks of cross-linked polymers, which are able to encapsulate large amounts of water.[26] By adjusting parameters like polymer chemistry and crosslinking density, hydrogels

can be optimized for tissue repair. Studies have shown that hydrogel crosslinking affects neocartilage formation, and different hydrogel compositions can impact chondrogenic outputs. As one prominent example, gelatin-methacrylamide (gelMA) hydrogels support chondrocyte viability and differentiation, with adjustable mechanical properties.[27] On the other hand, porous scaffolds and fibrous networks often allow for superior mechanical integrity and mass transport[28] while more recently, supramolecular hydrogels are also gaining attention for their ability to mimic the dynamic nature of cartilage native environment.[27,28] Overall, hydrogels provide an exceptional *in vitro* platform for advancing cartilage research.

1.3.2 Cell source selection in cartilage repair strategies

Even though primary articular chondrocytes seem to be the most obvious choice for regenerating hyaline cartilage, acquiring sufficient number of autologous cells still present a crucial restricting factor for the translation of these cells to the clinic.[29] Sufficient numbers could be possibly obtained by cell expansion *in vitro*, however this may lead to rapid loss of their chondrogenic phenotype.[30] Addition of growth factors to overcome this issue in vitro is somewhat possible. Chondrocytes expanded for up to passage 3 under the application of transforming growth factor beta 1 during expansion for instance, have been shown to significantly maintain the potential for autonomous cartilage-like tissue formation over the unstimulated control groups.[31]

Non-articular chondrocytes such as costal or nasal chondrocytes are also under investigation as alternative cell sources.[32,33] Similar to articular cartilage, the extracellular environment of costal cartilage is also rich with high amounts of collagen and glycosaminoglycans. In fact, neocartilage produced by costal chondrocytes exhibited mechanical and biochemical properties akin to those of native articular cartilage.[32] Furthermore, chondrocytes obtained from the nasal septum possess higher capacity to produce hyaline-like cartilage tissues and they also exhibit the plasticity to adapt to a ‘’loaded’’ environment. A nasal-chondrocyte based product is already in clinical trials for the repair of cartilage injuries in Europe.[34]

Stem cells have also been deemed as an elegant alternative option. The rationale in using these cells stems from the fact that they can be easily isolated, acquired and expanded *in vitro* to the desired number and then differentiate to chondrocytes. Regarding cartilage tissue, a lot of research has been focused on mesenchymal stem cells,[35,36] embryonic stem cells[37-39] and more recently to progenitor cells.[40] One problem that arises however, is that hypertrophy may occur during the chondrogenic differentiation of stem cells. Despite promising data, it seems that

their long-term efficacy should be further investigated.[41] Human fetal epiphyseal chondroprogenitors (hECPs) show great potential for advancing cartilage cell therapy. They outperform adult chondrocytes in chondrogenic potential in some cases, maintain stable phenotypes compared to stem cells, and possess low immunogenicity or tumorigenicity. Cultured hECPs maintain high viability, robustly proliferate in monolayer culture with high population doubling values, exhibit consistent surface marker profiles, and resist multilineage differentiation.[42]

All in all, the choice of cell type during cartilage research is a decision driven by research groups and study aims. Different research teams may prioritize certain cell types based on their specific goals and requirements. Factors such as cell availability, expansion potential, chondrogenic phenotype maintenance, and tissue properties play a crucial role in determining the most suitable cell source. Therefore, the selection of the optimal cell type varies and is tailored to the specific objectives of each research group or study.

1.3.3 From Mechanics to Regeneration: Harnessing the Power of Mechanobiology in Cartilage Tissue Engineering

Tissue engineering, a pioneering strategy at the nexus of engineering, medicine, biology, and biomaterials, has evolved into a multidisciplinary field that now encompasses a broad array of scientific principles. At the forefront of current challenges in the field is the formidable task of developing scalable tissue-engineered substitutes that can faithfully replicate the functional characteristics of native tissues, such as cartilage. This quest for functional mimicry is characterized by intricate, multi-faceted challenges that are at once demanding and exhilarating. Nevertheless, engineering approaches have made impressive strides in recent years, and biomechanical (physiologically relevant) cues have emerged as a critical driver of success, facilitating the creation of more advanced, functional, and biomimetic constructs. At the heart of these impressive advances is the pivotal role of mechanobiology, which illuminates the critical importance of the biophysical and biochemical interplay between cells and their environment in tissue formation and maintenance.

The use of biophysical factors to promote cartilage regeneration *in vitro*

Hyaline cartilage manifests as a biomechanically proficient tissue, adept at sustaining mechanical loads and affording frictionless articulation within articular joints. It can respond to various mechanical factors and even though the exact mechanisms involved in the

transduction of such forces are not completely elucidated, research highlights the beneficial impact of specific types of stimulation for cartilage synthesis and remodeling. Compressive loading for instance, presents a crucial component of normal stimulation inside the knee joint. Studies have utilized both static and dynamic compression to generate cartilage *in vitro*.[43,44] Indeed, mechanical stress emerges as a pivotal modulator of cellular physiology, wielding a profound influence on tissue regeneration and repair. Compelling evidence suggests that harnessing the power of biophysical stimuli through the precise application within sophisticated ‘’bioreactors’’ holds immense potential to optimize and expedite the process of tissue rejuvenation *in vitro*.

Using a custom-made apparatus, Lee and Bader for instance, showed that glycosaminoglycan levels were enhanced, under 15% compression at 1 Hz frequency for chondrocytes encapsulated in agarose scaffolds.[45] In another elegant study, Mauck and colleagues observed augmented collagen synthesis and increased equilibrium modulus of cell-seeded scaffolds under compressive loading, compared to free-swelling constructs.[46] Lee et al. also observed an increase of proteoglycan synthesis of chondrocytes when subjected to dynamic compressive loading, while static stimuli resulted in lower matrix deposition.[47] These results, are, in agreement with Wernike and associates, who also explored the effects of long-term dynamic compression stimuli on cartilage-engineered constructs. Apart from controlling the chondrogenic phenotype, they also achieved high GAG deposition and increased collagen type II expression in the engineered-constructs.[48]

Chondrocytes are also subjected to hydrostatic pressure following compressive stimulation of cartilage. More specifically, the low permeability of the tissue does not allow synovial fluid to run out of the tissue and inside the joint area. As a result, pressure inside cartilage cyclically augments during every instance of compressive loading.[49] This latter has inspired tissue engineers to utilize hydrostatic pressure to produce cartilage *in vitro*. In fact, hydrostatic pressure at a physiological range of 5-15 MPa, has been shown to enhance chondrocyte matrix deposition, *via* modulation of specific chondrocyte transporters.[50] Indeed, a significant improvement in proteoglycan (Aggrecan) production was observed on monolayer chondrocytes, following a 10 MPa static pressure for a period of four hours over free-swelling controls.[50,51] Studies utilizing constant pressure have reached physiological levels of biomechanical and biochemical properties in cartilage-engineered substitutes. When bovine chondrocytes were subjected to intermittent dynamic hydrostatic pressure, applied at 10 MPa and 1 Hz for 4 hours over a total period of 8 weeks, it led to an increase in collagen content

compared to the control groups. Furthermore, it helped mitigate the gradual decline in glycosaminoglycan content per construct observed in the control groups over time.[52] According to a 2021 study, transient receptor potential channels can transduce hydrostatic pressure and stimulate matrix formation.[49]

Improvements have also been shown when the culturing conditions of the engineered constructs include shear. Shear loading provides a form of stimulation during knee function. Shear bioreactor systems designed to simulate such loading typically hinge on the principle of generating constructs with heightened frictional attributes akin to those of cartilage. As in the case of compressive stimulation or hydrostatic pressure, dynamic shear seems to also yield promising results. Dynamic shear of around 2% could stimulate 40% higher collagen synthesis as well as 35% more proteoglycan production over the control groups.[53] This behavior could be attributed to the activation of primary cilia, a nanoantenna present in chondrocyte channelome. Polycystin I and II, two genes encoding the primary cilia, have been shown to be significantly upregulated when chondrocytes are subjected to shear.[54]

Oxygen concentration plays a crucial role in regulating cartilage homeostasis, with levels ranging from 7-10% at the tissue's superficial zone and around 1% near the subchondral bone, establishing cartilage as a hypoxic microenvironment. Primary human chondrocytes acquired from healthy[55] and diseased[56] regions of articular cartilage showed increased transcript levels of critical chondrogenic markers under low oxygen conditions. Transforming growth factor signaling pathways, which in turn regulate cartilage development and maintenance, have also been shown to be enhanced when culturing chondrocytes in hypoxia conditions. In fact, a 2.5% of oxygen concentration is considered optimal for boosting the transcription of such factors.[57] Studies have also utilized hypoxia conditions in synergy with mechanical loading. For example, the combination of dynamic compression and hypoxia led to a significant down-regulation of collagen type I expression, resulting in stabilization of the chondrogenic phenotype.[58] In this regard, hypoxia inducible factor 1 (HIF-1) has been deemed as a crucial element for hypoxic induction of chondrogenesis.[59]

As the pursuit of engineering functional cartilage constructs intensifies, researchers have pushed the limits of bioreactor technology to create increasingly sophisticated systems. These cutting-edge bioreactors integrate multiple mechanical stimuli, with the aim of eliciting an optimal response from cells. One example is the pioneering bioreactor system developed by Meinert and colleagues, which allows for defined uni- or bi-axial mechanical stimulation of

engineered cartilage constructs (see Figure 1.2 for details). Through this approach, researchers were able to assess the effects of individual physical cues on chondrocyte mechanobiology. Notably, the administration of intermittent biaxial stimulation to cartilage-engineered constructs, utilizing clinically relevant cells and biomaterials, was found to dramatically enhance matrix accumulation, leading to the production of cartilage constructs with superior, natural-like biomechanical characteristics.[60]

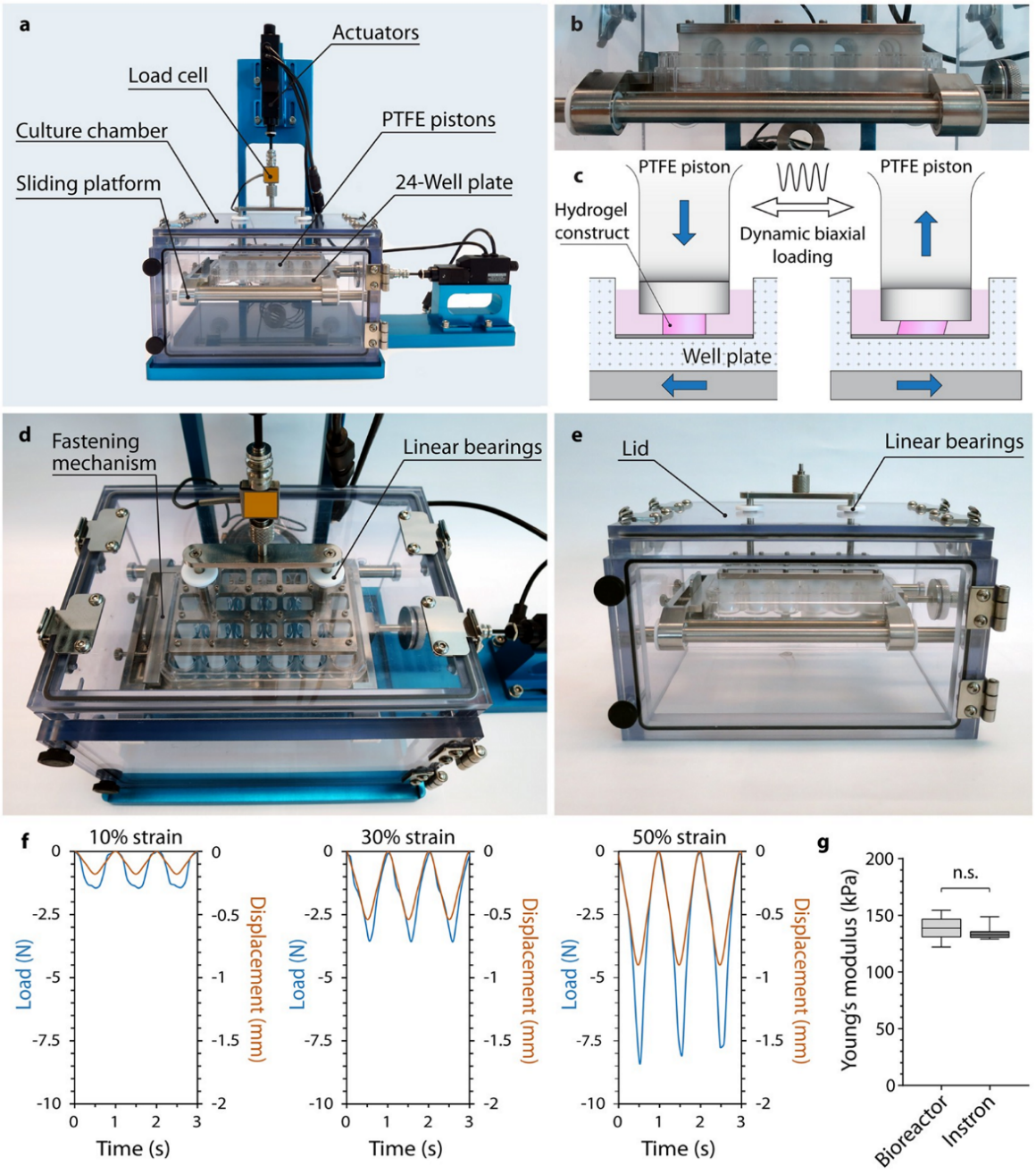

Figure 1.2 - (a) Front view depicting the bioreactor device, (b) close up view of the pistons lowered into a 24-well plate, (c) compressive loading is achieved via actuator-driven pistons,

while the movement of the sliding platform lead to shear deformation, (d) top view of the culture chamber, (e) culture chamber, (f) representative load-displacement curves, (g) Young's modulus measurement immediately through the bioreactor. Reproduced from [60]

The use of biochemical factors to promote cartilage regeneration *in vitro*

The use of biochemical stimulation in three dimensional cultures have also been explored to enhance the proliferation rate and to restore the chondrogenic capacity of articular chondrocytes. Application of insulin-like growth factor 1 (IGF-1) concomitantly with osteogenic protein 1 (OP-1) has been shown to improve matrix secretion in both osteoarthritic and normal chondrocytes, when seeded in alginate.[61] Additionally, culturing primary human chondrocytes with transforming growth factor 2 (TGF-β2) and insulin growth factor notably improved expression of type II collagen, when compared to control groups.[62] A synergy between transforming growth factor 1 (TGF-β1) and fibroblast growth factor 2 (FGF-2) has also been suggested. More specifically, when adipose mesenchymal stem cells were treated with the aforementioned factors, a significant up-regulation of cartilage-related genes was observed.[63] It is thought that such factors stimulate critical metabolic programs in chondrocytes, leading to improved matrix synthesis.

Taken together, the potential of bioreactors in improving the quality and efficiency of cartilage engineered constructs has been realised for quite some time. By providing a compendious level of control over a wide range of biophysical and biochemical factors, bioreactors offer the technological means to conduct studies, aiming at revealing which mechanical cue regulates which biological function.[64] Such data will provide crucial information in engineering cartilage tissue. This vital, mechanobiology-related research will offer the basis for tailoring the essential environmental conditions necessary for the generation of functional cartilage tissue.

1.4 From Neglect to Prominence: Shedding Light on the Physiological Relevance of Loading-Induced Evolved Temperature

Viscoelastic materials possess a unique capacity to transform mechanical energy into heat via friction mechanisms between polymeric chains, culminating in the process known as ''self-heatin''.[65] Indeed, once dynamically stimulated, friction mechanisms between the polymeric chains of the material result in the dissipation of a part of the total mechanical strain energy. A fraction of the dissipated energy is subsequently converted into heat, which results in local temperature increase inside the material. Cartilage tissue, characterized by a highly viscoelastic

nature, is no exception to this phenomenon. Indeed, experimental evidence has indicated that heat accumulation within the knee joint following cyclic loading is possible. Remarkably, it has been shown that cartilage temperature can rise from its normal intra-articular knee temperature of ~32.5 °C to ~39 °C following a mere one-hour jogging session.[1] Astonishingly, the scientific community has largely overlooked the impact of such a natural heat source on chondrocyte behavior, despite extensive evidence highlighting their responsiveness to temperature stimuli.

So far, studies examining the effects of a constant culture temperature on chondrocyte function have demonstrated that proteoglycan synthesis is enhanced when chondrocytes are cultured at 37 °C compared to 32 °C.[66] Such findings are further corroborated by research indicating that chondrogenesis-related genes are up-regulated at higher temperatures.[67] Nevertheless, these studies fail to consider the dynamic thermal environment in which chondrocytes thrive. Capitalizing on this concept, recent research in our laboratory has shown how the combination of a biomimetic temperature evolution and mechanical loading synergistically conveys chondro-inductive cues to human chondroprogenitor cells in 3D culture systems.[68] The stimulation of cartilage self-heating conditions was shown to be more effective in promoting chondrogenic markers (at gene level only and short-term studies) compared to mechanical stimuli at constant culture temperatures (both 32.5 °C and 37 °C).

Yet, the potential of this biomimetic temperature signal, alone or in combination with other biophysical cues like mechanical loading, oxygen tension, or extracellular matrix dynamics, to stimulate matrix synthesis in adult chondrocytes remains unclear. It is precisely this concept that this research thesis aims to explore, thereby shedding light on the unexplored potential of this promising avenue in cartilage tissue engineering.

1.5 Thesis Overview

The prospect of utilizing tissue-engineered articular cartilage as a formidable tool for treating cartilage injuries is an alluring one. However, despite the emergence of rudimentary tissue engineering approaches, involving a combination of scaffolds, cells, and biochemical signals, the ability to fully replicate the anatomical and functional characteristics of healthy native tissue remains an elusive goal. In this regard, biophysical stimulation during tissue culture has been viewed as a potent strategy for the development of more biomimetic constructs. Tissue

engineers have been keen to exploit mechanotransduction signaling pathways to enhance *in vitro* cartilage repair strategies.

Hyaline cartilage, a connective tissue with remarkable dissipative capabilities, can elevate its temperature from 32.5 °C to around 39 °C during daily activities, a condition known as ‘’self-heating’’ (Figure 1.3). Despite its potential significance, this thermal dimension of cartilage mechanobiology remains largely ignored within the scientific community. Grounded in the premise that biophysical cues are critical for cartilage development and maintenance *in vivo*, this thesis seeks to explore how loading-induced evolved temperature signals can be harnessed *in vitro* to enhance chondrocyte biosynthetic performance.

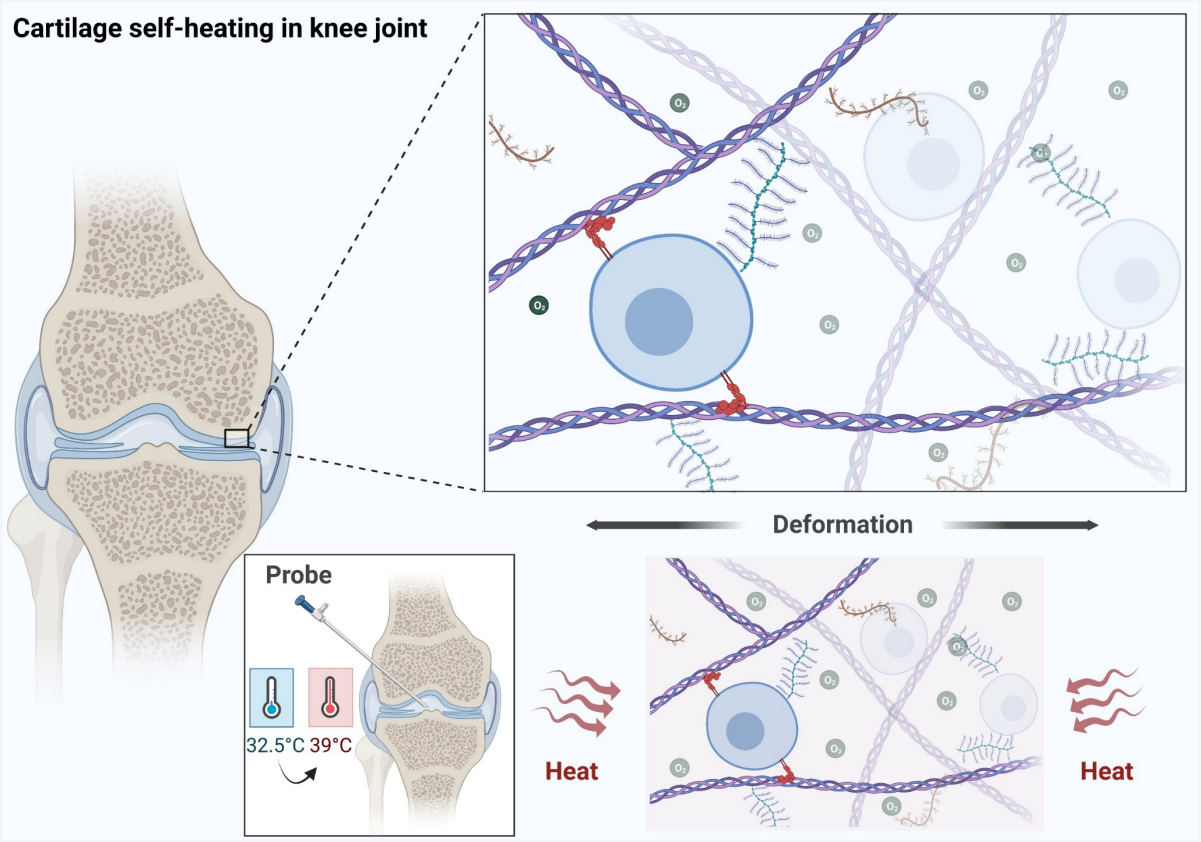

Figure 1.3 – Cartilage self-heating in knee joint. Cartilage tissue in knee joint is composed of a dense network of collagen type II fibers and negatively charged proteoglycans. Such structure gives rise to the tissue's viscoelasticity. Upon loading, part of the mechanical strain energy is dissipated through heat resulting in a local temperature increase inside the knee joint. Resting temperature and maximum temperature following self-heating have been measured in vivo via intra-articular probes in previous studies.[1] This condition, self-heating is the subject of this research thesis. Schematic was created with BioRender.com.

To achieve our research goals, we have structured the present work into three main studies, each deserving of its own chapter. Through a meticulous examination of the mechanistic underpinnings of thermal and mechanical stimuli in cartilage tissue culture, this thesis aims to establish a foundation for a new era of more efficacious and biomimetic tissue engineering perspective, with the potential to revolutionize the field of cartilage regeneration.

Chapter 2: This chapter seeks to investigate whether the loading-induced evolved temperature signal can act as an independent or complementary regulator of chondrocyte function. By examining the response of chondrocytes to mechanical and thermal cues, we aim to assess the impact of these biophysical stimuli on matrix biosynthesis in two distinct polymeric scaffolds, each exhibiting unique mechanical attributes. *The overarching hypothesis posits that a synergistic application of load and heat, will elicit a superior chondrocyte biosynthetic response compared to control groups subjected to free swelling or single-form stimulus, regardless of the scaffold's inherent biomechanical properties.*

Chapter 3: Chapter 3 presents a fundamental investigation of the interplay between low oxygen tension (hypoxia), mechanical stimulation, and biomimetic thermal cues, aiming to unlock the secrets of a unique regulatory microenvironment that can ‘’supercharge’’ chondrocyte function. *As previous studies have highlighted the effectiveness of low oxygen culture in regulating chondrocyte biosynthesis, the hypothesis of this chapter is that the concomitant use of hypoxia and thermomechanical stimulation will more closely replicate the natural in vivo environment of cartilage tissue, leading to a significant increase in the production of extracellular matrix components by chondrocytes.*

Chapter 4: This chapter intricately examines the interplay between intermittent thermomechanical cues, oxygen tension, and dynamic supramolecular hydrogels in influencing chondrocytes. While researchers have often turned to covalently crosslinked systems to enhance hydrogel mechanical strength for cartilage tissue engineering, these systems fall short in replicating the native matrix's dynamic nature. In contrast, dynamic supramolecular hydrogels have captured attention for their inherent dynamic qualities, akin to those of native tissue. *The chapter's hypothesis suggests that replicating the dynamic crosslinks observed in soft tissues like cartilage, alongside physiologically-relevant stimuli, could prompt chondrocytes to enhance matrix biosynthesis. A deeper grasp of mechanobiological cues governing chondrocyte function holds promise for refining cartilage tissue engineering strategies.*

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Chapter 2: Mimicking Loading-Induced Cartilage Self-Heating *in Vitro* Promotes Matrix Formation in Chondrocyte-Laden Constructs with Different Mechanical Properties*

This chapter has been published in ACS Biomaterials Science & Engineering Journal (Stampoultzis, T., Guo, Y., Nasrollahzadeh, N., Karami, P., and Pioletti, D.P. (2022). Mimicking Loading-Induced Cartilage Self-Heating in Vitro Promotes Matrix Formation in Chondrocyte-Laden Constructs with Different Mechanical Properties. ACS Biomater. Sci. Eng. 10.1021/acsbiomaterials.2c00723.).* ***Author's Contributions:* *T.S conceptualized the work and performed the experiments, Y.G assisted with part of experimental work, N.N previously developed the bioreactor, P.K assisted with the schematics, D.P.P supervised and reviewed the work.*

Abstract

Articular cartilage presents a mechanically-sensitive tissue. Chondrocytes, the sole cell type residing in the tissue, perceive and react to physical cues as signals that significantly modulate their behavior. Hyaline cartilage is a connective tissue with high dissipative capabilities, able to increase its temperature during daily activities, thus providing a dynamic thermal milieu for the residing chondrocytes. This condition, self-heating, which is still chiefly ignored among the scientific community, adds a new thermal dimension in cartilage mechanobiology. Motivated by the lack of studies exploring this dynamic temperature increase as a potential stimulus in cartilage-engineered constructs, we aimed to elucidate whether loading-induced evolved temperature serves as an independent or complementary regulatory cue for chondrocyte function. In particular, we evaluated chondrocytes response to thermal and/or mechanical stimulation in two types of scaffolds exhibiting dissipation levels close to healthy and degenerated articular cartilage. It was found, in both scaffold groups, that the combination of dynamic thermal and mechanical stimuli induced superior effects in the expression of major chondrogenic genes, such as *SOX9* and *LOXL2*, compared to either signal alone. Similar effects were also observed in proteoglycan accumulation over time, along with increased mRNA transcription and synthesis of *TRPV4*, and for the first time demonstrated in chondrocytes, *TREK1* ion channels. Conversely, the chondrogenic response of cells to isolated thermal or

mechanical cues was generally scaffold-type dependent. Nonetheless, the significance of thermal stimulus as a chondro-inductive signal was better supported in both studied groups. Our data indicates that the temperature evolution is necessary for chondrocytes to more effectively perceive and translate applied mechanical loading.

2.1 Introduction

Hyaline cartilage develops within a biomechanical milieu which allows the formation of a unique structural tissue architecture, befitted to its biomechanical function.[1, 2] Sensitivity of chondrocytes to external biophysical stimuli and subsequent adaptive responses to extracellular matrix (ECM) present critical features of articular cartilage.[3] In this way, and since mechanical cues regulate cell responses, dynamic mechanical stimulation has been deemed highly attractive during *in vitro* engineering of articular cartilage.[4] Indeed, over the past decade, a strong correlation has been established among dynamic biophysical cues, such as dynamic compression, shear or hydrostatic pressure and chondrocyte biological responses.[4-6] Nonetheless, up to now, studies utilizing such cues during tissue culture normally consider a constant culture temperature, that is, however, far from reflecting the normal intra-articular knee temperature.

Different types of cells have been shown to respond to environmental temperature.[7] However, only recently an expanding body of work has been addressing the potential role of this cue, specifically, on chondrocyte fate (phenotype and anabolism).[8,9] Indeed, given that cell metabolism is temperature dependent, potential thermal changes in the microenvironment surrounding chondrocytes could constitute a signal that could significantly modulate their behavior. Chondrocytes thrive within a highly viscoelastic environment.[10] Viscoelastic materials in turn can dissipate part of mechanical energy through heat production, under dynamic loading, a phenomenon known as self-heating.[11] It is therefore possible, that a natural heat source could be developed, resulting in heat accumulation over time within healthy cartilage tissue. Besides cartilage dissipation, ambient temperature or heat transfer from surrounding tissues could contribute to the temperature evolution in the joint, to an extent, as well. Notably, in 2008 Becher and associates convincingly demonstrated that cartilage temperature can progressively increase from ~32.5 °C (normal intra-articular knee temperature at rest) to ~39 °C after one-hour jogging, confirming the hypothesis that an internal heat source can be generated within the tissue, during cyclic loading.[12]

Accordingly, another study produced strong evidence that cartilage viscoelastic properties could induce a temperature rise in the tissue, optimal for proteoglycan production.[13] Strikingly, osteoarthritic tissue was not capable of inducing the aforementioned temperature increase due to its reduced dissipative capacity. Recent *in vivo* studies also demonstrate a strict correlation among cartilage degeneration and cartilage dissipative capabilities. In 2019 for instance, Maier and colleagues showed that even early-stage degenerated cartilage tissue samples with an ostensible normal appearance in structure and composition, can dissipate up to 50% less energy compared to healthy tissue.[14]

The inherent incapacity of degenerated cartilage to dissipate energy should be considered. This inability essentially denotes that the already proven capacity of cells to generate the proper extracellular components may not be enough to cope with degeneration, since chondrocytes can no longer perform under an appropriate ‘’dynamic thermal environment’’. This insight conceptually reveals a new distinct route that could be explored. Acknowledging the fact that the intra-articular cartilage temperature could significantly evolve during daily activities, could lead to the concept of using this natural thermal increase as an additional biophysical cue/signal, to more accurately mimic the dynamic environment of the tissue *in vitro*; thus, improving chondrocyte functionality.

Up to date, none of the conducted studies pertaining chondrocytes is centered upon a biomimetic temperature evolution regime. Hence, the overarching purpose of the current research was to investigate the role of dynamic thermal signal in regulating the function of human chondrocytes, in the presence of mechanical cues. Building upon our prior work that showed positive effects of coupled thermomechanical cues on human progenitor cells at transcriptional level only,[15] this study sought to promote matrix/protein accumulation within the engineered constructs (Figure 2.1). In particular, we aimed to answer whether the observed temperature evolution, as an indirect effect of mechanical loading in healthy cartilage, is necessary for effective mechanotransduction and subsequent chondrogenesis for adult chondrocytes as well. Towards mimicking the natural self-heating phenomenon *in vitro*, we utilized a bioreactor apparatus that we have recently designed, where both the evolution of temperature and mechanical load could be independently controlled.[15] We then considered two poro-viscoelastic poly-(2-hydroxyethyl methacrylate-co-ethylene glycol dimethacrylate)-based hydrogels, that we designed to exhibit comparable viscoelastic properties (energy dissipation level) to healthy and degenerated cartilage respectively, seeded with adult chondrocytes. We selected this type of hydrogels as no other formulation allow us to reach

mechanical properties comparable to those of healthy tissue. Next, we evaluated chondrocytes response following external application of isolated thermal or mechanical cues, or the combination of thereof.

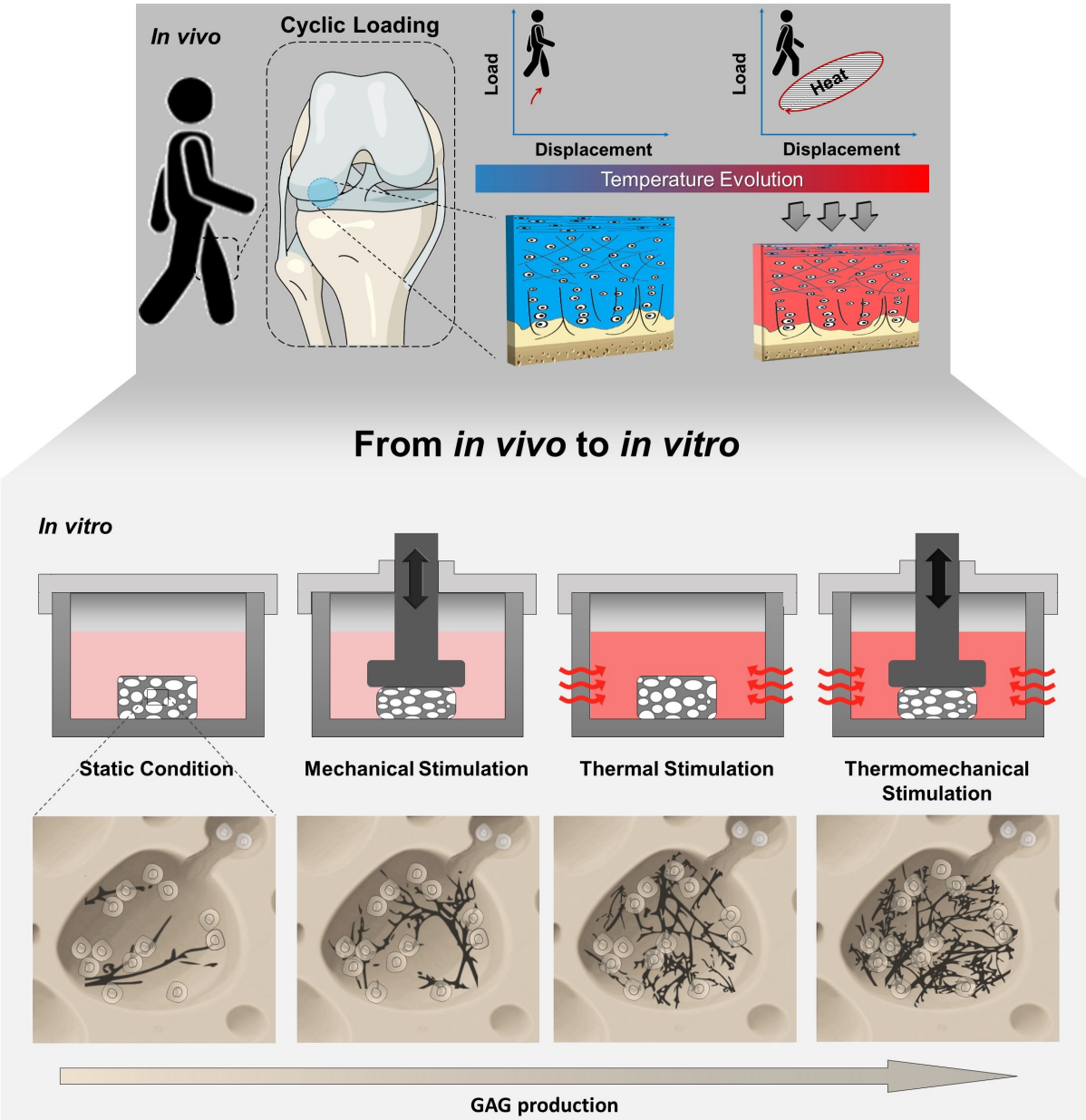

Figure 2.1 - Loading-induced self-heating in cartilage tissue. Energy dissipation, following joint loading, leads to a local heat accumulation inside cartilage tissue over time. Using a custom-made apparatus, cartilage self-heating can be mimicked in vitro to study its role on cartilage mechanobiology. Chondrocytes seeded in poro-viscoelastic hydrogels (mimicking both osteoarthritic and healthy cartilage tissue) produced greater amount of glycosaminoglycans (GAGs) when subjected to a combination of dynamic mechanical and thermal signal, compared to isolated forms of stimulation and control groups.

We hypothesized that a synergistic, biomimetic thermomechanical stimulation will augment the accumulation of hyaline cartilage-specific extracellular matrix, following enhanced chondrogenic expression of cells, regardless of the construct's mechanical characteristics. We consistently found that chondrocytes can positively transduce the externally applied mechanical loading and maintain their biosynthetic capacity only in the presence of thermal stimulus. Conversely, the effect of dynamic mechanical loading alone on cells could vary depending on hydrogel's level of dissipation. Overall, our study shows that combined thermal and mechanical cues in chondrocytes microenvironment contextualize each other to elicit a broad range of responses.

2.2 Materials and Methods

2.2.1 Fabrication of Poly-(2-hydroxyethyl methacrylate-co-ethylene glycol dimethacrylate)-based hydrogels (p(HEMA-co-EGDMA)-based hydrogels) and functionalization with RGD peptides

Two different Poly-2-hydroxyethyl methacrylate (HEMA, ≥99%, Sigma Aldrich) based hydrogels were fabricated via the salt leaching method, so as to exhibit different dissipation levels when exposed to identical loading parameters. The levels of energy dissipation in turn, were chosen to span the levels of dissipation presenting in healthy and degenerated cartilage samples. This was achieved by changing the pore size among the hydrogels. Polymer solution (hydrogel precursor) was prepared as previously described,[16] by mixing HEMA monomer with sodium metabisulfite/bi-distilled water solution (0.526 M) (Reagent grade 97%, Sigma Aldrich), ammonium persulfate/bi-distilled water solution (0.438 M) (Biorad) and with (4.8%) ethylene glycol dimethacrylate crosslinker (EGDMA, 98%, Sigma Aldrich). Hydrogel precursor was poured into cylindrical moulds prefilled with salt crystals (150-250 μm and 300-400 μm respectively) and polymerization was achieved by heating at 65 ˚C, for 2 hours. The resultant hydrogels were extensively rinsed with deionized water for 5 days to dissolve the trapped salt, ultimately resulting in macroporous structures. Morphological characterization of scaffolds was achieved through Micro-CT scan (See supplementary information for experimental details). Hydrogel RGD functionalization was performed following a two-step process described in detail elsewhere.[15]

2.2.2 Mechanical characterization of scaffolds

Cylindrical specimens (Ø:6, t: 2.2 mm) immersed in bi-distillated water were subjected to unconfined compression experiments (10% prestrain, 1 Hz sinusoidal load of 10% amplitude) via an Electropuls Dynamic Test System (Instron E3000, Instron, Norwood, Massachusetts, USA), at room temperature. The level of energy dissipation was calculated by measuring the area embedded by the hysteresis loop and it was further normalized to the volume of each sample.

2.2.3 Human chondrocyte expansion and seeding

Primary human articular chondrocytes were acquired from the knee joint of a 22-year-old Caucasian, male donor, with no known musculoskeletal pathology (Innoprot, P10970). Chondrocytes were seeded and expanded on T75 plastic tissue culture flasks, up to passage 4, in chondrogenic culture medium (Alpha minimum essential medium (α-MEM)), supplemented with 10% foetal bovine serum (FBS), 1% L-glutamine, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM nonessential amino acids (NEEA), 1% penicillin, 1% streptomycin, with bioactive factors (5 ng/ml fibroblast growth factor (FGF) and 1 ng/ml transforming growth factor beta 1 (TGF-β1)).

Cell seeding inside the macroporous hydrogels (~$0.8x10^6$ cells / scaffold, at passage 4) was achieved through an optimized dynamic cell-seeding method developed in our laboratory.[17] Cell-hydrogel constructs were cultured at 32.5 °C and 5% CO_2. The medium was refreshed every other day. After a preliminary 5-day culture period, constructs were treated with FBS-free α-MEM basal chondrocyte medium, supplemented with Insulin Transferrin Selenium (ITS-IV, 10%), L-ascorbic acid (VC, 1%), 5 ng/ml FGF and 1 ng/ml TGF-β1.

2.2.4 Applied modes of stimulation

The cell-seeded constructs were categorized into 4 different groups; 1) samples that were mechanically conditioned under dynamic compression (termed as M), 2) samples that were subjected to dynamic thermal stimulation alone (termed as T) or 3) to a combined thermomechanical stimulus (termed as MT) and 4) control groups that remained at free swelling state at 32.5 °C (termed as C). All forms of stimulation were re-enacted via a custom-made bioreactor developed in our laboratory.[15] The compressive regime was designed to mimic a normal physical activity. Dynamic mechanical stimulation involved subjecting engineered

hydrogels to 20% compressive strain at 1 Hz frequency for 1.5 hours. The scheme of the temperature increase following cyclic compression was modelled by a curve-fitting on reported *in vivo* data during jogging over a period of 1.5 hours.[12] When referring to combined thermomechanical stimulation, both dynamic compression and thermal stimulation were applied simultaneously. After each stimulation (a total of 3 rounds), specimens were allowed to recover. Stimulation was carried out every other day. A detailed experimental schematic can be seen in supplementary file (Figure S2.1).

2.2.5 Assessment of chondrocyte growth and viability

Prestoblue assay (ThermoFisher Scientific) was employed to determine chondrocyte proliferation inside the scaffolds. The Prestoblue reagent was diluted 10 times inside normal basal medium and samples were incubated for 1 hour inside Prestoblue measurement solution at 32.5 °C. The fluorescent signal was measured via Wallac microplate reader. Chondrocyte viability was monitored with calcein AM and ethidium homodimer (EH) staining (Biotium, Fremont, CA) and imaged by an inverted Leica SP8 confocal microscope.

2.2.6 RNA isolation and qPCR

After the last loading cycle had ceased, each sample was placed in a 2 ml Eppendorf tube containing 300 µl Trizol. RNA from each sample was isolated via Nucleospin® RNA XS kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Hydrogels were homogenized in Trizol via polytron (Kinematica, Switzerland), while maintaining the tube cold on dried ice. Next, chloroform (100µl) was added and samples were centrifuged for 10 minutes at 12000 rpm at 4°C. The aqueous phase was carefully transferred to 1.5 ml Eppendorf tubes and the extraction was completed by adding 6.5 µl RNA carrier and following the XS kit protocol. Total RNA amount was quantified via Nanodrop Lite Spectrophotometer (Thermo Scientific) and reversed transcribed into cDNA utilizing Taqman® Reverse Transcription Reagents (Applied Biosystems) in 50 µl reaction volume containing master mix, random hexamer and RNA sample. Quantitative polymerase chain reaction (qPCR) was executed using the Fast SYBR® Green PCR Master Mix (Applied Biosystems) in a final volume of 20 µl and containing 1 µl of synthesized cDNA. Respective primers were synthesized by Microsynth (Balgach, Switzerland). All sequences, as well as details pertaining to the thermal cycle condition during PCR can be seen in supplementary file (Table S2.1).

Cycle threshold values were converted to fold expression changes (ΔΔCt method), following normalization to the housekeeping gene.

2.2.7 Biochemical analysis

For the biochemical assessment of glycosaminoglycans (GAGs) and DNA content, hydrogels were digested overnight at 65 °C inside papain buffer solution (pH=6.5), containing 100 mM Na_2HPO_4, 10 mM L-cysteine, 10 mM EDTA and 6 μl ml^{-1} papain enzyme (Sigma Aldrich). The GAG content was determined using the 1,9-dimethylmethylene blue assay, at pH 1.5. Sample absorbance was measured at 530 and 590 nm and compared to standard curve of bovine chondroitin sulphate (Sigma Aldrich). DNA content was quantified using Hoechst 33258 DNA intercalating dye method (ThermoFisher Scientific) and purified Calf Thymus DNA was used as standard.

2.2.8 Immunofluorescence imaging

Immunofluorescence staining was performed to verify the expression of TREK1 and TRPV4 ion channels at protein level, and to visualize the accumulation of aggrecan following 11 days of culture. Briefly, samples were fixed in paraformaldehyde (4%), permeabilized with Triton X-100-PBS (0.25%) for 10 min and blocked in BSA (1%) within an hour. Next, samples were subjected to overnight incubation with the relevant primary antibodies at 4 °C. After washing with PBS/Tween, samples were incubated in relevant secondary antibodies for 1 h in the dark. Subsequently, cell nuclei were counterstained with DAPI (1:10000, Thermo Fisher) within 10 minutes. The relevant primary and secondary antibodies used can be found in supplementary file (Table S2.2). Fluorescent slides of different samples were imaged using tile scan method with 20x magnification in Olympus VS120 whole slide scanner. During the imaging process, the laser intensity and exposure duration were set consistent for all the samples. Tile scanned images were then imported into QuPath v0.3.2 before they were stitched for further analyses. Representative region of interest (ROI) were selected manually for each sample within which nucleus of cells were detected and counted using DAPI channel. Finally, total intensity of fluorescent signal of the corresponding channel (FITC) in the ROI were quantified and normalized by the number of cells detected.

2.2.9 Statistical analysis

Analysis of variance (ANOVA), followed by Tukey's *post hoc* tests, was performed to compare biochemical (n=3) and gene expression (n=3) data for multiple group comparisons. Data are presented as mean + standard deviation. (*) indicates p<=0.05, (**) indicates p<=0.01, (***) indicates p<=0.001. Statistics were performed in Origin Pro 2021 software. Three independent experiments, considering three biological replicates for each experimental group, have been conducted to verify gene expression (PCR) and biochemical results (GAG & immunofluorescence).

2.3 Results

2.3.1 Dynamic thermomechanical stimulation altered gene categories encoding various signaling pathways

Cell-seeded constructs with different viscoelastic properties (energy dissipation level) (Figure 2.2a) were cultured at 32.5 °C under static conditions for 5 days, before subjecting to 1.5 hours of dynamic stimulation. Prior to investigating temporal changes of gene expression following application of isolated or combined cues, we first assessed the response of human chondrocytes inside the RGD-modified hydrogels, before, as well as after stimulation. In particular, during the preculture period cell metabolic activity was assessed using a resazurin assay. An increasing trend in the average fluorescent PrestoBlue signal was observed over time (Figure 2.2b), indicating a relative firm attachment and proliferation of the cells inside the porous hydrogels. Next, confocal microscopy was utilized to further assess the effects of intermittent dynamic stimulation on chondrocyte fate. Our results (Figure 2.2b) demonstrate a high viability and chondrocyte attachment inside the porous scaffolds following application of different stimuli.

In the current study we were interested in examining the immediate changes on gene expression and the accumulation of proteins within the constructs. To elucidate the effects of thermal and mechanical cues on biological responses, we analyzed chondrocyte gene expression with regards to different stimuli. More specifically, quantitative real time PCR analysis, on samples that were collected immediately after the last loading cycle had ceased, revealed that dynamic thermal and thermomechanical stimulation resulted in changes in the expression of genes related to cellular signaling pathways (SRY-related HMG-box gene 9, *SOX9* & Twist Family

BHLH Transcription Factor 1, *TWIST1*), matrix remodeling enzymes (Lysyl oxidase homolog 2, *LOXL2*) and structural proteins (Aggrecan, *ACAN*).

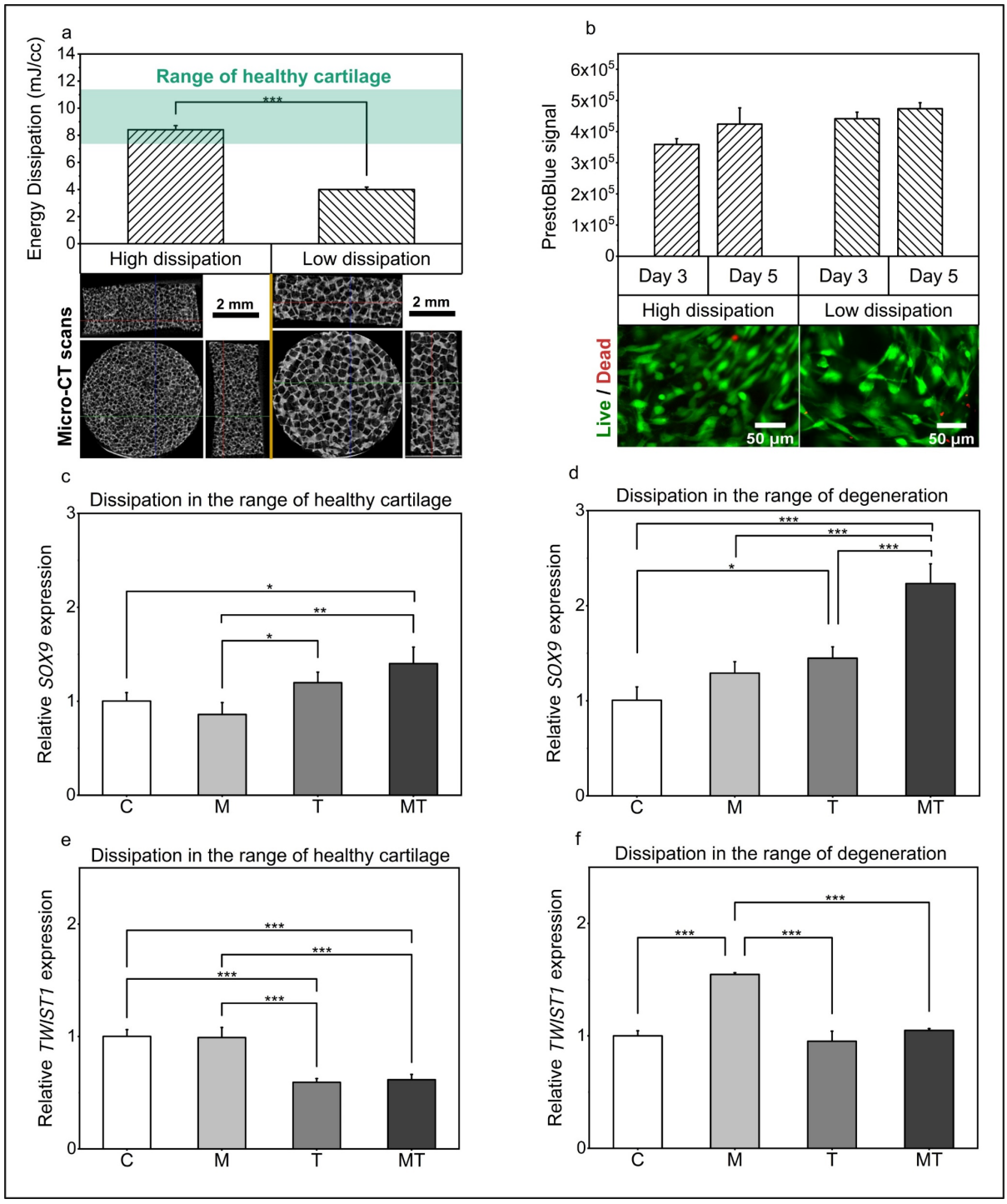

Figure 2.2 – Chondrocyte behavior within RGD-modified hydrogels. a) Energy dissipation level of the porous hydrogels together with the values reported for cartilage tissue and representative micro-CT scans of freeze-dried samples from different views,[18] b) Chondrocyte proliferation as quantified via PrestoBlue assay and typical live/dead confocal images at day

11, following thermomechanical stimulation, c-f) Comparison of the relative expressions of genes of interest SOX9 and TWIST1 are shown for the different forms of stimulation and for the different levels of energy dissipation normalized to the control group.

In particular, a positive combined enhancement following thermomechanical stimulation was observed, as indicated by the increased expression of *SOX9*, a factor considered as the master regulator for chondrogenesis (Figure 2.2c). This trend was evident for all constructs, despite the level of energy dissipation. Interestingly, the effect of coupled thermomechanical stimulation was significantly more pronounced in the case of scaffolds showing reduced dissipation level, leading to almost a 2.5-fold increase of *SOX9* expression over the unstimulated control groups (Figure 2.2d). Additionally, expression of *TWIST1* that is known to hinder chondrogenesis, was more than 40% decreased following thermal and thermomechanical stimuli, relative to both the control and mechanical groups (Figure 2.2e). This stimulatory response however, was highly dependent on construct's mechanical characteristics. Indeed, decreased dissipation level was shown to somewhat hinder the effect of isolated thermal or combined thermomechanical cues, as no significant changes were observed in the mRNA levels of *TWIST1* when compared to the control groups (Figure 2.2f). On the other hand, however, in the latter samples, dynamic thermal and thermomechanical stimulation allowed for an almost 50% lower expression of *TWIST1* relative to the samples that were stimulated only with dynamic mechanical loading.

Lysyl oxidase homolog 2 (*LOXL2*), an enzyme that serves to crosslink collagen fibers inside the extracellular matrix of cartilage tissue, has been shown to significantly modulate chondrocyte differentiation.[19] The expression of this enzyme following application of isolated forms of stimulation was scaffold-type dependent (Figure 2.3a and 2.3b). In particular, application of intermittent dynamic compression could significantly promote *LOXL2* expression only in the case of low dissipative constructs, whereas no effects were observed as the dissipation level matched that of healthy tissue. It is also noticeable, that the externally applied thermal increase over time could not affect the expression levels of *LOXL2* when compared to the unstimulated control groups, however a positive or negative average fold value could be observed depending on the scaffold type. Surprisingly a remarkable upstream of messenger RNA expression of *LOXL2* (by 42% for the case of high dissipative constructs and by 60% for the case of ''degenerated'' samples, over the control groups) can be seen when dynamic mechanical cues act concomitantly with dynamic thermal signal regardless of the scaffold's mechanical attributes.

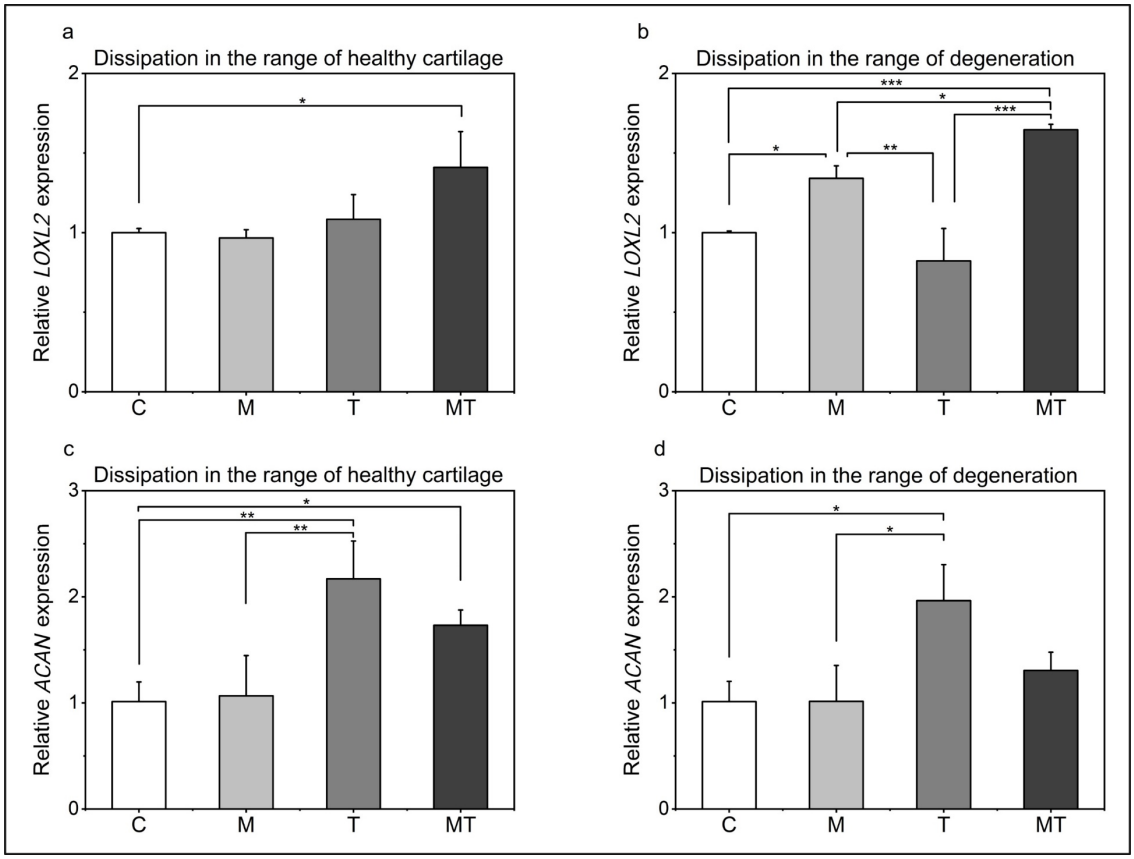

Figure 2.3 - Comparison of the relative expressions of genes of interest a-b) LOXL2 and c-d) ACAN are shown for the different forms of stimulation and for the different levels of energy dissipation normalized to the control group.

Structural molecules such as aggrecan (*ACAN*) play a major role in the maintenance of hyaline cartilage. In line with studies exposing primary chondrocytes in different thermal environments,[20] temperature evolution over time in the present study resulted in ~90% to ~120% more *ACAN* expression over the unstimulated control groups, as well as in ~85% to ~110% relative to the mechanical groups (Figure 2.3c and 2.3d). Additionally, unlike other genes, the level of energy dissipation did not alter the effect of each form of stimulation on *ACAN* expression. Nonetheless, a significant increase in *ACAN* expression was observed for constructs mimicking cartilage dissipative capacity under thermomechanical stimulation.

2.3.2 A combination of dynamic compression and thermal stimulation generated synergistic enhancements in proteoglycan synthesis, along with increased transcription and synthesis of TREK1 and TRPV4 channels

Once the benefits at gene level were identified, we investigated the effects of isolated and combined cues on glycosaminoglycan (GAG) accumulation within the hydrogels. Dynamic

compression has, in some cases, shown to favor proteoglycan synthesis in scaffold-based constructs. It was therefore of interest to investigate whether dynamic mechanical cues worked in tandem with dynamic thermal signals to stimulate additive improvements on chondrocytes biosynthetic responses. Constructs that were stimulated only with mechanical loading showed mixed results. In particular, GAG production following dynamic compression decreased in a dissipation-dependent manner and had no significant effect on the total GAG content. This trend is quite similar to what we observed at gene level. Samples exposed to isolated thermal stimulus exhibited a significantly higher amount of glycosaminoglycans compared to the free swelling controls, but not statistically different to those that were treated with dynamic mechanical loading alone. When dynamic compression was combined with dynamic thermal stimulation during culture, we observed the highest improvements in GAG content, when compared to the control groups. Even though the amount of GAG accumulated in the latter constructs is not statistically different to that of hydrogels that were treated only with dynamic thermal stimulation, these results show that by providing more biomimetic cues *in vitro*, we can augment the production of GAGs within the engineered constructs (Figure 2.4a and 2.4b).

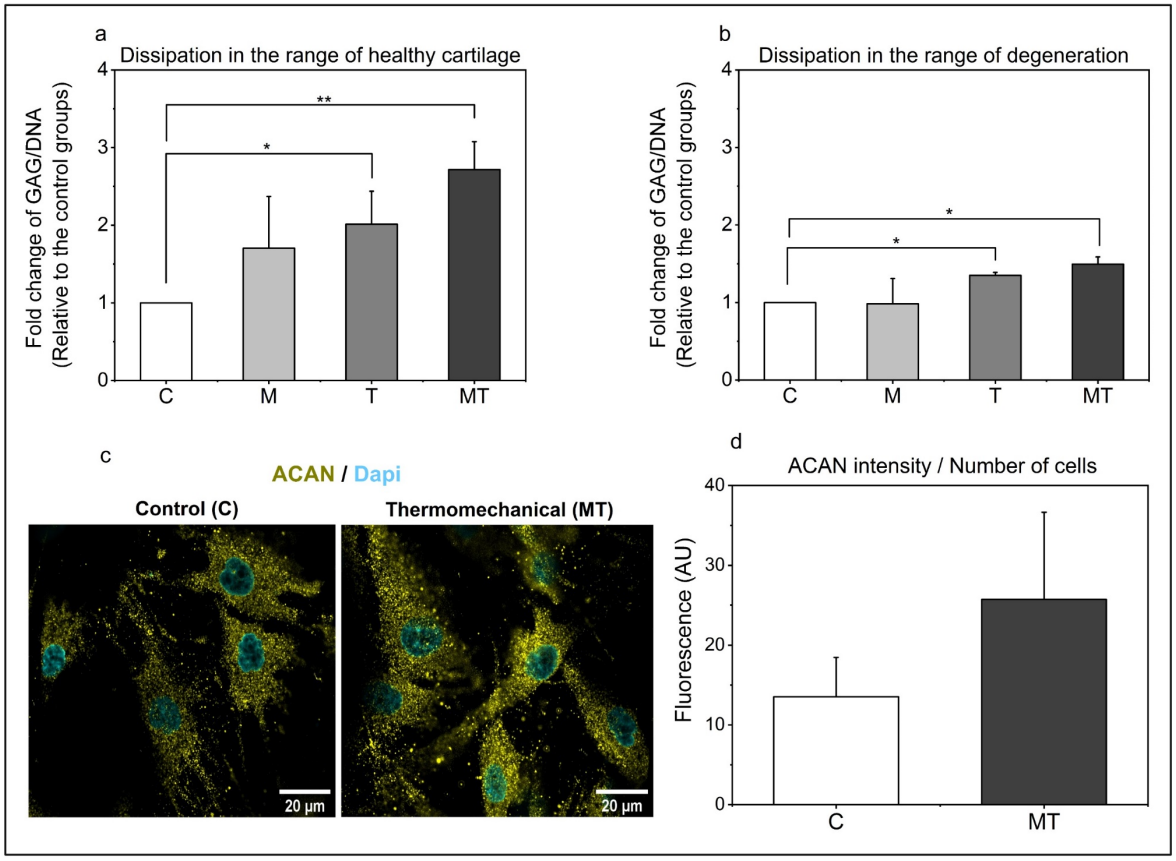

Figure 2.4 – a-b) Fold change of [Glycosaminoglycan/DNA] over the culture period of 11 days for the different dissipative hydrogel constructs following different forms of stimulation. c)

Typical confocal images of human chondrocytes seeded in dissipative in control and stimulated groups, d) Integrated fluorescent intensities for aggrecan at 63x objective. Scale bar at 20 µm.

Furthermore, the average biosynthetic content accumulated in the hydrogels following application of isolated form of stimuli was generally affected by the level of construct's dissipation. Interestingly, a higher level of dissipation allowed for enhanced average values of glycosaminoglycan accumulation. A similar trend was also observed when mechanical loading was applied concomitantly with dynamic thermal stimulus, resulting in more pronounced differences between the different hydrogels. Ultimately, despite the different mechanical characteristics of the hydrogels, application of mechanical loading could lead to significant changes in GAG content, only when applied together with dynamic thermal stimulation. Deposition of aggrecan, a high molecular weight proteoglycan in cartilage tissue, comprising of a protein core bearing many unbranched, negatively charged sulfated GAGs, was monitored with immunofluorescence staining. In line with the calorimetric data, aggrecan synthesis of chondrocytes was strongly enhanced by thermomechanical stimulation in 3D hydrogels, over the free swelling controls (Figure 2.4c and 2.4d).

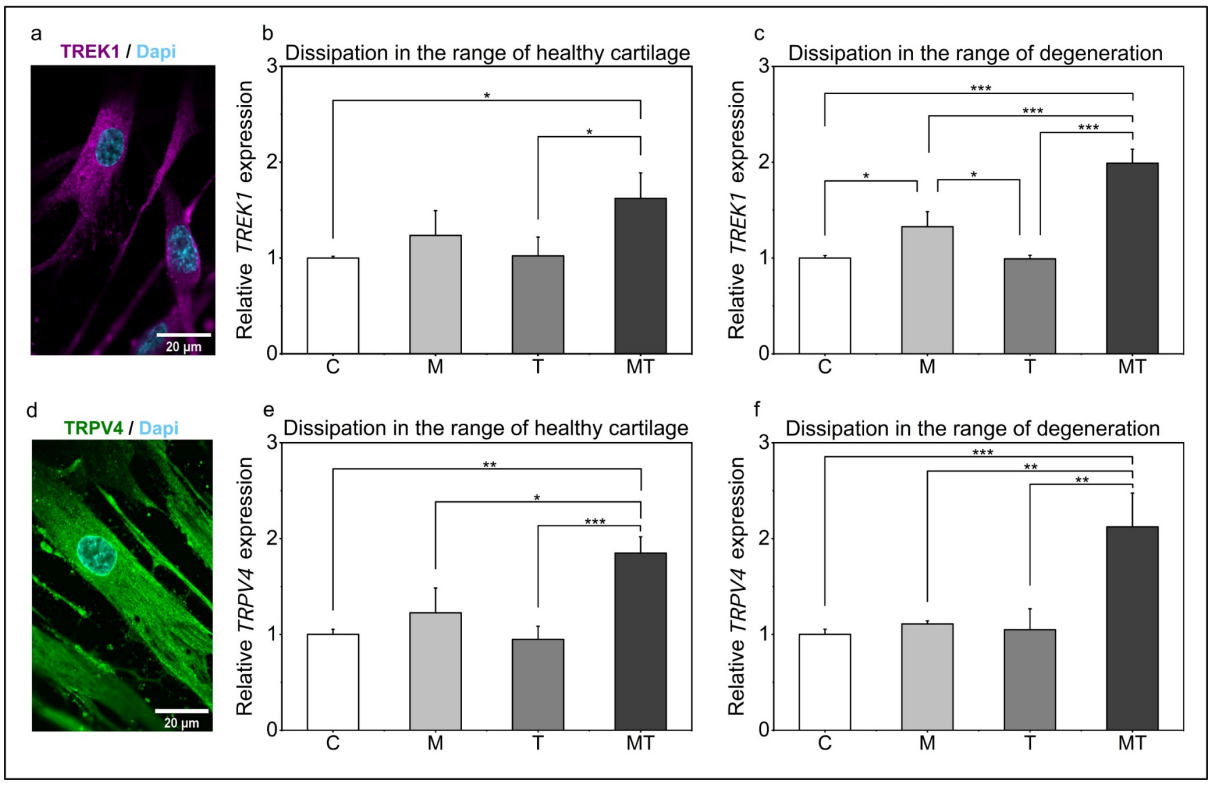

Figure 2.5 – a) Typical confocal image of TREK1 channel in RGD-modified hydrogels seeded with primary human chondrocytes, b-c) Comparison of the relative expressions of genes of interest TREK1 are shown for the different forms of stimulation and for the different levels of

energy dissipation, d) Typical confocal image of TRPV4 channel in RGD-modified hydrogels seeded with primary human chondrocytes, e-f) Comparison of the relative expressions of genes of interest TRPV4 are shown for the different forms of stimulation and for the different levels of energy dissipation. Scale bar at 20 µm at 63x objective.

Towards examining the mechanism behind the enhanced GAG secretion following thermomechanical stimulus, we evaluated expression of the candidate signal mediators of thermomechanical cues. Our data imply that the ''background K^+ channel'' (*TREK1*) and/or the transient receptor potential vanilloid 4 (*TRPV4*) channel may be involved in the transduction of self-heating. *TREK1* channel has been widely studied in neurons and research has shown that it can be activated by heat.[21-23] Our immunostaining results showed for the first time that this specific channel is expressed in primary human chondrocytes as well (Figure 2.5a). Transient receptor potential vanilloid (*TRPV4*) is also known to play a mechanosensory role in various musculoskeletal tissues.[24, 25] Expression of this channel was also verified in our studied system (Figure 2.4d). Thermomechanical stimulation significantly upregulated *TREK1* and *TRPV4* expression as illustrated in (Figure 2.5b and 2.5c) and (Figure 2.5e and 2.5f), suggesting that these ion channels are very likely involved in thermo-mechanotransduction.

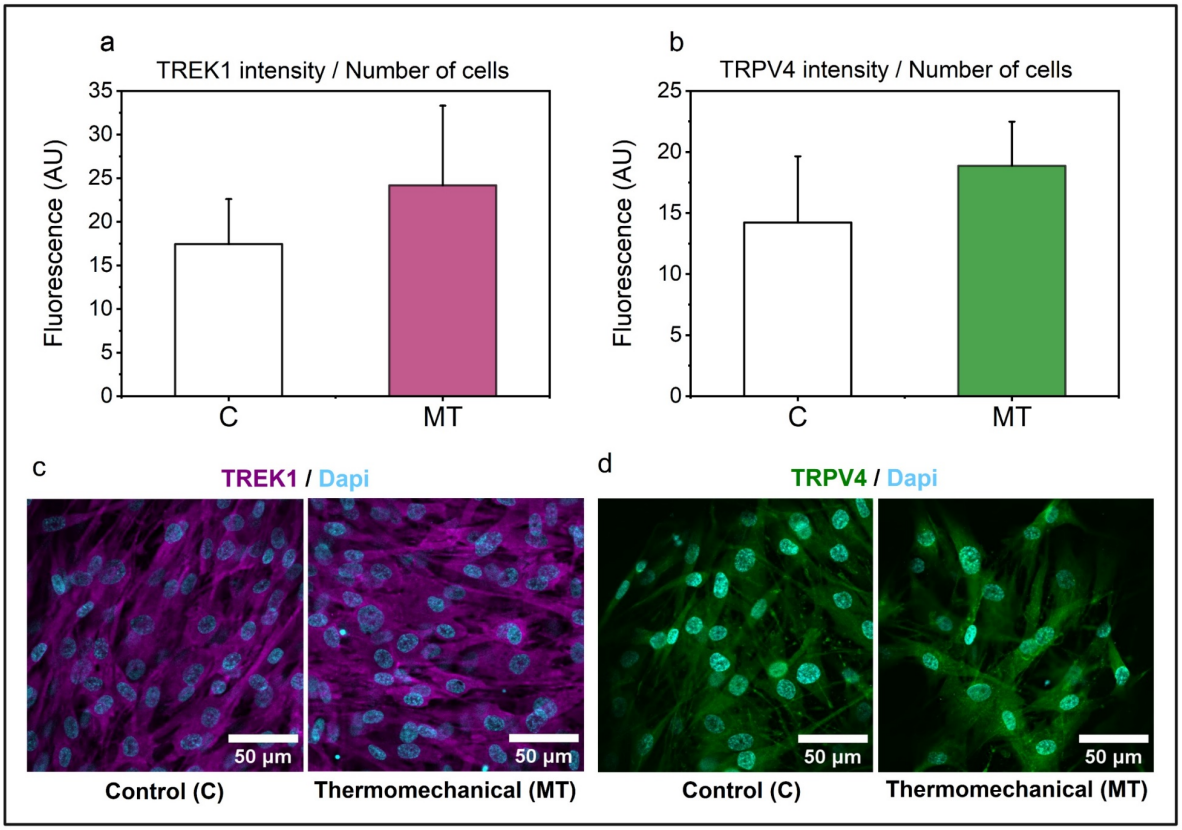

Figure 2.6 - a-b) Integrated fluorescent intensities for TREK1 and TRPV4 ion channels, c-d) representative confocal images of human articular chondrocytes seeded in pHEMA-based hydrogels for TREK1 and TRPV4 channels after 12 days of culture for Control and Thermomechanically stimulated groups at 20x objective. No differences were observed based on the level of energy dissipation.

To gain a better understanding on these observations, we further analyzed these effects at protein level. In line with the PCR outcomes, our results clearly demonstrate an upward trend in the average fluorescence intensity of both ion channels (Figure 2.6a-d) when constructs are subjected to biomimetic thermomechanical cues, strengthening the hypothesis that both ion channels are very likely participating in thermo-mechanotransduction process.

2.4 Discussion

Even though an appropriate strategy to engineering cartilage tissue analogues incorporates a broad array of variables that might be decisive, the goal of this study has been to examine one of them. More specifically, the already established influence of mechanical stimulation on chondrocyte metabolic response has been investigated in synergy with a naturally occurring dynamic temperature rise, measured *in vivo* during jogging and referred to as self-heating. Multiple research studies have previously described how different biochemical and biomechanical cues have been used to promote or even enhance chondrocyte metabolism.[26-28] However, to the best of our knowledge, this inherent ability of the native cartilage to increase its temperature following a physical activity, is still largely ignored by the scientific community. Our group is the first to pioneer and introduce this latter concept.[11, 12, 15] Urged by the central role of the biophysical interactions between chondrocytes and their environment in tissue formation and maintenance, this study was directed at elucidating the role of this new thermal signal (transient temperature increase from ~32 °C to ~39 °C) on cartilage-mimicking constructs. Previous studies have already considered the effect of static culture temperature on chondrocytes anabolism. Culturing adult chondrocytes under 32 °C for instance, allowed for a significant increase in the expression of major metabolic markers when compared to the standard culture conditions (37 °C).[9] Similarly, another study also showed, that the expression of matrix metalloproteinase-13 (*MMP-13*), a factor related to cartilage degradation, is significantly inhibited as the culture temperature is reduced to physiological values.[8]

Nonetheless, despite tantalizing hints that temperature regulates chondrocyte biological functions, not a single study is based on a biomimetic dynamic temperature regime. Temperature evolution following joint loading can be ascribed to different factors. Energy dissipation in avascular cartilage following a physical activity generates heat, resulting in temperature evolution over time. The absolute temperature increase, within cartilage milieu however, could also be affected by the ambient temperature and heat flux from surroundings tissues. Despite of the actual heat source inside the joint, such varied thermal environment might affect chondrocyte biological functions. To reproduce such conditions *in vitro*, we seeded primary human chondrocytes into porous hydrogels that were placed inside a bioreactor apparatus designed for thermomechanical stimulation of cell-laden hydrogels.[15] The hydrogels in turn, were engineered to capture the viscoelastic nature of healthy as well as degenerated cartilage, in terms of the energy dissipation level. It was of interest to understand whether the application of dynamic thermal signal in constructs with viscoelastic properties close to those of degenerated tissue, would also respond to the externally applied cues.

Our 3D scaffold system presents some limitations e.g., RGD modification of synthetic scaffolds resulted in a less round morphology of chondrocytes. Nevertheless, considering that we utilized identically prepared samples during all of our experimental studies as well as growth factors to prevent chondrocyte de-differentiation, we assume that the role of externally applied stimuli on cells' chondrogenic response could be evaluated regardless of the morphology.

In the current mechanobiological study, cell-seeded constructs were pre-cultured for 5 days, before subjecting to intermittent dynamic stimulation. Dynamic stimulation has been shown to generate matrix biosynthesis much more efficiently in systems where relatively denser matrix-related components are present.[29] Therefore, it was initially hypothesized that a pre-accumulated extracellular matrix might better facilitate the transmission of thermomechanical stimulus in the dissipative hydrogels. Previous investigations have shown that preculture of approximately 5 days is adequate for a significant up-regulation of cartilage-specific genes, such as cartilage oligomeric matrix protein, proteoglycan 4, collagen type II and aggrecan following a relatively short-term stimulation, as well as for the formation of a pericellular matrix.[29, 30] We have confirmed these observations by showing that chondrocytes can produce functional proteins (such as collagen type 2) during 5 days of preculture (See Figure S2.4). Given that there is currently no available ''universal'' stimulating protocol and based on the

fact that chondrocytes can produce collagen type 2 during five days preculture, we decided to proceed with the experimental protocol we used in our previous published study.[15]

To investigate the immediate changes on gene expression, constructs were sampled for total RNA isolation directly after (0 h) stimulation had ceased. Overall, application of a physiologically relevant compressive loading regime of 20% strain alone, could not significantly stimulate the expression of critical chondrogenic marker genes such as *ACAN* or *SOX9*. This trend was consistent, irrespective of the construct's structural characteristics. Even though mechanical cues (dynamic compression) have been previously utilized to successfully induce chondrogenic markers expression and subsequent matrix deposition in mesenchymal stem cells,[31] or in progenitor cells,[18] a great corpus of evidence suggests that mature chondrocytes are less sensitive to this type of stimulation.[32, 33] This behavior is probably due to fast adaptations to such stimuli and subsequent desensitization.[34] As for the constructs subjected to isolated thermal stimulus, our findings show that this naturally occurring temperature increase, as a by-product of loading in healthy tissue, could possibly serve two main purposes. First and foremost, enhancing the transcription of genes that encode major structural components in the tissue, such as aggrecan and secondly suppressing the expression of factors that could potentially hamper chondrogenesis, such as in the case of *TWIST1*.

Major gene categories related to cartilage normal function appeared to be responsive to concomitant application of thermomechanical cues. Our results showed that, the expression of *SOX9* and *LOXL2* peaked only when dynamic compression acted together with dynamic thermal stimulation. Additionally, with regards to *LOXL2* expression, in some cases, individual forms of stimulation were ineffective or yielded even adverse effects in the expression of this enzyme. Spectacularly, when dynamic compression was combined with biomimetic temperature increase there was always a direction towards highest expression. This trend is consistent and overall similar to the responsive pattern of *TREK1* channel, encouraging us to believe that there is a link among potassium channels and lysyl oxidase enzymes. This latter observation is extremely important, and illustrates that the obtained coupled improvements are distinct, and exert a more pronounced effect on chondrocytes behavior over pure mechanical cues, confirming the complementary role of this thermal cue. Such additive enhancements were evident regardless of biomechanical construct's differences.

Glycosaminoglycan content was also highly dependent on the type of stimulation, but also on the construct's dissipative capacity. In agreement with the PCR outcomes, dynamic mechanical

loading could not bring significant improvements in glycosaminoglycan synthesis over the control groups. In fact, dissipation level resembling cartilage degeneration resulted in decreased average GAG content following this form of stimulation. Furthermore, similar to findings with chondrocytes,[20] between the stimulated groups, there was always a direction towards elevated glycosaminoglycan level in the groups containing dynamic thermal signal. Ultimately, when dynamic thermal stimulation was coupled with mechanical loading, significant GAG synthesis was observed, irrespective of the level of energy dissipation. Our results indicate that chondrocytes can perceive and translate the externally applied mechanical strain much more effectively when thriving in a dynamic thermal environment.

Herein it could be argued that it's unclear whether a difference in "absolute" temperature could not also explain the observed additive effects in the current study. With respect to the latter, we have already showed in chondroprogenitor cells, at transcriptional level, that loading-induced self-heating condition can better enhance major chondrogenic markers compared to mechanical stimulus at constant temperatures (32.5°C or 37°C).[15] Additionally, culturing cells at high temperatures of approximately 39 °C have been shown to significantly prevent chondrocytes' biosynthetic capacity, therefore, such conditions were not considered during our experimental design.[8] Collectively, these results indicate that cells can sense the transient temperature rise.

Furthermore, glycosaminoglycan production following thermomechanical stimulation was higher in the hydrogels exhibiting increased dissipation levels. Energy dissipation is directly related to scaffold's microstructure,[35, 36] which may affect cell behavior. Previous research has shown that polymeric scaffolds with enhanced energy dissipation levels improved expression of major chondrogenic markers.[16] In agreement with the latter observations, our results show that the transmission of externally applied thermomechanical cues to cells can be significantly affected by the construct's level of energy dissipation, resulting in different GAG contents within the constructs.

The increased GAG synthesis following combined thermal and mechanical stimuli led us to explore cellular mechanisms by which self-heating may affect the engineered constructs. The ultimate goal is to identify potential pathways through which mechanotransduction occurs to induce production of extracellular matrix components. It has already been shown that primary cilia activation in shear or Piezo channel activation during compression are key mediators for transduction of dynamic mechanical stimulation on primary chondrocytes.[37, 38] For the first time, we now add to this list the activation of the mechanically gated potassium channel

TREK1, through thermomechanical stimulation. In peripheral sensory neurons, this polymodally-gated ion channel allows for signal transmission and temperature perception.[39] For chondrocytes, it has been proposed that related potassium channels allow for the influx of ions, stabilizing membrane potential.[40] To the best of our knowledge, this study is the first to identify a specific type of potassium ion channel (*TREK1*), at protein level, that was not known to be expressed on primary human chondrocytes. Since our immunostaining data show that *TREK1* actually exists in primary human chondrocytes, our results add to this body of work in connecting the *TREK1* channel in transducing thermomechanical stimulation as well.

The current research work demonstrates that *TRPV4* channels can be triggered by thermomechanical stimulation as well. Previous research in our lab showed that *TRPV4* channels are potent modulators for the expression of major chondrogenic genes (e.g collagen type 2) in human chondroprogenitor cells, in response to thermomechanical cues.[15] In case of primary chondrocytes, we hypothesize that application of thermomechanical stimulation involves concurrent modulation of both thermo-mechanotransducers, *TREK1* and *TRPV4*. Even though additional research, including antagonizing these channels to demonstrate loss or gain of function, is required to conclusively identify thermomechanical stimulation mechanism of action on chondrocytes, this research study demonstrated that dynamic thermomechanical stimulation increases expression of potential signal mediators to facilitate thermo-mechanotransduction process and augment extracellular matrix secretion.

Furthermore, since we examined chondrocyte transcriptional response following isolated and combined thermomechanical cues only in short-term studies, further long-term experiments are required to fully explore the role of dynamic thermal or thermomechanical loading on the quality of the newly formed tissue. Nonetheless, this work was far from the scope of this research study. Even though supplementary studies are required to explore rigorously the modes of action of thermomechanical loading, our preliminary gene expression and matrix deposition findings denote that the early response to thermomechanical loading is involved in alterations in cellular signalling pathways (*SOX9* & *TWIST1*), matrix remodelling (*LOXL2*) and structural proteins (ACAN and GAGs) which, eventually, may result in neo-cartilage formation with enhanced biomechanical properties.

2.5 Conclusion

In conclusion, this study aimed to answer whether the observed temperature evolution, as an indirect effect of mechanical loading in healthy cartilage tissue could be used to promote chondrogenesis in tissue-engineered constructs *in vitro*. Cellular response was evaluated through varied expression of major chondrogenic genes and subsequent downstream protein synthesis, following an applied stimulus. The results shown in this work notably demonstrate that biomimetic thermomechanical cues can induce tissue maturation through enhanced mRNA transcription, and an improvement in the content of major functional proteins. Overall, the current study places self-heating into the realm as a prominent approach for stimulating the metabolic as well as biosynthetic activity of primary human chondrocytes.

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Supporting Information

Mimicking loading-induced cartilage self-heating in vitro promotes matrix formation in chondrocyte-laden constructs with different mechanical properties

Morphological characterization of scaffolds in hydrated state – Micro-CT scans

Hydrated scaffold was put inside a 2 ml Eppendorf tube and carefully frozen in liquid nitrogen while avoiding direct contact of scaffold and nitrogen. Then the frozen samples were vacuumed dried for 72 hours and scanned afterwards for morphological characterization in dried state. The measurement parameters were set as 40 kV for voltage, 100 mA for current, 18 mm for spatial resolution, 400 mS for exposure time and 0.42° for rotation steps without using any filter.

Mechanobiological study – Modes of stimulation

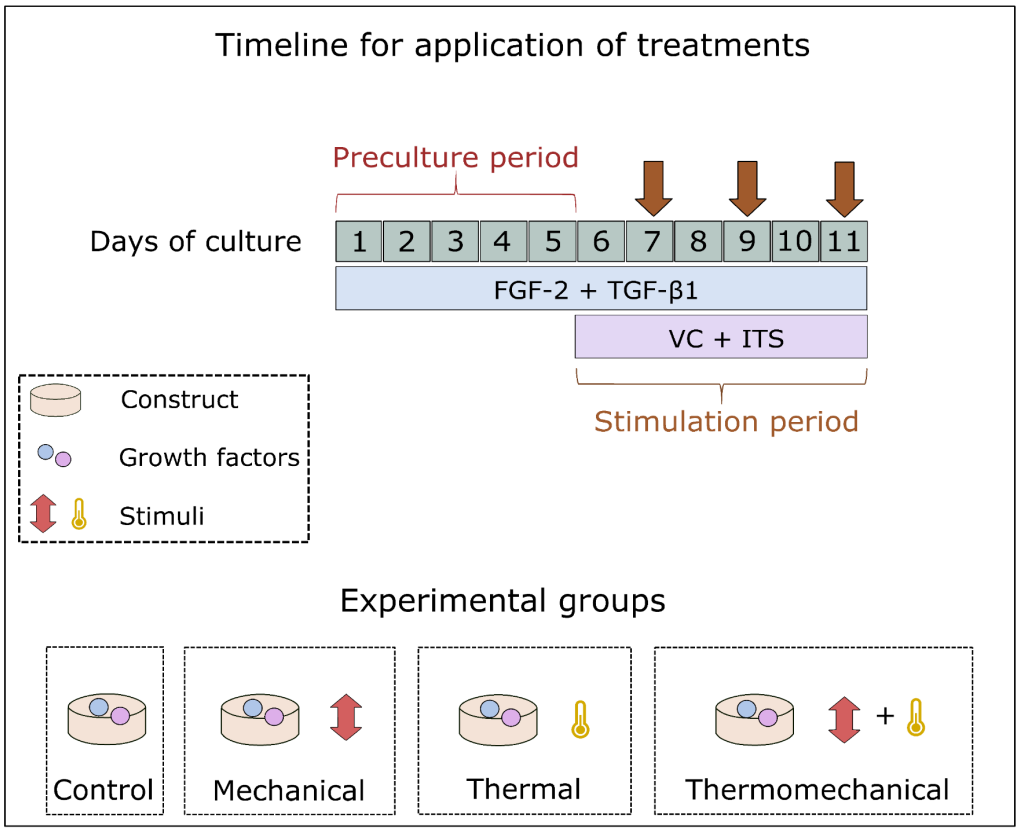

Figure S2.1 - A schematic illustration of the mechanobiological study timeline is shown. On day 0 (Not shown), chondrocytes are seeded on scaffolds and remain at free swelling condition

up to Day 5. On Day 6, foetal 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 7,9 and 11. Constructs were then collected for gene expression (n=3) and biochemical analysis (n=3).

Chondrocyte viability following different forms of stimulation

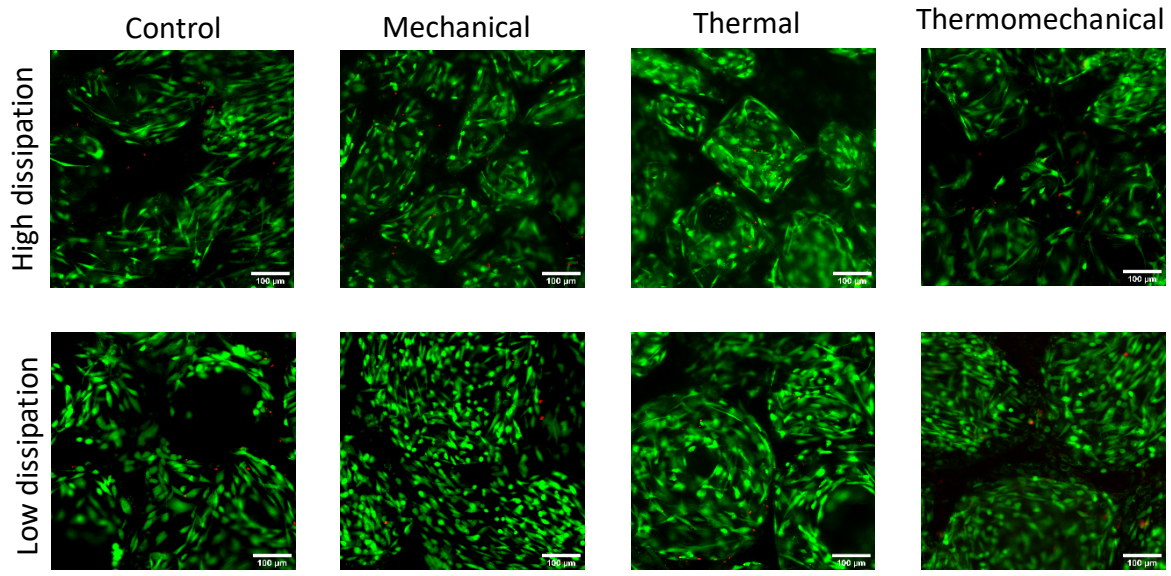

Figure S2.2 – Typical Live/Dead staining for samples with different levels of energy dissipation and following different forms of stimulation. Green stains for Live cells. Scale bar at 100 µm.

Chondrocyte metabolic response to physiologically relevant thermomechanical stimulus

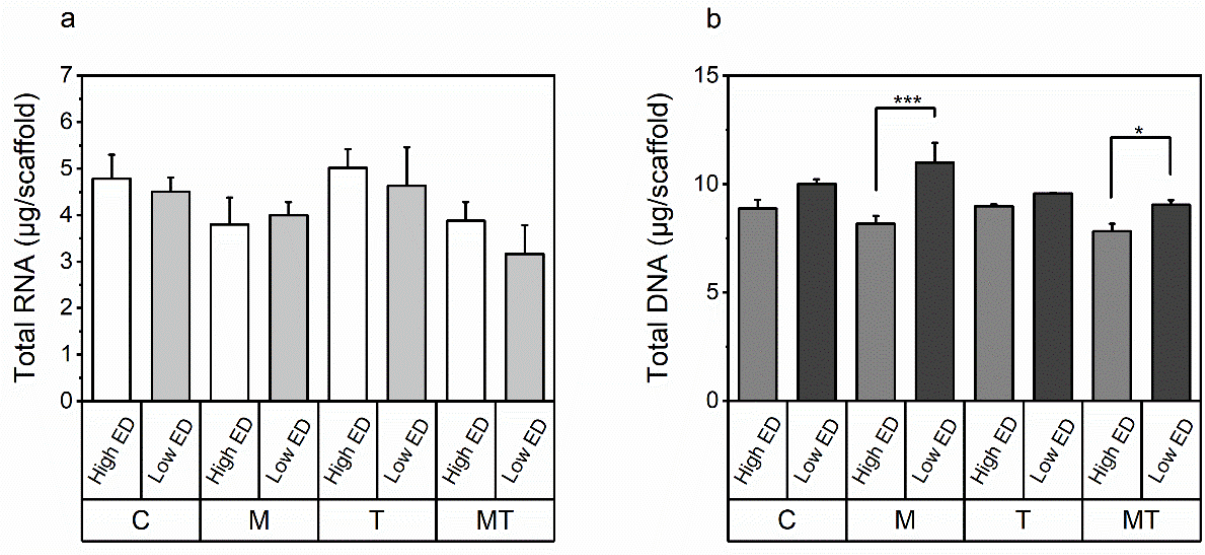

Figure S2.3 – a) Total RNA and b) Total DNA content for the different groups of scaffolds following different forms of stimulation. Total amount of RNA per construct was determined

and compared between the different groups of scaffolds. Overall, no significant changes were observed following the application of different types of stimulation. It is, however, noticeable that there is always a direction towards reduced RNA content in the groups containing dynamic mechanical loading. This trend was evident regardless of the level of energy dissipation. Furthermore, construct cellularity, as quantified from measuring DNA content in each sample, was mainly unaffected by the different forms of stimulation and slightly enhanced in the constructs with higher pore size (lower dissipation level). Additionally, dynamic thermomechanical stimulation resulted in lower DNA mean content when compared to free swelling controls.

Collagen type 2 expression during the 5-day preculture period and aggrecan accumulation within the engineered constructs after the last loading cycle was ceased.

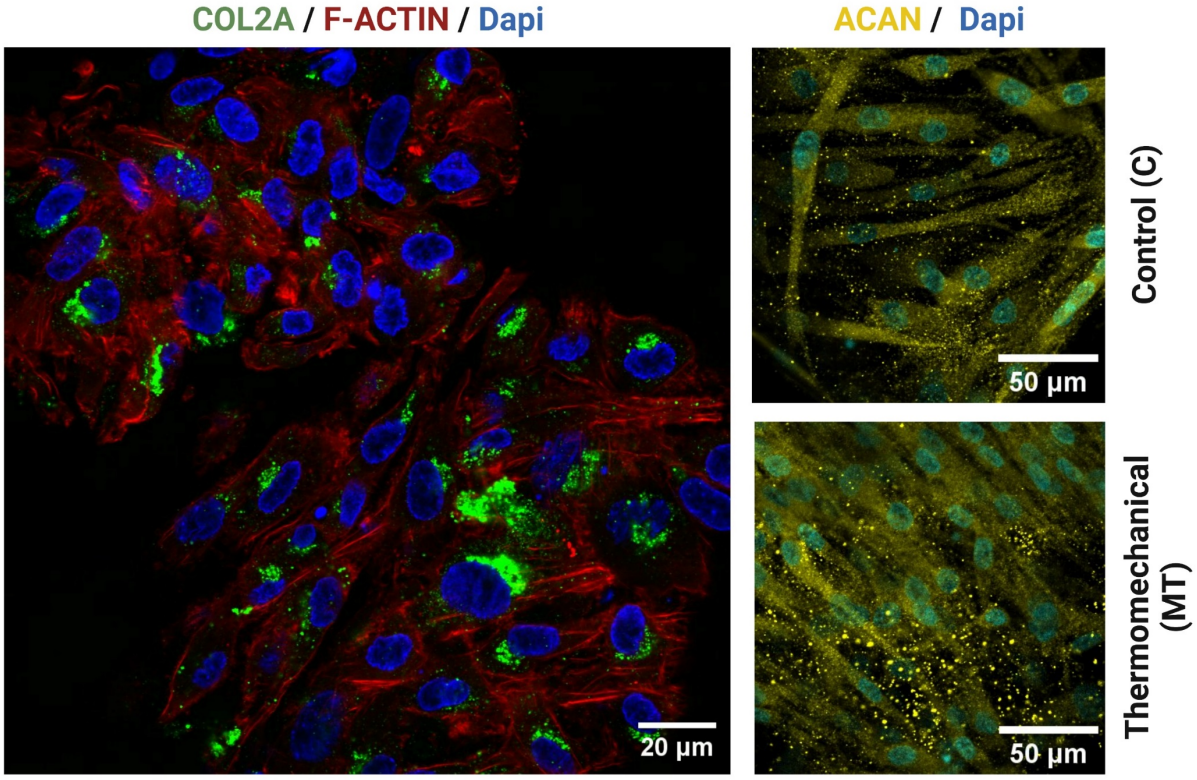

Figure S2.4 - Representative 3-color immunofluorescence staining of pHEMA-based hydrogels at 63x (left) and 20x (right), depicting accumulation of Collagen type 2, F-actin, Aggrecan and Dapi during culture for control and stimulated groups.

RNA extraction and PCR

Different annealing temperature and concentration were used for primers to optimize the process resulting in efficiency range of 88 to 108%. The PCR amplification was carried out in duplicate for each sample by StepOnePlus Real-Time PCR platform (Applied Biosystems). The thermal cycling condition was defined as an initial 95°C step for 1 min followed by 40 cycles of 95 °C for 5s and corresponding annealing-extension temperature of gene for 30s. Gene expression data were analyzed using the comparative ΔΔCt method with RPL13a as the reference gene. Corresponding free swelling hydrogels in each group were used as the biological reference for the stimulated hydrogels (n=3).

Table S1. Primers data used for qRT-PCR

Gene	Primer concentration (nM)	Efficiency (%)	Sequence 5'-3'
RPL13a	175	104	**F:**TAAACAGGTACTGCTGGGCCG **R:**CTCGGGAAGGGTTGGTGTTC
SOX9	275	108	**F:**TGGAAACTTCAGTGGCGCGGA **R:**AGAGCAAAAGTGGGGGCGCTT
TWIST1	250	91	**F:**AGCAGGGCCGGAGACCTAGATGTCA **R:**ACGGGCCTGTCTCGCTTTCTCT
ACAN	175	99	**F:**GGTACCAGTGCACAGAGGGGTT **R:**TGCAGGTGATCTGAGGCTCCTC
TREK1	250	99	**F:**CAATTCGACGGAGCTGGATG **R:**CTTCTGTGCGTGGTGAGATG
LOXL2	250	88.7	**F:** TGACGACTTCTCCATCCACG **R:** GTGTGCTTGCAGTCAGTGAC
TRPV4	250	90	**F:**TCCACCCTATATGAGTCCTCGG **R:**TAGGTGCCGTAGTCAAACAGT

Immunofluorescence staining

Table S2. Antibodies/dye used in immunofluorescence staining

Reagent	Designation	Source	Dilution
Primary antibody	Mouse anti-TREK1	(F-6): sc-398449, Santa Cruz Biotechnology	1:250
Primary antibody	Rabbit anti-TRPV4	ab191580, Abcam	1:250
Primary antibody	Mouse anti-ACAN	MS X HU AGGRECAN (969D4D11), Lifetechnologies Thermo Fisher	1:250
Primary antibody	Mouse anti-COLL2A	Invitrogen, MA5-12789	1:250
Phalloidin	Alexa Fluor 568 phalloidin	Invitrogen, A12380	1:400
Secondary antibody	ALEXA FLUOR 488 PHALLO	Lifetechnologies	1:250

Chapter 3: Low oxygen tension augments chondrocyte sensitivity to biomimetic thermomechanical cues in cartilage-engineered constructs*

** This chapter has been published in iScience – Cell Press Journal (Stampoultzis, T., Guo, Y., Nasrollahzadeh, N., Rana, V.K., Karami, P., and Pioletti, D.P. (2023). Low-oxygen tension augments chondrocyte sensitivity to biomimetic thermomechanical cues in cartilage-engineered constructs. iScience 26, 107491. 10.1016/j.isci.2023.107491). **Author's Contributions:** T.S conceptualized the work and performed the experiments, Y.G assisted with part of experimental work, N.N previously developed the bioreactor, V.K.R assisted with the writing of the manuscript P.K assisted with the schematics, D.P.P supervised and reviewed the work. All authors commented on the manuscript.*

Abstract

Chondrocytes have been shown to respond to a host of biophysical cues of the environment that surrounds them. Phenomenological studies suggest that certain of these cues, such as oxygen tension, transient thermal and mechanical signals could yield profound effects on chondrocyte function. Nonetheless, up to now, how these individual factors interact with each other to establish a unique regulatory microenvironment for chondrocyte function is largely unknown. Herein, we explore these interactions by means of our joint-simulating bioreactor that allows for the independent control of the culture oxygen concentration, evolution of temperature, as well as applied mechanical loading, to gain broad insights into the transcriptional and biosynthetic profiles affected by well-defined combinations of biophysical parameters. The outcomes of this work reveal significant coupling between these physiologically-relevant cues, as indicated by the ~14-fold increase in collagen type II (*COL2a*) and aggrecan (*ACAN*) mRNA expression. Motivated by the molecular analysis, we also found that glycosaminoglycan synthesis following dynamic thermomechanical stimulation was enhanced, while the magnitude of the biosynthetic changes was oxygen-dependent. Similar responsive trends were also observed for collagen type II protein synthesis. Finally, to attain insight into the beneficial outcomes of combined thermomechanical stimulation and low

oxygen tension, a mechanistic study was performed, showing a major regulator of chondrogenic response on chondrocytes (SRY-Box Transcription Factor 9, SOX9) that is expressed by the combined application of the latter cues. Overall, these series of studies shed light into how isolated and combined mechanobiological cues are integrated by chondrocytes and offer strategies to tissue engineers for improving the extracellular matrix content of cartilage-engineered constructs.

3.1 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. 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 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]

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 crosslinking 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 neglect 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 been also recognized as an indispensable element of 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 human chondrocytes microenvironment, ii) to investigate hypothesis pertaining to the coupling of these signals, and iii) to 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 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.

3.2 Results

3.2.1 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 knee joint, up to 16 days (Figure 3.1A). 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 (Figure 3.1B-E). 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 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).

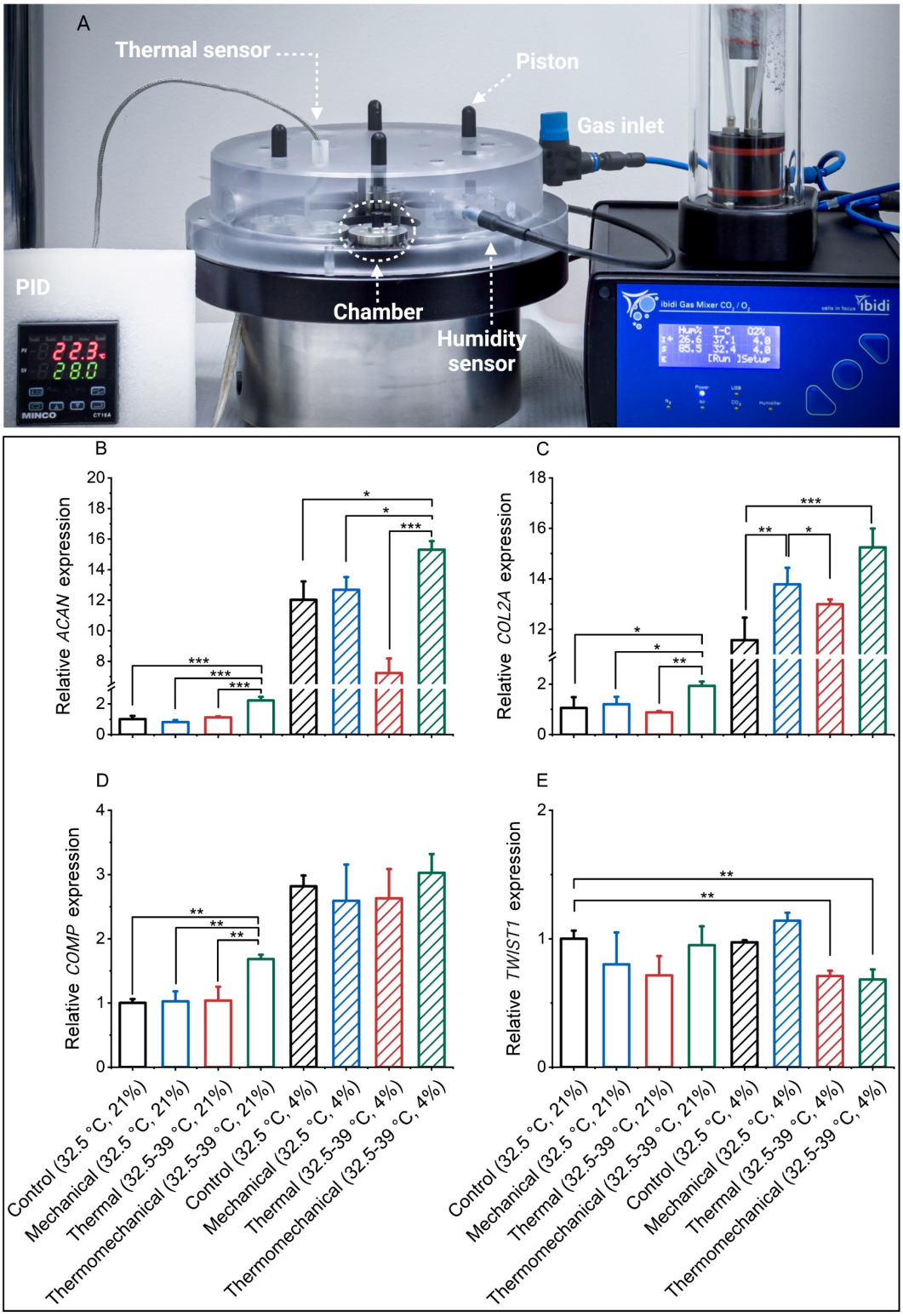

Figure 3.1 – A) Bioreactor apparatus consisting of three modules. A PID controller regulates the temperature profile applied in the engineered constructs through a thermal sensor. A gas

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.

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 3.1B and 3.1C 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 to 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.

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

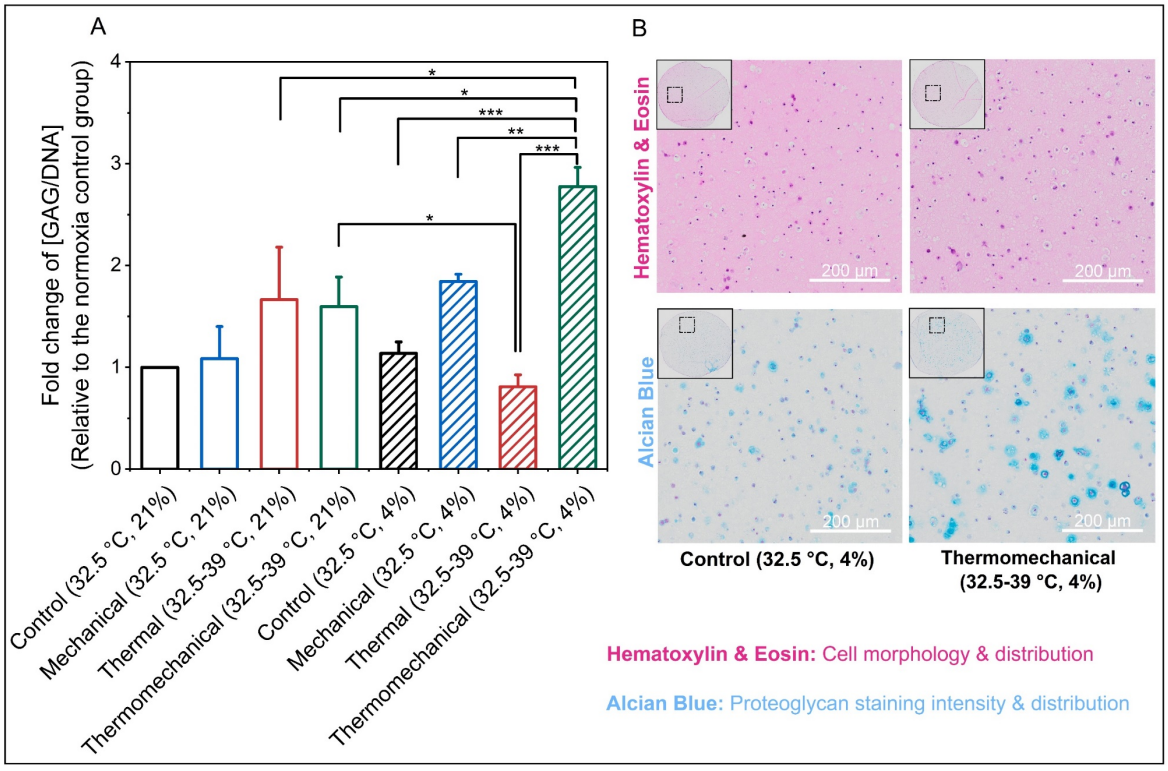

Figure 3.2 – a) Fold change of [Glycosaminoglycan/DNA] over the culture period of 16 days for the different forms of stimulation normalized the normoxia free-swelling control groups, b) H&E and Alcian blue staining for the realistic physiologically-relevant conditions.

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 3.2A. This trend was further supported by alcian blue staining as shown in Figure 3.2B.

The morphology and location of chondrocytes in the GelMA constructs was also assessed 16 days after encapsulation and was 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.

3.2.3 A combination of thermomechanical stimulation and hypoxia generated enhancements in COL2A expression

Functional collagenous matrix content also reflected the trends seen in gene expression (Figures 3.3A-3.3C). Collagen content of the engineered constructs was assessed using histology and immunofluorescence 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 3.3A). 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 3.3B). No significant differences were detected

between the experimental groups denoting that the [COL2A/total collagen] ratio is higher when samples receive combination of three biomimetic signals (Figure 3.3C).

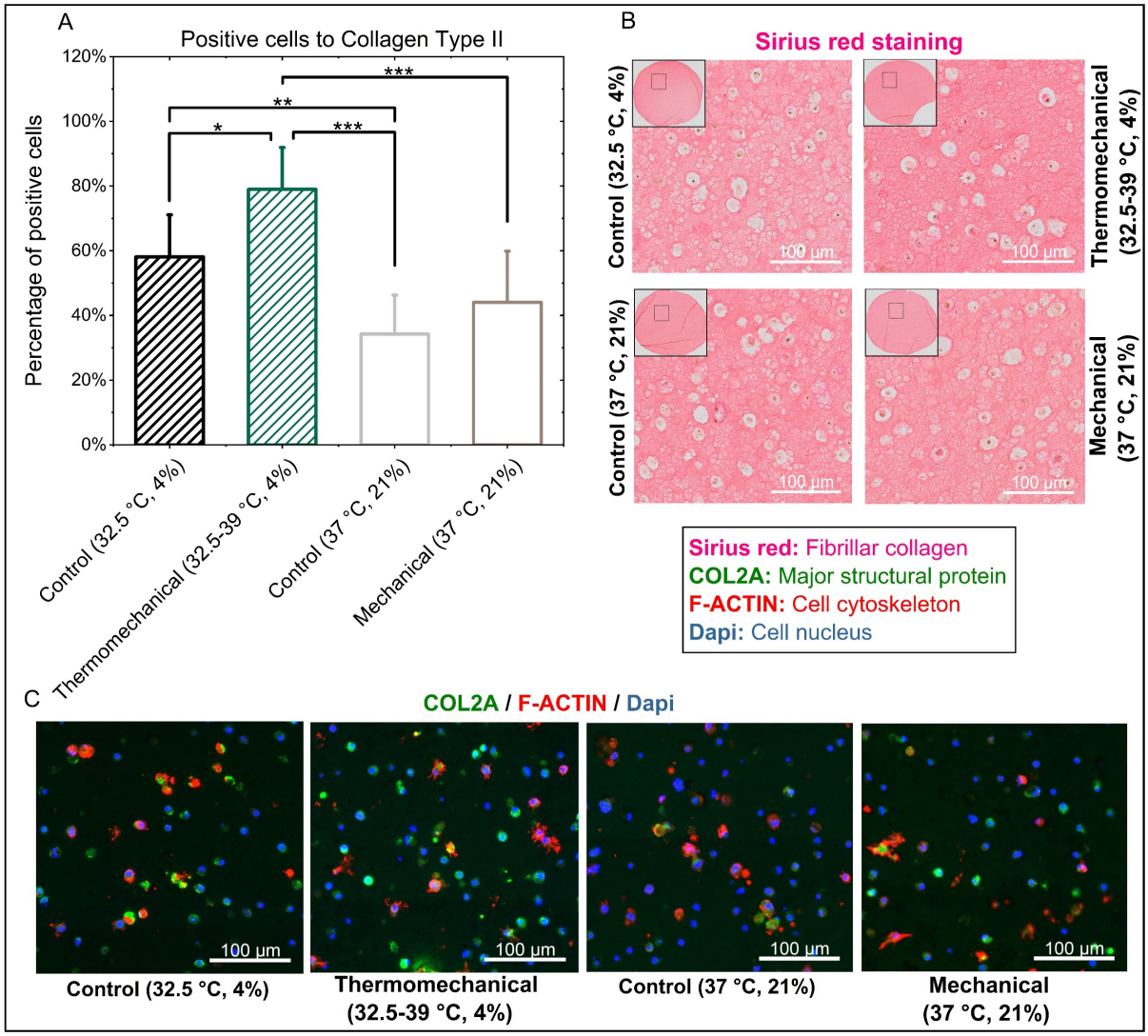

Figure 3.3 - A) Percentage of positive COL2A-cells for the different experimental groups, B) Sirius red staining for total collagen visualization, C) representative confocal images of human articular chondrocytes seeded in GelMA-based hydrogels channels after 16 days of culture.

3.2.4 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 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 dedifferentiation 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).

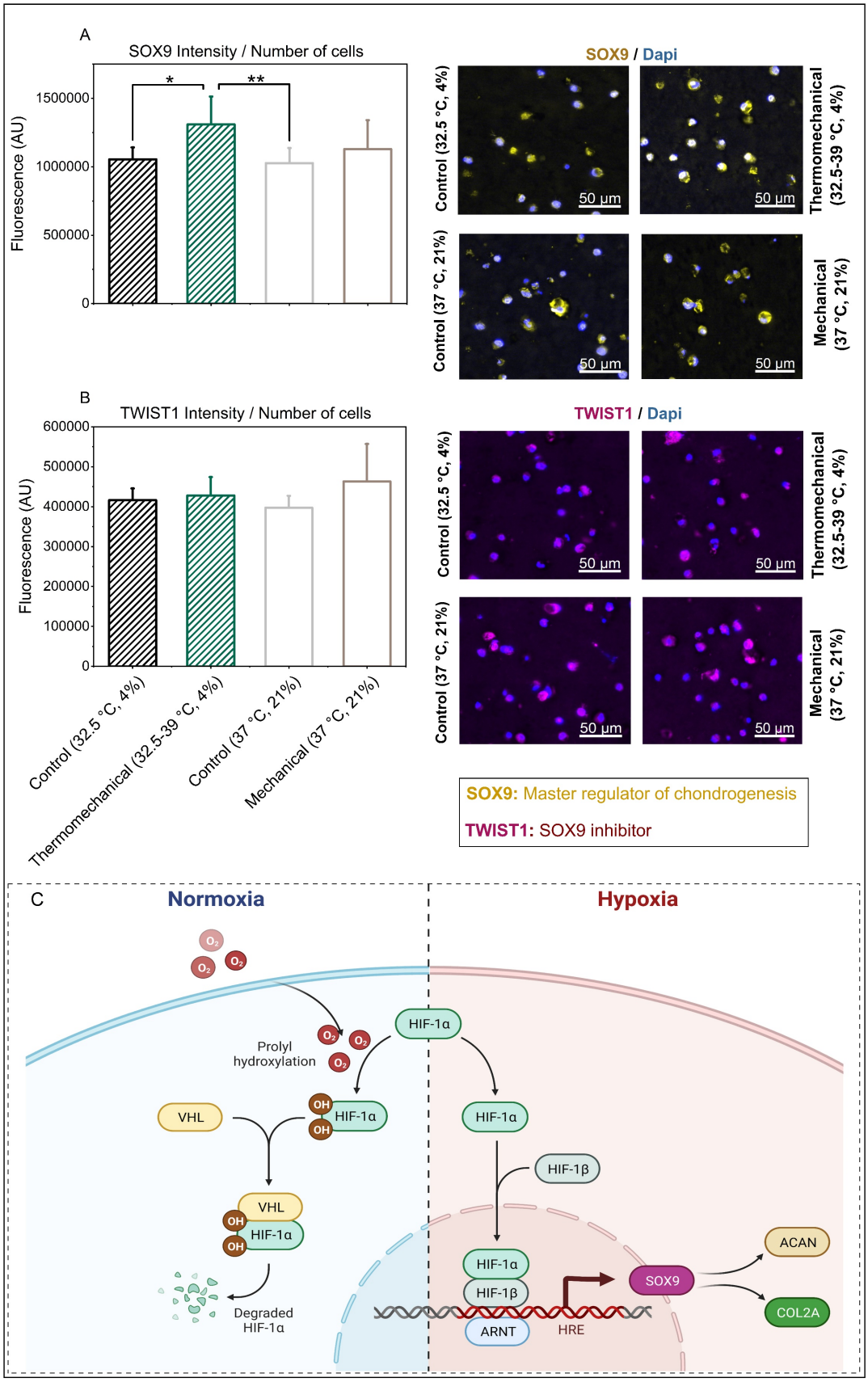

Figure 3.4 – A) Integrated fluorescent intensities for SOX9 with representative confocal images of human articular chondrocytes seeded in GelMA-based hydrogels channels after 16 days of culture, B) Integrated fluorescent intensities for TWIST1 with representative confocal images of human articular chondrocytes 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 produced using BioRender.com.

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 3.4A).

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 3.4B). 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 S3.2)

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 3.5C 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 concomitant application of 3 stressors can significantly improve the expression of pivotal transcription factors that may ultimately result in neo-cartilage with enhanced glycosaminoglycan and collagen type II content.

3.3 Discussion

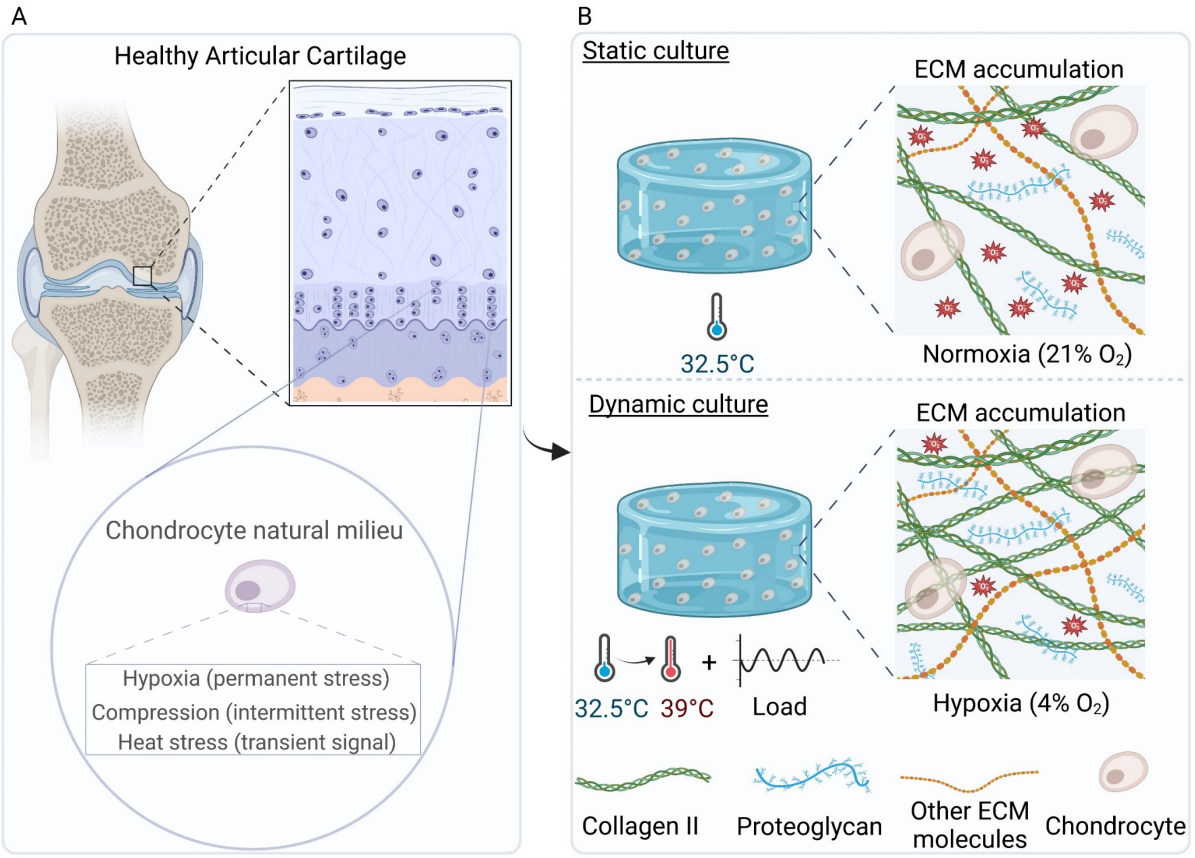

Figure 3.5 – Cartilage thermo-mechanobiology under hypoxia. A) Chondrocytes in cartilage tissue develop under hypoxia conditions, where intermittent mechanical or transient thermal cues can occur. B) By re-enacting these biophysical cues in vitro tissue maturation is accelerated through enhanced biosynthetic content. Schematic was produced using BioRender.com

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. 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 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 hours per day instead of 1.5 hours, 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.

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 suggest 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 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 static hypoxia condition which leads to suboptimal/confilicting 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 normoxia 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 Figure 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 degrees). 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.

3.4 Limitations of research work

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.

3.5 Materials and Methods

3.5.1 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.

3.5.2 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 beta 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, 3 mm × 4 mm × 6 mm). Chondrocyte/hydrogel suspension was crosslinked under illumination of 405 nm wavelength at w/cm^2 intensity for 2 minutes. 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.

3.5.3 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 hours. Similarly, the dynamic thermal stimulus was meant to simulate the natural temperature increase inside the knee joint, over a period of 1.5 hours.[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% (vol/vol) 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 can be seen in supplementary data (Figure S3.1).

3.5.4 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] 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. Further details can be found in supplementary information.

3.5.5 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.

3.5.6 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 hours 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 Haematoxylin 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 Supplementary Information. Fluorescent and non-fluorescent images of various specimens were obtained utilizing a 20x 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.

3.5.7 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.

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Supporting Information

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

Mechanobiological study – Modes of stimulation

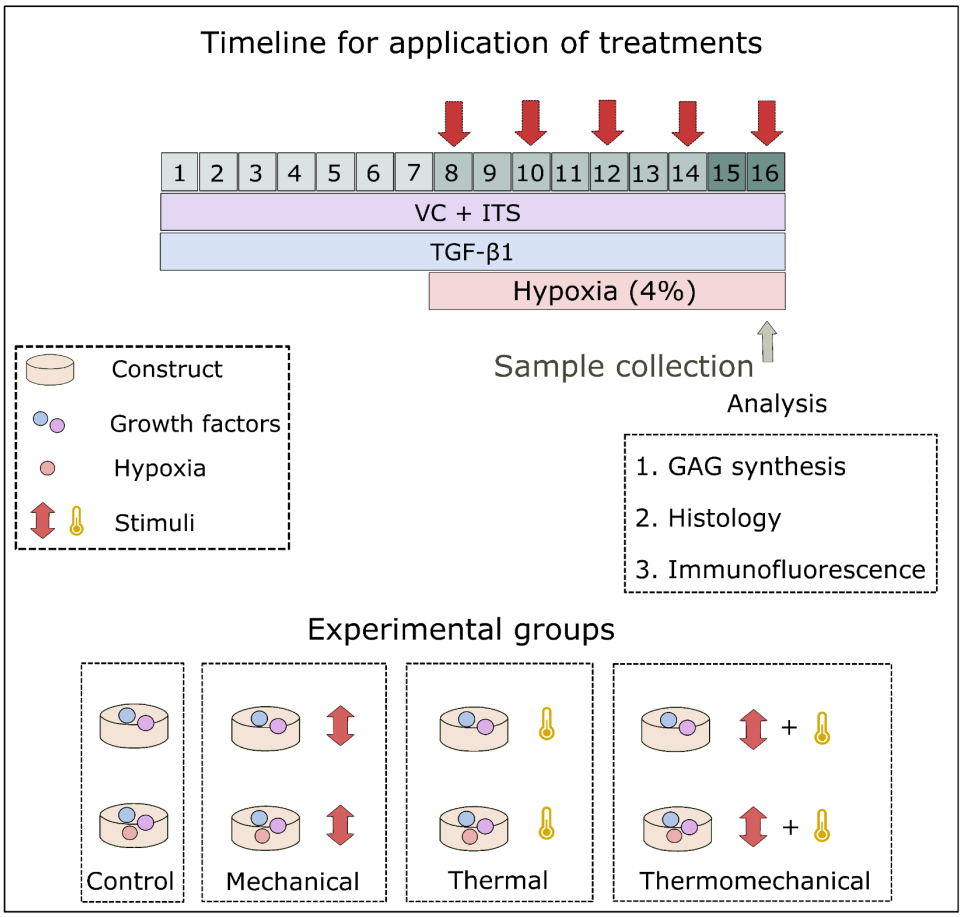


Figure S3.4: A schematic illustration of the mechanobiological study timeline is shown, related to STAR Methods. *On day 0 (Not shown), chondrocytes are seeded on scaffolds and remain at free swelling condition up to Day 7. On Day 7, foetal bovine serum is removed from the medium, while vitamin C and 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 (n=3) and biochemical analysis (n=3).*

HIF1A & *SOX9* mRNA expression

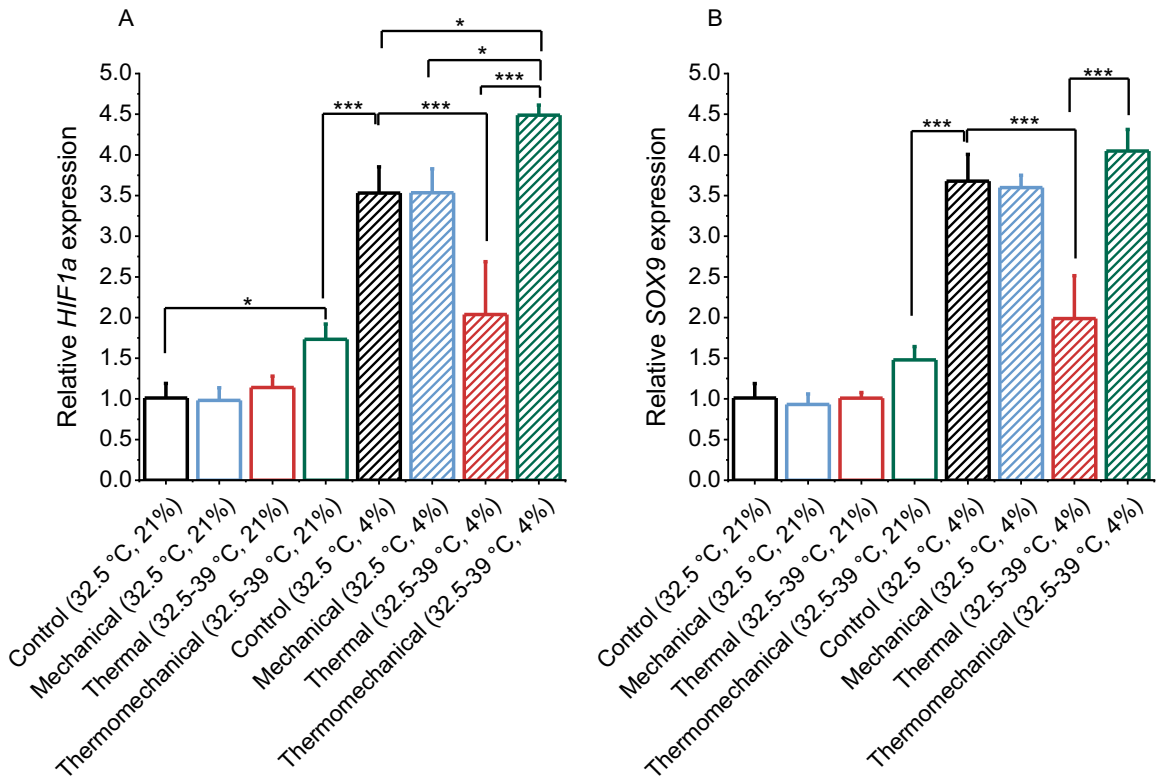


Figure S3.2: HIF1a and SOX9 gene patterns, related to Figure 4. *A.) Relative HIF1a & B.) SOX9 expression with respect to isolated and combined forms of stimulation. Both genes follow similar responsive patterns with aggrecan (Figure 1a of initial version) indicating a potential pathway.*

Table S1: Primers data used for qRT-PCR, related to STAR Methods

Gene	Primer concentration (nM)	Efficiency (%)	Sequence 5'-3'
RPL13a	150	104	**F:**TAAACAGGTACTGCTGGGCCG **R:**CTCGGGAAGGGTTGGTGTTC
COL2a	175	108	**F:** GGCAATAGCAGGTTCACGTACA **R:** GATAACAGTCTTGCCCCACTTACC
TWIST1	250	91	**F:**AGCAGGGCCGGAGACCTAGATGTCA **R:**ACGGGCCTGTCTCGCTTTCTCT

ACAN	175	99	**F:**GGTACCAGTGCACAGAGGGGTT **R:**TGCAGGTGATCTGAGGCTCCTC
COMP	250	101	**F:** TGCTTCGGGAACTGCAGGAAAC **R:** GCACGCGTCACACTCCATCACC

Immunofluorescence staining

Table S2: Antibodies used in immunofluorescence staining, related to STAR Methods

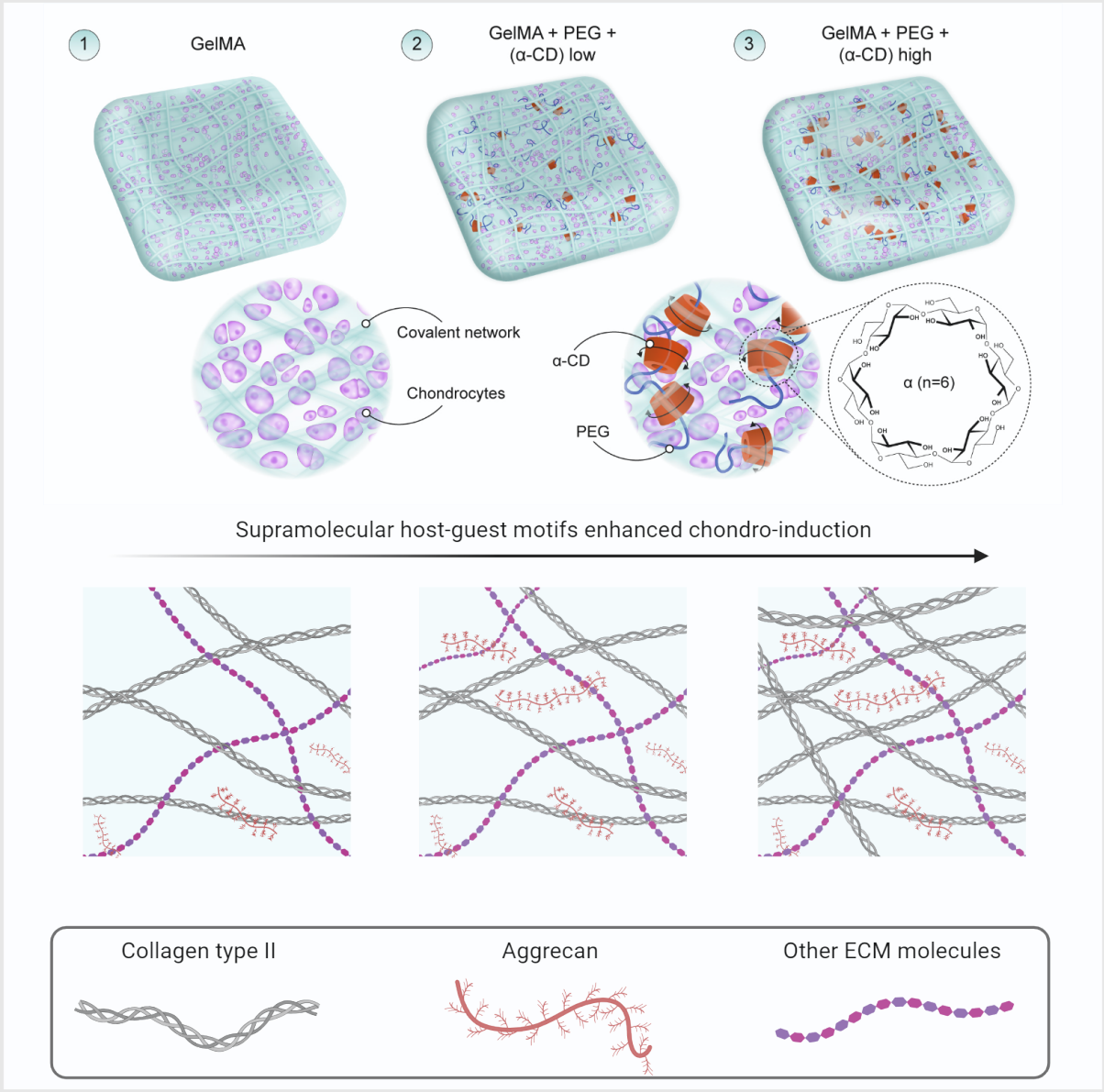
Reagent	**Designation**	**Source**	**Dilution**
Primary antibody	Mouse anti-SOX9	ABCAM, ab76997	1:250
Primary antibody	Mouse anti-COL2A	Invitrogen, MA5-12789	1:250
Phalloidin	Alexa Fluor 568 phalloidin	Invitrogen, A12380	1:400
Secondary antibody	ALEXA FLUOR 488 PHALLO	Lifetechnologies	1:250

Chapter 4: Impact of Molecular Dynamics of Polyrotaxanes on Chondrocytes in Double Network Supramolecular Hydrogels under Physiological Thermomechanical Stimulation*

*This chapter has been submitted to *BioRxiv* (doi: https://doi.org/10.1101/2023.09.01.555869). Currently it is under peer-review in *ACS Biomacromolecules* journal.

Abstract

Hyaline cartilage, a soft tissue enriched with a dynamic extracellular matrix, manifests as a supramolecular system within load-bearing joints. At the same time, the challenge of cartilage repair through tissue engineering lies in replicating intricate cellular-matrix interactions. This study attempts to investigate chondrocyte responses within double-network supramolecular hydrogels, tailored to mimic the dynamic molecular nature of hyaline cartilage. To this end, we infused non-covalent host-guest polyrotaxanes, by blending α-cyclodextrins as host molecules and polyethylene glycol as guests, into a gelatin-based covalent matrix, thereby enhancing its dynamic characteristics. Subsequently, chondrocytes were seeded into these hydrogels to systematically probe the effects of varied concentrations of introduced polyrotaxanes (instilling different levels of supramolecular dynamism in the hydrogel systems) on cellular responsiveness. Our findings unveiled an augmented level of cellular mechanosensitivity for supramolecular hydrogels compared to pure covalent-based systems. This is demonstrated by an increased mRNA expression of ion channels (*TREK1, TRPV4, PIEZO1*), signaling molecules (*SOX9*) and matrix remodeling enzymes (*LOXL2*). Such outcomes were further elevated upon external application of biomimetic thermomechanical loading that brought a stark increase in the accumulation of sulfated glycosaminoglycans and collagen. Overall, we found that matrix adaptability plays a pivotal role in modulating chondrocyte responses within double-network supramolecular hydrogels. These findings hold potential for advancing cartilage engineering within load-bearing joints.



Graphical abstract – Double network supramolecular hydrogels enhanced chondro-induction significantly compared to purely covalent-based hydrogels.

4.1 Introduction

In articular cartilage, chondrocytes and their dynamic local microenvironment constantly interact and communicate through biophysical and biochemical cues to regulate and guide various cell behaviors, such as cell differentiation.[1] It is now globally accepted that dynamic temporal interactions predominantly mediated by chondrocyte adhesion to extracellular matrix and applied biomechanical stimuli, present a crucial role in transferring forces to and between cells that ultimately control chondrocyte function and tissue homeostasis.[2] It is assumed that such interactions can be leveraged to alter cartilage disease and directly promote

regeneration. Investigating the mechanisms by which physical cues and the *nature* of the cellular microenvironment are sensed by chondrocytes and how these are converted into biochemical signals is believed to be the gatekeeper to understand cartilage mechanobiology.[3]

Hydrogels are deemed highly attractive candidate materials to study how physical cues affect the chondrocyte responses *in vitro,* due to their inherent simplicity in terms of starting constituents and preparation, allowing for precise control of their chemical and physical properties.[4] Nonetheless, thus far, previous studies have primarily focused on utilizing hydrogels crosslinked through covalent bonds to enhance compression resistance in case of applications for articulating joints. Despite their merits, covalent bonds tend to confine the synthesis of the extracellular matrix predominantly within the pericellular space.[5] Conversely, biological tissues predominantly embrace the dominance of non-covalent interactions, presenting a stark divergence from the primarily covalent crosslinking strategies often observed in hydrogel investigations.[6] In such regard, there is a growing interest in dynamic cell-adaptable hydrogels that can adjust and reorganize in response to mechanical stress or strain. As one example, supramolecular host-guest hydrogels are well-suited to study cartilage mechanobiology where the reversibility of the crosslinks occurs under physiological conditions.[7] Supramolecular chemistry is entrenched in the rational design of specific and reversible molecular recognition motifs capitalizing on dynamic non-covalent interactions to create organized systems.[8] Because of the dynamic nature of non-covalent interactions, supramolecular materials can rapidly respond to multifarious external stimuli, thereby recreating aspects of the dynamics present in living systems; making them a suitable candidate for cartilage studies.[9]

Although covalent hydrogels can be engineered to possess elastic and/or viscoelastic mechanical properties,[10] they fail to replicate the inherent dynamics of the extracellular matrix found in tissues like hyaline cartilage. Herein, we have devised an approach that combines a covalent-based hydrogel with supramolecular polyrotaxane motifs, aiming to capitalize on the beneficial properties of supramolecular host-guest interactions (dynamic reversibility) within the covalent network. This integration enables the creation of a more permissive environment for encapsulated cells to interact with the hydrogel matrix during externally applied deformation, while simultaneously upholding the desirable characteristics, such as robustness, inherent in covalent bonding. By incorporating supramolecular polyrotaxanes, we enable the hydrogel to undergo reorganization and adaptability, mimicking the natural behavior of the extracellular matrix in hyaline cartilage.

So far despite its significance, a link between the temporal hierarchy of gel dynamics and adult chondrocyte behavior, particularly in response to externally applied biomimetic stimulation, remains elusive. Understanding how mechanobiological signals affect chondrocyte behavior is crucial for enhancing the outcomes of tissue engineering approaches.[11] Thus, we further sought to examine mechanobiological interactions among dynamic matrix characteristics upon externally applied biomimetic thermomechanical load using host-guest supramolecular hydrogels. To the best of our knowledge this research work represents the first and only study to explore interactive effects between dynamic reversible crosslinks (polyrotaxanes) and physiologically relevant biomimetic thermomechanical loading for cartilage tissue engineering.

To this end, primary human chondrocytes encapsulated in supramolecular hydrogels were maintained in free swelling condition (static) as well as subjected to a long-term culture *via* a custom-made bioreactor apparatus designed to simulate transient thermomechanical stimuli as experienced in knee joint.[12] PCR analysis was employed to investigate the early transcriptional interactions among hydrogels with static and dynamic host-guest crosslinks (polyrotaxanes), revealing significant transcriptional changes between the experimental groups in free swelling condition. Rates of biosynthesis were also analyzed by quantifying the deposition of sulfated glycosaminoglycans (sGAGs) and total collagen type following thermomechanical stimulation. Histological analysis was further utilized to visualize and detect the spatial distribution of these molecules. Overall, this study underscores the significance of a dynamic extracellular matrix (ECM) akin to that found in native cartilage, accentuating how mechanobiological cues intricately guide chondrocyte biosynthetic responses within the dynamic hydrogel milieu.

4.2 Materials & Methods

α-Cyclodextrin was purchased from Sigma Aldrich (C4642, ≥98%). Polyethylene glycol 2000 was purchased from Sigma Aldrich (Mn = 2000 Da, for synthesis). Gelatin Type A from porcine skin (ref. G2500) and methacrylic anhydride (ref. 276685) were purchased from Sigma Aldrich.

4.2.1 Preparation of host-guest molecules and supramolecular hydrogels

Host-guest supramolecular complexes were prepared by dissolving two different concentrations of alpha cyclodextrin (α-CD) (12 mg and 36 mg) in a PEG/PBS solution (6.5%

wt). The mixture was thoroughly mixed and allowed to equilibrate for a specific period (1 day prior to experiments) to ensure the formation of the complexes. Methacrylated gelatin (GelMA) was synthesized as previously described[13] and then dissolved in the [PEG-α-CD]/PBS system at a final concentration of 7% wt. The solution was gently stirred and heated at 37 °C until complete dissolution of GelMA was achieved. For the preparation of non-supramolecular hydrogels, GelMA was dissolved in PBS at a final concentration of 7% wt.

4.2.2 NMR analysis and Isothermal Titration Calorimetry (ITC)

^{1}H-NMR spectra were acquired using a Bruker Avance NMR spectrometer (400 MHz) with a BBI probe and processed with MestReNOVA software, as previously described.[11] Chemical shifts were reported in ppm, rounded to the nearest 0.01 ppm for 1H NMR.

ITC experiments were conducted in phosphate buffered saline (PBS) at 298.15 K using the MicroCal PEAQ-ITC. The sample cell contained the guest PEG_{2k} solution, and the injection syringe contained the host alpha-CD solution. The concentrations of both alpha-CD and PEG_{2K} were calibrated using ITC titration in a blank PBS solution. Heat evolution was observed when a specific concentration of host or guest was titrated into the blank PBS solution or vice versa to know the correct concentration for the final measurements.

4.2.3 Experimental groups

This study encompassed three distinct phases. In Phase I, the objective was to assess the effects of incorporating dynamic host-guest polyrotaxanes into a covalent network and their subsequent impact on chondroinduction, particularly on the expression of key chondrogenic genes. This evaluation involved a comparative analysis with motif-absent (single network) hydrogels under free-swelling conditions. Advancing to Phase II, the focus shifted to the identification of potential enhancements observed in Phase I, now at the protein level. Subsequently, Phase III involved subjecting the hydrogels to biomimetic thermomechanical stimulation under hypoxia as previously described[14]. The overarching goal was to enhance chondrocyte biosynthesis by more accurately mimicking cartilage *in vivo* milieu. Phase I and Phase II were executed over a relatively condensed timeframe (Day 16), while Phase III extended over a more extended duration (Day 21).

4.2.4 Cell expansion, encapsulation and bioreactor culture

Primary human chondrocytes were derived from a 22-year old male donor (P10970 Innoprot, Spain). Cells were expanded in T-75 culture flasks inside chondrocyte basal medium (Alpha minimum essential medium (α-MEM)), supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM nonessential amino acids (NEEA), 1% penicillin, 1% streptomycin, 5 ng/mL fibroblast growth factor (FGF) and 1 ng/mL human recombinant transforming growth factor beta 1 (TGF-β1), up to passage 4.

Hydrogel precursor solution were prepared, as described earlier, inside phosphate buffered saline (PBS) containing LAP photoinitiator (Lithium phenyl-2,4,6-trimethylbenzoylphosphinate, at a final concentration of 0.1 mg/ml). Passage 4 chondrocytes, at a seeding density of 10^7 cells per ml, were resuspended in the hydrogel precursor and carefully pipetted into a custom-designed mold. The chondrocyte/hydrogel suspension was then crosslinked using a 405 nm wavelength light source for 2 minutes. The resulting constructs were cultured in a differentiation medium composed of FBS-free Dulbecco's Modified Eagle's medium supplemented with insulin–transferrin–selenium (ITS-IV, 10%), L-ascorbic acid (VC, 1%), 10 ng/mL TGF-β1, and other additives (10 mM HEPES and 10 mM NEAA). The cell-seeded constructs were prepared as a batch and subsequently randomly distributed among different study groups. Following the seeding step, all samples were pre-cultured for 7 days in a cell growth medium within standard incubators (32°C, 5% CO_2, 21% O_2). Next, the constructs were transferred to a bioreactor culture system where intermittent biomimetic thermomechanical signals (32–39 °C, 5% pre-strain, 10% amplitude at 1 Hz every other day) and a low oxygen tension environment (4% O_2) were applied until day 21. We replicated all forms of stimulation by utilizing a custom-made bioreactor that we developed in our laboratory.[12] The compressive regime was specifically designed to imitate normal physical activity. To model the temperature increase resulting from cyclic compression, we applied curve-fitting based on *in vivo* data during jogging over the same 1.5-hour period and as previously described.[14] After stimulation was ceased, constructs were allowed to recover within standard incubators (32°C, 5% CO_2). Constructs were collected for analysis on day 16 and day 21.

4.2.5 Quantitative real time PCR

After a 16 and 21 days of bioreactor culture, both stimulated and non-stimulated samples were promptly immersed in 0.3 ml ice-cold TRIzol (Invitrogen) and stored at −80 °C for subsequent RNA isolation. To prepare the samples, TRIzol was added to each sample on ice, followed by vigorous homogenization. Subsequently, 0.1 ml of chloroform was added, and the mixture was hand-shaken for 15 seconds and then centrifuged at 4 °C for 10 minutes. The aqueous layer, containing the RNA, was carefully collected and combined with an equal volume of 70% ethanol through pipetting. RNA isolation was performed using the Nucleospin XS kit according to the manufacturer's instructions. The isolated RNA was quantified using a NanoDrop 1000, and then reverse-transcribed into cDNA using the Taqman Reverse Transcription Reagents (Applied Biosystems) in a 50 µL reaction volume as previously described.[15] The reaction mixture included the master mix, random hexamer, and RNA sample.

For qRT-PCR analysis, the Fast SYBR Green PCR Master Mix (Applied Biosystems) was employed in a final volume of 20 µL. Each reaction contained 1 µL of synthesized cDNA. The target genes selected for qRT-PCR included background potassium channel (*TREK1*), lysyl oxidase-like 2 (*LOXL2*), aggrecan (*ACAN*), sex-determining region Y-type (SRY, *SOX9*), Piezo type mechanosensitive ion channel component 1 (*PIEZO1*), transient receptor potential cation channel subfamily V member 4 (*TRPV4*) and collagen type II (*COL2A*). To ensure accurate normalization, Ribosomal protein L13a (*RPL13A*) was utilized as the housekeeping gene. The relative gene expression of the experimental samples was determined using the ΔΔCT method. Primers were synthesized by Microsynth (Balgach, Switzerland).

4.2.6 Sample preparation for histology

To assess the production and distribution of the extracellular matrix (ECM), we employed histology. After 16 and 21 days, cell-hydrogel samples were harvested, and the chondrocytes were fixed in 4% paraformaldehyde overnight at 4 degrees Celsius. The following day, the samples were sequentially transferred to 15% and 30% sucrose solutions (Sigma) before being snap frozen in optimal cutting temperature compound (Sakura Tissue-Tek). Subsequently, the samples were sectioned into 7-µm thick slices using a standard cryostat. Imaging of histologic slides from different samples was performed using the tile scan method with a 20× magnification on an Olympus VS120 whole slide scanner. Throughout the imaging process,

the laser intensity and exposure duration were consistently set for all samples to ensure accurate comparisons.

4.2.7 Mechanical characterization of hydrogels

Cylindrical specimens with dimensions Ø: 6 mm and h: 3 mm were submerged in bidistilled water and underwent unconfined compression experiments. The experiments were conducted using an Electropuls Dynamic Test System (Instron E3000, Instron, Norwood, Massachusetts, USA) at room temperature. The compressive loading was performed at a rate of 0.1 mm s−1, with load-displacement data recorded. Swollen state samples underwent 30 compression cycles. The compressive modulus of the hydrogels was calculated through linear interpolation of the stress–strain curve during the last loading cycle, specifically between 12% and 15% strain (mm/mm). To determine the level of energy dissipation, the area enclosed by the hysteresis loop was measured and subsequently normalized to the volume of each sample.

4.2.8 Statistical analysis

Statistical analysis was conducted using analysis of variance (ANOVA) followed by Tukey's post hoc tests for comparing mechanical data (n = 5) and gene expression data (n = 4) in multiple group comparisons. The results are presented as mean + standard deviation. The significance levels are denoted as (*) for $p \leq 0.05$, (**) for $p \leq 0.01$, and (***) for $p \leq 0.001$. Statistical computations were performed using Origin Pro 2021 software. Additionally, to validate gene expression patterns, two independent experiments were conducted each of at least 3 biological replicates.

4.3 Results

4.3.1 Inclusion complex formation of linear polyethylene glycol (PEG) and α-cyclodextrin (α-CD)

Figure 4.1a shows a schematic illustration of typical polyrotaxanes consisting of a linear polymer chain (PEG) of 2kDa molecular weight and threaded rings (α-CD). The formation of inclusion complexes in an aqueous solution was confirmed and monitored by isothermal titration calorimetry (ITC). Figure 4.1b shows a typical ITC sigmoid thermogram indicating stable complex formation, while the stoichiometric ratio was close to 2.

^{1}H NMR spectroscopy was further utilized to investigate the relative position of the host-guest complexation between α-CD (a host) and PEG_{2k} chain (a guest). Figure 4.1c shows the NMR

data of free α-CD (black line), free PEG_{2k} (blue line), and α-CD + PEG_{2k} complexes (red line). It is evident that upon incorporation into the host, the protons of the PEG_{2k} chain experienced a complete upfield shift (shielded), while the protons of the α-CD cage exhibited peak broadening compared to the free α-CD. This observation confirms the occurrence of host-guest complexation between α-CD and the PEG_{2k} polymer chain.

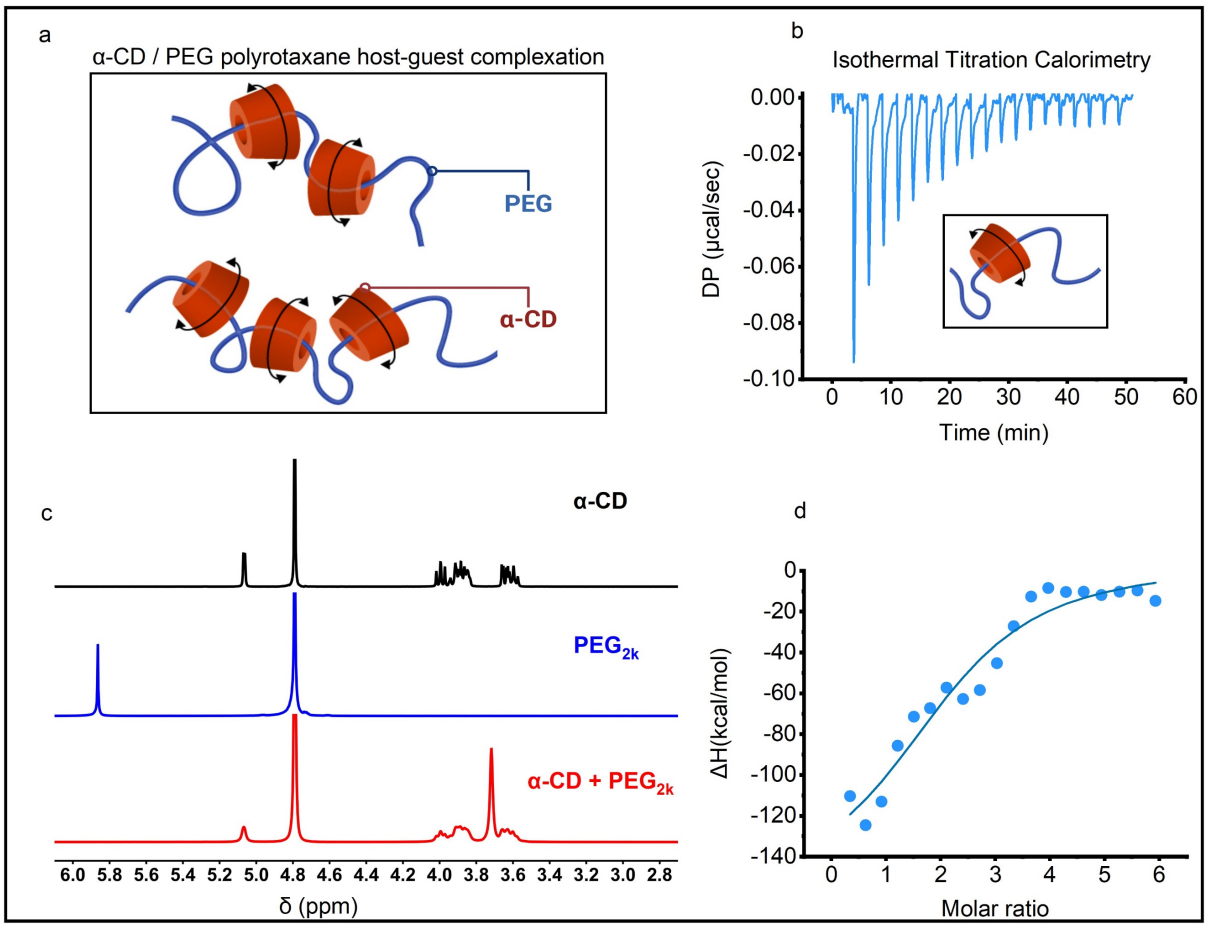


Figure 4.1 – a) Schematic illustration of elemental motion of polyrotaxanes, b) measured heat of interaction obtained from ITC from sequential α-CD injections into an PEG_{2k} solution, c) representative ^{1}H NMR spectra of free α-CD (black line), free PEG2k (blue line), and α-CD + PEG2k complexes (red line), d) fitting of heat mathematical integration as a function of molar ratio for the PEG_{2k} / α-CD inclusion complex.

4.3.2 Phase I: Dynamic polyrotaxane host-guest complexation altered gene categories encoding multiple signaling pathways in free-swelling hydrogels

Quantitative real-time PCR (qPCR) analysis was conducted on free-swelling hydrogels (labeled as 1-3) to discern the impact of dynamic host-guest complexation (polyrotaxanes) at transcriptional level on day 16 of culture.

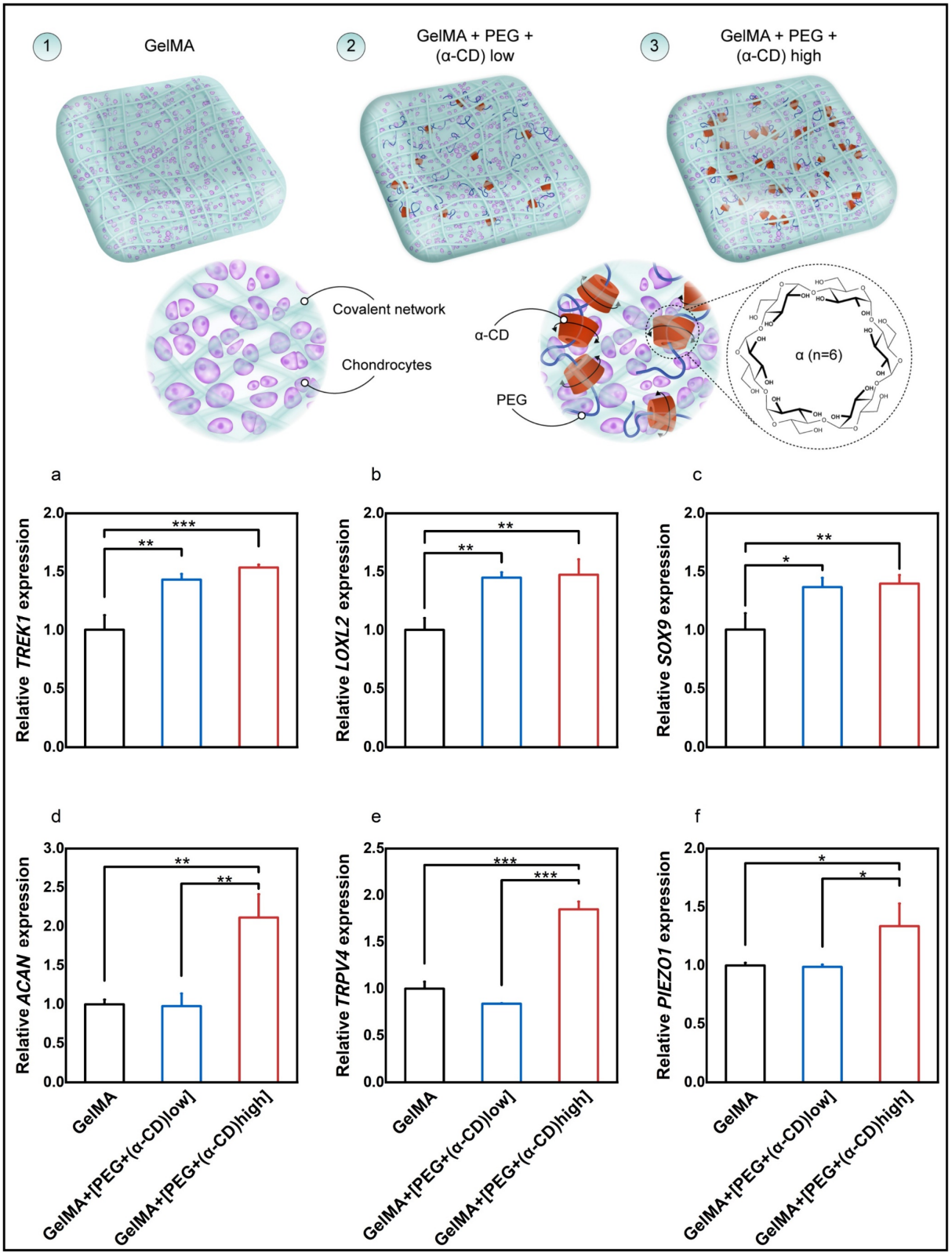


Figure 4.2 – Schematic illustration depicting the various types of hydrogels employed in the study. The polyethylene glycol (PEG) concentration remained consistent across all supramolecular hydrogels, while the alpha cyclodextrin (α-CD) concentration increased

progressively from left to right (a-f). Comparison of the relative expressions of genes of interest, with RPL13a serving as the housekeeping gene.

The findings unveiled profound changes in the expression of genes implicated in crucial cellular signaling pathways (SRY-related HMG-box gene 9, SOX9), genes associated with matrix remodeling enzymes (Lysyl oxidase homologue 2, LOXL2), extracellular matrix proteins (including Aggrecan, ACAN) and thermo-mechano-regulated ion channels (including potassium and calcium transducers, TREK1, TRPV4, PIEZO1) as illustrated in figures 2a-f.

Overall, the inclusion of α-CD/PEG polyrotaxanes within the gelatin-based covalent network yielded mixed results worth noting. At lower concentrations, TREK1, LOXL2, and SOX9 genes displayed significant increases in expression, indicating a positive impact. However, the transcription plateaued with higher concentrations, not contributing to further improvements in these genes (Figure 2a-c). Conversely, other major chondrogenic genes such as ACAN, PIEZO1, and TRPV4 exhibited no discernible changes at lower concentrations. Yet, an astonishing revelation emerged; these three genes demonstrated a synchronized response pattern, undergoing a remarkable increase in expression at higher concentrations spanning from 30 to 200% (Figure 2c-f).

These compelling results highlight the transformative influence of dynamic host-guest complexation on the gene expression profile, underscoring the potential of this approach in modulating vital cellular pathways involved in chondrocyte physiology.

These compelling results highlight the transformative influence of dynamic host-guest complexation on the gene expression profile, underscoring the potential of this approach in modulating vital cellular pathways involved in chondrocyte physiology.

4.3.3 Phase II: The impact of dynamic host-guest polyrotaxanes on sulfated proteoglycans and total collagen synthesis and distribution during free-swelling culture of hydrogels

In cartilage tissue engineering, scaffolds must provide mechanical support while facilitating the deposition of extracellular matrix (ECM) by embedded chondrocytes to promote the development of neo-cartilaginous tissue.[17,18] In this series of experiments, we aimed to investigate the influence of double network supramolecular hydrogels on the uniform secretion of the cartilaginous matrix during free-swelling conditions (without external stimulation up to day 16).

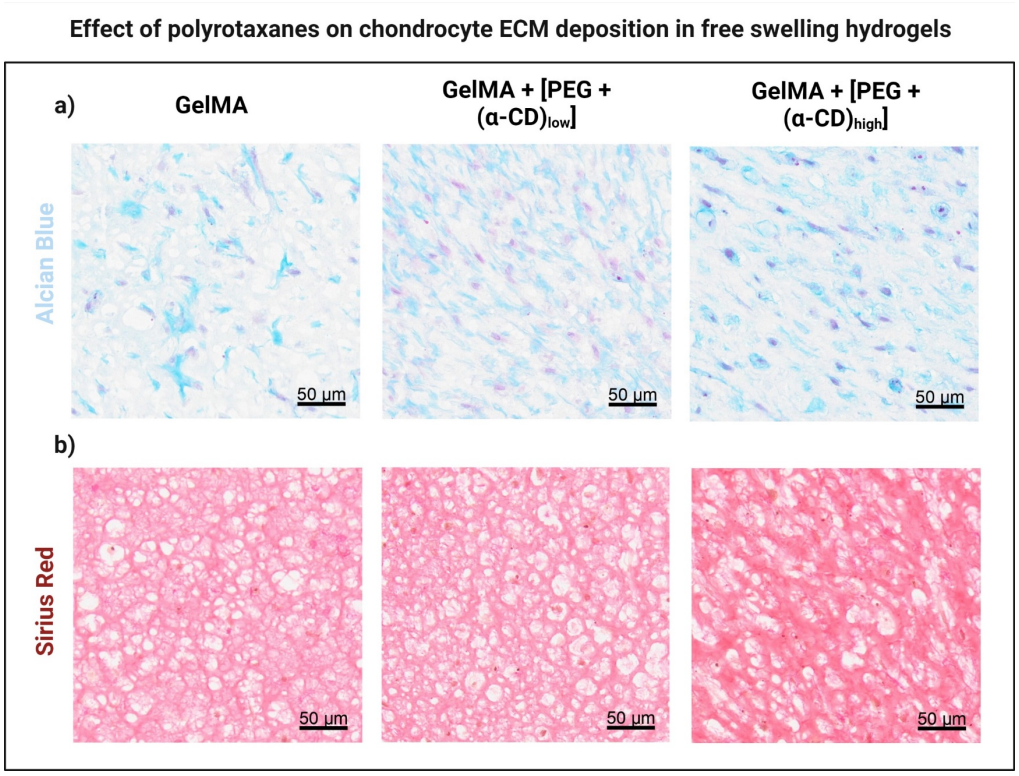


Figure 4.3 – Histological analysis of neo-cartilage constructs under free-swelling conditions. a) representative images of Alcian Blue staining for sulfated glycosaminoglycan & glycoproteins content (blue), b) representative images of Sirius Red staining for general collagen content (red). The intensity of Alcian blue staining is noticeably higher in supramolecular hydrogels with a higher concentration of alpha cyclodextrin (α-CD) molecules. Additionally, Sirius Red staining reveals a localized increase as the concentration of α-CD molecules increases. Scale bar: 50 µm, objective 20x.

Collagens, as fibrillar proteins, play a crucial role in determining the shape and microarchitecture of articular cartilage.[19] Sulfated glycosaminoglycans (sGAGs), major components of proteoglycans such as aggrecan, contribute to water retention and provide compressive strength.[20] We utilized histological staining techniques to examine the deposition of these key cartilage matrix molecules and assess the development of neo-cartilaginous tissue over time. In agreement with the gene expression data, our findings have unveiled remarkable disparities in both collagen and sGAG content, intricately linked to the concentration of α-CD/PEG polyrotaxanes within the hydrogels. Astonishingly, higher concentrations of α-CD which translates to higher dynamism in the hydrogels system (due to higher level of α-CD/PEG complexations) have been discovered to foster a substantial accumulation of aggrecan, vividly exemplified by intensified alcian blue staining. Furthermore, the diverse compositions of the

hydrogels have unraveled distinct patterns in total collagen deposition, with higher concentrations of α-CD showcasing an evident surge in overall collagen accumulation.

4.3.4 Phase III: Supramolecular host-guest complexation modulates the effects of biomimetic thermomechanical stimulation on chondrocyte biosynthesis in a dose-dependent manner

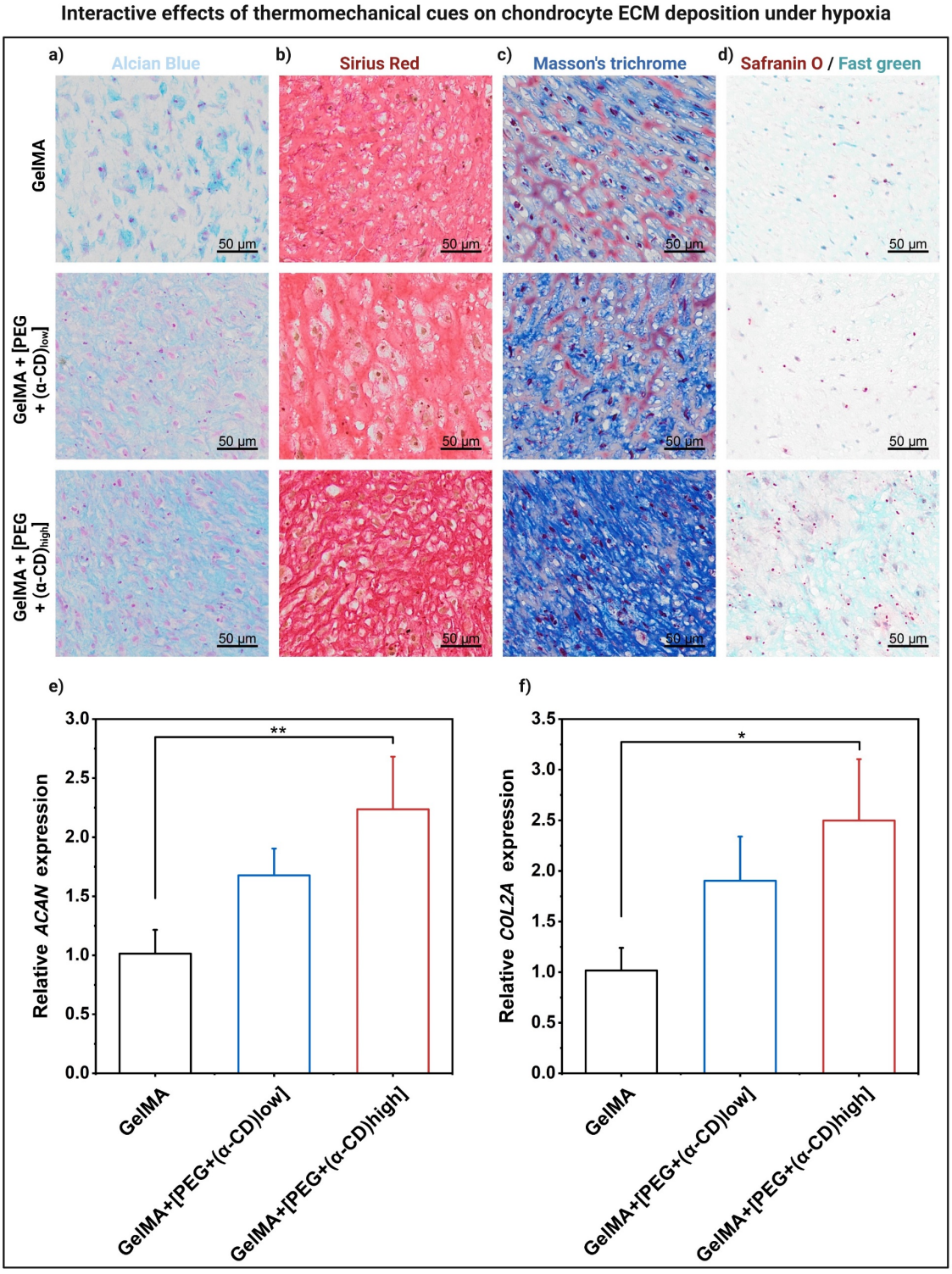


Figure 4.4 - Interactive effects of mechanobiological cues on chondrocyte ECM deposition. a) representative images of Alcian Blue staining for sulfated glycosaminoglycan & glycoproteins content (blue), b) representative images of Sirius Red staining for general collagen content (red), c) representative images of Masson's trichrome staining for total collagen content (blue) d) representative images of Safranin-O/Fast green staining for sulfated glycosaminoglycan content are shown for the different types of hydrogels after the last loading cycle was ceased on day 21. Application of biomimetic thermomechanical loading under hypoxia significantly enhanced cartilage-related matrix accumulation especially in the case of supramolecular hydrogels. Alcian Blue: sulfated GAGs and glycoproteins are stained blue, and the nuclei and cytoplasm pink, Sirius Red: collagen is stained red and the nuclei dark brown, Masson's trichrome: collagen is stained blue, nuclei are stained dark brown and the cytoplasm light purple, Safranin-O/Fast green: cartilage matrix will be stained orange to red, the nuclei will be stained black, and the background light green. Scale bar: 50 μm, objective 20x. e) Comparison of the relative expressions of aggrecan (ACAN) and f) collagen type II (COL2A) after the last loading cycle was ceased, with RPL13a serving as the housekeeping gene.

In this series of experiments, we extended the culture period from 16 days to 21 days while applying biomimetic thermomechanical stimulation upon hypoxia treatment to the chondrocyte-laden hydrogels. Notably, our findings reveal consistent and compelling trends in the total collagen and glycosaminoglycan (GAG) content. Increasing concentrations of α-CD within the covalent-based network (while keeping the PEG concentration the same) demonstrated a remarkable capacity to promote augmented collagen accumulation (as shown in Sirius Red and Masson's Trichrome stainings, Figures 4.4b,c), accompanied by significant deposition of sulfated GAGs (as shown in Alcian Blue and Safranin-O stainings Figures 4.4a,c) compared to pure covalent-based hydrogels. These outcomes underscore the pivotal role of host-guest supramolecular motifs in governing collagen and GAG secretion, thereby emphasizing the potential of double network supramolecular hydrogels, to facilitate the development of functional neo-cartilaginous tissue especially upon biomimetic thermomechanical stimulation.

In addition to the histological stainings, we analyzed the mRNA transcription of aggrecan (*ACAN*, Figure 4.4e) and collagen type II (*COL2A*, Figure 4.4f) genes among the different hydrogels. The inter-group comparisons aligned with the histological findings, demonstrating a substantial upregulation of these pivotal genes, with an increase of 200-250%, respectively.

4.4 Discussion

The interdependence of chondrocytes and their extracellular matrix (ECM) is a fundamental aspect of cell behavior and function.[21] To comprehend these relationships *in vitro*, hydrogels are a promising candidate due to the possibility of precisely controlling their chemical and physical properties. However, existing studies have predominantly utilized purely covalently crosslinked hydrogels, which fail to confer the essential dynamicity found in the ECM of native tissues.[22,23] Supramolecular hydrogels offer a dynamic non-covalent alternative that more accurately reflects the native environment of hyaline cartilage.[24] Utilizing supramolecular non-covalent motifs such as polyrotaxanes, this study aimed to engineer dynamic hydrogels with the ability to reorganize under free-swelling conditions. More specifically, we have developed a method that integrates a covalent-based hydrogel with supramolecular polyrotaxane patterns, with the intention of harnessing the advantageous attributes of supramolecular host-guest interactions (characterized by dynamic reversibility) within the covalent network. The addition of dynamic polyrotaxanes into the covalent network hydrogels was intended to enhance the biosynthetic capacity of neocartilage constructs. The underlying hypothesis suggested that by replicating the natural characteristics observed in cartilage extracellular matrix (e.g inherent dynamic network of molecules), chondrocytes would be prompted to produce essential matrix proteins. Subsequently, the investigation delves into the biophysical impacts of applied thermomechanical loads on chondrocytes encapsulated in these double network hydrogels. Given that native cartilage experiences transient thermal cues during joint loading upon a hypoxic environment, replicating these interactions *in vitro* became a focal point for assessing the potential acceleration of chondrocyte biosynthesis. The thermo-mechanical stimulation protocol used was similar to that used in our previous investigations.[14, 27]

Through our rigorous investigation, we have uncovered a fascinating interplay between double network supramolecular hydrogels, thermomechanical loading, and hypoxic conditions in regulating chondrogenesis. Our research unveiled that when polyrotaxanes are incorporated into the covalent network, especially at increased concentrations, alongside thermomechanical loading and reduced oxygen tension, a significant increase in chondrogenic markers and cartilage-related proteins is observed. This synergistic effect suggests a unique interplay between these cues that holds significant promise in the field of cartilage tissue engineering, highlighting the importance of a multifaceted approach to this complex challenge.

Remarkably, our findings indicate a substantial augmentation in chondrogenesis within the double network supramolecular hydrogels, surpassing that observed in the covalently crosslinked (single network) hydrogel counterparts. Moreover, a discernible relationship emerges, whereby the extent of host-guest complexation (polyrotaxanes) exhibits a direct correlation with the magnitude of chondrogenic differentiation. This positive correlation is substantiated through quantifiable variations in mRNA expression levels of critical chondrogenic marker genes, namely SOX9, ACAN, and LOXL2. Such improvements were detected at protein level as well. These findings are consistent with previous research[25, 7, 26] supporting the notion that supramolecular hydrogels, particularly those featuring stronger host-guest interactions, offer a promising platform for the development of tissue-engineered constructs with superior chondrogenic potential.

In our investigation, the hydrogel formulations were also subjected to bioreactor culture conditions that incorporated thermomechanical signals under low oxygen tension. Our previous studies have extensively elucidated the significance and relevance in replicating cartilage self-heating *in vitro* and demonstrated thoroughly how loading-induced evolved temperature can be harnessed *in vitro* to accelerate tissue maturation through expression of major structural proteins.[12, 14, 27] Building upon our prior findings where load, heat and hypoxia interactions were studied, herein we aimed to introduce the inherent dynamic nature of the culture environment as an additional parameter in this multifaceted equation. The relevance of including PEG/alpha cyclodextrin-based polyrotaxanes into the robust covalent network stems from their capacity to partially replicate the dynamic interactions found in natural cartilage.[28,29] Through the incorporation of dynamic polyrataxanes, we aim to mimic the innate non-covalent interactions inherent in cartilage tissue, playing a pivotal role in its mechanical composure and potential signals they transfer to chondrocytes. Our analysis of the resulting protein synthesis levels in the stimulated samples revealed that the double network hydrogels exhibited a notable upregulation of cartilage matrix proteins compared to the pure covalently crosslinked hydrogel.

Our findings strongly support the notion of the pivotal role of ion channels in mediating the effects of thermomechanical loading on chondrocytes. We identified three ion channels, namely TREK1, TRPV4, and PIEZO1, whose expression was significantly upregulated in the presence of host-guest reversible polyrotaxanes. While it could be argued that our channel analysis has only been performed in free-swelling constructs and not in stimulated hydrogels, we have previously shown that thermomechanical loading increases the expression of these channels at protein levels as well.[12,27] Hence, we contend the validity of our hypothesis,

suggesting that the observed effects likely arise from the interplay between various stimuli and the multifaceted responses of chondrocytes.

We also conducted a systematic investigation into the distinct influences stemming from the separate incorporation of PEG and alpha cyclodextrin moieties into the covalent network. Interestingly, we found no significant changes in the expression of major genes among the different experimental groups, indicating that the observed effect primarily arises from the complexation of alpha cyclodextrin and PEG (See supplementary figure 1). To ensure the validity of our observations, we diligently measured the bulk mechanical properties of the hydrogels, specifically focusing on energy dissipation levels and hydrogel stiffness before cell encapsulation (See supplementary figure 2). Encouragingly, our results demonstrated consistent mechanical attributes across the experimental groups (prior to cell encapsulation), suggesting that no other parameter significantly influenced the observed effects. These findings lend strong support to the relevance and reliability of our conclusions.

In conclusion, this study provides crucial insights into the dynamic nature of cartilage, even under free-swelling conditions, and the role of dynamic polyrotaxanes, thermomechanical loading, and hypoxia in regulating chondrogenesis. By incorporating supramolecular motifs into a covalent-based hydrogel system, we aimed to partially mimic the intricate dynamic and non-covalent interactions present in natural cartilage. Supramolecular hydrogels with high amount of host-guest crosslinks, when subjected to thermomechanical stimulation and hypoxic conditions, enhance chondrogenic markers and proteins. This highlights their potential as a promising platform for advanced tissue-engineered constructs with improved chondrogenic capabilities

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Supporting Information

Unraveling the Impact of Polyrotaxanes on Chondrocytes in Double Network Supramolecular Hydrogels under Physiological Thermomechanical Stimulation

Effect of pure PEG and pure α-CD on gene expression

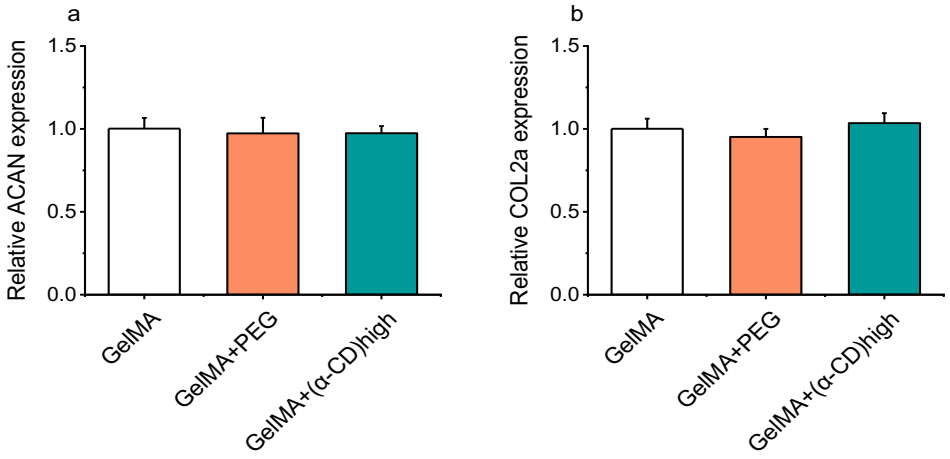

Figure S4.1 – Relative expression of genes of interest to assess the effects of individual PEG chains and α-CD molecules.

Energy dissipation level of different hydrogel formulations

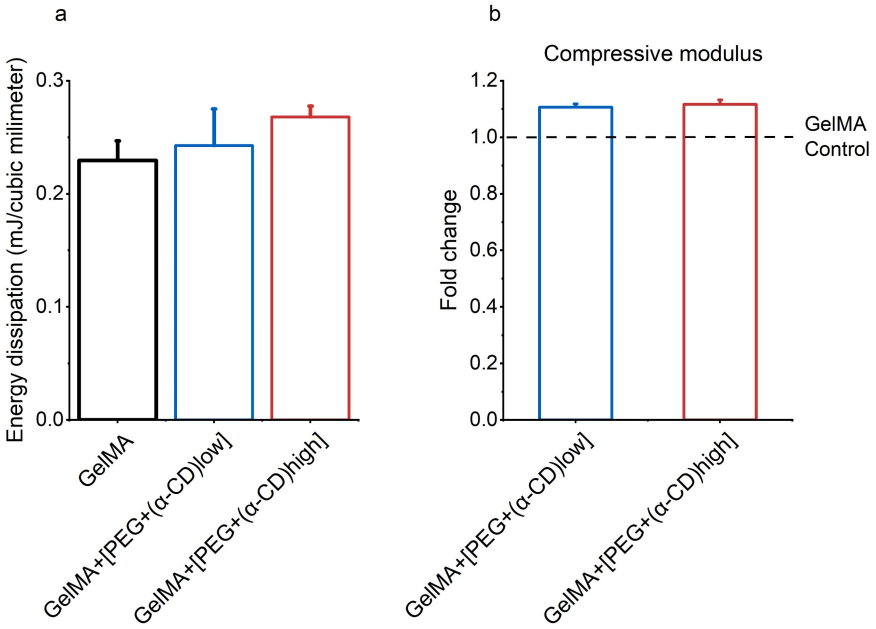

Figure S4.2 – Energy dissipation of hydrogel formulations calculated from the load-displacement curves of each sample. No significant differences were detected.

Chapter 5: Concluding Remarks and Future Perspectives

5.1 Major findings of this thesis

Throughout this thesis, a profound exploration into the enigmatic realm of cartilage has unraveled the hidden influence of transient temperature increase on chondrocyte functionality, shedding light on the complex interplay between mechanical and thermal stimuli in cartilage physiology. By adopting a biomimetic approach, the intricate effects of temperature increase during cyclic loading have been meticulously investigated, revealing a previously neglected thermal dimension in cartilage mechanobiology.

The research findings have demonstrated the remarkable amplification of major chondrogenic markers (up to 3-fold increase at protein level) when a synergistic combination of mechanical signals and biomimetic thermal increase is applied. Moreover, the upregulation of temperature-gated (TREK1) and mechano-gated ion channels (TRPV4) has provided invaluable insights into the underlying mechanisms of thermo-mechanotransduction, unraveling some of the mechanisms by which chondrocytes perceive and respond to temperature variations.

Building upon these insights, the thesis has also ventured into exploring the intricate interplay between loading-induced evolved temperature and hypoxia, challenging conventional paradigms and emphasizing the pivotal role of multiple co-existing stressors in shaping chondrocyte behavior. By carefully controlling and analyzing these factors within a state-of-the-art multi-modal bioreactor, the research findings have further enhanced our understanding of the regulatory microenvironment in which chondrocytes operate, highlighting the significance of considering temperature and oxygen tension alongside mechanical attributes.

Additionally, the thesis has extended its efforts into the sphere of biomaterials by developing double network supramolecular hydrogels, that can, to some extent, replicate the non-covalent (dynamic) interactions present in native tissue. Through the application of dynamic thermomechanical loading along with hypoxia, these innovative hydrogels have demonstrated synergistic enhancements in chondrocyte biosynthetic activity, surpassing the capabilities of conventional single network hydrogels. This novel approach presents a compelling perspective on the interplay between biomimetic temperature evolution and cartilage mechanobiology, paving the way for advanced bioengineering strategies in the field of cartilage repair.

The practical significance of our research lies in its potential to contribute to the advancement of strategies in the field of cartilage repair and regeneration. By investigating the effects of hypoxia and effectively integrating thermal and mechanical stimulation into cartilage-mimicking hydrogel systems, this thesis aims to develop *in vitro* models that closely mimic the physiological environment. This approach shows promise in guiding the development of innovative tissue engineering approaches and refining culture conditions tailored to the complexities of cartilage engineering. Our study represents a step forward in this trajectory, contributing to the ongoing progress of the field toward practical applications aimed at enhancing the outcomes of cartilage repair and regeneration.

5.2 Future perspectives

5.2.1 Towards reversing cartilage degeneration through biomimetic thermomechanical stimulation

A promising avenue to combat cartilage degeneration involves combining controlled heating with specialized physiotherapy protocols. This innovative approach harnesses the benefits of targeted heat and mechanical stimuli, offering the potential to revolutionize cartilage repair. While heat and physical therapy have individually shown benefits for pain relief and mechanotransduction,[1] their synergistic impact on healing remains largely unexplored. Our advanced bioreactor, alongside comprehensive studies using cartilage explants and animal models, provides a unique platform to investigate the effects of thermomechanical stimuli. The overall aim is to explore their potential to reverse osteoarthritis and propose tailored patient treatment methods.

Immediate Research Direction

Our research would first focus on utilizing human osteochondral explants in our bioreactor experiments. By directly comparing the effects of external stimulation on healthy and degenerated explants, we could establish a baseline for the beneficial impacts of our biomimetic approach. Concurrently, we would develop an *in vivo* rat model of osteoarthritis in the knee, accompanied by custom thermal and mechanical stimulation devices. Accurate control of these stimuli is crucial for assessing their effects on osteoarthritic conditions, requiring separate application of mechanical loading and thermal stimulation.

Methodology and Insights

This study would primarily involve periodic analyses of the rat model, including μCT imaging and histological/immunofluorescence staining of the osteochondral knee units. The insights garnered would enable the evaluation of the effects of thermomechanical stimuli across various stages of osteoarthritis. If our hypothesis is validated, the potential clinical implications would be undeniably significant.

5.2.2 Towards cartilage tissue engineering via supramolecular hydrogels primed with thermomechanical loading.

Towards Safe Clinical Implementation

In advancing cartilage engineering, ensuring the safety and compatibility of cartilage implants is paramount. To prepare for potential clinical applications, it's essential to evaluate the biocompatibility and safety of these implants. The Yucatan minipig, known for its resemblance to human histology and physiology, serves as a valuable model for preclinical safety assessments.[2,3,4]

Proposed Approach

Our proposed approach would involve conducting experiments on Yucatan minipigs using cartilage-mimicking supramolecular implants loaded with animal-derived cells. These constructs would undergo thermomechanical stimulation under hypoxic conditions before implantation. We would test different fixation methods, including microfracture, fibrin, and superficial fibrin, with unstimulated constructs serving as controls.

Comprehensive Assessments

Our assessments would cover a range of factors, including immune responses, morphology, and histopathology. By evaluating these aspects, we could gain insights into the biocompatibility and safety of the implants. This rigorous examination is crucial to ensure that the engineered cartilage implants adhere to safety standards required for clinical trials.

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UNIVERSITY COLLEGE LONDON (UCL): MASTER IN BIOMATERIALS AND TISSUE ENGINEERING

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Faculty of Engineering Sciences, Department of Mechanical Engineering
One-year Master Degree (Full time), 180 ECTS
The taught module consists of 8 compulsory modules and a dissertation
Master Thesis: "Preparation of Composite scaffolds, via direct ink writing for Bone repair"
Degree Grade: Distinction (81.86/100)
Prizes: Dean's list for outstanding academic performance & Biomaterials Award

UNIVERSITY OF PATRAS, GREECE: 5-YEAR DEGREE IN MECHANICAL ENGINEERING AND AERONAUTICS

09/2013 – 06/2018: http://www.mead.upatras.gr/lang_en/
Faculty of Engineering, Department of Mechanical Engineering and Aeronautics
Five-year Degree (Diploma) equivalent to undergraduate and Master qualification (300 ECTS)
- Stream: Applied Mechanics, Technology of Materials and Biomechanics

Diploma Thesis: "*Preparation of Nanocomposite Biomaterials for Tissue Engineering implants - Influence of substrate's stiffness on cell's behavior*"
Student Project: "*Preparation of two-dimensional films and control of their mechanical properties for use in tissue engineering applications"*
Degree Grade: 8.50 out of 10 "Excellent"

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Apolytirio Grade: 19.4 out of 20 "Excellent" (Date acquired 25/06/2013)
- Annual Award of Excellence for each of the three years of study due to top school performance

LANGUAGES:

Greek:	Native
English:	IELTS was taken on 11/11/2017 – Overall Band Score: 7, Michigan ECPE (26/11/2011), City & Guilds Level 1 Certificate in ESOL International (Spoken) (Pass)
French:	Beginner

LAB SKILLS:

Mechanical characterization: Mechanical stimulation (tensile, compression) of polymeric and composite materials

Rheological characterization: Steady and oscillatory rheology (Rotational tests, amplitude sweep & frequency sweep)

Biological characterization: Bioreactor, dynamic cell culture, RNA extraction, PCR, Protein quantification, Next generation sequencing (transcriptomics)

Imaging modalities: Confocal microscopy (immunofluorescence staining & histology)

COMPUTER SKILLS:

MS Office: Expert user

App. Software: MATLAB, AutoCAD, Patran Nastran, SolidWorks, Minitab, OriginLab

SCIENTIFIC RECORD

PUBLICATIONS:

1. **Stampoultzis, T.,** Guo, Y., Nasrollahzadeh, N., Rana, V.K., Karami, P., and Pioletti, D.P. (2023). Low oxygen tension augments chondrocyte sensitivity to biomimetic thermomechanical cues in cartilage-engineered constructs. iScience 26, 107491. 10.1016/j.isci.2023.107491.

2. **Stampoultzis, T.,** Guo, Y., Nasrollahzadeh, N., Karami, P., and Pioletti, D.P. (2023). Mimicking Loading-Induced Cartilage Self-Heating *in vitro* Promotes Matrix Formation in Chondrocyte-Laden Constructs with Different Mechanical Properties. ACS Biomater. Sci. Eng. 10.1021/acsbiomaterials.2c00723

3. Karami, P., **Stampoultzis, T.,** Guo, Y., and Pioletti, D.P. (2023). A guide to preclinical evaluation of hydrogel-based devices for treatment of cartilage lesions. Acta Biomater. 158, 12–31. 10.1016/j.actbio.2023.01.015.

4. **Stampoultzis,** T., Karami, P., and Pioletti, D.P. (2021). Thoughts on cartilage tissue engineering: A 21st century perspective. Curr. Res. Transl. Med. 69, 103299. 10.1016/j.retram.2021.103299.

5. F.K., Kozaniti, A.G., Papanikolaki, **T. Stampoultzis,** D., Deligianni. (2017). Nanotubes Reinforcement of Degradable Polymers for Orthopedic Applications. Adv. Tissue Eng. Regen. Med. Open Access 2, 266–272. 10.15406/atroa.2017.02.00047.

SUBMITTED & PEER-REVIEWED PUBLICATIONS:

1. Guo, Y., **Stampoultzis, T.,** Nasrollahzadeh, N., Karami, P., Rana, V.K., Applegate, L. and Pioletti, D.P.Unraveling Cartilage Degeneration : Synergistic Effects of Hydrostatic Pressure and Biomimetic Thermal Increase. (Frontiers in Medical Engineering Journal)

2. **Stampoultzis, T.,** Rana, V.K., Guo, Y., and Pioletti, D.P. (2023). Impact of Molecular Dynamics of Polyrotaxanes on Chondrocytes in Double Network Supramolecular Hydrogels under Physiological Thermomechanical Stimulation. 1–22. (ACS Biomacromolecules journal)

3. Uslu, E., Rana, V.K., Guo, Y., **Stampoultzis, T.,** and Pioletti, D.P. Enhancing Robustness of Adhesive Hydrogels Through PEG-NHS Incorporation. (ACS Applied Materials & Interfaces Journal)

SCIENTIFIC CONFERENCES/EVENTS ATTENDED:

- **09/06/2023:** Paris, France
- **Topic:** Symposium on Computer Methods in Biomechanics and Bioengineering (CMBBE)
- **14/07/2022:** Taipei International Convention Center
- **Topic:** World Congress of Biomechanics (WCB)

CAREER RECORD

01/09/2017 – 30/09/19: ILMO, Electrical Energy Production Private Company

	18 Kapetan Mpoua str., Panorama 55236, Thessaloniki, Greece / Tel: +30 2310 984728
Position:	External Associate (Part Time)
Tasks:	Technical support
01/07 – 31/08/2017:	Egnatia Construction & Real Estate http://egnatiarealestate.gr/3/131/
	106, Makrigianni Str., P.C. 546 31, Stavroupoli, Thessaloniki, Greece / Tel: +30 2310 589624
Position:	Intern (Full Time)
Tasks:	Designed photovoltaic power stations, prepared case folders for the installation of photovoltaic systems, prepared supportive sub-folders for the installation of photovoltaic power stations (materials, measurements, supplies)
01/07 – 31/08/2015:	Dienergia Energy Implementations
	15 -17 Antoni Tritsi, Pylaia 57001, Thessaloniki, Greece / Tel: +30 2310 481120
Position:	Intern (Full Time)
Tasks:	Trained to design electrical networks and photovoltaic projects of high scale (>1MW) via AUTOCAD 2014 Trained in the preventive electromehanical support of a photovoltaic panel of 5,5MW (I-V curve measurements using SEAWARD, SOLAR INSTALLATION PV 150, solar radiation and temperature measurements Riso measurements, character strings direct current cablings using MEGGER MIT 520 Trained in Re measurements of the photovoltaic power station using MEGGER DET4TCR2