Modeling the genetic and mechanical interplay in osteoarthritis: from in vitro systems to mechanistic insights

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CHAPTER 6

Summary, general discussion and future perspectives

General discussion and future perspectives

Summary
Osteoarthritis (OA) is a multi-factorial degenerative disease of joint tissues in which the etio-pathophysiology revolves around the interplay between genetic and environmental risk factors. In current years, several robust genetic studies have identified robust etio-pathophysiological pathways. The focus of this thesis was to close the gap between pathway identification and in-depth insight into underlying mechanisms of action, by applying functional follow-up studies. Hereto sustainable 3D *in vitro* organoid models of articular cartilage were set up, using both primary articular chondrocyte (hPACs) and human induced pluripotent stem cells (hiPSCs). Moreover, to accommodate the exploration of both genetic and OA-relevant environmental perturbations in interaction, hyper-physiologic mechanical loading was incorporated into the models. Multi-modal molecular landscapes were used as readouts that facilitated unbiased insight into molecular pathophysiological processes involved in OA onset.

In **Chapter 2** we presented a human *in vitro* neo-cartilage pellet model of primary articular chondrocytes (hPACs) and studied the behavior of OA risk genes after hyper-physiologic mechanical loading-induced cartilage damage. We showed consistent catabolic responses through upregulation of *ADAMTS5* gene expression, increased apoptosis, and early cartilage damage as reflected by proteoglycan loss. Furthermore, the model was then successfully used to confirm that several known OA risk genes showed similar expression patterns in response to hyper-physiologic mechanical loading as what is observed in OA articular cartilage ().

In **Chapter 3** we characterized the molecular landscape of hiPSC-derived neo-cartilage organoids from two different cell sources, skin fibroblasts (hFiCs) and articular chondrocytes (hCiC), and compared this to hPACs. By using hiPSCs generated from two different tissue sources we explored whether the epigenetic memory of articular chondrocytes could result in superior *in vitro* chondrogenesis. The high similarity among neo-cartilages generated from the two different hiPSC lines indicated that the articular cartilage epigenetic memory retained in hCiC does not further improve the consistency and quality of the *in vitro* chondrogenesis when compared to hFiC-derived *in vitro* chondrogenesis (30–32). Nonetheless, by subsequently determining discordant aspects of stable set-points of gene expression between neo-cartilage from hCiC relative to hPAC, we identified relevant differences in epigenetically regulated setpoints of gene expression between chondrocytes derived from hiPSCs and hPACs. These insights could be exploited to improve the quality, purity, and maturity of hiPSC-derived neo-cartilage matrix further, ultimately to realize the introduction of sustainable, hiPSC-derived neo-

cartilage implantation into clinical practice.

In **Chapter 4** we determined the effect of hyper-physiological mechanical loading on changes in stable set points of epigenetically regulated gene expression (ML-tCpGs) that could contribute to the long-lasting, detrimental changes in chondrocytes that might affect the propensity to enter an OA phenotype. To this end, we employed two human induced pluripotent stem cell (hiPSC)-derived neo-cartilage organoid models for robust readouts and studied the methylome- and transcriptome-wide changes in response to hyper-physiological mechanical loading conditions. We showed that hyper-physiological loading evokes consistent changes in ML-tCpGs associated with expression changes in OA-relevant genes and pathways. Moreover, by comparing the ML-tCpGs and their associated pathways to tCpGs in OA pathophysiology, we observed a modest but interconnected overlap with notable genes such as *CD44* and *ITGA5*. These genes could represent lasting detrimental changes to the phenotypic state of chondrocytes due to mechanical perturbations that occurred earlier in life. The latter is further suggested by the association between methylation levels of ML-tCpGs mapped to *CD44* and OA severity.

In **Chapter 5**, we identified a missense variant in *COL6A3* (c.4510C>T, R1504W) in a patient of the GARP study affected with symptomatic OA in two or more joint sites (*1*), that was predicted to affect the function of collagen type VI (COLVI) in the pericellular matrix. By introducing this damaging variant in hiPSCs using CRISPR-Cas9 genome engineering while employing these cells in an established 3D in vitro neo-cartilage organoid model, we showed that the variant decreased cartilage matrix integrity, as reflected by a reduction in abundance and size of sGAGs, Moreover, by subsequently isolating mutated COLVI protein, we showed that the variant reduced binding to fibronectin. Analysis of the transcriptome-wide gene expression changes with the *COL6A3* variant showed overlap with transcriptomic changes due to OA-pathophysiology (*2*) indicating that an osteoarthritic chondrocyte state appeared secondary to alterations in PCM function due to variant *COL6A3*. By exposing the neo-cartilage organoids to hyper-physiological mechanical loading, we demonstrated that variant *COL6A3* abolished the characteristic upregulation of inflammatory signaling after mechanical loading (*3-5*) with *PTGS2, PECAM1*, and *ADAMTS5*, as most central and notable genes. Finally, by integrating epigenetic regulation of protein-coding gene expression, we have identified lncRNA *MIR31HG* as a key regulator of inflammatory signaling in response to mechanical loading, which was abolished in the *COL6A3* variant. Together, our findings demonstrated that the identified variant in *COL6A3* resulted in impaired binding between COLVI and the PCM protein fibronectin and abolished the initial stress response to hyper-physiologic mechanical loading, potentially affecting

the ability of chondrocytes to properly respond to mechanical loading and maintain cartilage homeostasis.

Creating reliable and sustainable 3D in vitro organoid models of OA articular cartilage for functional follow-up studies.

To bridge the gap between pathway identification by genetic studies and in-depth insight into underlying mechanisms of action sustainable 3D *in vitro* organoid models of articular cartilage were set up, using both hPACs and hiPSCs. To accommodate the exploration of both genetic and OA-relevant environmental perturbations in interaction, hyper-physiologic mechanical loading was incorporated into these models. Multi-modal molecular analyses provided facilitated unbiased insight into molecular pathophysiological processes involved in the onset of OA.

Reliable human 3D in vitro organoid models of articular cartilage

In **chapter 2**, we applied a human *in vitro* 3D organoid model of primary chondrocytes of preserved OA cartilage. Previously, it was confirmed that hPACs isolated from preserved articular cartilage areas of OA patients who underwent a joint replacement surgery readily deposit neo-cartilage *in vitro* that exhibits a DNA methylation landscape nearly identical (99% similarity) to autologous cartilage (*6*). Moreover, as shown in **chapter 3**, DNA methylation in hPAC-derived neo-cartilage organoids is highly consistent across donors, with **92%** similarity observed in samples from 10 different individuals. As shown in **chapters 2** and **3**, these hPAC-derived neo-cartilage organoids readily deposit high-quality matrix as shown by the IHC staining of proteoglycans and collagen II. Upon expansion, primary chondrocytes are, however, prone to dedifferentiate and lose their characteristic articular phenotype, resulting in the deposition of a non-specific, mechanically inferior extracellular matrix which is less suitable for large-scale human disease modeling or applications in tissue regeneration like of the shelf allograph production. To overcome the loss of proliferative and chondrogenic capacity of hPACs upon expansion and enable genome engineering approaches like CRISPR-Cas9, hiPSCs, and respective chondrogenic differentiation protocols were introduced for disease modeling**.** Before applying hiPSCs to our disease models we first set out in **chapter 3** to obtain insight into factors that determine the purity and quality of hiPSC-derived neo-cartilage relative to the neo-cartilage deposited by hPACs. There we demonstrated that cellular and tissue structure as determined by Alcian Blue and COL2 staining of hiPSC-derived neo-cartilages were very similar to hPAC-controls, however, final expression levels of these matrix molecules were ~10% lower in hiPSC-derived neo-cartilage.

Introduction of hyper-physiological mechanical loading to human 3D in vitro models

To accommodate the study of the OA-relevant environmental perturbations we

incorporated injurious mechanical loading in the 3D *in vitro* organoid models. Hereto in **Chapter 2** we initiated the use of hyper-physiologic mechanical loading to our hPAC-derived neo-cartilage constructs using the MACH1 mechanical indenter. We determined that hPAC-derived neo-cartilage constructs perturbed by a mechanical loading regime of 2x10 minutes 20% strain with a frequency of 5hz at two consecutive days were injurious as marked by the strong significant increase in *ADAMTS5* expression by RT-qPCR concomitant with loss of Alcian Blue staining as measures of sGAGs in the neo-cartilage but without consistent changes in *MMP13* expression. This is different from the demonstrated effects in 65% mechanically stressed human osteochondral explants, which showed a strong increase in *MMP13* expression but no response of *ADAMTS5*. It highlights that the catabolic responses to mechanical loading strongly depend on the strain, stress, exposure time, and type of model being used. Since the upregulation of *ADAMTS5* was highly consistent in our neo-cartilage organoid model we put forward *ADAMTS5* as an important marker for hyper-physiologic mechanical loading of neo-cartilage. Subsequently, in **chapters 4 and 5**, the mechanical loading regime was used to mechanically load hiPSC-derived neo-cartilage organoids, which again showed consistent upregulation of *ADAMTS5.* The consistent upregulation of ADAMTS5 in mechanically loaded hiPSC-derived neo-cartilage organoids served two important purposes: it validated our mechanical stress paradigm across different model systems and demonstrated the robustness of our hiPSC differentiation protocol. This consistency in mechanical response suggests that our hiPSC-derived chondrocytes achieved a functional state comparable to primary chondrocytes. While our results indicated that, irrespective of cell source, we were able to produce neo-cartilage with deposition of major matrix proteins such as proteoglycans, collagen II, and collagen VI, there was a slightly lower deposition of these matrix proteins in hFiC and hCiC-derived neo-cartilage compared to hPAC derived neo-cartilage. To potentially improve on the hiPSC-derived neo-cartilage formation further we subsequently analyzed differences in epigenetically regulated set-points of gene expression between hFiC-, hCiC-, and hPAC-derived neo-cartilage organoids.

Multi-modal molecular analyses of 3D in vitro neo-cartilage models

Epigenome wide landscape of hiPSC and hPAC derived neo cartilages

To assess and potentially improve the quality of hiPSC-derived neo-cartilage we investigated in **chapter 3** whether the epigenetic memory of the somatic cell type used for the generation of hiPSCs is retained as a unique DNA methylation signature that facilitates differentiation toward the hiPSC tissue of origin. Henceforth we tested whether hiPSCs derived from hCiCs may differentiate more readily to neo-cartilage and with higher similarity to articular cartilage than hiPSCs derived from hFiCs while

taking neo-cartilage deposited by human primary articular chondrocytes as the golden standard. We used the methylome-wide landscape as a critical biological level of information on (on/off target) cell fate decisions. (*7*) To this end, we performed chondrogenic differentiation of hiPSCs generated from hFiCs, hCiC, and hPACs. First, our chondrogenic differentiation protocol proved highly consistent regardless of the hiPSC source. Both hFiCs and hCiCs showed remarkable consistency across independent differentiations, with Jaccard similarity indices of 96% and 94% respectively. This high consistency validates the robustness of our differentiation protocol. Second, neo-cartilage produced from preserved OA cartilage showed exceptional consistency across patients. Despite the inherent heterogeneity of preserved OA cartilage, samples from ten different individuals achieved 92% methylation similarity, confirming the reliability of our model system.

Identifying potential targets for improvement of hiPSC-derived chondrogenic differentiation

Contrary to our initial hypothesis, the cell source for hiPSC generation did not significantly impact chondrogenic potential. Both hFiC and hCiC-derived neo-cartilage achieved similar methylation profiles compared to hPAC-derived tissue (84% and 86% similarity respectively), indicating that epigenetic memory provides no significant advantage in chondrogenic differentiation. While the high similarity between hiPSC and hPAC-derived cartilage (84-86%) demonstrates the quality of our differentiation protocol, the remaining 15% difference warrants further investigation for potential improvements. To understand these differences at a biological level, we integrated transcriptome data with our methylation analysis. This led us to prioritize transcriptionally active methylation sites, as shown in **chapter 2**, providing specific targets for the optimization of our differentiation protocol.

Our integrative analysis suggests that the observed difference could potentially reflect an overall immature or lower-grade quality of the hiPSC-derived neo-cartilage. Indeed, discordant CpG-gene pairs in hCiC-derived neo-cartilage versus hPAC-derived neo-cartilage were enriched in pathways related to extracellular matrix organization, chondrogenic differentiation, and cartilage development. Alternatively, this could be explained by the presence of a subpopulation of off-target cells in the neo-cartilage derived from hiPSCs. We have identified discordant CpG-gene pairs that were upregulated in hCiC-derived neo-cartilage relative to hPAC-derived neo-cartilage. These genes were enriched in neurogenesis-related pathways, which may suggest off-target differentiation towards a neurogenic lineage, consistent with findings by Wu et al.(*8*)
Due to the nature of the bulk-sequencing method applied in this thesis, we were not able to distinguish different cell populations within a sample, as it masks cell-to-cell

variability. As such we could not test the hypothesis of off-target cells versus overall lower chondrogenic capacity in the hiCiC-derived neo-cartilage. By applying single-cell sequencing to hiPSC-derived chondrocytes, we could better understand the dynamic processes underlying differentiation, identifying cellular heterogeneity, transient cell states, and epigenetic changes that occur as cells progress toward a mature chondrocyte phenotype. This approach will allow us to directly link specific CpG methylation sites with gene expression changes in individual cells, which would enable more precise identification of key regulatory CpG-gene pairs. Such granularity could uncover subtle differences between hiPSC-derived chondrocytes and primary articular chondrocytes (hPACs) that are not evident in bulk analyses. Furthermore, single-cell approaches could identify off-target cell populations within hiPSC-derived cartilage models that may contribute disproportionately to the hiPSC to chondrocyte differentiation. These insights could be crucial for optimizing tissue engineering protocols and refining in vitro models of osteoarthritis, ultimately advancing both basic research and therapeutic applications in cartilage regeneration.

We prioritized N=195 discordant genes that were highly significant differentially methylated and expressed between hCiC and hPACs, with large effect sizes, and with high correlation between methylation and expression, as eligible targets to improve hiPSC chondrogenic cell fate. The genomic regions plotted for compelling CpG-pairs highlighted that the mapping of these CpG sites coincided with high-confidence transcription factor binding sites. That added to the validity that our prioritization scheme, although primarily based on association, has indeed identified differential set-points of transcriptionally active methylation hence potential epigenetically modifiable target genes. A full exploration of the N=195 prioritized genes is required to implement strategies that improve the quality, purity, and maturity of hiPSC-derived neo-cartilages. We envision that such an exploration requires high throughput experimental validation by methodologies such as CRISPR-dCAS9 activation and interference (CRISPRi/a) during hiPSC chondrogenic differentiation with single-cell read-out and followed by system biological approaches to model interactions. Moreover, using single-cell sequencing data enables us to further finetune neural networks for in silico gene regulatory network analysis and in silico gene-perturbation experiments to find the key drivers in this set of 195 discordant genes. (9-11) Next, these in silico selected key drivers of chondrogenesis can then be validated using methodologies such as dCas9-DNMT/TET.

Assessing epigenetic differences in mechanical loading into 3D in vitro organoid models of OA cartilage for functional testing of environmental stimuli

To assess the downstream effects of hyper-physiologic mechanical loading, we performed a comprehensive transcriptome analysis of chondrocytes exposed to hyper-physiologic

mechanical loading. This analysis revealed not only the enrichment of expected stress-related pathways but also the regulation of developmental and biological processes. Epigenetic mechanisms, particularly DNA methylation, are key regulators of these signaling cascades and can adapt to environmental stimuli. In muscle tissue, methylation levels appeared to adapt to exercise-induced mechanical loading, poising the myocytes to an anabolic state. (*9, 10*) While it has been shown that OA is associated with a loss of transcriptional control (*11*), it has not been studied how mechanical loading affects the epigenetic landscape in chondrocytes. Based on these observations, we hypothesized that mechanical loading could induce persistent epigenetic modifications in chondrocytes, and thus in part explain the OA-associated loss of transcriptional control. The accumulation of such methylation changes in response to mechanical stimuli could potentially drive a progressive drift in the chondrocyte phenotype toward a maladaptive state, contributing to disease progression. Therefore, in **chapter 4** we tested the hypothesis that the loss of epigenetic control of transcription (*11*) in osteoarthritis is in part explained by an accumulation of damaging mechanical cues and subsequent changes in set points of epigenetically regulated transcription, which could potentially lead to a maladaptive state of chondrocytes. To this end, we have studied the epigenetically regulated transcriptome in chondrocytes in response to mechanical loading, which showed differential epigenetic regulation of genes that were central in the OA-associated t-CpG landscape, and genetic OA risk signals. Examples of these genes were *ITGA5*, *CD44,* and *CAV1*, suggesting that differential epigenetic regulation in response to injurious mechanical cues potentially plays a role in the propensity of chondrocytes to enter an OA disease state. Mechanistically, the relevance of mechanically induced expression of *ITGA5* is that the binding of matrix fragments such as fibronectin to integrin α5β1 heterodimer activates a pro-catabolic response (*12*). Also, *CD44* plays a role in matrix catabolism by degrading hyaluronic acid in articular cartilage (*13*), while catabolic stress has been shown to upregulate *CAV1*, coding for caveolin-1, which has been linked to chondrocyte senescence (*14*). Similar to OA-associated DNA methylation quantitative trait loci (mQTLs) (*15-19*), these mechanical-induced changes in epigenetic set-points of gene expression may alter the threshold and magnitude of key signaling pathway activation, thereby contributing to OA pathogenesis. To establish causality between these mechanical load-induced changes in DNA methylation and subsequent alterations in gene expression, future studies could employ targeted epigenetic editing using dCas9-DNMT/TET systems within our established 3D organoid model. This approach would allow direct manipulation of methylation at specific sites to validate their role in OA-associated phenotypes.

In current years, several robust genetic studies have elucidated robust etio-pathophysiological pathways. To close the gap between pathway identification and

in-depth insight into underlying mechanisms of action, we now applied functional follow-up of a genetic variant using the optimized in vitro disease models as defined in **chapters 2**, **3,** and **5**. First, we applied exome sequencing in a patient with symptomatic OA at multiple joint sites. The advantage of using genetic targets identified by exome sequencing is that they generally have larger effects than genetic variants identified by GWAS studies. While these high-impact mutations hold significant potential for understanding OA pathogenesis, they rarely undergo functional validation studies. Their strong effects make them particularly valuable for identifying causal disease pathways that may also be relevant to more common forms of OA. Furthermore, their pronounced effects make them especially suitable for modeling disease states in human in vitro tissue systems, where they can help elucidate fundamental OA pathways. To prioritize the identified genetic variants in the patient with symptomatic OA at multiple joint sites we relied on an extensive prioritization scheme as outlined in **chapter 5**. In short, we selected missense mutations that were both predicted to have a damaging effect on protein function using *in silico* analysis tools and were located in genes associated with OA pathophysiology. This is the reason why in our study we have focused on the

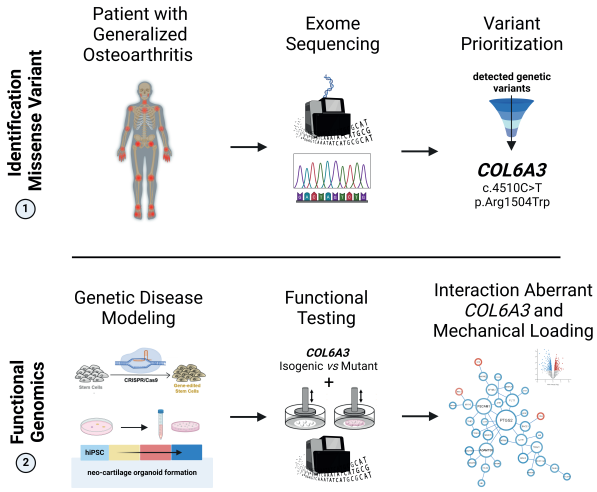

Figure 1 | Schematic overview of the experimental workflow used to identify and functionally validate the *COL6A3* variant in osteoarthritis. The workflow consists of two main phases: (1) Identification of the missense variant through exome sequencing of a patient with generalized osteoarthritis, followed by variant prioritization that identified the *COL6A3* c.4510C>T (p.Arg1504Trp) mutation. **(2)** Functional genomics approach using CRISPR-Cas9 engineered hiPSC-derived neo-cartilage organoids to study the molecular effects of the variant and its interaction with mechanical loading.

molecular effects in our *in vitro* neo-cartilage organoid models genetically engineered with CRISPR-Cas9 to contain the identified likely damaging *COL6A3* mutation. We measured PCM and ECM integrity, chondrocyte phenotype, and chondrocyte phenotype in response to hyper-physiological mechanical cues i.e., in a more general manner.

Downstream effects of the identified COL6A3 mutation.

In **chapter 5** we showed that the R1504W mutation in the N-terminal VWA domain of *COL6A3* resulted in deleterious effects on the level of COLVI protein with downstream effects on the PCM and ECM. This is in line with previous results showing the role of COLVI in regulating the biomechanical environment of the chondrocyte via calcium signaling (*20*), as well as the induction of an OA phenotype secondary to dysregulated mechanotransduction in a COLVI sub-unit alpha 1 murine knockout model. (*21*) First, *in silico* analysis with Polyphen 2 (*22*), Provean, and PANTHER-PSEP (*23*) confirmed that the predicted effect of the mutation on protein function was highly damaging. Next, upon performing a binding assay to study whether the mutated VWF-A domain of *COL6A3* affected binding to PCM (fibronectin) or ECM (hyaluronan) (*24-26*), we demonstrated that binding of mutated COLVI to particularly fibronectin was reduced. In contrast to previous research, we were not able to detect the binding of COLVI to hyaluronan. Finally, we showed that the COLVI mutation caused a loss of sGAG deposition in the neo-cartilage organoids. Using machine learning on TEM data we further confirmed a reduced abundance of sGAGs in the ECM as well as a reduction of sGAG aggregate size. These findings suggest that the *COL6A3* mutation R1504W causes alterations in ECM (e.g., reduced quantity and size of sGAG) and PCM properties (e.g. reduced COLVI binding to fibronectin) which could cause an altered transcriptome-wide landscape, as well as a dysfunctional mechanical signal transduction in response to mechanical loading. Additionally, preliminary data showed that the *COL6A3* mutation disrupted the normal response to hypoosmotic stimuli, similar to a loss of COLVI in a knock-out mouse model. (*27*) These findings suggest that the *COL6A3* mutation R1504W causes alterations in PCM and/or ECM properties that lead to dysfunctional mechanical signal transduction.

To get more insight into the molecular pathophysiology incurred by the *COL6A3* mutant we performed transcriptome-wide sequencing. We demonstrated an aberrant chondrocyte gene expression profile in the *COL6A3* mutant, of which notable highly significant DEGs were related to development and cartilage metabolism, with downregulation of structural PCM and ECM proteins such as *COL27A1* and *PRG4*, increased catabolic activity such as *MMP9*, and ECM mineralization such as *SPP1*. Moreover, pathway analysis showed enrichment of developmental processes, cell-cell adhesion/anatomical structure morphogenesis, and ECM organization in the mutant

condition. The relevance of the altered transcriptome in the *COL6A3* mutant was confirmed by an overlap of the DEGs with the transcriptional changes associated with OA pathophysiology, which particularly contained pathways related to skeletal system development and inflammation. While these findings clearly show the relevance of the R1504W *COL6A3* mutation for OA pathophysiology, a direct investigation of the genotype-phenotype would require an in-depth study on the (extended) pedigree, penetrance, age of onset, and focused phenotyping e.g., on myopathy of carriers. Due to ethical constraints, we were not able to go back to the GARP study participants hence we could not study this aspect of the mutation other than the phenotype ascertained during inclusion.

Dysfunctional mechano-transduction in *COL6A3* mutant neo-cartilage organoids.

Given that the *COL6A3* mutant showed a loss of sGAGs and reduced binding of COLVI to fibronectin we hypothesized that dysfunctional COLVI altered the response to hyper-physiologic mechanical loading. Hereto, two different organoid models were applied and jointly analyzed; cylindrical constructs in which hiPSC-derived pellets were digested to obtain single-cell chondrocytes that were then encapsulated in an agarose gel, and spherical constructs with neo-cartilage deposited by hiPSC-derived chondrocytes. Both these organoid models were exposed to hyper-physiologic mechanical loading conditions (20% sinusoidal peak-to-peak strain at 5hz for 10 minutes) cartilage organoids were harvested at 12 hours post-loading. Hyper-physiological loading resulted in a moderate reduction of proteoglycan content whereas in the *COL6A3* mutated organoids large reduction was observed. Most notable, when exposing the neo-cartilage organoids to hyper-physiological mechanical stress, we demonstrated that the *COL6A3* variant abolished the characteristic inflammatory signaling response after mechanical loading, with *PTGS2 (i.a., COX-2)*, *PECAM1*, and *ADAMTS5* as central genes. The lack of this inflammatory response could affect tissue repair mechanisms, as a controlled inflammatory response is needed for proper tissue maintenance and repair (*28*). This was further evidenced by studying the long-term effects 4 days after mechanical loading, showing sustained alterations in the mutant condition compared to controls. Of note is that the sample size used to test this long-term effect was relatively small (n=5-6). Future studies should investigate the accumulative and sustained effects of this lacking initial inflammatory response in *COL6A3* mutants by repeated bouts of hyper-physiologic mechanical loading. Nonetheless, these results point towards the necessity of an initial inflammatory response after hyper-physiologic mechanical loading and thus highlight the dualistic role of inflammation on OA.

Epigenetic regulation of the inflammatory response by lncRNA *MIR31HG*

By integrating epigenetic regulation of protein-coding gene expression, we identified

lncRNA *MIR31HG* as a key regulator of the inflammatory signaling response to mechanical loading, a response that was abolished in the *COL6A3* variant. *MIR31HG* appeared as a central hub in a correlation network accounting for 32 out of 72 significant correlations between lncRNAs and protein-coding genes. It showed strong associations with stress response and inflammation-related genes such as *PTGS2*, *PTGER4*, *IRAK2,* and *IL1R1*. The regulatory role of *MIR31HG* was experimentally validated using LNA-GapmeR mediated knockdown in primary chondrocytes, confirming its control over these inflammatory genes. A potential explanatory mechanism for this result is the previous work showing that *MIR31HG* acts as a co-activator of HIF1-α and its p300 co-factor (*29*), which is associated with the regulation of inflammation. (*30*) *In vitro,* HIF1-α deficient chondrocytes showed accelerated apoptosis in response to catabolic signaling (*31*), which was confirmed in a murine conditional HIF1-α knock-out model. (*32*) Together, these results identify *MIR31HG* as a potential therapeutic target for restoring proper inflammatory responses in mechanically stressed cartilage.

The role of non-infectious inflammation in tissue repair and homeostasis

The abolished inflammatory response we observed in *COL6A3* variant neo-cartilage organoids raises fundamental questions about the role of inflammation in tissue repair and homeostasis. While chronic inflammation is widely associated with OA pathology (*33-37*), our findings suggest that the complete absence of an initial stress response may also be detrimental to post-injury cartilage repair. This concept challenges the traditional view of inflammation as purely harmful to joint disease.

In physiological conditions, mechanical loading triggers a controlled inflammatory response that initiates a cascade of cellular events necessary for tissue adaptation and repair (*3*). Our studies demonstrate this through the precise temporal regulation observed in control neo-cartilage, where mechanical loading induced a transient upregulation of inflammatory mediators like *ADAMTS5* and *PTGS2*. This response was followed by a return to homeostasis and matrix recovery. The *COL6A3* variant tissue, lacking this initial response, showed impaired recovery and sustained alterations in phenotype, suggesting that the inflammatory phase serves as a crucial signal for initiating proper repair mechanisms. While preliminary, our observations at different time points post-loading suggest that proper tissue repair requires not only the correct magnitude of inflammatory response but also appropriate timing. The transient nature of this response in healthy tissue, contrasting with its absence in *COL6A3* variant tissue, suggests that both the initiation and resolution of inflammation are actively regulated processes essential for tissue homeostasis. These findings connect to a broader biological principle: the same mechanisms that enable tissue adaptation and repair can when dysregulated, contribute to disease progression. (*28, 33, 35, 36, 38-40*) In the context

of cartilage, this manifests as a balance between necessary inflammatory signaling for tissue repair and the risk of chronic inflammation leading to tissue destruction. The fact that the *COL6A3* variant neo-cartilage organoids show both an impaired stress response and compromised repair capacity suggests these processes are tightly linked. Potentially, this understanding has significant implications for therapeutic approaches in OA. Rather than broadly suppressing inflammation, future interventions might need to focus on restoring proper inflammatory regulation. The identification of specific regulatory molecules like *MIR31HG* provides potential targets for such interventions, though modulating these pathways will require careful consideration of timing and context. The complexity of inflammatory regulation in tissue repair also highlights the limitations of current therapeutic approaches. While anti-inflammatory treatments may provide symptomatic relief in OA, they might inadvertently interfere with natural repair processes if not properly timed or targeted. Indeed, clinical trials have shown only marginal effects of celecoxib (a COX-2 inhibitor) (*34*), while ADAMTS-5 inhibitors showed no effects on cartilage degradation or pain and disability in a phase II clinical trial (*41*), underscoring the complexity of targeting inflammatory pathways in OA.

Key questions remain about how to precisely modulate these pathways in a clinical setting, how to identify the optimal timing for interventions, and how to account for individual variation in inflammatory responses. The answers to these questions will be crucial for developing more effective treatments for OA and other degenerative joint conditions.

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