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Development of innovative therapeutic strategies for osteoarthritis: exploring thermosensitive hydrogels, hiPSC-derived cells and cell-products, and novel drugs in preclinical models

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Citation
Sayedipour, S. S. (2026, May 7). *Development of innovative therapeutic strategies for osteoarthritis: exploring thermosensitive hydrogels, hiPSC-derived cells and cell-products, and novel drugs in preclinical models*. Retrieved from https://hdl.handle.net/1887/4303298

Version:	Publisher's Version
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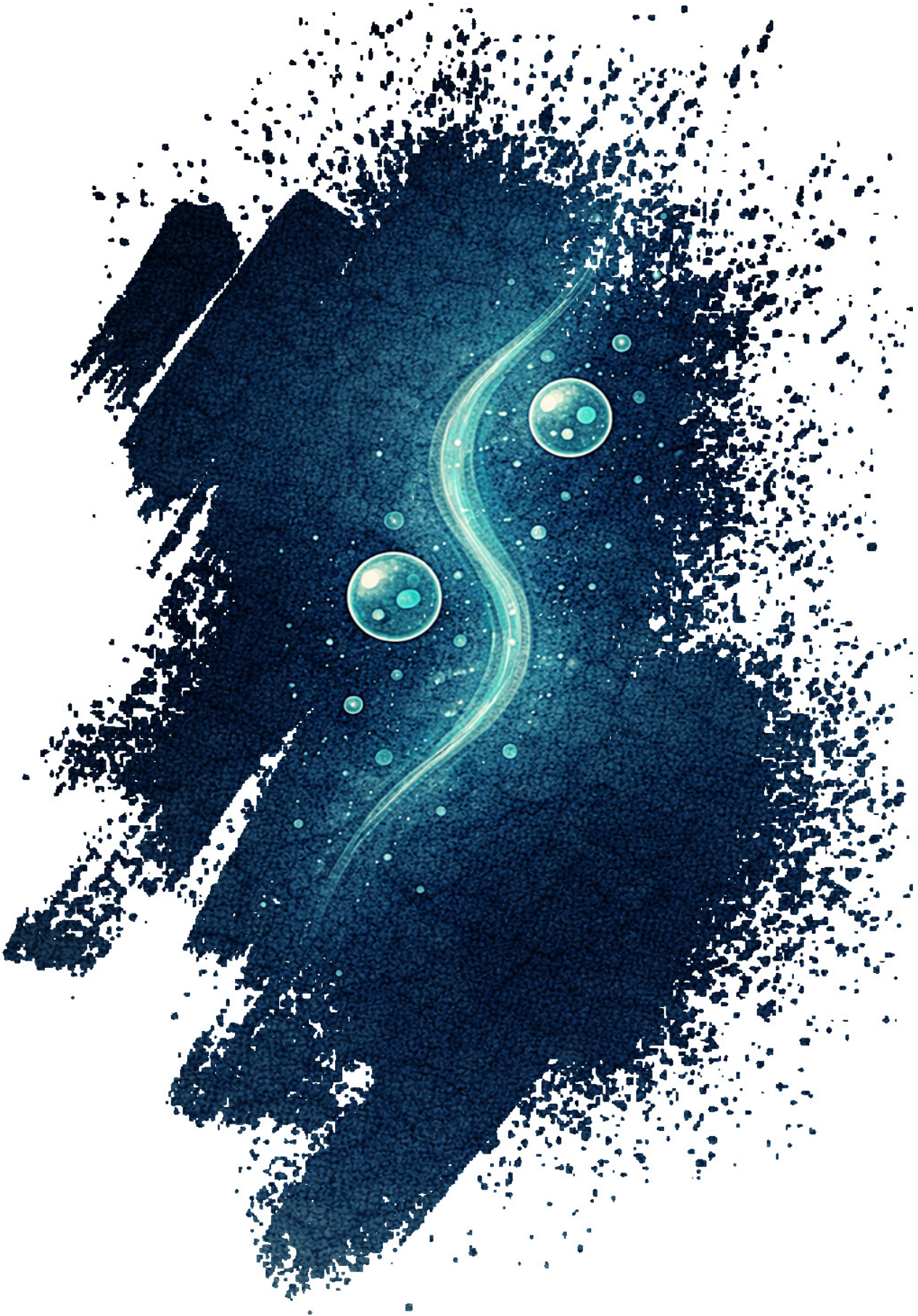
Note: To cite this publication please use the final published version (if applicable).



Chapter 6

General discussion and recommendations

Summary

With the rising prevalence of OA in the global population, there has been growing interest in developing novel treatment strategies. This thesis aimed to design and develop a novel thermosensitive hydrogel for intra-articular (i.a.) delivery of therapeutics and evaluate the therapeutic efficacy of stem cell-based approaches: with hiMSCs, with cell-free therapies using hiMSC-derived EVs, and with IOP used as repurposed treatment to prevent OA-related damage by using a DMM mouse model of OA and lesioned osteo-chondral explants from human OA patients. In **Chapter 2**, we optimized a thermosensitive hydrogel by exploring various concentrations of poloxamer 407 in combination with a self-assembling peptide. This formulation empowered the hydrogel to deliver cells, cell-derived exosomes, and therapeutic agents directly into the joint, by providing controlled and sustained release at the site of cartilage damage. On the road towards utilizing stem cell therapy for OA, in **Chapter 3**, in the DMM OA mice model we evaluated the therapeutic efficacy of hiMSCs in comparison to hBMSCs, both independently and encapsulated within the optimized thermosensitive hydrogel. While both cell types demonstrated similar effects on OARSI damage scoring, immunohistochemical analyses revealed that hiMSCs outperformed hBMSCs by promoting chondrocyte anabolic activity (as indicated by increased Col2 expression) and reducing catabolic markers (such as Mmp13). **Chapter 4** focused on the use of hiEVs as a cell-free therapeutic strategy. HiEVs were isolated from hiMSCs cultured in either serum-containing or in serum-free (xeno-free) medium, and their therapeutic potential was also evaluated in the DMM mouse model. Both, serum-derived and xeno-free hiEVs demonstrated significant chondroprotective effects; however, xeno-free hiEVs have an advantage for clinical application due to the absence of animal-derived components. Finally, **Chapter 5** explored the therapeutic efficacy of IOP in OA using both the DMM mouse model and lesioned osteochondral explants from human OA patients. IOP was shown to partially restore cartilage integrity in both models. In the DMM mouse model, histological analyses using OARSI scoring demonstrated that IOP enhanced proteoglycan retention, reduced structural cartilage damage, and promoted tissue repair compared to untreated controls. Similarly, in human lesioned osteochondral explants, Mankin scoring revealed improvements in cartilage matrix integrity and reduced tissue degradation following IOP treatment.

Thermosensitive hydrogel-delivery in OA

Thermosensitive hydrogels generally offer a minimally invasive and highly effective method for delivering therapeutic agents to the joint cavity (1). These hydrogels remain liquid at room temperature and below and form a gel when exposed to body temperature, allowing easy i.a. injection and subsequent solidification at the site of joint damage. Herein, P407 is widely used as a thermosensitive hydrogel (2). The unique characteristics of P407 make it a promising platform for injectable hydrogels in therapeutic applications such as drug delivery for OA. However, despite its potential for localized and minimally invasive delivery, the use of P407-based hydrogels in OA and other therapies is still limited by challenges related to mechanical strength, controlled release, and stability (3). Across the chapters of this thesis, the hydrogel's properties including thermosensitivity,

biocompatibility, potential as a minimally invasive delivery system for cells and therapeutic agents, and capacity for localized sustained release, played distinct roles in optimizing various therapeutic applications for OA as summarized in **table 1**.

Table 1: Applications of the thermosensitive hydrogel across thesis Chapters

Chapter	Therapeutic Cargo	Hydrogel role	Key funding
2	None (Hydrogel developement)	Developed and characterized P407 + self-assembling peptide hydrogel	Achieved injectable, thermosensitive gelation at ~37 °C with controlled degradation and sustained release profile
3	hiMSCs vs. hBMSCs	Vehicle for intra-articular cell delivery	Supported hiMSC retention and viability; enhanced Col2 expression, reduced Mmp13; modest independent protective effect
4	hiMSC-derived EVs (serum and xeno-free)	Sustained release system for EVs	Enabled gradual release of hiEVs *in vitro* and *in vivo*; significantly reduced cartilage damage; xeno-free EVs favorable for clinical translation
5	Iopanoic Acid (*DIO2* inhibitor)	Localized drug depot for sustained delivery	Improved OARSI scores, reduced Mmp13 and CCDC80 expression

In **Chapter 2**, we developed a thermosensitive injectable hydrogel based on P407 in combination with a self-assembling peptide to tune its biochemical properties for i.a. injection in DMM mouse model of OA. The ideal injectable hydrogel should rapidly form a gel at body temperature (37 °C) for proper delivery and positioning. However, the gelation temperature should not be too low as it will cause handling problems at room temperature with clogging of the needle before injection (4). Through iterative testing of P407 concentrations and blending with self-assembling peptides, we achieved a formulation that transitions from liquid to gel at physiological temperatures. The hydrogel's properties were validated, showing a controlled degradation profile in PBS over nine days. This slow degradation facilitated the sustained release of encapsulated molecules which was confirmed *in vitro* through gradual IR780 release patterns that correlated with hydrogel breakdown. In **Chapter 3**, the hydrogel was investigated *in vivo* for its potential as a vehicle for stem cell delivery in the DMM OA mouse model. The gel's injectable form allowed direct application to the joint cavity, while its thermosensitivity enabled solidification at the injury site. This characteristic is critical for retaining

hiMSCs at the target site. The hydrogel helped retain hiMSCs at the injection site in the knee and facilitated their gradual release over time, aligning with observed therapeutic improvements such as increased Col2 expression and reduced Mmp13 activity in treated groups. Furthermore, statistical analysis of OARSI scoring revealed a modest independent therapeutic effect of the hydrogel, even in the absence of cells. In **Chapter 4**, the hydrogel was also used for controlled hiEV delivery in the mouse knee joint. Labeling the hiEVs with IR780 confirmed their gradual release both *in vitro* and *in vivo*, supporting a localized and sustained therapeutic effect within the affected joint. This release pattern aligns with clinical needs for prolonged exposure in joint tissues.

However, further refinements are required to optimize its mechanical properties, stability, and long-term degradation profile. Incorporating bioactive molecules or cross-linking strategies could enhance its mechanical strength, while additional testing in larger animal models would provide insights into its scalability and clinical relevance. Combining the hydrogel with other biomaterials, such as hyaluronic acid (HA), may further broaden its therapeutic potential for OA. The hydrogel's capacity to reduce mechanical stress and tension within the articular joint likely contributes to the observed therapeutic improvements. Future work will explore whether the hydrogel can mimic the ECM by replicating its mechanical properties, which may further enhance its effectiveness in OA treatment.

Reliable preclinical framework for evaluating OA therapeutics

The DMM mouse model

In addition to designing and developing an injectable hydrogel as a carrier for therapeutic agents, it is important to use pre-clinical models of OA. This, to facilitate studying pathophysiology of the disease and development of new therapies. For this purpose, the destabilization of the medial meniscus (DMM) mouse model was employed throughout all chapters as a representative OA model. The DMM model reliably induces OA-like symptoms in mice by surgically creating mechanical instability, leading to a gradual breakdown of articular cartilage. Histological analyses of the DMM model revealed characteristic features of OA, including roughened cartilage surfaces, proteoglycan loss, and matrix thinning, closely mirroring the clinical progression of human OA (5-7). A key advantage of the DMM model is its ability to replicate different stages of OA, from early to late-stage disease, depending on the duration post-surgery. In this study, OA was allowed to develop for three weeks following DMM surgery, representing the early stage of OA. This stage is particularly valuable for evaluating therapeutic interventions, as treatments are most effective during disease initiation and progression (8). In this thesis, male C57BL/6 (Black 6) mice were selected for inducing the DMM OA model due to their reliability and suitability in OA research. Studies have shown that male C57BL/6 mice develop OA more rapidly and severely compared to females and other strains, due to hormonal fluctuations that influence cartilage and bone metabolism (9). However, this introduces a potential limitation, as the findings may not directly translate to female mice. Estrogen is known to exert chondroprotective effects by promoting anabolic pathways in chondrocytes and suppressing inflammatory mediators such as MMPs and ADAMTS. In contrast, testosterone may modulate immune responses differently, potentially influencing OA pathogenesis in males (10). Since this study did not include molecular analyses beyond histology and IHC, the extent to which hormonal fluctuations would alter the

observed therapeutic effects remains unclear. Future studies should investigate sex-dependent responses to hiMSC and EV treatment by incorporating female and ovariectomized models to account for estrogen depletion. This would provide a more comprehensive understanding of the efficacy of these therapies across both sexes and enhance the translational relevance of the findings.

In **Chapter 2**, the DMM model enabled testing of the newly developed thermosensitive hydrogel as a delivery system for therapeutic agents. The hydrogel's potential as an injectable treatment was evaluated by assessing its ability to sustain the release of cells and drugs *in vivo*. By employing the DMM mouse model, cartilage degradation was monitored using OARSI scoring and Safranin O-fast green staining, providing a reliable framework for comparing treated and untreated joints. OARSI scoring stands for the Osteoarthritis Research Society International histological grading system, which is used to assess the severity of cartilage degradation in histological samples from the DMM model. This scoring system evaluates specific histological features, such as proteoglycan loss, cartilage structural integrity, and surface roughness, providing a quantitative measure of cartilage damage (11, 12). The DMM mouse model demonstrated consistent and reproducible observations of cartilage degradation across all *in vivo* experiments, as evidenced by OARSI scoring. In the DMM/PBS control groups, an average OARSI score of 4.48 ± 0.75 was consistently recorded, underscoring the model's reliability and its suitability for studying OA progression. This consistency provided a robust control baseline, enabling the confident evaluation of the therapeutic efficacy of treatments such as hiMSCs, hiEVs, and IOP.

Bone decalcification is a crucial step in preparing joint tissues for histological staining. Common methods for decalcification include acidic solutions (e.g., formic acid, HCl, nitric acid) and chelation-based approaches using EDTA (5–10%). Acidic methods offer faster processing but may compromise tissue integrity, while chelation methods like EDTA are preferred for preserving molecular and structural quality, particularly for glycosaminoglycans (GAGs) and proteoglycans. The choice of method depends on whether the priority is rapid processing or high-quality tissue preservation. In **Chapter 2** and **Chapter 3** of this thesis, 10% EDTA at room temperature was employed for decalcifying mouse joints. This method effectively preserved proteoglycan content, ensuring high-quality Safranin O-Fast Green staining and reliable OARSI scoring. However, the decalcification process required up to 21 days, posing a significant time limitation. To address this, a commercially available Mol-Decalcifier was used in **Chapter 4** and **Chapter 5**, reducing the decalcification time to just 5 days. Despite the time savings, the rapid decalcification process was found to affect the staining quality, particularly for Safranin O and other GAG-sensitive stains, leading to inconsistencies in OARSI scoring. To resolve this issue, we introduced an alternative damage scoring system to evaluate cartilage degradation. The damage scoring system demonstrated a strong positive correlation with OARSI scoring ($r = 0.763$, $p < 0.01$), confirming its reliability and comparability. This adjustment ensured continuity in assessing treatment effects across the different experiments while accommodating the faster decalcification process. However, maintaining consistency across all steps is crucial. Given that 10% EDTA preserves proteoglycan integrity and ensures high-quality histological analysis, it remains the preferred method despite its time-consuming nature. If a switch to Mol-Decalcifier is considered for future studies, additional validation steps are necessary. This includes systematic testing at multiple decalcification time points, followed by Safranin O staining and GAG assessment to determine the optimal conditions that minimize tissue damage while maintaining staining quality. Despite its strengths, the DMM mouse

model has certain limitations. It primarily represents mechanically induced OA and may not fully capture other forms of the disease driven by metabolic or inflammatory processes. Moreover, the surgical procedure can introduce variability, influenced by factors such as surgical skill and post-surgical complications, including infection, which can affect overall outcomes. In this study, standardized surgical methods helped minimize variability.

Osteochondral lesioned human OA explants

Another OA model used in this thesis was the *ex vivo* (osteo)chondral lesioned explants derived from human OA patients. These explants provide a highly relevant platform for investigating human OA pathophysiology and evaluating potential therapies due to their direct human origin, which eliminates interspecies differences and potential underlying pathophysiology dissimilarities, and their preservation of the natural cartilage-subchondral bone interface. This enables detailed studies of tissue interactions involved in OA progression Lesioned explants closely replicate OA's degenerative and inflammatory processes, such as matrix remodeling, proteoglycan loss, and altered chondrocyte metabolism, while retaining the ECM and aged chondrocytes within their natural microenvironment (13).

In **Chapters 4** and **Chapter 5**, we investigated the efficacy of hiEVs and IOP for treating OA using both the *in vivo* DMM mouse model and human lesioned osteochondral explants. This dual approach allowed us to comprehensively evaluate therapeutic potential in preclinical models relevant to both human and animal OA research. Our findings from *ex vivo* experiments with hiEVs derived from PS medium differed significantly from those observed in the mouse model. While histological evaluation in the *in vivo* setup demonstrated therapeutic benefits, the *ex vivo* setup with human lesioned osteochondral explants showed no significant changes in the gene expression levels of *Mmp13* or *COL2A1*. This discrepancy highlights the reliance of hiEVs' therapeutic effects on the joint microenvironment, including synovial tissues and immune system components, which are absent in the explant model. Conversely, the data from lesioned osteochondral explants treated with IOP aligned closely with the outcomes observed in the *in vivo* DMM mouse model in histological level. In both models, IOP demonstrated significant therapeutic benefits, including improved histological scoring (damage scoring), enhanced proteoglycan retention, and reduced expression of Mmp13. In the *ex vivo* explant model, IOP effectively preserved ECM integrity and mitigated chondrocyte hypertrophy, confirming its ability to counter OA-associated cartilage damage. However, similar to hiEVs, the gene expression data from IOP-treated explants did not fully align with the histological findings. This inconsistency may be attributed to explant culture positively conditions affecting tissue quality and cellular responses over time

The inclusion of lesioned osteochondral explants alongside the DMM mouse model provided a robust evaluation of therapeutic efficacy. The DMM model allowed for dynamic, *in vivo* assessment of OA progression and therapeutic intervention, while the explant model offered precise control over experimental variables such as drug concentration and mechanical stress. Additionally, explant models are more cost-effective and ethically favorable than animal studies, making them a practical choice for routine preclinical research (14). Future research should aim to standardize explant culture conditions and mechanical loading protocols to ensure reproducibility and reliability. Integration of

explant models with advanced imaging techniques, such as micro-CT and confocal microscopy, could provide more detailed assessments of cartilage and subchondral bone changes during therapy. By addressing these areas, future studies can bridge the gap between preclinical research and clinical application, ensuring the development of safe, effective, and targeted therapies for OA treatment.

From hiMSCs to hiEVs: Advancing cell-cased and cell-free therapies for OA treatment

In **Chapter 3**, we explored hiMSCs as a sustainable alternative cell source to hBMSCs for OA treatment, using the DMM mouse model of OA. Comparative analysis revealed that, while both cell types significantly reduced OA-associated cartilage damage as indicated by OARSI scoring, hiMSCs exhibited superior therapeutic efficacy compared to hBMSCs. Specifically, immunohistochemical analysis of anabolic marker Col2 and catabolic marker Mmp13 showed that hiMSCs preserved proteoglycan levels and maintained cartilage structure more effectively, supporting their potential as a viable cell-based therapy for OA.

Recognizing the potential influence of culture conditions, both hiMSCs and hBMSCs were cultured in serum-containing and serum-free media before administration. Our results revealed no significant differences in histological or immunohistochemical outcomes between both conditions, suggesting that serum-containing media are sufficient for early-stage research applications. However, it is important to note that the hiMSCs used in our study were used at early passages (P8-P9). Prolonged culture of hiMSCs lead to morphological changes and a heterogeneous differentiation potential, as hiMSCs are prone to alteration during long-term *in vitro* expansion (15, 16). A recently published systematic review emphasized the importance of addressing such variability through the implementation of consistent culture protocols and good manufacturing practices (GMP). These approaches are critical for achieving scalable, reliable, and clinically translatable MSC-based therapies (17). Future research should prioritize evaluating the stability and differentiation potential of hiMSCs over extended culture periods to improve their therapeutic consistency and reliability. Extended culture studies will provide critical insights into how hiMSCs maintain their regenerative properties and functionality over time. Additionally, testing hiMSCs in human OA-relevant models, such as lesioned osteochondral explants, could offer valuable insights into their therapeutic mechanisms within a native extracellular matrix environment. These investigations will be crucial in validating the clinical translatability of hiMSCs and optimizing their application for OA treatment.

Given that EVs inherit the functional properties of their parent cells (15, 18), hiEVs were investigated in **Chapter 4** as a cell-free alternative for OA therapy. A key advantage of hiEVs over their parental hiMSCs is their non-living nature, which eliminates the need for expensive and complex measures to maintain viability during manufacturing, storage, transport, and administration (19, 20). Unlike MSCs, hiEVs cannot differentiate, thus avoiding the expression of potentially immunogenic differentiation antigens (21). This enhances their safety profile while retaining the regenerative and anti-inflammatory properties of their parent cells, making them a more practical option for therapeutic applications (22). In our research, hiEVs derived from hiMSCs cultured in both serum-containing and serum-free (PurStem, PS) media were evaluated in the DMM mouse model, with and without a

thermosensitive hydrogel for localized delivery. Both hiEV_serum and hiEV_PS treatments significantly reduced cartilage damage, as demonstrated by histological analysis and damage scoring The chondroprotective capacity of hiMSC-derived EVs was evident in their ability to modulate key inflammatory and catabolic markers. Mmp13 expression, indicative of cartilage degradation, was significantly reduced, while Col2, an anabolic marker for cartilage synthesis, was upregulated. This dual effect demonstrates hiEVs' ability to both inhibit cartilage degeneration and promote cartilage formation. These results confirm hiEVs as an effective therapeutic option for OA. This therapeutic effect likely requires support from the joint environment (synovial tissues, immune system). However, it is important to note that hiEVs derived from serum-containing media, particularly fetal bovine serum, may include non-EV particles such as lipoproteins and bovine-derived EVs, which introduce variability and potential contamination (23). Hence, the inclusion of proper controls is essential to rule-out the possibility that the observed therapeutic effects are, in part, attributed to components unrelated to hiEVs. In this respect, serum-free conditions, such as PS formulations, offer a more suitable platform for clinical applications by minimizing contamination and ensuring consistency (20).

Future research should focus on optimizing the production and functionality of hiEVs to maximize their therapeutic potential. Preconditioning hiMSCs with stimuli such as hypoxia or inflammatory signals prior to hiEV isolation may further enhance their regenerative and anti-inflammatory capabilities(24). Additionally, while this study employed a single intra-articular injection of hiEVs in the DMM OA mouse model, further studies should explore the effects of multiple injections with varying dosages to determine the optimal delivery strategy for sustained therapeutic outcomes.

In conclusion, both hiMSCs and hiEVs show potential for treating OA, but each of them has advantages and limitations. hiMSCs facilitate direct cartilage regeneration through both differentiation and paracrine signaling. However, they face challenges such as immune reactions when injected into joints (25). On the other hand, hiEVs, eliminate many of these risks. However, hiEVs lack differentiation potential and are quickly cleared from the body without effective delivery systems. Furthermore, variability in isolation methods can affect their therapeutic consistency. Future research should focus on integrating both therapies to take advantage of their synergistic benefit, with hiMSCs being better suited for advanced OA or severe cartilage defects requiring structural support and regeneration.

Therapeutic potential of IOP: Insights from DMM mouse model of OA and human explant models

Previously in our group, the importance of thyroid signaling in OA pathogenesis has been well-documented, with *DIO2* serving as a key regulator of T3 activation (26). This activation promotes chondrocyte maturation and hypertrophy, key processes in OA progression. In human explants subjected to OA-like conditions through injurious mechanical stress and in *Dio2*-knockout mice, inhibition of *DIO2* using IOP showed significant chondroprotective effects (27). For that matter, in **Chapter 5**, we investigated the efficacy of IOP for treating OA using the DMM mouse model and lesioned osteochondral explants derived from human OA patients. *In vivo* evidence demonstrated the effectiveness of IOP in reducing OA-related damage, as indicated by significantly increased Col2 expression and decreased Mmp13 expression in immunohistochemical analyses. Additionally, a

notable reduction in CCDC80 levels was observed, suggesting that IOP plays a role in mitigating the terminal maturation of articular cartilage. These results were in line with the findings in lesioned human explants, where sGAG release, assessed via DMMB assay, indicated effective prevention of excessive matrix degradation during the initial six days of treatment in comparison with untreated lesioned explants the way there was no significant result between IOP treated lesioned explants and preserved ones. Histological analyses assessed by Mankin scoring further supported IOP's protective role, showing partial restoration of cartilage integrity and reduced structural damage in treated explants compared to untreated lesioned explants (P= 0.012). However, gene expression level analysis of RT-qPCR results after 12 days of culture showed no significant differences in the expression of chondrocyte phenotype-related genes across the experimental groups. These findings highlight IOP's therapeutic potential in the early stages of OA treatment and emphasize the need for further studies focusing on earlier time points, such as days 3 and 6, to better understand IOP's early molecular effects on chondrocyte phenotype and matrix preservation.

Future work should focus on investigating IOP's effects at earlier time points in both human explant will help clarify its mechanisms of action and identify optimal treatment windows. Additionally, exploring IOP's potential synergistic effects with hiMSCs or EVs could reveal combination therapies that maximize chondroprotection and cartilage regeneration.

Overall conclusions and future perspectives

Osteoarthritis remains a significant clinical challenge due to its progressive nature and the lack of disease-modifying treatments. This thesis has demonstrated the potential of a thermosensitive hydrogel as a versatile delivery system for OA therapies, as well as the efficacy of hiMSC-based approaches and IOP treatment. Through a combination of *in vivo* and *ex vivo* models, we systematically evaluated these therapeutic strategies and provided insights into their mechanisms of action and limitations.

One of the most reassuring findings of this work is the potential of hiMSCs over hBMSCs in preserving cartilage integrity, supporting their potential as a cell-based therapy for OA. However, given the limitations associated with direct implantation of allogenic hiMSCs as cell therapy, hiEVs emerged as a promising cell-free alternative, showing strong chondroprotective effects in the DMM mouse model. Importantly, hiEVs derived from serum-free cultures offer a clinically relevant approach by avoiding animal-derived contaminants. The findings from this thesis further emphasize the role of the joint microenvironment in modulating therapeutic outcomes, as demonstrated by the discrepancies between *in vivo* DMM models and *ex vivo* human explants. These results underscore the robustness of our research and the need for multi-model validation to enhance translational potential. Additionally, this thesis highlighted the therapeutic promise of IOP as a repurposed drug for OA treatment. IOP demonstrated beneficial effects across both models by reducing cartilage degradation and preserving proteoglycan content, though molecular analyses suggested differences in treatment responses. These findings reinforce the importance of exploring early intervention strategies and optimizing treatment timing.

From a methodological perspective, this work has contributed to advancing preclinical OA models. The thermosensitive hydrogel enabled localized and sustained delivery of both hiEVs and IOP, demonstrating its feasibility for intra-articular applications. The introduction of a damage scoring system helped address challenges related to decalcification inconsistencies, ensuring robust histological assessments. Additionally, the comparison of DMM and human explant models highlighted their respective strengths and limitations, advocating for a combined preclinical approach to better mimic OA pathophysiology.

While this thesis provided valuable insights, several areas warrant further investigation to enhance the translational impact of these therapies:

1. **Optimizing hiMSC and hiEV therapies** – Future studies should focus on standardizing EV isolation and preconditioning strategies to enhance their therapeutic consistency. Evaluating sex-dependent responses in human explants will further clarify their clinical applicability.
2. **Refining IOP-based OA treatments** – Investigating early treatment time points and alternative delivery methods, such as nanoparticle formulations, could enhance its efficacy. Exploring synergistic effects of IOP with hiMSCs or hiEVs may offer combination therapies with enhanced chondroprotective potential.
3. **Enhancing hydrogel performance** – Modifying hydrogel formulations to improve mechanical strength and degradation control will be essential for its long-term application in OA treatment.
4. **Expanding preclinical models** – Incorporating mechanically loaded explant cultures and large-animal models will be critical to bridging the gap between preclinical studies and clinical trials.

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