UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

SYNTHETIC CHONDROINDUCTIVE BIOMATERIAL FOR HYALINE CARTILAGE REGENERATION

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

Degree of

DOCTOR OF PHILOSOPHY

By

BOUSHRA AJEEB
Norman, Oklahoma
2024

SYNTHETIC CHONDROINDUCTIVE BIOMATERIAL FOR HYALINE CARTILAGE REGENERATION

A DISSERTATION APPROVED FOR THE
STEPHENSON SCHOOL OF BIOMEDICAL ENGINEERING

BY THE COMMITTEE CONSISTING OF

Dr. Michael Detamore, Chair

Dr. Handan Acar

Dr. John Clegg

Dr. Andrew Madden

Dr. Amgad Haleem

Acknowledgments

I extend my gratitude to everyone who supported me throughout this unexpected journey and contributed to the completion of my thesis.

First, I express my sincere thanks to my advisor, Dr. Michael Detamore, for his mentorship, guidance, encouragement, and profound insights both academically and personally. He taught me that family and health come first, and he helped me gain confidence in my potential. His continuous support allowed me to successfully navigate a Ph.D. thesis, a pandemic, and parenthood.

I am grateful to my committee members, Dr. Handan Acar, Dr. John Clegg, Dr. Andrew Madden, and Dr. Amgad Haleem for their support and constructive feedback that enriched this work. Their expertise has been valuable for the rigor of this research, and I have enjoyed all the discussions we had in the past 5 years.

I thank all the faculty and staff in the Stephenson School of Biomedical Engineering (SBME), specifically Cherie Hudson and Shayla Glover, who helped me navigate the requirements and challenges of the Ph.D. I thank the Stephenson Research & Technology Center (SRTC) staff, specifically Jacquelyn Middleton for her help with orders and deliveries and Dora Rapp, for her support, especially during my postpartum period.

I acknowledge the support of the University of Oklahoma Gallogly College of Engineering PhD Recruitment Excellence Fellowship (PREF), the Oklahoma Center for Adult Stem Cell Research (OCASCR), and the National Institutes of Health (NIH; NIH/NIAMS grants R21 AR077800 and R21 AR079705) for funding this work.

I thank my lab members, my friends, and the undergraduates who helped me and taught me throughout my time in the lab. Specifically, I express my appreciation for Peggy Keefe, our lab manager, for her kindness, and support, and for all the chats that made the long days more bearable. To Dr. Emi Kiyotake, my lab partner and friend, thank you for always being there to help inside and outside the lab. Thank you for never forgetting a birthday or anniversary and for reminding me to celebrate my accomplishments. I thank Dr. Jakob Townsend, for all the discussions and for reminding me to stay focused on the end goal, and Dr. David Nedrelow, for always listening to my complaints. I thank current and previous undergraduates, especially Carter Oostmeyer, for his help, willingness to learn, and dedication.

I thank my husband, Karim Saadeddine, for his love and care throughout these years. Your unconditional support made this journey possible. My son, Kayan, and my daughter, Talia, who brought joy, meaning, and happiness to our home and helped me put things in perspective.

Finally, I thank my mom and dad for their love and support and my sisters, Layal, Farah, Balsam, Hiba, and Rana for being part of who I am today and my journey here. I thank my friends in Lebanon and US, especially Samar, Sara, Roula, and Lamis for all the chats and laughs.

Table of Contents

List of Figures

Chapter 1

No figures

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Tables

Chapter 1

No Tables

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Abstract

Avascularity, low cell count, and low proliferative potential constitute the triad that defines the limited self-regenerative potential of articular cartilage. Consequently, the repair of articular cartilage defects presents a highly challenging task for researchers and orthopedic surgeons. Despite the progress in currently available treatments, including surgical and regenerative cell therapy techniques, the regeneration of a tissue that completely mimics the biochemical and mechanical properties of articular cartilage has not yet been successful.

Among a multitude of approaches being investigated to induce hyaline cartilage regeneration, the design of an acellular chondroinductive biomaterial would provide a safe, cost-effective, and translational approach toward successful true hyaline cartilage regeneration.

In this dissertation, the objective was to design a chondroinductive hydrogel to induce the chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells (hBMSCs). The base of the biomaterial was a pentenoate-functionalized hyaluronic acid (PHA) hydrogel along with a conjugated chondroinductive factor.

The search for a chondroinductive factor started with the evaluation of two peptides and two small compounds from the literature with human and equine BMSCs; however, no evidence of chondroinduction was observed with any of the peptides or compounds. The search continued with the evaluation of the chondroinductive potential of 11 newly discovered peptides with no success. Hence, and as a contingency plan, I resorted to the

use of a peptide (Peptide D), based on preliminary results (from others on our team) with hBMSC spheroid cultures, which suggested that Peptide D could enhance the chondrogenic differentiation of hBMSCs in the presence of transforming growth factor (TGF)-β3. Therefore, the final objective of this dissertation was to synthesize a PHA hydrogel with conjugated Peptide D and conjugated TGF-β3. Varying concentrations of Peptide D and TGF-β3 were evaluated, and I identified a combination that resulted in the upregulation in the gene expression of collagen II and SOX-9 compared to hydrogels with conjugated TGF-β. This outcome signifies the successful synthesis of a promising chondroinductive biomaterial.

The results obtained in this dissertation were the basis to select promising hydrogel groups to evaluate in a currently ongoing *in vivo* cartilage regeneration study in rabbits. Future work beyond this thesis will focus on advancing the hydrogel-peptide-TGF-β3 system based on the outcome of the current *in vivo* study, and to refine the sequence of Peptide D in efforts to enhance its chondroinductive potential.

Chapter 1: Introduction

The long-term goal of this dissertation is to create a synthetic chondroinductive biomaterial for *in situ* scaffold-based cartilage regeneration. Cartilage fails to regenerate following an injury, and despite recent advances in surgical and cell therapy techniques, the outcome in most cases is the formation of an inferior fibrous tissue that lacks the structural organization, matrix composition, and mechanical performance of hyaline cartilage. Acellular biomaterials are becoming increasingly popular as they rely on the endogenous mobilization of BMSCs through microdrilling, thus simplifying the regulatory barriers, and the tediousness and high cost of *in vitro* cell expansion. However, we are yet to develop a synthetic chondroinductive biomaterial capable of inducing cartilage regeneration. The key to developing a chondroinductive hydrogel is to create the material to be chondroinductive by itself by conjugating peptides that can mimic the action of growth factors and induce cartilage repair. This thesis considered peptides from the literature in addition to peptides discovered within our research group, including a peptide that was named “Peptide D”.

The specific aims for this dissertation are (1) : Evaluate the chondrogenic potential of promising synthetic chondrogenic factors (CFs) with equine bone marrow-derived stem cells (eBMSCs), and equine umbilical cord mesenchymal stromal cells (eUCMSCs) in spheroid culture. (2) Evaluate the chondrogenic potential of promising synthetic chondrogenic factors (CFs) and their combinations with hBMSCs. (3) Synthesize and evaluate the chondroinductive potential of a bioactive PHA hydrogel with conjugated TGF-β3 and synergistic Peptide D.

Chapter 2 provides an exhaustive review of peptides used for cartilage regeneration. I categorized peptides based on two major distinctions. The first distinction is a clear separation of peptides reported to independently induce chondrogenesis from those peptides reported to synergize with other growth factors for cartilage regeneration. The second distinction focuses on whether peptides underwent evaluation *in vitro* or *in vivo*. This chapter provides valuable insight on the necessity of establishing proper positive and negative controls, both *in vitro* and *in vivo*, to allow rigorous evaluation of chondroinductive peptides. Additionally, chapter 2 provides the rationale for selecting peptides CM10 and CK2.1, and compounds kartogenin and SM04690 in subsequent chapters.

Chapter 3 addresses the first part of **Aim 1** by evaluating and comparing the chondroinductive potential of equine BMSCs and equine UCMSCs in response to two compounds, kartogenin and SM04690, and two peptides, CM10 and CK2.1, in parallel with a TGF-β3 positive control. Furthermore, I compared the chondroinductive potential of eBMSCs in hypoxia and normoxia in both monolayer and spheroid culture in response to dexamethasone and/or TGF-β3. Although subsequent chapters do not include the use of equine cells, chapter 3 provides confirmation that BMSCs from different species and cells from other sources (e.g., umbilical cord) respond differently to growth factors and culture conditions.

Chapter 4 addresses **Aim 2** by evaluating the chondroinductive potential of two compounds, kartogenin and SM04690, and two peptides, CM10 and CK2.1, with hBMSCs. I assessed the chondrogenic differentiation of the hBMSCs based on the gene

expression of chondrogenic markers (i.e., ACAN, collagen II, and SOX-9) in addition to collagen I. Additionally, I ran a donor study that evaluated the chondrogenic differentiation of hBMSCs from three distinct donors in response to selected concentrations of kartogenin, SM04690, CM10, and CK2.1. Chapter 4 emphasizes the need to identify peptides and compounds that are not situation dependent.

Chapter 5 addresses **Aim 2,** or more specifically, a contingency plan for Aim 2. Based on the findings that are discussed in chapter 4, I screened the chondroinductive potential of 11 newly discovered peptides from our research group using hBMSCs in spheroid culture *in vitro*.

Chapter 6, addresses **Aim 3** by synthesizing and evaluating the chondroinductive potential of a bioactive PHA hydrogel. Based on the findings that are discussed in chapters 4 and 5, I moved on to a second contingency plan whereby I employed a newly discovered peptide from our research group named Peptide D, which others in our group had shown had enhanced the chondrogenic differentiation of hBMSC spheroids in the presence of TGF-β3. Hence, I synthesized a PHA hydrogel with both conjugated TGF-β3 and conjugated Peptide D. This chapter was the basis on which we selected promising groups to be evaluated *in vivo* in a cartilage defect model that is outside the scope of this thesis.

Chapter 7 is the conclusion to the dissertation, which summarizes the key findings of all three aims, addresses limitations of the studies, and provides recommendations for future research.

Chapter 2: Chondroinductive Peptides for Cartilage Regeneration[1]

Abstract

Inducing and maintaining a hyaline cartilage phenotype is the greatest challenge for cartilage regeneration. Synthetic chondroinductive biomaterials might be the answer to the unmet clinical need for a safe, stable, and cost-effective material capable of inducing true hyaline cartilage formation. The past decade witnessed an emergence of peptides to achieve chondrogenesis, as peptides have the advantages of versatility, high target specificity, minimized toxicity and immunogenicity, and ease of synthesis. Here, we review peptides as the basis for creating promising synthetic chondroinductive biomaterials for *in situ* scaffold-based cartilage regeneration. We provide a thorough review of peptides evaluated for cartilage regeneration while distinguishing between peptides reported to induce chondrogenesis independently, and peptides reported to act in synergy with other growth factors to induce cartilage regeneration. Additionally, we highlight that most peptide studies have been *in vitro,* and appropriate controls are not always present. A few rigorously-performed *in vitro* studies have proceeded to *in vivo* studies, but the peptides in those *in vivo* studies were mainly introduced via systemic, subcutaneous, or intraarticular injections, with a paucity of studies employing *in situ* defects with appropriate controls. Clinical translation of peptides will require the evaluation of these peptides in well-controlled *in vivo* cartilage defect studies.

[1] Published as: Ajeeb, B., Acar, H. & Detamore, M. S. Chondroinductive Peptides for Cartilage Regeneration. *Tissue Eng Part B Rev* (2021) doi:10.1089/ten.TEB.2021.0125.

In the decade ahead, we may be poised to leverage peptides to design devices that are safe, reproducible, cost-efficient, and scalable biomaterials, which are themselves chondroinductive to achieve true hyaline cartilage regeneration without the need for growth factors and other small molecules.

Introduction

Articular cartilage regeneration manifests as a tough challenge for researchers and clinicians globally. The loss of cartilaginous tissues affects all age groups and is mainly due to arthritis and traumatic injuries. According to the 4th edition of the Burden of Musculoskeletal Diseases in the United States (BMUS), published in 2018, approximately 78 million Americans will develop arthritis by 2040, and the annual direct medical costs attributed to arthritis are roughly $81 billion in the United States.[1]

Articular cartilage cannot self-regenerate mainly due to the lack of vascularization and the low density of chondrocytes. Non-surgical treatments include intra-articular injections, with standard examples such as corticosteroids or hyaluronic acid (HA) (FDA approved). However, intra-articular injections are an advanced pharmacological intervention to manage the pain for patients with persistent osteoarthritis (OA) symptoms and are typically used as a last resort before or in lieu of surgical intervention. A recent review by Jones *et al.*[2] highlighted the currently available intra-articular injections for knee OA and emphasized the need to have significant clinical data to support the effectiveness of these treatments as compared to placebo.

Currently available surgical approaches include microfracture (MF), which was introduced in the early 1980s[3] and is still one of the first choices for treating cartilage injury due to its feasibility and lower cost as compared to alternative surgical approaches. However, MF is mainly employed for small defects, and the repair tissue is usually fibrocartilage. Other options include autologous osteochondral grafts (mosaicplasty) and osteochondral allografting (OCA). Mosaicplasty is becoming less popular due to donor site morbidity and variability of outcomes,[4] as for OCA, the main drawbacks are cost and graft availability.[5]

Regenerative surgical techniques emerged approximately 30 years ago[6,7] with the primary technique being autologous chondrocyte implantation (ACI). ACI has been modified for 2nd, 3rd, and 4th generation.[7,8] The 3rd generation refers to the matrix-assisted chondrocyte implantation (MACI), involves the use of scaffolds, and in December 2016,[9] the FDA approved Vericel's MACI® for full-thickness cartilage defects. Clinical outcomes of regenerative surgical techniques are affected by several variable such as, lesion size, site of defect, sex and age; however, most reviews addressing MF, ACI, and MACI conclude that ACI or MACI are recommended when lesions are >3 cm.[27,10] 4th generation ACI overcomes the limitation of two surgical procedures in previous generations, and involves chondrocyte and BMSC harvest and implantation in one intervention.[7] Single treatment autologous chondrocyte implantation (STACI) and INSTRUCT[11] are currently available 4th generation ACI techniques; however, as of May 2021, they were not FDA approved.

However, despite the pain relief and enhancement of knee function, the outcome of regenerative surgical techniques in most cases is an inferior fibrous tissue that lacks the structural organization, matrix composition, and mechanical properties of hyaline cartilage. Therefore, the question that remains is, how can we induce true hyaline cartilage regeneration? Several approaches to answer this question have been extensively studied by the tissue engineering community, which is reflected by the number of reviews that have been published in the past couple of years to address cell therapy,[4,12–21] scaffolds,[4,22] biomaterials,[15,23,24] hydrogels,[25–28] 3D printing,[20] and gene therapy,[16] etc. However, there remains an unmet clinical need for the development of small synthetic molecules that have the potential to induce chondrogenesis (i.e., chondroinductive) and promote cartilage regeneration without the fear of immunogenicity of naturally-derived components, the high cost of surgical and cell therapy procedures, and the potential variability of extracellular matrix (ECM) products. Such device-only, synthetic chondroinductive materials would provide a safe, cost-effective, and translational approach toward successful true hyaline cartilage regeneration.

Two main promising categories of synthetic chondroinductive materials are currently recognized, small molecules and peptides, of which peptides will be the focus of this review. Chondroinductive pathways of stem cells are initiated via protein-protein interactions (i.e., between cell surface receptors, growth factors, and extracellular matrix proteins). The small binding pocket of the cell membrane protein or the binding ligand of the exogeneous protein may be identified and generated as a peptide to trigger a given pathway, even without the ligand protein. The particular binding pocket may additionally

be triggered with small molecules, only if the binding site has a defined “hot spot” to target.[29] However, protein-protein interactions can be wide and have more than one “hot spot” to target. Therefore, many protein-protein interactions remain as “undruggable” by the small molecules.[30] Peptides, by covering the wide binding pocket and interacting with the “hot spots” may be more specific and more effective.[31]

Small molecules have been widely investigated for their ability to induce stem cell differentiation, chondrocyte proliferation, and to maintain chondrocyte proliferation. A recent review by Li *et al.*[32] provided an extensive list of natural and synthetic small molecules that have been evaluated for their applications in cartilage tissue engineering and regeneration. Among the reported synthetic small molecules, KA-34[33] (kartogenin analog) and lorecivivint[34–38] (SM04690) may perhaps be the most promising so far. Lorecivivint is already in phase 3 clinical trials as a disease-modifying osteoarthritic drug (DMOAD),[39] whereas KA34 has a phase 1 trial completed, but no results or plans for phase 2 are yet published.

On the other hand, several peptides have been studied for their chondroinductive potential, and a few reviews[40–42] have addressed the use of these peptides. A previous review from our group provided a concise review of chondrogenic peptides related to cell-adhesion sequences.[42] Liu *et al.*[40] focused on the different applications of peptides in cartilage regeneration and distinguished peptides based on their function (e.g., transforming growth factor (TGF)-β mimics, affinity, cell-penetrating, self-assembly, and degradable peptides).[40] Gonzalez-Fernandez *et al.*[41] focused on materials used for musculoskeletal regeneration and listed peptides that can be employed for

chondrogenesis, osteogenesis, and myogenesis.[41] However, the previous reviews did not provide a clear distinction between peptides that are chondrogenic and those that facilitate or synergize with other factors to induce chondrogenesis. Additionally, none of the previous reviews highlighted the *in vitro* controls and animal models used to evaluate the chondroinductive potential of peptides. The current review provides a comprehensive overview of peptides involved in cartilage regeneration, with an emphasis placed on the *in vitro* and *in vivo* controls, animal models, and defect types used to evaluate the peptides. **Figure 2.1** presents an overview of the different sources of peptides reported to be used for cartilage regeneration. Additionally, we divide peptides into 2 categories, the first includes peptides reported to be chondroinductive without the addition of growth factors *in vitro* or *in vivo*. The second category includes peptides that were reported to be used in cartilage regeneration, but where chondrogenesis was only observed in the presence of growth factors. Tables 1 and 2 provide a summary of *in vivo* and *in vitro* studies, respectively, for peptides reported to exhibit chondroinductive activity. Tables 3 and 4 provide a summary of *in vivo* and *in vitro* studies, respectively, for peptides reported to be used in cartilage regeneration.

Finally, we aim from this review to highlight the currently existing peptides capable of inducing chondrogenesis and to emphasize the gaps that need to be filled to drive the field of synthetic chondroinductive biomaterial devices forward.

CK2.1 (QIKIWFQNRRKWKKMVPSDPSYEDMGGC)

In 2017, two studies were reported by Akkiraju *et al.*[43,44] that addressed the regenerative potential of peptides inspired by the protein-protein interaction between the

protein casein kinase II (CK2) and the bone morphogenetic protein (BMP) receptor type Ia (BMPRIa). CK2 is bound to the intracellular domain of BMPRIa, but is released inside the cell when BMPRIa binds to BMP-2, with the release of CK2 playing a role in downstream signaling in carrying the BMP-2 signal forward. CK2.1, CK2.2, and CK2.3 peptides were designed with the intent to block the BMPRIa-CK2 interaction by binding to CK2 intracellularly, thereby keeping CK2 in circulation within the cell to maintain the downstream signaling as it does when the cell is bound to BMP-2.[45] In the first study,[43] the peptides were designed with the Antennapedia homeodomain signal sequence **(**QIKIWFQNRRKWKKMVPSDP) for cellular uptake. The authors evaluated the effect of the three CK2 peptides on chondrogenesis, and among these three peptide variants, only CK2.1 exhibited a chondrogenic potential. Specifically, CK2.1-stimulated C3H10T1/2 cells showed an increase in proteoglycan and collagen II synthesis equivalent to that of the BMP-2 positive control, as indicated by Alcian blue and immunostaining. Notably, immunostaining indicated a lower expression of collagen X and osteocalcin hypertrophy markers as compared to BMP-2-treated cells. Interestingly, and in contrast, BMP-2, CK2.2, and CK2.3 all induced mineralization in the C3H10T1/2 cells, but CK2.1 did not. Therefore, CK2.1 appeared to be selective for chondrogenesis, whereas CK2.2 and CK2.3 appeared to be selective for osteogenesis. It is unclear why this contrast among these three peptide variants was observed, given that all three were designed to operate via the same mechanism (i.e., inhibiting CK2 binding to BMPRIa).

For an *in vivo* evaluation with CK2.1, the authors then performed a systemic injection of CK2.1 into the tail vein of C57BL/6J mice that resulted in enhanced articular

cartilage formation around the femurs. Increased articular cartilage formation based on Saf-O/fast green staining and collagen II and IX immunostaining was observed at levels equivalent to a BMP-2 positive control. Furthermore, an increase in expression of the hypertrophy marker collagen X was observed with BMP-2 but not with CK2.1.[43]

In the second study, Akkiraju *et al.*[44] evaluated the performance of CK2.1 when conjugated to hyaluronic acid hydrogel particles (HGP) in a mouse intra-articular injection model. An induced OA-like condition was created by destabilization of the medial meniscus (DMM). Mice receiving intra-articular injections of HGP-CK2.1 following OA showed higher levels of collagen II and IX immunostaining along with low immunostaining of collagen X and osteocalcin as compared to PBS and HGP controls.[44]

CK2.1 appears to be a promising and potentially chondroinductive peptide, although it was employed as a drug as opposed to in a regenerative medicine context. I.e., CK2.1 was delivered as a drug via intra-articular injection in an OA model, as opposed to being conjugated to a material to fill a cartilage defect. Therefore, future studies could perhaps investigate the performance of this CK2.1 peptide in a full articular cartilage defect model as compared to a positive control to fully establish its chondroinductive potential. However, given that the proposed mechanism of action with CK2.1 is intracellular, it is unclear how CK2.1 might fare in such an approach.

HSNGLPL

HSNGLPL was discovered in 2010 by phage display as a peptide sequence with binding affinity to TGF-β1.[46] Shah *et al.*[46] engineered a self-assembling peptide amphiphile (PA) molecule, specifically a TGF-binding PA (TGFBPA) that formed

nanofibers with a high density of TGF-β1 binding epitopes exposed on the surface. The TGFBPA included the HSNGLPL sequence inside of the sequence of HSNGLPLGGGSEEEAAAVVV(K)-CO$(CH_2)_{10}CH_3$. These amphiphilic molecules assembled into hydrogels comprised of an interconnected network of nanofibers in the presence of calcium chloride.

In vitro analysis with human mesenchymal stem cells (we assume from bone marrow) showed that one TGFBPA group exhibited higher aggrecan gene expression levels after 4 weeks of culture relative to a filler control (i.e., similar to TGFBPA except without the HSNGLPLGGGS (the TGF binding domain) of the sequence), but only when cultured with TGF-β1, as no difference was identified between the TGFBPA groups and the filler control group in the absence of TGF-β1. Time points from 2 to 4 weeks for all groups containing TGF-β1 showed similar upregulation over time regardless of whether they were the TGFBPA groups or the filler PA group. As for GAG production, there was no difference observed between the TGFBPA and filler PA groups in the presence of TGF-β1 after 3 weeks. Moving *in vivo* with rabbits, 2 mm diameter, full-thickness chondral defects were created in the femoral trochlear groove and followed by microfracture. In this 12-week study, there were four groups: TGF-β1 alone, the filler PA hydrogel + TGF-β1, the TGFBPA hydrogel + TGF-β1, and the TGFBPA hydrogel alone. There was no sham (i.e., unfilled, microfracture-only) control. Based on gross morphology, collagen II immunostaining, and GAG staining by Saf-O, it appeared the two groups with the TGFBPA hydrogel achieved superior regeneration. Notably, although there was no functional mechanical testing of regenerated cartilage, the TGFBPA hydrogel group

achieved excellent structural regeneration without TGF-β1. In retrospect, a group of the filler PA hydrogel without TGF-β1 would have been a valuable point of comparison.[46]

In 2018, Chen *et al.*[47] incorporated the HSNGLPL peptide at high concentrations into a porous chitosan sponge scaffold via a carbodiimide linker, and investigated the chondrogenic differentiation of porcine bone marrow-derived MSCs *in vitro* and *in vivo*. For the *in vitro* studies, the chitosan sponges were pre-loaded with TGF-β1 for 3 hrs before culturing in TGF-β1-free medium. Scaffolds with the highest peptide concentration, i.e., a chitosan to peptide mass ratio of 10:3, exhibited the highest gene expression levels of SOX9, collagen II, and aggrecan, even outperforming the positive control group (i.e., chitosan and TGF-β1 without peptide). In contrast, collagen X gene expression was not upregulated compared to the chitosan material negative control or positive control. It remains unknown how the HSNGLPL-conjugated chitosan scaffolds would have performed without the preloading of TGF-β1, which would be an appropriate comparison in future investigations.

In vivo, the authors evaluated the chitosan-HSNGLPL (10:3 mass ratio) scaffolds in a rabbit model with 4 mm diameter and 4 mm deep osteochondral defects in the femoral trochlear groove. There were three groups: a negative sham (i.e., empty defect) control, the chitosan scaffold alone, or the 10:3 chitosan-HSNGLPL scaffold. Cartilage regeneration was assessed after 3 and 6 months. The chitosan group alone appeared to have a detrimental effect compared to the sham control, and the addition of the peptide appeared to ‘rescue’ the performance of the chitosan scaffold to put the regeneration more back on par with the negative control. The authors noted that the International

Cartilage Regeneration & Joint Preservation Society (ICRS) scores were higher in the peptide group than in the negative sham control, but these results were not indicated to be statistically significant, perhaps due to the low sample number (i.e., n = 3) or the relative standard deviations. Nevertheless, the Saf-O staining showed a fairly consistent and continuous staining across the surface in the peptide group that was not as apparent in the negative control.

The intriguing question would be how the HSNGLPL peptide, which may have rescued the detrimental performance of chitosan alone, may fare under different circumstances such as conjugation to a different material, or if placed in a more weight-bearing region (e.g., femoral condyles), or if placed in a chondral-only defect instead of osteochondral.

In summary, the HSNGLPL peptide has shown some promise *in vitro* in conjugation to chitosan scaffolds but required a pre-loading of TGF-β1 to achieve this effect and has shown some promise *in vivo* in rabbit trochlear groove defects as a self-assembling hydrogel (2 mm diameter chondral-only defects with microfracture) or via conjugation to chitosan sponges (4 mm diameter osteochondral defects). However, further evaluation will be required before conclusions can be drawn for this TGF-β1-binding peptide. Specifically, the TGFBPA group was not compared to a filler PA control group, so it could not be determined whether the peptide itself was responsible for the quality of regeneration in the absence of TGF-β1. In the chitosan scaffold, the peptide may have helped to recover the detrimental performance of chitosan, but it remains to be seen whether the peptide in another biomaterial or defect type/location would significantly

outperform a negative sham control. Moreover, functional mechanical testing (e.g., indentation stress relaxation), would be a valuable addition to future outcome analyses.[48] Further investigation of this intriguing peptide is warranted in cartilage regeneration.

HAV

His-Ala-Val (HAV) is a conserved sequence in the first extracellular domain (ECD1) of N-cadherin, a transmembrane protein that plays a vital role in cell-cell interactions during mesenchymal condensation, a prerequisite to cartilage formation. Synthetic peptides containing the HAV domain have been shown to possess N-cadherin–like binding activity.[49,50]

In 2013, Bian *et al.*[51] incorporated HAV (as HAVDIGGGC) into a methacrylated hyaluronic acid (HA) hydrogel and evaluated the role of the functionalized hydrogel in the chondrogenic differentiation of encapsulated hBMSCs (we assume from bone marrow). In a 28-day culture period in chondrogenic medium containing TGF-β3, the gene expression of collagen II, aggrecan, and SOX9 in the cadherin peptide group on days 1 and 3 was significantly higher than the control and scrambled groups; however, by day 7, there were no significant differences among the groups. Following 28-day culture with hBMSCs, peptide-functionalized hydrogels possessed higher GAG and collagen content relative to the control and scrambled groups. The stand-alone chondroinductivity of HAV was not evaluated, as the medium for each group contained TGF-β3.

Moving *in vivo* with nude mice, the subcutaneous injection of HAV-functionalized hydrogels along with TGF-β3-loaded microspheres and hBMSCs resulted in a higher content of GAGs and collagen compared to the control and scrambled groups and more

intense staining of collagen II and chondroitin sulfate. The authors concluded that there was an enhancement of early chondrogenesis of BMSCs and cartilage-specific matrix production.[51] We emphasize that the stand-alone chondroinductivity of HAV was not evaluated, but instead its ability to enhance the chondrogenesis of TGF-β3 was evaluated, and indeed the data supported an enhancement.

In 2016, Vega *et al.*[52] used single-cell imaging to demonstrate that the incorporation of the HAV peptide augments the β-catenin recruitment to the cell membrane in hMSCs (we assume from bone marrow), followed by translocation to the nucleus.[52] In a follow-up study by the same group in 2018, Kwon *et al.*[53] investigated the effect of dosage and timing of the HAV peptides in HA hydrogels in the presence of TGF-β3 and showed that the effect of these N-cadherin peptides strongly depended on the dosage. Specifically, a dose-dependent increase in collagen II gene expression was observed during the first 7 days of culture; however, there were no significant differences among groups (including the peptide-free negative control) at 14 days. Following 56 days of culture, increased levels of GAGs and collagen II staining in HAV-containing groups was observed as compared to unfunctionalized HA groups and scrambled peptide groups.[53] As highlighted by the authors, the chondroinductive potential of HAV alone without any additional growth factors is still an area to investigate, given that TGF-β3 was included with all groups.

In 2019, Cimenci *et al.*[54] designed a self-assembling amphiphilic peptide nanofiber system containing the HAV peptide to induce the chondrogenic differentiation of rat BMSCs. The nanofiber systems' activity was evaluated by seeding rat BMSCs on HAV-

nanofibers, non-bioactive nanofibers, or uncoated tissue culture plates (TCP) in a commercially available chondrogenic medium. Saf-O staining and DMMB assay indicated that cells cultured on HAV-nanofibers exhibited abundant GAG accumulation after culture for 14 days compared to cells cultured on non-bioactive nanofibers, and TCP. Gene expression analysis indicated an increase in collagen II, aggrecan, and SOX-9 expression on days 3, 7, and 14 in cells cultured on HAV-nanofibers compared to cells cultured on non-bioactive nanofibers and TCP. The authors concluded that the HAV peptide and nanofiber system facilitated chondrogenesis.[54]

In 2020, Feng *et al.*[55] fabricated an HAV-conjugated, aggrecanase-1 cleavable hydrogel and evaluated its regenerative potential in a rabbit osteochondral defect model. HAVDIGGGC peptide was conjugated into a hyperbranched PEG-based multi-acrylate polymer (HBPEG) and mixed with aggrecanase-1 cleavable peptide (ACpep) and cysteamine-modified chondroitin sulfate (CS) to form (HAV-HBPEG)-CS-ACpep hydrogels. Rabbit BMSC-encapsulated hydrogels were evaluated in a rabbit model with 4 mm diameter and 4 mm deep osteochondral defects in the patellar groove. There were three groups: a negative sham control, the (HAV-HBPEG)-CS-ACpep with rabbit BMSCs, or HBPEG-CS-ACpep (with no HAV) with rabbit BMSCs. The repair potential of the hydrogels was evaluated after 12 weeks and 18 weeks. Based on the gross morphological view, μ-CT imaging, and histological analysis the subchondral bone was partially repaired after 12 weeks and fully repaired after 18 weeks in all three groups, with higher bone volume and mineral content in the HAV-conjugated hydrogel group. As for the cartilage layer, immunostaining indicated that after 12 weeks, the HBPEG-CS-

ACpep/BMSCs hydrogel group exhibited more collagen II deposition than collagen I and more intense GAG staining by Saf-O, and periodic acid-Schiff (PAS) staining, as compared to the sham and HAV-conjugated hydrogel group. Interestingly, after 18 weeks the HAV-conjugated group exhibited intense Saf-O and PAS staining, indicating abundant deposition of GAGs and glycoproteins, in addition to intense collagen II immunostaining with no collagen I immunostaining as compared to sham and HAV-free groups.[55] The presence of aggrecanase 1 cleavable peptide and HAV peptide appeared to have had a synergistic effect to enhance cartilage regeneration. It would have been interesting to evaluate the hydrogel's regenerative potential in the presence and absence of each of the components, i.e., CS, HAV peptide, and aggrecanase 1 degradable peptide.

In 2021, Mohammed *et al.*[56] designed self-assembling hydrogels with tunable mechanical stiffness based on five different peptide sequences that mimicked the HAV motif with fluorenylmethyloxycarbonyl (Fmoc) as an aromatic assembling group into fibrillar structures. The five peptide sequences were Fmoc-GGHAV, Fmoc-GGHAVD, Fmoc-GGHAVS, Fmoc-GGHAVDI, and Fmoc-GGHAGDI. The chondroinductive potential of the soluble peptides and self-assembled peptide hydrogels was evaluated with hMSCs-3A6 cells. After 21 days in culture, the Fmoc-GGHAVDI peptide solution group exhibited the highest gene expression of collagen II compared to other peptide solutions and chondroinductive medium (containing TGF-β1). Aggrecan gene expression was equivalent between all peptide groups; SOX-9 gene expression was mainly increased with chondroinductive media alone and with Fmoc-GGHAVDI. However, when hMSCs-3A6 cells were encapsulated in the peptide hydrogels, the Fmoc-GGHAVS with greater

stiffness resulted in more intense Alcian blue staining indicating increased GAG deposition, and a significant increase in collagen II /collagen I ratio as compared to other peptide hydrogels.

In 2021, Teng *et al.*[57] synthesized functionalized a methacrylated hyaluronic acid hydrogel (MeHA) as a matrix for hBMSC chondrogenesis. Six hydrogel groups were synthesized: MeHA alone as a control (HA), MeHA+RGD (RHA), MeHA+HAV (HHA), MeHA+RGD+HAV (HR), HR loaded with kartogenin (K@HR), and HR hydrogel with KGN encapsulated in PLGA microspheres (K@PM-HR). Following 14 days of culture in chondrogenic medium, gene expression analysis indicated that the HR group significantly upregulated the gene expression of aggrecan, collagen II, and SOX-9, compared to MeHA alone and MeHA functionalized with a single peptide. The kartogenin groups (K@HR and K@PM-HR) resulted in a significant increase in gene expression of aggrecan, collagen II, and SOX-9. As for matrix synthesis, a significant increase in GAG content (DMMB assay) and collagen II content (hydroxyproline assay) was observed with all RGD functionalized groups as compared to HA and HHA. At 28 days, HR hydrogels significantly upregulated the gene expression of aggrecan and collagen II as compared to HA and HHA hydrogels. Interestingly, the K@PM-HR group resulted in the highest gene expression of aggrecan, collagen II, Sox-9, and collagen X. The GAG and collagen contents in all RGD functionalized groups were significantly higher than HA and HHA groups.

Transitioning to *in vivo,* the subcutaneous injection of HA, HR, K@HR, or K@PM-HR in nude mice showed after 56 days that the GAG (DMMB assay) and collagen

contents (hydroxyproline assay) were significantly higher in the kartogenin groups as compared to the HA and HR groups with the highest collagen content being for K@PM-HR. Additionally, a significant increase in GAG and collagen content was observed with the HR group as compared to the MeHA control group.

In summary, the HAV peptide was shown to be promising *in vitro*; however, it has been evaluated in the presence of TGF-β3 or TGF-β1, so it remains unknown whether the peptide alone is capable of chondroinduction. *In vivo,* the subcutaneous studies in nude mice suggested that the HAV peptide has enhanced chondroinduction in the presence of TGF-β3 or kartogenin. In the rabbit osteochondral defect study, the HAV peptide appeared to be promising when combined with aggrecanase 1 cleavable hydrogels. Additional evaluation will be required to determine whether the peptide alone or in combination with other biomaterials would be chondroinductive. Based on the studies performed so far, the major application of the HAV peptide is to facilitate chondrogenesis by enhancing cell-cell interactions.

Cytomodulins-(CM)

Cytomodulins (CMs) are TGF-β1 mimicking peptides that have been reported to exhibit TGF-β1-like activity by enhancing the expression of collagen I and improving the wound healing effect of fibroblasts.[58] The original source of these “cytomodulins” can be traced back to patents from the late 1990s,[59,60] which perhaps were meant to mimic a β bend in the protein, as the sequences of LIANAK (a.k.a. cytomodulin 10 or CM-10) and ANVAENA (a.k.a. cytomodulin 1, or CM-1) do not actually appear in TGF-β. In 2015, Zhang *et al.*[61] conjugated the CM-10 (LIANAK) peptide to functional nanofibrous hollow

microspheres (FNF-HMS), which were leveraged as a delivery system for the CM-10 peptide. The microspheres were made from a poly(L-lactic acid) (PLLA)-based block copolymer. 3-week culture of CM-10-functionalized microspheres with rabbit BMSCs resulted in positive Saf-O staining, and the appearance of round cells encased in lacunae, suggesting chondrogenic differentiation of rabbit BMSCs as compared to a material-only control and in the absence of additional growth factors. Moving then to a 2-week *in vivo* study, the subcutaneous injection of CM-10-functionalized microspheres in mice, along with rabbit BMSCs, resulted in ectopic cartilage-like tissue formation as demonstrated by intense Saf-O staining of GAGs and collagen II immunostaining, with no apparent mineralization, as compared to no cartilage formation in the material control.[61]

In 2019, Park *et al.*[62] used a click-crosslinked hyaluronic acid (Cx-HA) hydrogel as a scaffold, and physically loaded or covalently linked CM-10 to the hydrogel and tested the chondrogenic potential of both on human periodontal ligament stem cells (hPLSCs). *In vitro* culture of hPLSCs for 4 weeks with soluble CM-10 or TGF-β (type 1/2/3 not specified) chondrogenic medium showed increased staining of collagen II and GAGs as compared to the peptide-free medium. Furthermore, hPLSCs cultured for 4 weeks with soluble CM-10 exhibited an increased gene expression of SOX9, aggrecan, and collagen II at a comparable fold increase to a positive control TGF-β group. Transitioning to an *in vivo* study, hPLSCs and hydrogels were subcutaneously injected in mice, with three groups being the HA hydrogel alone, with CM physically entrapped, or with the CM conjugated. There was no TGF-β positive control for this *in vivo* study. The CM was much more effective when conjugated than when included in soluble form. Specifically,

increased staining of collagen II and GAGs and increased gene expression of SOX9, aggrecan, and collagen II, was observed for the covalently-linked CM group relative to the physically loaded CM and peptide-free hydrogels. Worth-mentioning is that Park *et al.*[62] refer to the peptide as cytomodulin-2; however, the sequence of the peptide provided in the paper is LIANAK which is that of CM-10, and CM-2 is LIAEAK as per Lam *et al.*[58] In summary, the TGF-β mimicking peptide CM-10 demonstrated upregulation of chondrogenic markers both *in vitro* and *in vivo* (subcutaneous injection in mice) relative to negative controls. However, comparison to a TGF-β positive control was generally absent with the exception of one *in vitro* study that demonstrated chondrogenesis comparable to TGF-β. The CM-10 peptide may be a promising peptide that warrants investigation in an *in vivo* cartilage defect study.

B2A

B2A (a.k.a. B2A2-K-NS) is a synthetic multidomain peptide ((H-AISMLYLDENEKVVLKK(H-AISMLYLDENEKVVLK)-Ahx-Ahx-AhxRKRLDRIAR-NH2) that was recognized as a BMP-2 receptor modulator by Lin *et al.*[63] in 2005. The B2A design includes a heparin-binding domain (RKRKLERIAR), a hydrophobic domain, and a receptor-targeted domain (AISMLYLDENEKVVL), with binding to both type I and II receptors, with selectivity for BMPR-Ib. B2A was found to enhance BMP-2 activity *in vitro* synergistically and was hypothesized that it might improve bone repair. Knowing the role of the BMP pathway in chondrogenesis, Lin *et al.*[64] evaluated the chondrogenic potential of B2A peptide *in vitro* and *in vivo*. Polymerase chain reaction (PCR) array analysis of B2A-treated murine multipotential embryonic stem cell line C3H10T1/2 cells showed

significant upregulation of fibroblast growth factor receptors 1 and 2 (Fgfr1, Fgfr2) and moderate upregulation of Fgf1 genes. The upregulation of matrix genes (collagen I and II), and Smad1, Smad4, and Twist1 genes was additionally observed compared to the peptide-free group.

Furthermore, Alcian blue and collagen II staining indicated that B2A stimulated the production of GAGs and collagen II in hBMSC and human chondrocytes (passage number not identified) in micromass culture. Transitioning to an *in vivo* rat model, the authors evaluated the activity of B2A by performing a pilot study in a chemically-induced OA model. Following induction of OA, rats were injected intra-articularly with either saline or 500 ng of B2A. There appeared to be a significant repair of articular cartilage in the B2A-treated group vs. saline group based on H&E and Saf-O staining.[64]

In summary, it appears there may be some chondroinductive effect with B2A *in vitro*, and some chondroprotective effect in a rat OA model, but it remains to be seen whether B2A would elicit a chondroinductive effect in a cartilage defect model.

SPPEPS

In 2019, we identified the SPPEPS peptide as a similar sequence between two chondroinductive molecules, aggrecan and the TGF-β3 pro-protein.[65] The chondroinductive potential of SPPEPS was assessed with rat BMSCs, and it was determined that the soluble peptide at 100 ng/mL increased the expression of collagen II as compared to the negative control with negligible cytotoxic effects. In addition, proteomic analysis revealed that after 7 days in culture, the insulin signaling pathways were activated through the GSK-3β gene, which is involved in the maintenance of

chondrocyte phenotype and cartilage extracellular matrix, in both SPPEPS and positive control groups. In addition, collagen II expression was increased when rat BMSCs were cultured on the surface of pentenoate-functionalized hyaluronic acid (PHA) hydrogels when a combination of both SPPEPS and the adhesion peptide RGD was provided, relative to either peptide alone. The SPPEPS peptide may be a promising peptide; however, additional studies are required to confirm its chondroinductive potential.[65]

Link N (DHLSDNYTLDHDRAIH)

Link N peptide was first discovered in 1993 by Martin and Dean[66] by enzymatic cleavage of the link protein, and it was hypothesized that Link N might play a role in proteoglycan synthesis regulation. In 2003, Mwale *et al.*[67] reported that Link N peptide stimulated the production of proteoglycans, collagen II, and IX in intervertebral (IVD) pellet-cultured cells. Similarly, Wang *et al.*[68] investigated the effect of Link N peptide in an *ex vivo* 3D culture of rabbit IVD cells. Real-time PCR, ELISA, and western blotting confirmed that Link N protein significantly upregulated the gene expression and synthesis of SOX9, aggrecan, and collagen II as compared to a negative control and a scrambled peptide group. The authors showed that Link N peptide interacted with BMP-RII receptor and significantly increased the protein production of BMP-4 and BMP-7, but not BMP-2 or BMP-6.

In 2018, He *et al.*[69] studied the effects of Link N protein on the proliferation and chondrogenic differentiation of rat cartilage stem/progenitor cells (CSPCs). *In vitro*, the 2D culture of CSPCs with increased concentrations of Link N protein (0-500ng/mL) resulted in increased gene expression of SOX9, collagen II, and aggrecan, with no

increase in Runx2 or collagen X expression as compared to peptide-free groups. 3D pellet culture showed that Link N stimulated the chondrogenic differentiation of CSPCs, based on collagen II and SOX9 immunostaining; however, the best performance was for the peptide + TGF-β3 group as compared to peptide-free and peptide groups.

GFOGER

The GFOGERGVEGPOGPA peptide was identified in 1998 as a sequence of residues 502-507 of the α1 collagen chain, located in the α1(I)CB3 fragment, based on its high binding affinity to α2β1 integrin.[70] In 2000, Knight *et al.*[71] determined that the actual recognition site is mainly in the sequence GFOGER and that this peptide sequence signifies a high-affinity binding site in collagen I and IV for α2β1 integrin, and in collagen I for α1β1 integrin.

In 2010, Liu *et al.*[72] incorporated a collagen mimetic peptide (CMP) containing GFOGER into a PEG hydrogel and evaluated its chondroinductive potential with encapsulated hBMSCs. In the presence of TGF-β3, peptide-containing hydrogels promoted the chondrogenesis of hBMSCs indicated by the enhanced staining of GAG, collagen II, and aggrecan as compared to peptide-free hydrogels. Gene expression analysis showed an upregulated of SOX9 and downregulation of collagen X. Based on the *in vitro* data, the authors concluded that the presence of GFOGER induced chondrogenic activity in the presence of TGF-β3 and prevented or delayed hypertrophy.

In 2014, Mhanna *et al.*[73] incorporated GFOGER and MMP-sensitive motifs into PEG hydrogels, creating a functionalized degradable hydrogel, and compared the chondroinductive potential of this hydrogel with RGD-functionalized hydrogels and MMP-

free (non-degradable) hydrogels. In the presence of TGF-β3, GFOGER-modified degradable gels provided the highest hBMSCs proliferation rate, as compared to peptide-free gels and RGD-gels. GAG and DNA content was higher but not statistically significant in the GFOGER-modified degradable gels as compared to peptide-free and RGD hydrogels. Gene expression of collagen II was highest in GFOGER-modified degradable gels as compared to peptide-free gels.

Worth mentioning is that the GFOGER was used as a PCL scaffold coating to enhance bone formation.[74] Reyes *et al.*[75] coated titanium surfaces with GFOGER to promote α2β1 integrin binding and found that the presence of GFOGER triggered osteoblastic differentiation and mineral deposition in bone marrow stromal cells in an osteogenic medium, as compared to peptide-free titanium. Recently, Clark *et al.*[76] found that GFOGER-functionalized hydrogels based on 4-arm PEG macromers with terminal maleimide groups (PEG-4MAL) hydrogels prolonged hBMSC survival and improved bone repair in a mouse model.

In summary, GFOGER appears to elicit a favorable response, but since both bone and cartilage regeneration studies report favorable outcomes, the real question is how it may perform *in vivo* and whether osteogenesis versus chondrogenesis would be favored.

KIPKASSVPTELSAISTLYL

KIPKASSVPTELSAISTLYL was identified in 2003[77] as a potential candidate to improve bone formation, and it represents residues 73-92 of BMP-2's knuckle epitope. In 2012, Renner *et al.*[78] evaluated the effect of KIPKASSVPTELSAISTLYL on hMSCs (we assume from bone marrow), and observed an increased production of GAGs relative to

the negative control and at a value comparable to the BMP-2 positive control; however, when added with TGF-β3, this BMP-2-inspired peptide did not increase GAG production. A hydroxyproline assay showed a significant increase in total collagen with the KIPKASSVPTELSAISTLYL-treated cells but at levels lower than the BMP-2 positive control. Interestingly, KIPKASSVPTELSAISTLYL did not increase the alkaline phosphatase (AP) activity or collagen I deposition as compared to BMP-2 treated cells. Gene expression analysis showed an upregulation of aggrecan, COMP, and collagen II as compared to the negative control yet a reduced effect as compared to BMP-2 and TGF-β3. In 2013, a follow-up paper from the same group confirmed that KIPKASSVPTELSAISTLYL stimulated GAG production in hBMSCs to levels comparable to the positive control and in a full-factorial experiment, KIPKASSVPTELSAISTLYL stimulated GAG production without the need for other peptides or growth factors.[79]

RYPISRPRKR and YKTNFRRYYRF

RYPISRPRKR (termed “HAbind” by the authors) is a peptide derived from the HA-binding region of link protein. Link protein stabilizes the interaction between hyaluronic acid and the core protein of individual aggrecan molecules to form large aggrecan complexes in articular cartilage.[80,81] YKTNFRRYYRF (termed “CSbind”) was discovered by peptide array screening as a sequence that binds chondroitin 6-sulfate (C6S) and it was found to block the inhibitory activity of C6S on neurite outgrowth.[82]

In 2015, Parmar *et al.*[83] synthesized a biodegradable hydrogel from recombinant streptococcal collagen-like 2 (Scl2) proteins, functionalized with HAbind and CSbind peptides, and crosslinked with matrix metalloproteinase 7 (MMP7)-sensitive peptide. The

authors evaluated the chondrogenic differentiation of encapsulated human BMSCs, and in the presence of TGF-β3, both HAbind-MMP7-Scl2 and CSbind-MMP7-Scl2 hydrogels enhanced the gene expression of collagen II, aggrecan, and SOX9 in hBMSCs, with the highest gene upregulation of chondrogenic markers being for HAbind-MMP7-Scl2 at 10% functionalization as compared to unfunctionalized hydrogels. Collagen I and X gene expression was significantly lower in HAbind-MMP7-Scl2 and CSbind-MMP7-Scl2 as compared to unfunctionalized hydrogels. Furthermore, biochemical assays indicated that total collagen, GAG, and DNA content were highest for the HAbind (10%)-MMP7-Scl2 hydrogels as compared to all other groups.

GRVDWLQRNANFYDWFVAELG (insulin peptide)

GRVDWLQRNANFYDWFVAELG peptide was created by phage display and recognized by its affinity for insulin receptor and thought to mimic IGF-1.[84] In 2013, Renner and Liu.[79] investigated the chondrogenic effect of the insulin peptide in soluble form on human MSCs (we assume from bone marrow). In the presence of insulin and TGF-β3, the insulin peptide treatment resulted in an increased production of GAG as compared to the TGF-β3 positive control. Interestingly, the insulin peptide was combined with the TGF-β1-inspired peptide (ANVAENA) to mimic the synergy observed between the insulin peptide and full-length TGF-β3 protein; however, the presence of both peptides resulted in a decrease in GAG production as compared to having one peptide only. The authors hypothesized that this effect might have been due to a change in experimental conditions (culture time, passage number, rBMSCs expansion conditions), or it may be that the TGF-β1 peptide acted on different pathways than TGF-β3.

Other peptides

Several peptides have been reported to be used for cartilage regeneration, which are summarized in Tables 4 (*in vivo* studies) and 5 (*in vitro* studies). Among these peptides are self-assembling peptides such as KLD[85–90] and RADA[86,91] (commercially available as PuraMatrix™), which may provide chondro-supportive scaffolds, but were not designed specifically for chondroinduction. *In vivo* evaluation of KLD peptide in a full thickness cartilage defect rabbit model[85] showed that the KLD hydrogel alone resulted in significantly more intense Saf-O staining and collagen type II immunostaining as compared to KLD hydrogels containing chondrogenic factors or BMSCs. However, a later study performed in a 15-mm-diameter cartilage defect equine model[89] showed that KLD treatment improved the clinical outcome and filling. However, there were decreased levels of aggrecan and collagen type II, resulting in a poor repair tissue quality compared to MF.[89]

E7 peptide (EPLQLKM), identified in 2012,[92] is a strong MSC affinity peptide candidate. A biphasic scaffold made of demineralized bone matrix (DBM) and chitosan (CS) hydrogel was functionalized with E7 and implanted in an osteochondral defect rabbit model in combination with MF.[93] The E7-functionalized scaffolds resulted in superior cartilage regeneration with no signs of hypertrophy relative to MF and unfunctionalized control scaffolds.[93] In another study by the same group,[94] DBM particles were functionalized with E7 and combined with CS hydrogel (DBM-E7/CS). DBM-E7/CS scaffold implanted into the fossa iliaca subcutaneous region of athymic nude mice for 4

weeks resulted in the formation of a translucent cartilage-like tissue superior to that generated by material control groups (i.e., CS and DBM/CS).

WYRGRL, a collagen II affinity peptide, and KLER, a decorin-derived peptide, were evaluated for their chondroinductive potential *in vitro* and *in vivo* through subcutaneous injection in a mouse model.[95] These peptides were not found to be attractive for collagen II deposition. KLER was additionally assessed in 2009[96] and was found to promote chondrogenesis of hBMSCs in chondrogenic medium (+TGF-β1) when combined with RGD in a poly(ethylene glycol) (PEG) scaffold, as compared to control medium and scrambled peptide group. However, no chondroinductive activity was observed in the absence of TGF-β1.

ANVAENA, like CM-10 (reviewed above) is a TGF-β1-inspired peptide. ANVAENA was evaluated for its chondroinductive potential with hBMSCs in its soluble form, but unlike CM-10, it was found to decrease the synthesis of GAGs as compared to a negative control.[79]

Additionally, GTPGPQGIAGQRGVV (termed P15 by the authors) is a collagen I inspired peptide that was found to enhance the commitment of C3H10T1/2 cells toward the chondrogenic lineage in the presence of TGF-β.[97] GPPDWHWKAMTH peptide (termed R1-P1 by the authors) was inspired from FGFR1 and was found to exhibit chondroprotective effects.[98] CDPGYIGSR-modified scaffolds inspired by laminin supported the proliferation of bovine knee chondrocytes (BKCs) and increased GAG and collagen content.[99] Interestingly, a lot of progress has been made in the past few years with several laminin-mimetic peptides including YIGSR to modulate the behavior of the

nucleus pulposus (NP) of the intervertebral disc (IVD) in terms of cell attachment, morphology, signaling, and phenotype.[100–102] However, to the best of our knowledge, no additional studies reported the chondroinductive potential of these peptides *in vitro* with BMSCs or *in vivo* in a cartilage defect model in the absence of exogenous growth factors.

In addition to the previously discussed peptides, we highlight two additional peptides, TPX-100 and Engedi1000, which are currently in clinical trials as DMOADs. TPX-100 is a 23-amino acid peptide derived from matrix extracellular phosphoglycoprotein (MEPE) that has been evaluated in clinical studies as a DMOAD.[103–105] Based on a published abstract,[106] intra-articular injections of TPX-100 in goats resulted in hyaline cartilage formation. The abstract mentioned that based on the performed clinical trials, TPX-100 injections were safe, well-tolerated, and improved knee function.[106]

Engedi1000 (E1K) is a synthetic peptide from Ensol Biosciences Inc. (Daejeon, South Korea), which is currently in phase 1 clinical studies at Seoul National University Hospital.[107] The manufacturers claim that E1K blocks the Smad1/5/8 pathway, which promotes degeneration of cartilage tissue, through blocking TGF-β1, and maintains the Smad2/3 pathway that induces cartilage tissue regeneration. However, we have been unable to locate peer-reviewed publications evaluating the activity of TPX-100 and E1K peptides in full cartilage defect models.

Discussion

Among the peptides discovered to date, there exists great potential for synthetic peptides to induce the chondrogenic differentiation of stem cells. It is no surprise that the search for chondroinductive peptides has commonly been inspired by proteins that are

known to play a role in chondrogenesis. TGF-β1 or -β3 are widely used to induce chondrogenesis *in vitro* and are the most targeted growth factors with four peptides reported: HSNGLPL, CM-10, and ANVAENA from TGF-β1 and SPPEPS from both TGF-β3 and aggrecan. HSNGLPL, which is a TGF-β1 affinity peptide, seems promising based on the *in vivo* cartilage studies done in the absence of any exogenous growth factors. CM-10 looks promising as well; however, it has not yet been tested in a full articular cartilage defect model *in vivo*. ANVAENA did not appear to be chondroinductive based on the studies done so far.

BMP-2 is known to induce chondrogenesis and osteogenesis, and there are three peptides reported to be inspired by BMP-2, which are B2A, KIPKASSVPTELSAISTLYL, and CK2.1. Both B2A and KIPKASSVPTELSAISTLYL were initially identified for their ability to enhance bone formation, and their chondrogenic potential was later assessed. While there are no reported chondroinductive studies done *in vivo* for KIPKASSVPTELSAISTLYL, this peptide did show an advantage in comparison to BMP-2 *in vitro* as it did not lead to an increase of alkaline phosphatase (AP) activity or collagen I synthesis. B2A was tested *in vivo* in a chemically induced OA model; however, there are no reported studies in a full osteochondral defect model so far. CK2.1 is the most recently investigated peptide, and as compared to BMP-2, this peptide enhanced articular cartilage formation with no increase in collagen X expression following injection; however, CK2.1 assessment *in vivo* was done in a DMM model only. Additional investigation is necessary to fully identify the regenerative potential of these BMP-2-inspired peptides and to decipher their mechanisms of action.

Overall, there are a limited number of *in vivo* studies, and predominantly these are studies where the peptide was delivered by injection, either systemically, intra-articularly, or subcutaneously. Of the peptides designed to be chondroinductive, only two have been implanted in a cartilage defect, which are HSNGLPL and HAVDI (**Figure 2.2**). The call to action for the regenerative medicine community is therefore to evaluate chondroinductive peptides in well-controlled cartilage defect studies *in vivo*.

While significant progress has been made in terms of cell therapy, scaffolds, and growth factors in the regenerative medicine community, pharmaceutical companies have been in a race to provide the first human-use approved DMOAD. However, the ultimate breakthrough that can change the current standard of care (microdrilling) would be to develop a cost-effective synthetic biomaterial that would regenerate true hyaline cartilage without the addition of any exogenous cells or growth factors. Having clinical translation in mind, such a biomaterial will be advantageous in terms of safety, cost, and regulatory approvals. Furthermore, the discovery of synthetic chondroinductive materials would potentially enhance currently existing treatments (e.g., MACI, MF) or set the stage for a completely new treatment approach.

Synthetic chondroinductive agents are mainly small molecules or peptides. Small molecules are mainly evaluated as DMOADs and are usually administered via intra-articular injections, which can range from one to several injections over the course of several weeks depending on the drug, which might be a limiting factor. On the other hand, peptides evaluated for cartilage regeneration fall into two categories. In the 1st category are peptides that are not designed to induce chondrogenesis, and in the 2nd category are

peptides that are designed to induce chondrogenesis. Category 1 includes peptides that have yet to exhibit evidence of chondroinduction in the absence of a growth factor (e.g., WYRGRL, KLER, ANVAENA) and potential chondroprotective peptides (e.g., CDPGYIGSR). The first category additionally comprises promising self-assembling peptides that are themselves scaffolds (e.g., RADA, KLD) or MSC affinity peptides (e.g., E7). *In vitro* studies in this category did not include a positive control (except ANVAENA), and most of them relied on TGF-β to induce chondrogenesis. As for *in vivo* studies, only E7, RADA, and KLD were evaluated in a full-thickness cartilage defect, but no positive control was included in those studies.

The second category includes peptides focused on chondrogenesis, most peptides were evaluated *in vitro* only, and most had TGF-β added to the culture medium (e.g., HSNGLPL, GFOGER, RYPISRPRKR, YKTNFRRYYRF, GRVDWLQRNANFYDWFVAELG) or lacked a representative positive control (e.g., SPPEPS, CK2.1, B2A). *In vivo*, and for the exception of HSNGLPL and HAVDI all remaining peptides (i.e., CK2.1, CM-10, B2A) were evaluated via subcutaneous or intra-articular injection. Therefore, while these peptides seem promising, performing additional well-controlled cartilage defect studies *in vivo* is necessary to assess their chondroinductive potential.

Peptides represent promising synthetic chondroinductive agents that can be reproducibly and inexpensively produced and would allow the design of a synthetic chondroinductive material that can induce true hyaline cartilage regeneration. The main target for synthetic agents in cartilage regeneration is to mimic the protein-protein

interactions between growth factors, cells, and/or ECM components. Bearing the common wide surface area of the protein-protein interactions, peptides are more likely to achieve this goal by covering the surface area with high specificity. Such specificity reduces the side effects of the peptide therapeutics, as they are not likely to bind to the proteins other than their targets, compared to small drugs with promiscuous binding affinity. Peptides are advantageous in biocompatibility, as they are biodegradable into amino acids, and do not accumulate in the body.[108] Additionally, peptides can be easily modified with different functional groups without complex chemistry, and various peptide chains can be combined such as adding a cell penetrating sequence to a chondrogenic peptide sequence to enable intracellular delivery. Peptides can be conjugated to natural and synthetic polymers to form ECM-like scaffolds to trigger pathways associated with chondroinduction by binding to the appropriate surface receptor. Importantly, peptides allow the synthesis of a biomaterial device that is *itself* chondroinductive. Engineering of peptide-based therapeutics provides new and exciting opportunities that alternative chondroinductive agents cannot provide.

Conclusion

Chondroinductive peptides are a promising tool to design and engineer scalable synthetic chondroinductive biomaterials. We recommend further investigation of such peptide-modified biomaterials in well-designed cartilage defect models without the addition of any growth factors to evaluate chondroinduction *in vivo*. The cell biology of hyaline cartilage chondrocytes involves several interconnected pro-chondrogenic and anti-chondrogenic signaling pathways that interplay to maintain the hyaline phenotype;

therefore, several peptide candidates are yet to be evaluated and synergistic activities among different peptides are yet to be investigated. We additionally advocate for the continued discovery of new peptides, and for rigorously designed *in vivo* studies with appropriate positive and negative controls to evaluate their efficacy.

Chapter 3: Equine Comparison of the Chondrogenic Potential of eBMSCs and eUCMSCs in Response to Selected Peptides and Compounds[2]

Abstract

Background: Cartilage injuries pose significant challenges in horses and often lead to post-traumatic osteoarthritis (PTOA). Despite the advances in surgical and regenerative techniques, the result in most cases is the formation of a fibrocartilage repair tissue. Cell-based cartilage therapies are mainly focused on equine bone marrow-derived mesenchymal stem cells (eBMSCs) as they are easily accessible, and multipotent. Nonetheless, alternative allogeneic sources, for example equine umbilical cord matrix mesenchymal stromal cells (eUCMSCs), hold promise given their non-invasive and readily accessible nature. Considerable research has been dedicated to exploring chondroinductive factors (e.g., peptides and small compounds), aiming to replace growth factors for inducing chondrogenesis. However, these factors have not yet translated to the equine community. Therefore, in the current study, we selected from the literature two promising peptides, CM10 and CK2.1, and two promising compounds, kartogenin and SM04690, and assessed their chondroinductive potential with both eBMSCs and eUCMSCs. In addition, the chondroinductive potential of eBMSCs was evaluated in

[2] Boushra Ajeeb, Emi A. Kiyotake, Peggy A. Keefe, Nikki Phillips, Jennifer Hatzel, Laurie Goodrich, Michael S. Detamore. *BMC Veterinary Research*. To be submitted, 2024.

monolayer and spheroid culture in both hypoxia and normoxia in response to dexamethasone and/or TGF-β3.

Results: Following 21 days of culture, none of the evaluated chondrogenic factors resulted in a higher gene expression of chondrogenic markers compared to the positive or negative controls with eBMSCs or eUCMSCs. Interestingly, spheroid culture in hypoxia with dexamethasone treatment (without TGF-β or any compound or peptide) was sufficient to induce the chondrogenic differentiation of eBMSCs.

Conclusion: Based on cell response to the positive control, eBMSCs may be preferred over eUCMSCs for chondrogenesis. The current study therefore supports the use of spheroid culture, and the use of dexamethasone over TGF-β or any of the compounds or peptides tested here from the prior literature to drive chondrogenesis with eBMSCs.

Introduction

Equine musculoskeletal diseases are the most common debilitating diseases in horses, with osteoarthritis representing the most prevalent condition.[109,110] Currently used treatments include anti-inflammatory drugs, which are mainly nonsteroidal anti-inflammatory drugs (NSAIDS)[111,112] (intravenous) or corticosteroids (intraarticular).[112] Other treatments include intraarticular injections of hyaluronic acid or polysulfated polysaccharides.[112] Regenerative therapies have become more common in the past few decades and are referred to as orthobiologics,[112,113] and include autologous-conditioned serum,[114,115] autologous-protein solution,[116–118] platelet-rich plasma,[119–121] and mesenchymal stem cells (MSCs).[122–125] Surgical interventions[112,126–128] include bone-

marrow stimulation,[129] autologous chondrocyte implantation (ACI),[130] and osteochondral grafting.[131] In many cases, the combination of surgical techniques and intraarticular therapies provide a better outcome.[112]

In cell therapy, autologous equine bone marrow-derived stem cells (eBMSCs) are the primary cell type used for the treatment of equine musculoskeletal diseases in horses. However, in the past decade, the use of *allogeneic* eBMSCs increased because these allogeneic cells offer the value of an off-the-shelf therapy.[123] Given the limited supply of allogeneic eBMSCs that have been pre-screened for potential efficacy, there has been an interest in investigating the chondroinductive potential of other allogeneic cell sources, of which umbilical cord blood mesenchymal stem cells (UCB-MSCs) and umbilical cord matrix mesenchymal stromal cells (UCMSCs) represent attractive options that are readily available, do not require invasive procedures to collect, and have possible immunomodulatory potential.[132–134] While most of the literature on equine umbilical cord cells focuses on eUCB-MSCs, a few studies have evaluated the chondroinductive potential of eUCMSCs. For example, in 2011, Lovati *et al.*[134] compared the chondroinductive potential of eUCMSCs, equine amniotic fluid mesenchymal stem cells (eAF-MSCs), or eBMSCs in pellet culture (500,000 cells/pellet) in response to TGF-β1, and found that eUCMSCs showed limited chondroinductive potential compared to the other cell sources based on histological staining and biochemical assays. In a more recent (2018) example, Rakic *et al.*[133] evaluated the chondroinductive potential of eUCMSCs and eUCB-MSCs in response to BMP-2 with TGF-β1 in 3D on a collagen scaffold and found that eUCMSCs had a limited chondroinductive potential as compared

to eUCB-MSCs. Our previous work with human UCMSCs demonstrated some potential for chondrogenesis,[135–137] providing motivation to revisit UCMSCs from an equine source with new chondroinductive signals.

Peptides and small compounds have recently gained attention for cartilage regeneration.[32,138] Chondroinductive compounds could be used to enhance cell therapy or to precondition MSCs prior to intraarticular injection. Among the evaluated compounds in the literature, kartogenin[139–141] and SM04690[34,35] have shown some promise for chondrogenic differentiation of BMSCs. As for peptides, CM10[58,61,79] and CK2.1[43–45] appear to be promising candidates for the chondrogenic differentiation of BMSCs.[138] A previous study from our team showed no evidence of chondroinduction with human BMSCs[142] with kartogenin, SM04690, CM10, or CK2.1; however, as of February 2024, there are no published studies that have evaluated the chondroinductive potential of the aforementioned peptides or compounds with equine MSCs.

Hypoxia has been reported to enhance the chondrogenic differentiation of human BMSCs.[143,144] With equine MSCs, only one study evaluated eBMSCs in hypoxia macropellets (500,000 cells/pellet)[145] and another study evaluated eUCMSCs in hypoxia in a collagen scaffold.[133] Both studies found hypoxia and normoxia to be equivalent in terms of the gene expression of chondrogenic markers. More studies evaluating hypoxia versus normoxia for chondrogenesis with equine cells are needed, especially given that the native cartilage environment is under hypoxia, therefore, we evaluated both conditions in this study.

In the current study, we evaluated and compared the chondroinductive potential of kartogenin, SM04690, CM10, and CK2.1 to a TGF-β3 positive control with eUCMSCs and eBMSCs in hypoxia in spheroid culture. Additionally, knowing that BMSCs from different species respond differently to culture conditions and growth factors, we compared the chondroinductive potential of eBMSCs in monolayer and spheroid culture, in response to dexamethasone with or without TGF-β3 in both hypoxia and normoxia. Our hypotheses were that eUCMSCs would have chondroinductive potential comparable to eBMSCs, and that at least one of the peptides or compounds would induce the chondrogenic differentiation of eBMSCs and eUCMSCs. Additionally, we hypothesized that dexamethasone would induce the chondrogenic differentiation of eBMSCs in the absence of TGF-β3.

Methods

Cell culture

Umbilical cord tissue and bone marrow harvest: Upon normal parturition, umbilical cord samples were collected through a sterile process. In that process, approximately 12 inches from the fetal side were removed, while the majority of membranes were retained within the mare prior to natural expulsion. The umbilical cords were washed thoroughly with PBS, and then placed into a sterile urine sample cup and submerged in fresh PBS. Seven cords were collected immediately after birth, stored in phosphate-buffered saline (PBS), and shipped overnight cold (not frozen) on ice packs from Colorado State University (CSU) to the University of Oklahoma (OU). (Awaiting donor information from CSU) Equine UCMSCs were harvested within 48 hours of birth.

The harvest protocol was adapted from Wang *et al.*[146] with minor modifications. Once received, the umbilical cords were washed under running water, then moved into a biosafety cabinet, soaked in ethanol for 15 min, and then washed with PBS supplemented with 1% antibiotic-antimycotic (anti-anti, cat# 15240062, ThermoFisher Scientific, Waltham, MA). The blood vessels were then removed, and the umbilical cord matrix (i.e., Wharton's jelly) was minced into 1-2 mm^3 pieces and digested in Dulbecco's Modified Eagle Medium (DMEM, cat# 11885084, ThermoFisher Scientific) supplemented with 0.2% collagenase type II (cat# LS004176, Worthington Biochemical Corporation, Lakewood, NJ) for 16–18 h at 37°C. Following digestion, the medium was diluted with PBS, centrifuged, and then the supernatant was discarded. The pellet was resuspended in DMEM supplemented with 10% fetal bovine serum (FBS, cat# 16000044, Fisher Scientific) and 1% anti-anti, and then transferred to 75 cm^2 flasks. Bacterial or fungal contamination was detected at passage 0 or 1 in cells harvested from seven cords and were thus bleached. eUCMSCs from the seven cords were cryopreserved in Recovery™ Cell Culture Freezing Medium (cat# 12648010, ThermoFisher Scientific). Out of the seven cords, three were used (awaiting mares' information from CSU). eBMSCs were harvested as previously described[122] at CSU. Briefly, bone marrow aspirates were collected using 1000 U/mL of heparin from adult horses, and cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin (P/S, cat# 15140122, ThermoFisher Scientific), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (cat# 118-089-721EA, Quality Biological, Gaithersburg, MD). eBMSCs were cryopreserved in 95% FBS (cat# SH3091003, FisherScientific, Waltham, MA) and 5% dimethyl sulfoxide (DMSO,

ATCC 4-X, Manassas, VA). eBMSCs were shipped from CSU to OU at passage 1 or 2 on dry ice. Six eBMSCs donors were received in total. Donor 1 was a 4-year-old male mixed breed horse, donor 2 was a 2-year-old male Quarter horse, donor 3 was a 3-year-old female Quarter horse, donor 4 was a 2-year-old female mixed breed horse, donor 5 was a 2.5-year-old female mixed breed horse, and donor 6 was a 2.5-year-old gelding (castrated male) mixed breed horse.

Cell expansion: Once colonies were established, eBMSC and eUCMSC expansions were done in Minimum Essential Medium alpha (MEM-α, cat# 12561056, ThermoFisher Scientific) supplemented with 10% FBS, 1% anti-anti, 2 ng/mL fibroblast growth factor (FGF, cat# 100-18B, PeproTech) and 25 mM HEPES buffer. Cells were seeded at 3,300 cells/cm^2 and the medium was changed every 2-3 days. Cells were passaged at 80-90% confluency and used at passage 4. For each cell type, three donors were combined. For eBMSCs, for the experiment evaluating compounds and peptides, cells were used from donors 1, 2, and 3. For the experiment evaluating the effect of dexamethasone, the cells were used from donors 4, 5, and 6. For eUCMSCs, cells from horses A, B, and C were used.

Chondrogenic differentiation: Chondrogenic differentiation was induced as previously described.[142] For monolayer culture, cells were seeded at 4,000 cells/well (i.e., 12,500 cells/cm^2) in a flat-bottom 96-well plates (cat# 62406-081, VWR, Radnor, PA), and for spheroid culture, cells were seeded at 40,000 cells/well in U-bottom 96-well plates (cat# 10861-564, VWR). For spheroid culture, wells were pre-treated with Anti-Adherence Rinsing Solution (cat# 07010, StemCell Technologies, Vancouver, Canada) followed by

the centrifugation of the plates at 100 x g for 3 minutes for pellet formation. Negative control medium was prepared as previously described[142] using DMEM/high glucose/GlutaMAX™ (cat# 10566016, ThermoFisher Scientific), supplemented with 1% P/S, insulin with human transferrin and selenous acid (ITS)+ premix 1x (cat# 354352, Corning, Corning, NY), sodium pyruvate 1 mM (cat# 11360070, ThermoFisher Scientific), Minimum Essential Medium (MEM) non-essential amino acids 1x (cat# 11140050, ThermoFisher Scientific), and ascorbate-2-phosphate 50 µg/mL (cat# A8960, Sigma-Aldrich). For the experiment evaluating compounds and peptides, dexamethasone 100 nM (cat# D4902, Sigma-Aldrich) was included in the negative control medium. For the positive control, TGF-β3 10 ng/mL (cat# 8420-B3-005, R&D Systems, Minneapolis, MN) was added to the negative control medium. For the experiment evaluating compounds and peptides, we added KGN (cat# HY-16268, MedChemExpress, Monmouth Junction, NJ) at 1 or 10 µM, SM04690 (cat# HY-109049, MedChemExpress) at 30 or 100 nM, CM10 (Sequence LIANAK, GenScript, Piscataway, NJ) at 100 µM or 200 µM, and/or CK2.1 (Sequence: QIKIWFQNRRKWKKMVPSDPSYEDMGGC, GenScript) at 100 nM or 500 nM. For the experiment evaluating compounds and peptides, cells were incubated at 37 ^{o}C in hypoxia (5% O_2). For the experiment evaluating the effect of dexamethasone, cells were incubated in hypoxia or normoxia.

Flow cytometry

Equine BMSCs and eUCMSCs were evaluated for the expression of cell surface markers listed in Table 3.1 using the Cytek Northern Lights Flow Cytometer (Cytek Biosciences, Fremont, CA). Antibodies were selected based on their reported reactivity

with equine epitopes by the supplier, and based on the validation of the reactivity of the antibody clone with equine epitopes in previous publications (Table 1).[147,148] Briefly, the cultured cells were harvested and suspended at 10^6 cells/mL. Cells were incubated with conjugated primary antibodies for 25 min at 4°C. Unstained controls were used as negative controls. Data analysis was performed using the FCS Express 7 (De Novo software, Pasadena, CA). The percentage of positive cells was determined by gating on the negative control.

Real-time quantitative polymerase chain reaction

The Quick-RNA Miniprep Plus Kit (cat# R1058, Zymo Research, Irvine, CA) was used for the isolation and purification of total RNA on day 21, from cells grown in monolayer and from spheroids as previously described.[142] Afterward, the total RNA was transcribed to cDNA using the High-Capacity cDNA Reverse Transcription kit (cat# 4368813, ThermoFisher Scientific) and real-time quantitative polymerase chain reaction (RT-qPCR) was performed using the TaqMan® Fast Universal PCR Master Mix (cat# 4366073, ThermoFisher Scientific) on the Analytik-Jena QTower^3-G real-time thermocycler (AnalytikJena, Jena, Germany). The following TaqMan probes were used: GAPDH (Assay ID# Ec03210916_gH), aggrecan (ACAN, Assay ID# Ec03469667_m1), collagen I (COLI, Assay ID# Ec03469676_m1), collagen II (COLII, Assay ID# Ec02622868_m1), and SOX-9 (Assay ID# Ec03469763_s1). For spheroid cultures, eight samples from each group (n = 8) were tested in duplicate. For monolayer culture, four samples from each group (n = 4), with every sample prepared by combining two wells, were tested in duplicate. The ΔΔCt method was used to calculate relative levels of gene

expression. GAPDH was used as the endogenous reference gene. For the experiment evaluating compounds and peptides, the negative control group was the calibrator group. For the experiment evaluating the effect of dexamethasone, the control group without dexamethasone and without TGF-β3 was used as the calibrator group.

Biochemical assays

On day 21, cells were digested in papain solution consisting of 125 mg/mL papain from papaya latex, 5 mM N-acetyl cysteine, and 5 mM EDTA, in PBS. Samples were digested overnight at 60°C. The DNA content of the samples was measured using a PicoGreen assay kit (cat# P7589, ThermoFisher Scientific) following the manufacturer's protocol.

Glycosaminoglycan (GAG) content was determined using the dimethylmethylene blue (DMMB) assay. DMMB dye solution (pH = 3) was prepared as previously described[149] and chondroitin sulfate A sodium salt (cat# C9819, Sigma-Aldrich Corporation) was used for standard preparation. For spheroid cultures, eight samples from each group (n = 8) were tested in duplicate. For monolayer culture, four samples from each group (n=4), with every sample prepared by combining two wells, were tested in duplicate.

Statistical analysis

All graphs and statistical analyses were performed using GraphPad Prism 9 (GraphPad Software Inc., La Jolla, CA). One-way ANOVA or two-way analyses were performed followed by a Tukey post hoc correction. Results were considered significant at $p < 0.05$. Error bars on graphs show the standard deviation of the mean.

Results

Flow cytometry

Characterization of passage 4 eBMSCs and eUCMSCs was performed using flow cytometric analysis of cell surface markers CD29, CD44, CD45, CD73, CD79a, CD90, and CD105. For eBMSCs (**Figure 3.1**), all six donors were positive for CD29, CD44, CD90, and CD105. In addition, all six eBMSC donors were negative for CD73 and CD79a. While donors 1, 2, 3, and 6 were negative for the hematopoietic cell marker CD45, donors 4 and 5 surprisingly exhibited positive CD45 staining.

For eUCMSCs (**Figure 3.2**), cells harvested from the three different umbilical cords were positive for CD29 at a percentage greater than 98%. Donors 1, 2, and 3 were 92.8%, 96.3%, and 75.9% positive for CD44, respectively. For CD90 and CD105, more than 95% of the cells from all donors were positive. All three donors were negative for CD73 and CD45, whereas for CD79a, variable expression was observed among donors. Specifically, donor 2 was negative for CD79a, whereas donor 1 was 77.4% positive for CD79a and donor 3 was 45.7% positive for CD79a.

Gene expression

The gene expression of eBMSCs and eUCMSCs in response to kartogenin, SM04690, CM10, and CK2.1 are shown in **Figure 3.3**. Starting with eBMSCs (**Figure 3.3A**), no compound or peptide significantly increased chondrogenic gene expression of SOX-9, ACAN, or collagen II in eBMSCs compared to the negative control group. Interestingly, the TGF-β3 positive control did not induce any significantly higher gene expression of ACAN, collagen II, or SOX-9 compared to the negative control group (noting

that dexamethasone was present in both the negative and positive control media). SM04690 at 30 nM had 90% lower ACAN gene expression than the negative control ($p < 0.0001$), and no ACAN was detected for SM04690 at 100 nM. Additionally, the combination of CM10 and CK2.1 did not induce any significant gene change in ACAN gene expression compared to the negative control. For collagen II, no gene expression was detected with SM04690 at 100 nM. SM04690 had the lowest detectable collagen II gene expression at 30 nM; however, the lower value was not significantly different from the negative control, but was 92.4% lower than KGN-1 μM ($p < 0.05$), 94.2% lower than KGN-10 μM ($p < 0.0001$), 93.8% lower than CM10-200 μM ($p < 0.01$), and 93.0% lower than CM10/CK2.1 ($p < 0.05$). As for SOX-9, SM04690 at 100 nM resulted in the lowest gene SOX-9 gene expression including a 91% lower value ($p < 0.0001$) compared to the negative control. Interestingly, no collagen I gene expression was observed in any of the groups.

For eUCMSCs (**Figure 3.3B**), only SOX-9 gene expression was detected and there were no significant differences in SOX-9 expression among groups except for CM10-200 μM having an 87% lower value ($p < 0.05$) compared to KGN-10 μM. No collagen I gene expression was observed with any of the groups.

Due to the similar eBMSC responses between the negative and positive controls (**Figure 3A**), as a next step we transitioned to evaluate the effect of dexamethasone, with and without TGF-β3, with eBMSCs. In eBMSC spheroid culture in hypoxia (**Figure 3.4A**), for all genes there were no significant differences between eBMSC spheroids treated with dexamethasone with or without TGF-β3, consistent with the previous experiment (i.e., the

positive vs. negative controls). Likewise, in the absence of dexamethasone, no significant differences were observed between eBMSC spheroids treated with or without TGF-β3 for all genes. In contrast to TGF-β3, which had no significant effect in hypoxia, dexamethasone led to some increases of an order of magnitude or two for chondrogenic genes.

Specifically, and interestingly, large differences for eBMSC spheroids in hypoxia were observed compared to the negative control (i.e., no dexamethasone and no TGF-β3) for ACAN and collagen II. For example, eBMSC spheroids treated with dexamethasone alone had 43-fold ($p < 0.05$) and 181-fold higher ($p < 0.05$) ACAN and collagen II gene expressions, respectively, compared to the negative control (**Figure 3.4A**). eBMSCs treated with both dexamethasone and TGF-β3 had 45-fold ($p < 0.05$) higher ACAN gene expression compared to the negative control, but not a significantly different gene expression for collagen II gene expression. In the presence of TGF-β3, eBMSC spheroids treated with dexamethasone had 185-fold higher ($p < 0.05$) ACAN gene expression compared to eBMSC spheroids without dexamethasone; however, no significant differences were observed for the remaining genes. No significant differences were observed among groups for either collagen I or SOX-9 in hypoxia (**Figure 3.4A**).

In normoxia (**Figure 3.4B**), the eBMSC spheroids treated with both dexamethasone and TGF-β3 had the highest gene expression for ACAN and collagen II, with three orders of magnitude, i.e., 4,108-fold ($p < 0.0001$) and 4,705-fold ($p < 0.01$) higher gene expressions, respectively, compared to the negative control. For eBMSC spheroids treated with dexamethasone alone, despite a mean value that was 412-fold higher for

ACAN and 539-fold higher for collagen II compared to the negative control, the difference was not statistically significant. In the absence of dexamethasone, no significant differences were observed between eBMSC spheroids treated with or without TGF-β3 for all genes, similar to what was observed in hypoxia. However, in the presence of dexamethasone, eBMSC spheroids treated with TGF-β3 had 10-fold higher ($p < 0.001$) and 9-fold higher ($p < 0.01$) ACAN and collagen II gene expression, respectively, compared to eBMSCs treated with dexamethasone alone. In the presence of TGF-β3, eBMSC spheroids treated with dexamethasone had 48-fold ($p < 0.0001$) and 2,136-fold higher ($p < 0.01$) gene expression of ACAN and collagen II, respectively, compared to eBMSC spheroids treated without dexamethasone. No significant differences were observed among groups for SOX-9. As for collagen I, eBMSC spheroids treated with dexamethasone alone exhibited the highest gene expression with a 38-fold higher ($p < 0.05$) gene expression compared to the negative control and 448-fold higher ($p < 0.05$) gene expression compared to eBMSC spheroids treated with TGF-β3 alone. No other significant differences were observed among the remaining groups for collagen I gene expression.

The responses of eBMSCs in monolayer culture differed from those in spheroid culture. For the gene expression of eBMSCs in monolayer culture, in hypoxia **(Figure 3.5A)**, eBMSCs treated with TGF-β3 alone resulted in the highest gene expression of ACAN and SOX-9, including 2.0-fold higher ($p < 0.05$) and 2.1-fold higher ($p < 0.0001$) values, respectively, compared to the negative control. In the presence of dexamethasone, eBMSCs treated with TGF-β3 had 23-fold ($p < 0.0001$) higher SOX-9

gene expression compared to treatment with dexamethasone alone. For eBMSCs treated with dexamethasone alone, 2.8-fold higher ($p < 0.05$) collagen I gene expression was observed, compared to eBMSCs treated with both dexamethasone and TGF-β3, and 27.4-fold higher ($p < 0.01$) value compared to the negative control. In the absence of TGF-β3, eBMSCs treated with dexamethasone had 98.8% ($p < 0.05$), 98.9% ($p < 0.05$), and 94.1% ($p < 0.001$) lower expressions of ACAN, collagen II, and SOX-9, respectively. In the presence of TGF-β3, eBMSCs without dexamethasone treatment had 3-fold ($p < 0.01$) and 1.6-fold ($p < 0.01$) higher ACAN and SOX-9 gene expressions compared to eBMSCs treated with dexamethasone; however, no significant differences were observed for collagen II or collagen I gene expression.

In normoxia **(Figure 3.5B)**, in the absence of TGF-β3, eBMSCs treated *without* dexamethasone resulted in 8.6-fold ($p < 0.05$), 8.8-fold ($p < 0.05$), and 11.7-fold ($p < 0.01$) higher gene expression of ACAN, collagen II, and SOX-9, respectively, compared to treatment with dexamethasone. Again, in the absence of TGF-β3, eBMSC collagen I expression in contrast increased by 22.4-fold ($p > 0.01$) with dexamethasone compared to without dexamethasone. In the presence of TGF-β3 in normoxia, no significant differences were observed between eBMSCs treated with or without dexamethasone for all genes. In the presence of dexamethasone, no significant differences were observed between eBMSCs treated with or without TGF-β3 for ACAN and collagen II gene expression; however, 9-fold ($p < 0.05$) higher SOX-9 and 62.2% ($p < 0.05$) lower collagen I gene expressions were observed with eBMSCs treated with TGF-β3. Again, in the absence of dexamethasone in normoxia, no significant difference was observed with the

addition of TGF-β3 for SOX-9, and no collagen I was detected for eBMSCs treated with TGF-β3 alone.

Normalizing the gene expression to the normoxia control group to compare hypoxia and normoxia (**Figures 3.6 and 3.7**). In spheroid culture (**Figure 3.6**), eBMSCs treated with only dexamethasone in hypoxia resulted in 90.5% and 94.3% higher ACAN and collagen II gene expression, respectively, compared to eBMSCs treated with only dexamethasone in normoxia. For both aggrecan and collagen II expression, there were three groups that were significantly higher than all other groups, but not significantly different from each other: the TGF-β3 with dexamethasone groups in both normoxia and hypoxia, and the dexamethasone group in hypoxia. However, no significant differences were observed between normoxia and hypoxia for groups treated with TGF-β3 alone or both dexamethasone and TGF-β3. For SOX-9 and collagen I, no significant differences were observed between hypoxia and normoxia. In monolayer culture (**Figure 3.7**), eBMSCs treated with TGF-β3 alone in hypoxia resulted in 8.9-fold and 2-fold higher ACAN and SOX-9 gene expression, respectively, compared to eBMSCs treated with only TGF-β3 in normoxia. For eBMSCs treated with both dexamethasone and TGF-β3 in hypoxia a 3.9-fold higher ACAN gene expression was observed compared to eBMSCs treated with both dexamethasone and TGF-β3 in normoxia. Treatment of eBMSCs with dexamethasone alone in hypoxia resulted in 10.9-fold lower collagen II gene expression compared to eBMSCs treated with dexamethasone alone in normoxia. No other significant differences were observed between hypoxia and normoxia for the remaining groups.

Biochemical assays

The GAG contents of eBMSCs and eUCMSCs in response to kartogenin, SM04690, CM10, and CK2.1 are shown in **Figure 3.8**. For eBMSCs **(Figure 3.8A)**, following 21 days in spheroid culture, the TGF-β3 positive control had the highest DNA content including a 41.7% higher ($p < 0.001$) value compared to the negative control. SM04690 at 100 nM had 38.7% ($p < 0.01$) lower DNA content compared to the negative control. For GAG content, the TGF-β3 group had the highest GAG content including a 2.0-fold higher value ($p < 0.0001$) compared to the negative control. Almost every other group produced less GAG than the negative control. The SM04690 groups had the lowest GAG content among all groups; SM04690 at 30 nM had 68.5% lower ($p < 0.0001$) GAG content than the negative control, and no GAG content was detected in the SM04690 group at 100 nM. KGN at 1 μM and at 10 μM had 32% lower ($p < 0.0001$) and 48% lower ($p < 0.0001$) GAG contents, respectively, compared to the negative control. CM10 at 100μM and at 200 μM had 27% ($p < 0.0001$) and 34% ($p < 0.0001$) lower GAG contents, respectively, compared to the negative control. CK2.1 at 500 nM and CM10/CK2.1 had 24% ($p < 0.001$) and 23% ($p < 0.01$) lower GAG contents compared to the negative control, respectively. As for the GAG content normalized to DNA content, no significant difference was observed between the negative and positive control groups. The SM04690 groups produced significantly less GAG/DNA than all but one of the other groups; most notably SM04690 at 30 nM had 62% ($p < 0.0001$) less GAG/DNA compared to the negative control. KGN at 10 μM and CM10 at 200 μM had 46% ($p < 0.001$) and 33% ($p < 0.05$) lower GAG/DNA compared to the negative control. No significant differences in

GAG/DNA content were observed between the negative control and the remaining experimental groups.

For eUCMSCs **(Figure 3.8B)**, GAGs were not detected in any of the groups. Therefore, only DNA content was reported, with the negative control exhibiting the highest DNA content, which was significant compared to six groups, including 66.0% higher DNA content than the positive control ($p < 0.05$).

For the evaluation of the effect of dexamethasone on the GAG production in eBMSC spheroids **(Figure 3.9)**, in hypoxia, in the absence of TGF-β3, treatment with dexamethasone resulted in 1.9-fold higher ($p < 0.05$) DNA content compared to the negative control, with no significant differences observed for GAG and GAG/DNA content compared to the negative control. In the presence of TGF-β3, eBMSCs treated with dexamethasone had significantly higher DNA content (3.1-fold ($p < 0.0001$)), GAG content (21.6-fold ($p < 0.0001$)), and GAG/DNA content (4.9-fold ($p < 0.01$)) compared to eBMSCs not treated with dexamethasone. Additionally, treatment with both dexamethasone and TGF-β3 had significantly higher DNA content (4.6-fold ($p < 0.0001$)), GAG content (19.6-fold ($p < 0.0001$)), and GAG/DNA content (3-fold ($p < 0.05$)) compared to the negative control (i.e., no dexamethasone and no TGF-β3). Treatment of eBMSCs with TGF-β3 alone did not result in any significant differences for DNA, GAG, or GAG/DNA content compared to the negative control **(Figure 3.9A)**. In normoxia **(Figure 3.9B)**, in the absence of TGF-β3, treatment with dexamethasone alone did not result in any significant differences in DNA, GAG, or GAG/DNA content. In the presence of TGF-β3, eBMSCs treated with dexamethasone resulted in 3.0-fold ($p < 0.001$), 36.0-fold ($p <$

0.0001), and 11.7-fold ($p < 0.05$) higher DNA content, GAG content, and GAG/DNA content, respectively, compared to eBMSCs not treated with dexamethasone. Additionally, eBMSCs treatment with both dexamethasone and TGF-β3 resulted in 2-fold ($p < 0.01$), 50-fold ($p < 0.001$), and 35-fold ($p < 0.05$) higher DNA content, GAG content, and GAG/DNA content, respectively, compared to the negative control.

In monolayer culture, in hypoxia **(Figure 3.10A)**, no significant differences were observed among the experimental groups for DNA content. For GAG and GAG/DNA content, the only significant difference was between the negative control and the treatment with both dexamethasone and TGF-β3, which resulted in 2.6-fold ($p < 0.05$) higher GAG and GAG/DNA contents. In normoxia (**Figure 3.10B**), no significant differences were observed among groups for DNA content, GAG content, or GAG/DNA content.

Discussion

The current study was the first to evaluate the chondroinductive potential of reported chondroinductive compounds and peptides with eBMSCs or eUCMSCs *in vitro*, and the first to compare the chondroinductive potential of eBMSCs and eUCMSCs in response to TGF-β3 in pellet culture in hypoxia. Additionally, this is the first study to evaluate and compare the chondroinductive potential of dexamethasone and TGF-β3 in eBMSC spheroid culture, and the first to run the comparison in both normoxia and hypoxia. Our hypotheses addressing the chondroinductive compounds, and the chondroinductive potential of eUCMSCs, were not supported, as there were no chondroinductive compounds that outperformed TGF-β3 in chondrogenesis and there

was no evidence to support the chondroinductive potential of eUCMSCs. However, the hypothesis that dexamethasone would induce the chondrogenic differentiation of eBMSCs in the absence of TGF-β3 was supported in spheroid culture in hypoxia.

None of the evaluated peptides (i.e., CM10 and CK2.1) or compounds (i.e., kartogenin and SM04690) induced a higher gene expression of chondrogenic markers (ACAN, collagen II, and SOX-9) or increased GAGs production compared to the negative control for either eBMSCs or eUCMSCs following 21 days in spheroid culture. The limited efficacy of compounds from the literature with eBMSCs in the current study was in agreement with the results of our recent study that evaluated the chondrogenic potential of the same compounds and peptides with hBMSCs.[142]

No evidence of chondroinduction was observed with eUCMSCs, as no ACAN or collagen II gene expression was detected, and no GAGs were detected following 21 days in culture. The limited chondrogenic potential of eUCMSCs is in agreement with previous studies by Lovati *et al*.[134] that reported a similar outcome following the treatment of UCMSCs pellets with TGF-β1 for 15 days in normoxia, and by Rakic *et al*.[133] that reported a limited chondroinductive potential of eUCMSCs following BMP-2 + TGF-β1 treatment in both normoxia and hypoxia.

All eBMSC and eUCMSC donors exhibited markers associated with mesenchymal stem cells (i.e., CD29, CD44, CD90, and CD105) in accordance with reported literature,[147,148,150] and consistent with stem cell phenotype. Interestingly, while all eBMSC donors were negative for CD73 and CD79a, donors 4 and 5 exhibited positive CD45 staining. One possible explanation could be that the isolation of BMSCs based on their

adherence to plastic is known to result in a heterogenous population of cells, which might include subpopulations of CD45-positive cells, as previously reported with rat BMSCs.[151] Other factors that have been reported to influence the expression of CD45 in hBMSCs is the donor age, and culture duration[152] with some reports indicating that most BMSCs derive from $CD49a^{+}/CD45^{med.low}$ cells and turn into $CD49a^{+}/CD45^{-}$ cells when cultured.[152,153] Despite being CD45 positive, chondroinductive differentiation was observed with pooled eBMSC donors 4, 5, and 6. As for eUCMSCs, donors 1 and 3 exhibited a CD79a positive phenotype, which might be explained by the heterogeneity of the stem cell population obtained during harvest and culture. As a point of comparison, one reference has indicated that MSCs derived from human adipose tissue do exhibit positive CD79a staining.[154] Collectively, the cell surface markers observed in the current study indicated that more rigorous evaluation of stem cell markers for various sources of equine MSCs at different passages is warranted. Additional evaluation of hematopoietic markers such as CD34 and HLA markers might help to further characterize eUCMSCs and eBMSCs in future studies.

The lack of significant differences between the negative and positive controls in our first experiment, for both gene expression and GAG content, warranted additional investigation into the effect of dexamethasone and TGF-β3 on the chondrogenic differentiation of eBMSCs. The current study was the first to evaluate and compare the chondroinductive potential of dexamethasone and TGF-β3 in eBMSC spheroid culture, and that comparison was done in both normoxia and hypoxia. Interestingly, in eBMSC spheroid culture under hypoxia, significantly higher gene expression of ACAN and

collagen II was observed following treatment with dexamethasone alone compared to the negative control. Regardless of whether hypoxia or normoxia was used, addition of TGF-β3 did not lead to significantly higher chondrogenic gene expression, meaning that dexamethasone in hypoxia was sufficient to induce eBMSC chondrogenesis without TGF-β3. Furthermore, no significant differences were observed in GAG/DNA production between dexamethasone and dexamethasone + TGF-β3 groups, further reiterating that dexamethasone alone might be sufficient to induce the chondrogenic differentiation of eBMSCs in spheroid culture in hypoxia, without the need of additional growth factors.

Noteworthy is that no collagen I was detected in the study comparing the chondroinductive compounds in spheroid culture in hypoxia, whereas we did detect collagen I in the study evaluating the effect of dexamethasone under the same conditions. One possible reason for this discrepancy may be that different donors were used in the aforementioned studies, and two out of the three donors used in the dexamethasone study had positive CD45 staining.

In eBMSC spheroid culture in normoxia, dexamethasone alone did have a higher mean value than the negative control for the gene expressions of ACAN and collagen II; however, those differences were not significant, which could be due to a type II error (i.e., false negative due to small sample number). The treatment with dexamethasone and TGF-β3 in normoxia resulted in significantly higher ACAN and collagen II gene expression and higher GAG/DNA production compared to the negative control and dexamethasone groups, but again did not lead to significantly higher expression than with dexamethasone alone in hypoxia. In other words, a combination of both TGF-β3 and dexamethasone were

required to reach the highest aggrecan and collagen II expression in normoxia, but in hypoxia that highest expression level was possible with dexamethasone alone.

In the future, it may be worth evaluating markers of hypertrophy (e.g., collagen X) following dexamethasone treatment, as BMSCs can tend to acquire hypertrophic properties during chondrogenic induction.

In monolayer culture of eBMSCs under hypoxia, dexamethasone resulted in a significant decrease in the gene expression of ACAN, collagen II, and SOX-9, and the addition of TGF-β3 alone resulted in significantly higher gene expression of ACAN, collagen II, and SOX-9. However, in terms of GAG production, no significant differences were observed among groups. Interestingly, in normoxia in monolayer culture the control group had the highest gene expression for ACAN, collagen II, and SOX-9. It could be that in eBMSC monolayer culture, dexamethasone induces osteogenesis rather than chondrogenesis, which warrants future evaluation of osteogenic markers (e.g., Runx2, BGLAP). Notably in monolayer culture, in comparing normoxia and hypoxia, eBMSCs treated with both dexamethasone and TGF-β3 in hypoxia resulted in higher ACAN gene expression compared to eBMSCs treated with both dexamethasone and TGF-β3 in normoxia.

The findings of the current study do not support the use of eUCMSCs for cell therapies where chondrogenesis of the cells is the therapeutic goal, as we did not find any evidence of chondroinduction in the current culture conditions. However, there might still be an advantage of using eUCMSCs for their immunomodulatory potential.[155–157] eBMSCs remain the most attractive candidate for cell therapy, and in the current study,

dexamethasone in hypoxia was revealed to be a potent and sufficient chondroinductive signal for eBMSC spheroids, without the need for reportedly chondroinductive peptides, compounds, or even TGF-β3. Therefore, in cell therapies where cartilage regeneration in a defect area is the goal, there may an advantage to employing spheroid culture in hypoxia and priming with dexamethasone to enhance the chondroinductive potential of the eBMSCs. The use of spheroids instead of a cell suspension or monolayer has been shown to offer an advantage with chondrocytes, such as using the chondrospheres[158] (spherox) system, which is a 4th generation ACI that is currently approved for use in Europe.

As we look to the future, *in vivo* evaluation is essential to truly evaluate the advantages of using eBMSC spheroids for cartilage regeneration in equines. Additional *in vitro* studies may add value prior to *in vivo* evaluation, for example to explore different spheroid sizes, minimum culture durations for chondroinductive effect, dexamethasone concentrations, and suitable biomaterials to deliver eBMSCs. Longer term, a clinically translational vision may be for dexamethasone-primed, allogeneic eBMSC spheroids to be delivered via an injectable biomaterial capable of supporting weight-bearing and inducing cartilage regeneration in equine cartilage defects.

Chapter 4: Comparison of Multiple Synthetic Chondroinductive Factors in Pellet Culture Against a TGF-β Positive Control[3]

Abstract

Despite the advances in surgical and cell therapy regenerative techniques for cartilage repair, the challenge is to overcome an inferior fibrocartilage repair tissue. *In vitro,* TGF-β1 and TGF-β3 are the primary growth factors employed to induce chondrogenic differentiation. However, the clinical application of native proteins may present challenges regarding stability, cost, or reproducibility. Therefore, there remains an unmet clinical need for the identification of small chondroinductive synthetic molecules. From the literature, two peptides—CM10 and CK2.1—appear to be promising candidates; however, they have not been directly compared to TGF-β with human bone marrow-derived stem cells (hBMSCs). Similarly, two promising compounds—kartogenin and SM04690—have been reported in the literature to exhibit chondroinductive potential *in vivo* and *in vitro*; however, kartogenin was not directly compared against TGF-β. In the current study, we evaluated the chondroinductive potential of CM10, CK2.1, kartogenin, and SM04690, and directly compared them to each other and to a TGF-β3 positive control.

[3] Published as: Ajeeb, B. & Detamore, M. Comparison of multiple synthetic chondroinductive factors in pellet culture against a TGF-β positive control. *Osteoarthritis and Cartilage Open* **5**, 100369 (2023).

Following 21 days of culture, none of the evaluated chondrogenic factors, either individually or even in combinations of two, resulted in a higher gene expression of chondrogenic markers as compared to TGF-β3. Additionally, no collagen II gene expression was detected except in the TGF-β3 positive control group. Given that the evaluated factors have confirmed efficacy in the literature, but not in the current study with a positive control, there may be value in the future identification of new chondroinductive factors that are less situation-dependent, with rigorous evaluations of their effect on chondrogenesis using positive controls.

Introduction

The complete regeneration of functional articular cartilage following traumatic or arthritic lesions is unlikely to occur owing to the low regenerative potential of cartilage. Cartilage injuries are common, especially in athletes where prevalence is around 37%,[159] and it is currently estimated that 1 in 7 adults in USA suffer from osteoarthritis.[160]

Treatment of cartilage lesions is challenging, and a variety of surgical techniques are available, of which bone marrow stimulation is the most commonly performed procedure.[161] Other procedures include autologous osteochondral transfer, osteochondral allograft transplantation,[4,5] and matrix-assisted chondrocyte implantation autologous chondrocyte implantation (MACI).[7] However, the outcome from currently available procedures is often an inferior fibrocartilage tissue that may lack the mechanical and functional properties of healthy hyaline cartilage.

Recent advances in cartilage regeneration focus on three main directions. The first is the development of off-the-shelf biomaterials that would fit into defects. The

second direction is cell therapy, in which recent research focuses on using autologous or allogenic mesenchymal stem cells (MSCs) or chondrocytes or a combination of the two cell types to enhance cartilage regeneration. A third direction is focused on the search for chondroinductive factors, such as peptides or small compounds, to replace the current use of growth factors to induce the chondrogenic differentiation of MSCs and to regenerate cartilage.

The development of chondroinductive factors is an added value to both the biomaterials and cell therapy approaches. Peptides, for example, may be used to functionalize biomaterials to enhance their bioactivity;[42] additionally, chondroinductive factors may be added during cell therapy, or cells may be treated with chondroinductive factors prior to implantation to increase the likelihood of obtaining a repair tissue that resembles hyaline cartilage.

Several papers have reported the identification of peptides or small compounds with chondrogenic potential; however, comparison to a TGF-β3 positive control was lacking in most cases.[138] Additionally, different cell sources and culture methods have been used to evaluate different chondroinductive factors, rendering it challenging to compare the potency of different factors.

We recently presented an overview of peptides involved in cartilage regeneration,[138] where we summarized promising peptides that have been evaluated both *in vitro* and *in vivo* for their chondroinductive potential. Among these peptides, we identified two leading candidates from the literature, CM10 and CK2.1, to evaluate and compare their chondrogenic potential with TGF-β3 using hBMSCs. CM10 (LIANAK) is

a TGF-β1-simulating peptide that has been mainly evaluated for its wound healing potential[58] and collagen I synthesis, where it exhibited activity in the nanomolar and micromolar concentration range. Evaluation of CM10 as a chondroinductive peptide was first done by Renner and Liu in 2013[79] as a soluble peptide; however, no significant activity was observed. In subsequent publications, CM10 was evaluated while conjugated to microspheres[61] or hydrogels,[162] with the exception of one study[62] that evaluated soluble CM10 at a concentration of 100 μM with human periodontal ligament stem cells (hPLSCs), which found that a higher gene expression was observed for SOX-9, aggrecan, and collagen II at comparable values to a TGF-β positive control group.

CK2.1 is a peptide designed to block the interaction between the protein casein kinase 2 (CK2) and bone morphogenetic protein receptor type Ia (BMPRIa) inside the cell, thus leading to the activation of the BMP signaling pathway without the presence of a ligand. CK2.1 exhibited chondrogenic potential with C3H10T1/2 cells (i.e., a mouse cell line derived from C3H mouse embryos) following treatment with BMP-2. However, the *in vitro* evaluation with hBMSCs and comparison with TGF-β3 is yet to be done. Additionally, through a literature review of chondroinductive small compounds (unpublished), we identified two leading small compounds, SM04690[34] and kartogenin,[139] which have been reported to induce cartilage regeneration.

Kartogenin (KGN) was first reported in 2012 by Johnson *et al.*[139] as a heterocyclic drug-like molecule that induced the chondrogenic differentiation of hBMSCs; however, it was compared against dimethyl sulfoxide (DMSO) with no positive control. Kang *et al.*[140] enhanced the delivery of KGN by conjugating it to chitosan particles and found that

conjugated KGN induced chondrogenic differentiation of hBMSCs more effectively than unconjugated kartogenin; however, there was no positive control from the TGF-β family included in the study. Liu *et al.*[141] evaluated lubricin and glycosaminoglycan (GAG) accumulation by rat BMSCs following treatment with TGF-β1, BMP-7, and/or KGN in monolayer culture. While KGN induced a significant increase in lubricin production, only the combination of TGF-β1, BMP-7, and KGN resulted in a significant increase in GAG content as compared to the untreated control group. Liu *et al.*[141] additionally evaluated the chondrogenic differentiation of rat BMSC/chondrocyte pellet co-cultures at different ratios in combination with KGN; however, KGN was added to all combinations, and no positive control was included *in vitro.*

SM04690 was identified as a Wnt signaling pathway inhibitor by Deshmukh *et al.*[34] using high throughput screening. SM04690 is currently in phase 3 clinical trials as a disease-modifying osteoarthritic drug (DMOAD).[163] *In vitro*, SM04690 exhibited promising chondrogenic potential with hBMSCs, comparable to a TGF-β3 positive control.[34]

In the current study, we evaluated and compared the chondrogenic potential of KGN, SM04690, CM10, and CK2.1, alongside a TGF-β3 positive control using hBMSCs following 21 days in pellet culture. Several studies in the literature suggest that an improvement in the chondrogenic differentiation of BMSCs may be observed under hypoxic conditions;[143,144,164] therefore, following preliminary studies that confirmed enhanced collagen II gene expression in hypoxia, we elected to perform the current study in 5% hypoxia.

Given that previous studies have reported synergistic effects between growth factors to enhance chondrogenic differentiation,[165–168] we additionally evaluated synergistic effects between small compounds and peptides. Finally, knowing that chondrogenesis may be donor-dependent,[169] we evaluated the chondrogenic potential of select group concentrations with three different hBMSCs donors. Our hypothesis was that a combination of peptides and/or compounds would induce the chondrogenic differentiation of hBMSCs at levels equivalent or superior to TGF-β3.

Materials and methods

Cell culture

Human BMSCs (hBMSCs) were purchased from RoosterBio, Inc., Frederick, MD, cultured in RoosterBasal™-MSC (cat# SU-022, RoosterBio Inc.) and supplemented with 20% RoosterBooster™-MSC (cat# SU-003, RoosterBio Inc.) and 1% penicillin/streptomycin (P/S, cat# 15140122, ThermoFisher Scientific, Waltham, MA). The donor was a 23-year-old male. Cells were seeded at 3,300 cells/cm^2 and no medium exchange was required as per RoosterBio guidelines. Cells were passaged at 80-90% confluency and used at passage 3. For the multiple donors study, donor 1 was a 20-year-old female, donor 2 was a 19-year-old male, and donor 3 was a 25-year-old male.

Pellet formation: For chondrogenic differentiation, 100,000 cells were added to the wells of U-bottom 96-well plates (cat# 10861-564, VWR, Radnor, PA) following treatment with Anti-Adherence Rinsing Solution (cat# 07010, StemCell Technologies, Vancouver, Canada). The plates were centrifuged at 100 x g for 3 minutes for pellet formation.

Differentiation medium: Negative control medium was prepared using DMEM/high glucose/GlutaMAX™ (cat# 10566016, ThermoFisher Scientific), with 1% P/S, insulin with human transferrin and selenous acid (ITS)+ premix 1x (cat# 354352, Corning, Corning, NY), sodium pyruvate 1 mM (cat# 11360070, ThermoFisher Scientific), Minimum Essential Medium (MEM) non-essential amino acids 1x (cat# 11140050, ThermoFisher Scientific), ascorbate-2-phosphate 50 µg/mL (cat# A8960, Sigma-Aldrich, St. Louis, MO), and dexamethasone 100 nM (cat# D4902, Sigma-Aldrich). For the positive control, TGF-β3 10 ng/mL (cat# 8420-B3-005, R&D Systems, Minneapolis, MN) was added to the negative control medium. For experimental groups, KGN (cat# HY-16268, MedChemExpress, Monmouth Junction, NJ), SM04690 (cat# HY-109049, MedChemExpress), CM10 (Sequence LIANAK, GenScript, Piscataway, New Jersey),and/or CK2.1 (Sequence: QIKIWFQNRRKWKKMVPSDPSYEDMGGC) were added to the control medium at concentrations that were based on the literature and summarized in Table 1. Cells were incubated at 37 ºC in hypoxia (5% O_2) for 21 days.

Real-time quantitative polymerase chain reaction

Total RNA was isolated from the pellets on day 21 using Quick-RNA Miniprep Plus Kit (cat# R1058, Zymo Research, Irvine, CA) following the kit handbook. Briefly, pellets were stored in DNA/RNA shield, then digested with Proteinase K and digestion buffer for 4 hours at room temperature, followed by the addition of an equal volume of lysis buffer. DNA transcription was done using the High-Capacity cDNA Reverse Transcription kit (cat# 4368813, ThermoFisher Scientific). Real-time quantitative

polymerase chain reaction (RT-qPCR) was performed using a qTOWER (AnalytikJena, Jena, Germany) and TaqMan® Fast Universal PCR Master Mix (cat# 4366073, ThermoFisher Scientific). Preconfigured TaqMan probes were used and are listed in Table 2. Eight samples from each group (n = 8) were tested in duplicate. Relative levels of gene expression were calculated using the comparative ΔΔCt method for relative quantification. The negative control group was the calibrator group and GAPDH was used as the endogenous reference gene.

Statistical analysis

All graphs and statistical analyses were performed using GraphPad Prism 9 (GraphPad Software Inc. La Jolla, CA). One-way ANOVA analyses were performed followed by a Tukey post hoc correction. Results were considered significant at $p < 0.05$. Showing **** $p < 0.0001$, *** $p < 0.001$,** $p < 0.01$, and * $p < 0.05$. Error bars on graphs show the standard deviation of the mean.

Results

Pellet formation

Pellets successfully formed in all groups within 24 hours of culture. Over 21 days of culture, the pellets maintained their form in all groups.

Gene expression

For ACAN, no significant differences were observed among groups except for the TGF-β3 positive control group, which was significantly larger than all other groups, including a 10,000-fold ($p < 0.0001$) larger ACAN gene expression compared to the negative control **(Figure 4.1)**. Regarding SOX-9, TGF-β3 induced a 50-fold ($p < 0.0001$) higher gene expression compared to the negative control **(Figure 4.2)**, whereas no significant differences were observed among the remaining experimental groups and the negative control. Surprisingly, collagen II expression was only detected in the TGF-β3 positive control; therefore, relative gene expression could not be calculated for this single group.

Interestingly, no significant difference was observed in the collagen I gene expression between the negative control and the TGF-β3 positive control; however, a

variation was observed in response to the evaluated compounds and peptides. CM10 (100 μM) had the highest gene expression with a 2.5-fold ($p < 0.0001$) higher expression compared to the negative control group. CK2.1 had a collagen I gene expression that was 40% ($p=0.0978$) and 50% ($p=0.9782$) higher at 100 nM and 500 nM than the negative control, respectively, although these differences were not significant. The combination of compounds and peptides did not induce a significant difference in collagen I gene expression as compared to the negative control group.

For the multiple donors study **(Figure 4.4)**, no significant differences in the gene expression of ACAN were observed among groups, except for TGF-β3 group across all donors, which is in agreement with the results obtained in the first study. For SOX-9, CM10 exhibited a higher gene expression as compared to the negative control in donor 1; however, with donor 2 and 3 the gene expression of SOX-9 in the CM10 group was significantly lower than the negative control. For KGN, no significant differences compared to the negative control were observed with all 3 donors. As for SM04690 and CK2.1, no significant differences were observed compared to the negative control with donors 1 and 2, whereas a significantly lower SOX-9 gene expression was observed with donor 3. As for collagen II, and as observed in study 1, gene expression was only detected in the TGF-β3 positive control; therefore, relative gene expression could not be calculated.

Discussion

The current study was the first study to evaluate and compare the chondroinductive potential of multiple promising peptides and compounds with TGF-β3

as a positive control. Additionally, this is the first report that evaluates the combination of peptides and compounds on the chondrogenic differentiation of hBMSCs. Our hypothesis was not supported, as there were no individual factors or combinations of factors that outperformed TGF-β3 in chondrogenesis.

Interestingly, none of the evaluated KGN concentrations in the current study induced the gene expression of collagen II or enhanced ACAN or SOX-9 gene expression as compared to the negative or positive control following 21 days in spheroid culture. The absence of increased gene expression as compared to TGF-β3 is in agreement with what was observed by Music *et al.*[170] with hBMSC macropellet and micropellet cultures, which showed that KGN was inferior to TGF-β1 in terms of chondrogenic potential following 7 or 14 days of continuous KGN treatment. The first KGN paper by Johnson *et al.*[139] reported a higher gene expression of chondrogenic markers (ACAN and collagen II) on day 21 following 3 days of treatment with KGN at concentrations 100 nM, 1 μM, and 10 μM in hBMSCs pellet culture. While there was no TGF-β positive control in that study, it may be possible that continuous treatment with KGN for more than 3 days could hinder chondrogenesis; therefore, additional evaluation is warranted given that 3 days of TGF-β3 treatment is known to work as a chondrogenesis positive control. Interestingly, a 2020 abstract reported the synthesis of KA-34,[33] a more potent and stable KGN analog, and additionally a phase I clinical trial[171] has been completed with KA-34, but no publication is currently available. Hence, future work might include investigating the chondrogenic potential of KA-34.

SM04690 was reported as a promising DMOAD by Deshmukh *et al.*[34,35] and its chondroinductive potential was demonstrated with hBMSCs in pellet culture (150,000 cells/pellet) and in monolayer culture (40,000 cells/ well in 48 well plate) at 30 nM as compared to treatment with 20 ng/mL of TGF-β3 with medium changes done every 5 days.[34] In the current study, SM04690-treated groups surprisingly exhibited no significant difference in the gene expression of ACAN and SOX-9 relative to the negative control, and collagen II gene expression was not observed. One possible explanation for the differences in outcomes between studies is that Deshmukh *et al.*[34] used incomplete chondrocyte differentiation medium (iCDM; Lonza) and chondrogenesis was evaluated in normoxia, whereas in the current study, DMEM medium with individually prepared supplements was used and cells were cultured in hypoxia.

The current study was the first study to evaluate the chondrogenic potential of CM10 with hBMSCs as compared to a positive control. No significant difference in the gene expression of ACAN or SOX-9 was observed with CM10 treatment as compared to the negative control. On the other hand, treatment with 100 μM CM10 resulted in the highest gene expression of collagen I as compared to the negative control group that might indicate why CM10 was successfully used in wound healing applications.[58,172] The chondrogenic potential of CM10 was first evaluated in 2013 with hBMSCs at 0.05 and 0.1 μM;[79] however, no promising outcome was observed. Another study[62] reported enhanced gene expression of chondrogenic markers following treatment with 100 μM of CM10; however, hPLSCs were the cell source, which may respond differently from hBMSCs. Interestingly, other studies reported positive outcomes with CM10-

functionalized microspheres[61] and CM10-functionalized hydrogels;[162] however, no positive control was included in the first study. It could be that CM10 will exhibit a more prominent activity when presented on a scaffold versus as a soluble peptide, which requires additional investigation in the future.

The current study is the first report to evaluate the chondrogenic potential of CK2.1 with hBMSCs in comparison to TGF-β3. CK2.1 showed promising outcomes in the original publication;[44] however, the peptide was evaluated with C3H10T1/2 cells, which are a mouse cell line that are functionally similar to mesenchymal stem cells and undergo chondrogenesis when induced by cellular condensation and bone morphogenetic protein-2 (BMP-2).[173] The current study used the same concentrations reported in the original paper (100 nM and 500 nM); however, no significant difference in the gene expression of ACAN and SOX-9 chondrogenic markers relative to the negative control were observed. Additionally, the gene expression of collagen II was not observed. In addition to the difference in cell source, the original paper used DMEM medium with 10% fetal bovine serum (FBS) with no additional supplements, whereas the current study used a serum-free medium.

Combinations between growth factors have been shown to enhance the chondrogenic differentiation of MSCs.[165–168] The current study evaluated different combinations between CM10, CK2.1, KGN, and SM04690; however, none of the combinations resulted in a higher gene expression of chondrogenic markers as compared to the negative control.

To eliminate the effect of donor variation on the results obtained in the first study, we evaluated the chondroinductive potential of select group concentrations: CM10 (100 μM), CK2.1 (100 nM), KGN (10 μM), and SM04690 (30 nM), with 3 different donors. While we did observe donor-dependent variation in the gene expression of SOX-9 among experimental groups; however, and as seen with the first study, the lack of collagen II expression and absence of higher ACAN gene expression indicated that there was no evidence of chondrogenesis with any of the evaluated peptides and compounds.

Given that prior studies have demonstrated chondroinduction with CM10, CK2.1, KGN, and SM04690, and used a variety of different conditions as delineated above, we cannot conclude that these factors are not chondroinductive. Instead, we conclude that evidence of chondroinduction was not observed in the conditions employed in the current study, and therefore that *in vitro* chondroinduction with these factors may depend on the cell source and culture conditions (e.g., medium composition, monolayer vs. pellet vs. 3D scaffold, hypoxia vs. normoxia). Additionally, the evaluated factors could be chondroinductive if applied transiently[174–177] or have alternate modes of action such as stabilizing chondrogenesis if applied beyond day 7 or 14 of chondrogenesis, or could be fibrogenic rather than chondrogenic, which entreats additional investigation in the future. Furthermore, once we move *in vivo*, there are other parameters such as the immune system and/or mechanical stimulation that may affect the potency of chondroinductive factors.

TGF-β3 is the main growth factor employed for chondrogenesis, as it is known to induce chondrogenesis with different cell sources and culture conditions, including in the current study. Therefore, the current study underscores the importance of identifying factors (as alternatives to TGF-β3) that are not situation dependent, and identification of new factors might be the key to enhancing the outcome of biomaterial-based or cell therapy-based cartilage regeneration techniques. In future studies that do identify new factors for chondroinduction, comparing to other published factors is recommended if possible, and including a positive control is strongly recommended so that there is context for the extent of observed chondroinduction. Furthermore, as BMSCs tend to acquire hypertrophic properties during chondrogenic induction, it is imperative that hypertrophy markers (e.g., collagen X) be evaluated in future studies to clearly highlight the difference between various chondrogenic compounds.

Chapter 5: Screening and Evaluation of the Chondroinductive Potential of Promising Peptides

Abstract

The challenge to repair articular cartilage following a trauma has been tackled from several angles, for which the discovery of a synthetic chondroinductive factor would represent a breakthrough. Currently, growth factors including TGF-β1 and TGF-β3 are mainly used to induce chondrogenic differentiation of BMSCs *in vitro*; however, the clinical translation of growth factors in cartilage repair is yet to be established owing to challenges related to cost, variability between batches, and stability. Peptides are a potentially attractive group of chondrogenic factors as they can be chemically synthesized with high reproducibility, they are easily modified, and they can be conjugated to biomaterials. In this chapter, we investigated the chondroinductive potential of 11 peptides newly discovered by our group, which were selected based on their affinity to TGF-β receptors, alongside a TGF-β3 positive control with human bone marrow-derived mesenchymal stem cells (hBMSCs). Following 21 days in culture, none of the evaluated peptides resulted in a higher gene expression of chondrogenic markers compared to TGF-β3. Given the complexity of the pathways and receptors involved in chondrogenesis, the results suggest that secondary and tertiary structures may play an important role in the activation of TGF-β receptors. Therefore, longer peptide sequences and predictive peptide software packages might be valuable tools to identify a chondroinductive peptide.

Introduction

Articular cartilage injuries are observed in up to 62% of knees during arthroscopy.[178] Following an injury, cartilage lacks the potential to regenerate, owing to its avascularity and low cellularity, thus often leading to the onset of osteoarthritis.[179] Non-surgical treatments include intra-articular injections of corticosteroids or hyaluronic acid, and they provide a temporary relief of pain;[2] however, cartilage repair necessitates surgical intervention. Various surgical techniques are available, including microfracture, mosaicplasty, autologous chondrocyte implantation (ACI), and matrix-assisted autologous chondrocyte implantation (MACI),[4] with the choice contingent on several factors including defect size, patient's overall condition, and cost. Notably, microfracture has emerged as the most commonly used procedure in the USA for the treatment of cartilage lesions.[161] Despite the availability of various surgical techniques, the regeneration of hyaline-like cartilage remains an ongoing challenge.

In vitro, growth factors mainly from the TGF-β3 family have been used to induce the chondrogenic differentiation of stem cells. However, clinical translation of growth factors is challenging due to the high cost of production, low production yield, and potential adverse side effects. Alternatively, peptides represent an attractive replacement that has a lower cost and lower variability between batches. Additionally, peptides can be used to functionalize biomaterials, creating a chondroinductive biomaterial that can be used to induce the regeneration of cartilage. We recently reviewed peptides involved in cartilage regeneration (Chapter 2),[138] and identified two leading candidates from the literature, CM10 and CK2.1. We then evaluated their chondrogenic potential with

hBMSCs using TGF-β3 as a positive control (Chapter 4).[2] Unfortunately, no evidence of chondrogenesis was observed with CM10 or CK2.1 with hBMSCs (Chapter 4), or even with eBMSCs or eUCMSCs (Chapter 3).

Using a peptide discovery strategy that is common in the cancer field, but new to regenerative medicine, our team identified nine peptides derived from TGF-β3 or aggrecan. We named these peptides as numbers 1 through 9. Our team additionally identified another set of peptides, C1 through C5, derived from growth factors involved in chondroinduction, including TGF-β1 and TGF-β3. In the current study, we evaluated the chondroinductive potential of six peptides from the first group (i.e., Peptides 1, 2, 4, 5, 6 and 9) in addition to Peptides C1 through C5. The evaluation was conducted in spheroid culture in hypoxia with human bone marrow-derived mesenchymal stem cells (hBMSCs), with a TGF-β3 positive control for comparison. Our hypothesis was that at least one of the evaluated peptides would result in an increased gene expression of chondrogenic markers (i.e., ACAN, collagen II, and/or SOX-9) compared to the TGF-β3 positive control.

Materials and methods

Cell source and expansion

Human BMSCs were purchased from RoosterBio, Inc. (Frederick, MD). For the screening of peptides 1, 2, 4, 5, 6, and 9; and for the screening of peptides C1, C2, C3, C4, and C5 in the nM range; the donor was a 19-year-old male. For the screening of peptides C1, C2, C3, C4, and C5 in the μM range, the donor was a different 19-year-old male. Cells were expanded at 3,300 cells/cm^2 in RoosterBasal^{TM}-MSC (cat# SU-022, RoosterBio Inc.) and supplemented with 1% penicillin/streptomycin (P/S, cat# 15140122,

ThermoFisher Scientific, Waltham, MA) and 20% RoosterBooster™-MSC (cat# SU-003, RoosterBio Inc.). Cells were passaged at 90% confluency and used at passage 4.

Cell spheroid formation and culture

For chondrogenic differentiation of hBMSCs, 100,000 cells/well were added to U-bottom 96-well plates (cat# 10861-564, VWR, Radnor, PA) that were pre-treated with Anti-Adherence Rinsing Solution (cat# 07010, StemCell Technologies, Vancouver, Canada). Pellets were formed by the centrifugation of the plates at 100 x g for 3 minutes. Negative control medium was prepared as previously described[142] using Dulbecco's Modified Eagle Medium (DMEM)/high glucose/GlutaMAX™ (cat# 10566016, ThermoFisher Scientific), with 1% P/S, insulin with human transferrin and selenous acid (ITS)+ premix 1x (cat# 354352, Corning Inc., Corning, NY), sodium pyruvate 1 mM (cat# 11360070, ThermoFisher Scientific), Minimum Essential Medium (MEM) non-essential amino acids 1x (cat# 11140050, ThermoFisher Scientific), ascorbate-2-phosphate 50 µg/mL (cat# A8960, Sigma-Aldrich, St. Louis, MO), and dexamethasone 100 nM (cat# D4902, Sigma-Aldrich). For the positive control, TGF-β3 10 ng/mL (cat# 8420-B3-005, R&D Systems, Minneapolis, MN) was added to the negative control medium. Peptides 1-9 and C1-C5 were purchased from GenScript (Piscataway, NJ), and were added to the control medium at assay specific concentrations. Based on the sequence of ordering and delivery, peptides 1, 4, and 6 were run together in parallel, and peptides 2, 5, 9 were run together in parallel. Peptides 3, 7, and 8 were not evaluated because we were not able to receive the peptides at a purity greater than or equal to 95%. For the investigation of the effect of DMSO on the chondrogenic differentiation of hBMSCs, DMSO was evaluated

at 0.5% and 1% final concentration in the medium. The hBMSC spheroids were incubated at 37 ^{o}C in hypoxia (5% O_2) for 21 days.

Real-time quantitative polymerase chain reaction

Total RNA was isolated from the pellets on day 21 using Quick-RNA Miniprep Plus Kit (cat# R1058, Zymo Research, Irvine, CA) for studies evaluating Peptides 1, 2, 4, 5, 6, 9, and Peptides C1 through C5 in the nM range, or the Quick-RNA Microprep Plus Kit (cat# R1051, Zymo Research) for studies evaluating Peptides C1 through C5 in the μM range and the study evaluating the effect of DMSO, following the kit handbook. Briefly, pellets were stored in DNA/RNA shield, then digested with Proteinase K and digestion buffer for 4 hours at room temperature, followed by the addition of an equal volume of lysis buffer. DNA transcription was done using the High-Capacity cDNA Reverse Transcription kit (cat# 4368813, ThermoFisher Scientific) or iScript Reverse Transcription Supermix (cat# 1708841, Bio-Rad, Hercules, CA). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using TaqMan® Fast Universal PCR Master Mix (cat# 4366073, ThermoFisher Scientific) or SsoAdvanced Universal Probes Supermix (cat# 1725284, Bio-Rad) on the Analytik-Jena QTower^3-G real-time thermocycler (AnalytikJena, Jena, Germany) or CFX Opus 96 (Bio-Rad) were used to run the qPCR. Preconfigured TaqMan probes (listed in **Table 5.1**) or Bio-Rad duplexing probes (listed in **Table 5.2**) were used. Eight samples from each group (n = 8) were tested in duplicate. Relative levels of gene expression were calculated using the comparative ΔΔCt method. The negative control group was the calibrator group and GAPDH was used as the endogenous reference gene.

Flow cytometry

The interaction of Peptide C1 with hBMSCs was evaluated using the Cytek Northern Lights Flow Cytometer (Cytek Biosciences, Fremont, CA) and data analysis was performed using the FCS Express 7 (De Novo Software, Pasadena, CA). Briefly, cells were cultured in a 6-well plate and treated for 18-20 hours with Peptide C1 conjugated to FAM fluorophore (C1-FAM) at 10 and 100 μM or left untreated. Then cells were washed with PBS three times, harvested, and suspended at 10^6 cells/mL. Untreated cells were divided into two groups, one group was used as unstained controls and the other group incubated with Peptide C1-FAM for 25 min at 4°C. Peptide C1-FAM was purchased from GenScript.

Statistical analysis

All graphs and statistical analyses were performed using GraphPad Prism 10 (GraphPad Software Inc. La Jolla, CA). One-way ANOVA or two-way ANOVA analyses were performed followed by a Tukey *post hoc* correction. Results were considered significant at $p < 0.05$. Error bars on graphs show the standard deviation of the mean.

Results

Gene expression

For the screening of peptides 1, 4, and 6 **(Figure 5.1A)**, for both ACAN and SOX-9, the TGF-β3 positive control had significantly higher ACAN and SOX-9 gene expressions compared to all other groups, including 1269-fold ($p < 0.0001$) and 17-fold ($p < 0.0001$) higher values for ACAN and SOX-9, respectively, compared to the negative control. As for the peptide experimental groups, no statistically significant differences

were observed among groups or compared to the negative control. Collagen II was only detected in the positive control group; therefore, relative gene expression could not be calculated. As for collagen I, variable gene expression was observed among these peptide groups (differences not significant), and TGF-β3 had a 2.3-fold ($p < 0.01$) higher value compared to the negative control. Peptide 1 at 10 nM and at 100 nM had 61% ($p < 0.01$) and 47% ($p < 0.05$) lower collagen I gene expression, respectively, compared to the TGF-β3 positive control. Peptide 4 at 10 nM had 53% ($p < 0.05$) lower collagen I gene expression compared to the TGF-β3 positive control. Peptide 6 at 1 nM and 100 nM had 58% ($p < 0.01$) and 54% ($p < 0.01$) lower collagen I gene expression, respectively, compared to the TGF-β3 positive control.

For the screening of peptides 2, 5, and 9 **(Figure 5.1B)**, the TGF-β3 group had significantly higher ACAN and SOX-9 gene expression compared to all groups, including 884-fold ($p < 0.0001$) and 55-fold ($p < 0.0001$) higher values, respectively, compared to the negative control. None of these peptide groups exhibited any significant differences compared to the negative control. Interestingly, SOX-9 was not detected with Peptide 2 at 10 nM or with Peptide 5 at 100 nM. As for collagen I, no significant differences were observed between the negative and positive controls or between these peptide groups and the negative control. Peptide 2 at 10 nM had 1.3-fold ($p < 0.05$), 2.2-fold ($p < 0.05$), and 2.6-fold ($p < 0.05$) higher collagen I gene expression compared to Peptide 5 at 1 nM, Peptide 9 at 10 nM, and Peptide 9 at 100 nM, respectively. Peptide 5 at 100 nM had 2.2-fold ($p < 0.05$), and 2.4-fold ($p < 0.05$) higher collagen I gene expression compared to Peptide 5 at 10 nM, and Peptide 9 at 100 nM.

For the screening of peptides C1-C5 in the nM range **(Figure 5.2)**, all peptides were run in parallel along with the same negative and positive controls; however, each peptide was plotted on a separate figure for easier interpretation. For ACAN, for all peptides, no statistically significant differences were observed among groups except for TGF-β3, which had significantly higher ACAN expression compared to all other groups, including a 5.7-fold larger value ($P < 0.0001$) compared to the negative control. For SOX-9, no significant differences were observed among groups for Peptide C1, C2, C3, and C4. As for SOX-9 gene expression with Peptide C5 **(Figure 5.3E)**, significant differences were observed for Peptide C5 at 100 nM compared to all other groups with 2.6-fold ($p < 0.001$) higher value compared to the negative control. Collagen II was not detected in any of the peptide groups; therefore, relative gene expression could not be calculated except for the positive control group **(Figure 5.2F)**.

For the screening of peptides C1-C5 in the μM range **(Figure 5.3)**, there was no positive control included in the data analysis because samples were lost during RNA purification. For Peptide C1 **(Figure 5.3A)** for ACAN, Peptide C1 at 100 μM had a significantly lower ACAN expression compared to all other groups, including a 59% ($p < 0.0001$) lower value compared to the negative control. No other statistically significant differences in ACAN expression were observed among groups. For collagen II, significantly higher gene expression was observed with C1 at 1 and 10 μM with 2.7-fold ($p < 0.05$) and 2.8-fold ($p < 0.01$) higher values, respectively, compared to the negative control. For Peptide C2 **(Figure 5.3B)**, C2 at 100 μM had significantly lower ACAN expression compared to all other groups, including a 70% ($p < 0.0001$) lower value

compared to the negative control. No statistically significant differences were observed among the other groups. For collagen II, 3.2-fold higher gene expression was observed with C2 at 10 μM compared to the negative control ($p < 0.01$). As for SOX-9, no significant differences were observed among groups. For Peptide C3 **(Figure 5.3C)**, for ACAN, no statistically significant differences were observed among groups except for C3 at 100 μM, which had a lower value compared to two other groups, including a 42% lower ACAN expression compared to the control group ($p < 0.01$). No significant differences were observed among groups for the gene expression of collagen II and SOX-9. For Peptide C4 **(Figure 5.3D)**, the control group had 1.5-fold ($p < 0.05$), 1.6-fold ($p < 0.01$), and 1.4-fold ($p < 0.05$) higher gene expression compared to treatment with C4 at 1 μM, 10 μM and 100 μM, respectively. No collagen II gene expression was observed following treatment with Peptide C4. As for SOX-9, a significantly higher gene expression was observed with C4 at 100 μM compared to all other groups including a 2-fold ($p < 0.01$) higher value compared to the negative control. For Peptide C5 **(Figure 5.3E)** only 1 μM and 10 μM concentrations were evaluated, as C5 dissolves in DMSO and 100 μM concentration would have resulted in a high DMSO concentration that could be toxic to cells. Significantly higher ACAN gene expression was observed with C5 at 10 μM compared to the control and C5-1 μM groups, with 3.5-fold ($p < 0.001$) and 2.3-fold ($p < 0.01$) higher gene expressions, respectively. For SOX-9, a significantly higher gene expression was observed with both the C5 at 1 μM and C5 at 10 μM groups, with 1.7-fold ($p < 0.001$) and 1.5-fold ($p < 0.01$) higher values, respectively, compared to the negative

control. No collagen II gene expression was observed following treatment with Peptide C5.

Following the evaluation of peptides C1-C5, C1 had promising results with higher collagen II expression compared to the negative control and C5 had promising outcomes with higher ACAN and SOX-9 gene expression. Therefore, an additional study was run to evaluate the chondroinductive potential of C1 and C5 with and without TGF-β3.

Starting with Peptide C1 **(Figure 5.4A)**, for both and ACAN and collagen II, TGF-β3 had the highest gene expression with a 243-fold change ($p < 0.0001$) for ACAN and a 986-fold change ($p < 0.0001$) for collagen II compared to the negative control. No significant differences for the gene expression of ACAN or collagen II were observed for Peptide C1 alone. Additionally, no synergy was observed between Peptide C1 and TGF-β3, rather in TGF-β3 medium: the addition of C1 at 1 μM or 10 μM resulted in 47% lower ($p < 0.0001$) ACAN and 24% lower ($p < 0.01$) collagen II gene expression compared to TGF-β3 alone. For SOX-9, Peptide C1 at 1 μM had the highest gene expression with an 8.8-fold higher ($p < 0.05$) value compared to the negative control, and an 8.2-fold higher ($p < 0.05$) value compared to C1 at 10 μM. No significant differences were observed among groups in TGF-β3 medium for SOX-9 gene expression.

For Peptide C5 **(Figure 5.4B)**, in the absence of TGF-β3, no significant differences in the gene expression of ACAN, collagen II, or SOX-9 were observed among groups. The TGF-β3 positive control had the highest ACAN gene expression with a 243-fold higher value ($p < 0.0001$) compared to the negative control. In TGF-β3 medium, for

ACAN, the presence of C5 at 1 μM and 10 μM resulted in 50% ($p < 0.0001$) and 41% ($p < 0.001$) lower gene expressions, respectively, compared to TGF-β3 alone. The TGF-β3 positive control had a 987-fold higher value ($p < 0.0001$) compared to the negative control. For collagen II, in TGF-β3 medium, the presence of C5 at 10 μM resulted in 99.7% lower ($p < 0.0001$) gene expression compared to TGF-β3 alone. The presence of C5 at 1 μM did not result in a significant difference in the gene expression of collagen II compared to TGF-β3 alone. For SOX-9, in TGF-β3 medium, the presence of C5 at 1 μM and 10 μM resulted in 35% ($p < 0.001$) and 64% ($p < 0.0001$) lower gene expressions as compared to TGF-β3 alone. The TGF-β3 positive control had the highest SOX-9 gene expression, with a 4-fold higher value ($p < 0.0001$) compared to the negative control.

For the investigation of the effect of DMSO on the chondrogenic differentiation of hBMSCs **(Figure 5.5)**, DMSO did not result in a significant change in the gene expression of ACAN, collagen II, or SOX-9 compared to the control group. In TGF-β3 medium, for ACAN, 0.5% and 1% DMSO resulted in 59% ($p < 0.01$) and 54% ($p < 0.05$) lower gene expression, respectively, compared to TGF-β3 alone. For collagen II, in TGF-β3 medium, 0.5% and 1% DMSO resulted in 59% ($p < 0.0001$) and 99% ($p < 0.0001$) lower gene expression compared to TGF-β3 alone. For SOX-9, in TGF-β3 medium, 1% DMSO resulted in a 58% ($p < 0.05$) lower value compared to TGF-β3 alone, but no significant difference was observed between the 0.5% DMSO and TGF-β3 group.

For the evaluation of the interaction of Peptide C1 with hBMSCs using flow cytometry **(Figure 5.6)**, the overnight treatment of hBMSCs with 10 μM or 100 μM resulted in positive staining compared to the unstained control with the 100 μM resulting in a higher

staining intensity. Treatment of hBMSCs with 100 μM C1-FAM for 25 min, resulted in positive staining compared to the unstained control, with a high intensity exceeding a value of 10^6.

The treatment of hBMSCs with both TGF-β3 and Peptide C1 at the higher concentrations of 10 μM and 100 μM **(Figure 5.7)** did not result in any significant difference in the gene expression of ACAN, collagen II, or SOX-9.

Discussion

The current study was the first to evaluate peptides identified from a new peptide discovery strategy. Specifically, the current study is the first to evaluate and compare the chondroinductive potential of the 11 newly discovered peptides in spheroid culture with TGF-β3 as a positive control. Our hypothesis was not supported, as none of the peptides resulted in significantly higher gene expression of all chondrogenic markers (i.e., ACAN, collagen II, and SOX-9).

TGF-β3 was used as a positive control at a concentration of 10 ng/mL, equivalent to ~ 0.4 nM. Therefore, all peptides were first evaluated in the nM range to match the molar equivalency of TGF-β3. There was no promising upregulation of the gene expression of chondrogenic markers ACAN, collagen II, and SOX-9 in the nM range of Peptides 1, 2, 4, 5, 6, or 9. Therefore, we did not pursue any additional investigation of these peptides.

Peptides C1-C5 were first evaluated as soluble peptides in the nM range, and only C5 showed a promising outcome, with a higher gene expression of SOX-9 compared to the negative control. Given that the peptides exhibited promise based on the discovery method, and the cautiously promising outcome of C5 at 100 nM, we investigated the chondroinductive potential of peptides C1-C5 in the μM range. In the μM range, Peptides C1 (upregulated collagen II) and C5 (upregulated ACAN and SOX-9) resulted in promising outcomes compared to the negative control; hence, additional investigation was done by evaluating peptides C1 and C5 with and without TGF-β3 in the medium to determine whether there was any synergy between each individual peptide and TGF-β3. No synergy was observed with Peptide C1 or C5. Instead, and rather surprisingly, a *lower* gene expression of ACAN, collagen II, and SOX-9 was observed mainly with C5 + TGF-β3. We hypothesized that Peptide C5 was binding to TGF beta receptors and blocking the binding of TGF-β3, thus resulting in a lower gene expression of chondrogenic markers. However, since C5 was dissolved in DMSO with a final concentration of 1% in the C1-100 μM group, we ran a study to evaluate the effect of DMSO on the chondrogenic differentiation of hBMSCs. The results confirmed that DMSO at 1% did result in a lower gene expression of ACAN, collagen II, and SOX-9, suggesting that the inhibition may not have been due to the peptide binding to the receptor, but rather due to the alternative explanation for reduced gene expression with DMSO.

Since Peptide C1 dissolves in water, we evaluated the interaction of Peptide C1-FAM with hBMSCs using flow cytometry and found that a positive staining with C1-FAM following 18-20 hours or 25 min of incubation, which confirmed that Peptide C1 was

binding to the hBMSCs. This result warranted additional investigation of synergistic potential of Peptide C1 at 100 μM with TGF-β3. However, gene expression of chondrogenic markers following treatment with TGF-β3 and Peptide C1 (10 μM, and 100 μM) showed no synergistic potential of Peptide C1.

Given that Peptides C1-C5 may have a high binding efficiency to TGF receptors, including with evidence with flow cytometry with C1, future work could be focused on confirming whether the peptides do bind to the receptors in their native structures using ELISA. Alternatively, biolayer interferometry (BLI) could be used for the detection and characterization of molecular interactions between the peptide and the receptors.

Chapter 6: Evaluation of the Chondroinductive Potential of Peptide D with Conjugated TGF-β3 in a PHA Hydrogel

Abstract

The use of biomaterials is a promising approach for cartilage regeneration; however, the current limitation with biomaterials is that they are not independently chondrogenic. One approach to overcome this limitation is the conjugation of peptides that would induce or enhance the chondrogenic differentiation of bone marrow-derived stem cells (BMSCs). Our team had previously identified a new peptide that enhanced the chondrogenic differentiation of human BMSCs (hBMSCs) in spheroid culture (not published). Hence, in this chapter, I synthesized a pentenoate-functionalized hyaluronic acid (PHA) hydrogel with conjugated Peptide D and conjugated TGF-β3. Additionally, I evaluated the chondroinductive potential of the hydrogel with encapsulated hBMSCs in hypoxia. Results indicated that in the presence of conjugated TGF-β3, Peptide D enhanced the gene expression of collagen II and SOX-9 compared to hydrogels with conjugated TGF-β3 only. The successful synthesis of a chondroinductive biomaterial allowed us to select promising groups to be evaluated *in vivo* in an osteochondral cartilage defect model.

Introduction

Articular cartilage fails to regenerate due to several factors mainly, low density and proliferative capacity of chondrocytes, mechanical failure resulting from trauma, and inflammatory stress.[180] Despite substantial efforts in the development of new treatments, the clinical outcomes fall short of restoring a native hyaline-like cartilage.

In recent years, scientists have pursued several directions to repair cartilage, including advancing cell therapy techniques[181] and biomaterials.[182–184] In cell therapy techniques, the approach involves the use of several cell types, notably mesenchymal stem cells (MSCs) or chondrocytes, leveraging the ability of the cells to differentiate and secrete extracellular matrix to facilitate cartilage regeneration. Notably, the latest FDA approved cell-therapy technique is matrix-assisted chondrocyte implantation (MACI),[9] for full-thickness cartilage defects. Concurrently, another avenue of exploration focuses on the development of biomaterials, that act as a foundation used to fill up defects while supporting the regeneration of cartilage. A noteworthy advancement in the field of biomaterials is Agili-C™ [185] that was FDA approved in 2022.[186]

In our previous review,[138] we highlighted peptides that have been reported to induce the chondrogenic differentiation of stem cells. Of those peptides, HSNGLPL and HAVDI, showed promising chondroinductive potential when used with hydrogels or biomaterials. HSNGLPL was evaluated in self-assembling peptide amphiphile (PA gels)[46] and in chitosan gels[47] both *in vitro* and *in vivo*, and HAVDI have been evaluated in methacrylated-hyaluronic acid (MeHA) hydrogels[51,53] both *in vitro* and *in vivo*. While both peptides required the presence of TGF-β1 or TGF-β3 to promote their chondroinductive

function, none of those peptides were evaluated in the presence of conjugated TGF-β3. Additionally, in the case of HAVDI, no positive controls were used in the *in vitro* experiments.[51,53,57]

TGF-β3 is the main growth factor used to induce the chondrogenic differentiation of hBMSCs *in vitro,* and successful differentiation requires continuous supply of TGF-β3 in the medium. *In vivo,* TGF-β in its active form is known to have a half-life of few minutes,[187] posing a significant challenge for its application *in vivo,* as it would necessitate multiple injections of TGF-β3. One strategy to address this challenge is the controlled release of TGF-β3.[188] Previous research have reported the conjugation of TGF-β1 in hydrogels[189–192] at concentrations varying from 10 nM to 150 nM and found that TGF-β1 maintained its activity. Another study[193] reported the conjugation of TGF-β3 to poly(ethylene glycol) (PEG) hydrogel at 50 nM and found that TGF-β3 retains its activity. In this study, we evaluated the chondroinductive potential of tethered TGF-β3 at 25 nM, 50 nM, and 100 nM in a pentenoate-functionalized hyaluronic acid (PHA) hydrogel with encapsulated human bone marrow-derived mesenchymal stem cells (hBMSCs).

Recent unpublished *in vitro* work from our team identified Peptide D as a new peptide that could induce chondrogenesis in the presence of TGF-β3. Therefore, in the current study we conjugated Peptide D to PHA hydrogel. PHA hydrogels are an attractive biomaterial owing to their rapid crosslinking time of 2 minutes, compared to other hyaluronic acid-based hydrogels. Additionally, the paste-like rheology of the PHA precursor solution allows seamless material delivery and shaping onto defects during surgical procedures.[194] Peptide D was conjugated to PHA hydrogels with encapsulated

hBMSCs in the presence of conjugated TGF-β3. Our hypothesis is that in the presence of conjugated TGF-β3, Peptide D will enhance the gene expression of chondrogenic markers and increase the production of glycosaminoglycans (GAGs).

Materials and methods

Synthesis of pentenoate-functionalized hyaluronic acid (PHA)

Pentenoate-functionalized hyaluronic acid (PHA) was synthesized as previously described.[194] Briefly, 2 g of hyaluronic acid (HA, cat# HA15M-5, research grade, Mw = 1.67 MDa, Lifecore Biomedical, Chaska, MN) was dissolved in 400 mL (0.5% (w/v)) deionized water (DI). Once dissolved, dimethylformamide (DMF, cat# 319937, Sigma-Aldrich, St. Louis, MO) was slowly added to reach a final ratio of 3:2 (DI water : DMF). Then, 500 mg of 4-(dimethylamino)pyridine (cat# 107700, Sigma-Aldrich) was added, followed by the dropwise addition of 4.8 mL (5 M excess relative to HA) pentenoic anhydride (cat# 471801, Sigma-Aldrich). The pH was maintained between 8 and 9 using 1 M sodium hydroxide (NaOH, cat# S318, FisherScientific, Waltham, MA) until the pH became constant, and then the solution was left stirring overnight. Subsequently, 10 g sodium chloride (NaCl, cat# 746398, Sigma-Aldrich) was added to the solution followed by the addition of 2 L of acetone to precipitate the PHA. PHA pellets were recovered following centrifugation at 6000 x g. PHA pellets were then dissolved in DI water and dialyzed in DI water for 2 days using dialysis packets (MWCO: 6–8 kDa). Following dialysis, the PHA solution pH was adjusted to 7.4 using NaOH (1 M), then the solution was frozen, lyophilized, and stored at −20 °C.

Hydrogel precursor preparation

PHA was sterilized by ethylene oxide gas (AN7916, Anderson Sterilizers, Haw River, NC) then the hydrogel precursor was prepared at 4 wt% for all experiments. PHA was suspended in 2x PBS or PBS + peptide or PBS + peptide + thiolated TGF-β3 and left to dissolve overnight at 4°C. Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, 98 %, TCL0290-1G, TCI America, OR, USA) and DL-dithiothreitol (DTT, cat # D0632, Sigma-Aldrich) were added at a final concentration of 0.65 mg/mL and 0.7 mg/mL, respectively, on the same day of the experiment. Cells were mixed in at a final concentration of 10^6 cells/mL. The precursor solution was then transferred into a rectangular mold, crosslinked for 2 min using a 405 nm light, then punched using 8 mm biopsy punches.

NMR quantification of pentenoate modification

Percent quantification of pentenoate functionalization of PHA was done using nuclear magnetic resonance (NMR) on a VNMRS-500 MHz Spectrometer (Varian, Palo Alto, CA), with 3-(Trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt (TMSP, cat# 269913-1G, Sigma-Aldrich) as an internal standard as previously described.[195] The percent functionalization of PHA was determined to be 44%.

Thiolation of TGF-β3

Carrier-free TGF-β3 (cat# 8420-B3-100/CF, R&D Systems, Minneapolis, MN) was thiolated using Pierce™ Traut's reagent (cat# 26101, ThermoFisher Scientific, Waltham, MA) following the kit protocol. Zeba Spin Desalting columns (cat# 89891, ThermoFisher Scientific) were used to separate thiolated protein from excess Traut's Reagent. Following

thiolation, Micro BCA™ Protein Assay (cat #23235, ThermoFisher Scientific) was used to determine protein content, and Measure-iT™ Thiol Assay (cat# M30550, ThermoFisher Scientific) was run to determine degree of thiolation following the respective kit's protocols. Following thiolation and quantification, BSA was added to the eluted TGF-β3 at a ratio of 50 μg BSA: 1 μg TGF-β3. 7-day chondrogenic differentiation studies were run with hBMSCs using soluble thiolated TGF-β3 to confirm the activity of the thiolated protein prior to conjugating in hydrogels.

Cell culture

Cell culture: Human BMSCs (hBMSCs) were purchased from RoosterBio, Inc., Frederick, MD, cultured in RoosterBasal™-MSC and supplemented with 1% penicillin/streptomycin (P/S, cat# 15140122, ThermoFisher Scientific), and 20% RoosterBooster™-MSC. The Donor for study 1 was a 19-year-old male was used for first study. For studies 2 and 3 the donor was a 25-year-old male . Cells were seeded at 3,300 cells/cm^2, passaged at 90% confluency, and used at passage 4.

Chondrogenic differentiation: For chondrogenic differentiation, hBMSCs were encapsulated at 10^6 cells/mL. Hydrogels were added to the wells of a 48-well. Negative control medium was prepared as previously desrcribed[142] using DMEM/high glucose/GlutaMAX™, with 1% P/S, insulin with human transferrin and selenous acid (ITS)+ premix 1x, sodium pyruvate 1 mM, Minimum Essential Medium (MEM) non-essential amino acids 1x, ascorbate-2-phosphate 50 μg/mL, and dexamethasone 100 nM. For the positive control, TGF-β3 10 ng/mL was added to the negative control medium.

Peptide D-GCGYG was purchased from GenScript (Piscataway, New Jersey). Cells were incubated at 37 ^{o}C in hypoxia (5% O_2) for 21 days.

Biochemical assays

On day 21, hydrogels were digested in papain solution consisting of 125 mg/mL papain from papaya latex, 5 mM N-acetyl cysteine, and 5 mM EDTA, in PBS. Samples were digested for 72 hrs at 60°C. The DNA content of the samples was measured using a PicoGreen assay kit (P7589, ThermoFisher Scientific) following the manufacturer's protocol.

Glycosaminolglycan (GAG) content was determined using the Dimethylethylene blue (DMMB) assay. DMMB dye solution (pH=3) was prepared as previously described[149] and chondroitin sulfate A sodium salt (cat# C9819, Sigma-Aldrich Corporation) was used for standard preparation. Six samples from each group (n = 6) were tested in duplicate.

Cell Viability assay

Viability of encapsulated hBMSCs in PHA hydrogels was performed with PrestoBlue™ Cell Viability Reagent (cat # A13261, ThermoFisher Scientific) following the kit's protocol. In summary, on day 1, 3, 7, 14 and 21, following the removal of medium from each well, presto blue was diluted in prewarmed medium to a final concentration of 1X. Hydrogels were incubated with presto blue for 2 hours, followed by the transfer of 100 µl from every well into a black 96-well plate. Fluorescence (excitation: 540 nm, emission: 590 nm) was measured using a BioTek Cytation™ 5 plate reader (BioTek, Winooski, VT) and the fluorescence was normalized to the average fluorescence of the PHA group for each day.

Real-time quantitative polymerase chain reaction

Total RNA was isolated from the pellets on day 21 using Quick-RNA Miniprep Plus Kit (cat# R1058, Zymo Research, Irvine, CA) or the Quick-RNA Microprep Plus Kit (cat# R1051, Zymo Research) following the kit handbook. Briefly, pellets were stored in DNA/RNA shield, then digested with Proteinase K and digestion buffer for 4 hours at room temperature, followed by the addition of an equal volume of lysis buffer. DNA transcription was done using the High-Capacity cDNA Reverse Transcription kit (cat# 4368813, ThermoFisher Scientific) or iScript Reverse Transcription Supermix (cat# 1708841, Bio-Rad, Hercules, CA). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using TaqMan® Fast Universal PCR Master Mix (cat# 4366073, ThermoFisher Scientific) or SsoAdvanced Universal Probes Supermix (cat# 1725284, Bio-Rad) on the Analytik-Jena QTower^3-G real-time thermocycler (AnalytikJena, Jena, Germany) or CFX Opus 96 (Bio-Rad,) were used to run the qPCR. Preconfigured TaqMan probes were used were the screening experiments and are listed in Table 6.2. Bio-Rad duplexing probes listed in Table 6.3 were used for the experiment evaluating the inhibitory potential of C5 and the effect of DMSO. Eight samples from each group (n = 8) were tested in duplicate. Relative levels of gene expression were calculated using the comparative ΔΔCt method for relative quantification. The negative control group was the calibrator group and GAPDH was used as the endogenous reference gene.

Statistical analysis

All graphs and statistical analyses were performed using GraphPad Prism 9 (GraphPad Software Inc. La Jolla, CA). One-way ANOVA or Two-way ANOVA analyses

were performed followed by a Tukey post hoc correction. Results were considered significant at $p < 0.05$. Error bars on graphs show the standard deviation of the mean.

Results

For the evaluation of Peptide D in PHA hydrogels at three concentrations 1 μM, 10 μM, and 100 μM in control and TGF-β3 media **(Figure 6.1)**, for ACAN, no Ct values were detected for the groups with control medium, as for the groups with TGF-β3 medium, PHA with conjugated Peptide D-10 μM exhibited the highest gene expression with 5-fold ($p < 0.05$) higher value compared to PHA alone. Interestingly, no ACAN was detected with Peptide D at 100 μM group in the TGF-β3 medium. For collagen II, in control medium, PHA + Peptide D-100 μM had significantly higher collagen II gene expression with 1.8-fold higher ($p < 0.0001$) value compared to the negative control; however, no significant differences were observed between the negative control and PHA + Peptide D at 1 and 10 μM. The addition of TGF-β3 resulted in 1.4-fold ($p < 0.05$) higher collagen II gene expression in the PHA group compared to PHA in control medium. In TGF-β3 medium, PHA + Peptide D-100 μM had the highest collagen II gene expression with 1.4-fold higher ($p < 0.01$) value compared to PHA alone with TGF-β3, no other significant differences were observed among the remaining groups in TGF-β3 medium. For SOX-9, in control medium, PHA+D-100 μM had the highest gene expression with 1.9-fold ($p < 0.0001$) higher value compared to the negative control. In the presence of TGF-β3, PHA+D-100 μM had 1.8-fold higher ($p < 0.0001$) value compared to the negative control. In TGF-β3 medium, PHA+D-100 μM had 1.8-fold higher ($p < 0.0001$) SOX-9 gene expression compared to both PHA+D-1 μM and PHA+D-10 μM.

For the evaluation of the chondrogenic differentiation of encapsulated hBMSCs in PHA hydrogels with conjugated TGF-β3 conjugated Peptide D **(Figure 6.2A)**. PHA + soluble TGF-β3 induced the highest gene expression of ACAN and collagen II with 230-fold ($p < 0.0001$) and 64-fold ($p < 0.0001$) higher values, respectively, compared to the negative control (PHA in control medium); however, no significant differences in the gene expression of SOX-9 were observed between PHA + soluble TGF-β3 group and the negative control group. For ACAN, T25 (PHA + TGF-β3-25 nM) and T50 PHA + TGF-β3-50 nM) had 170-fold ($p < 0.0001$) and 86-fold ($p < 0.0001$) higher values, respectively, compared to the negative control. No significant differences were observed for the remaining groups for ACAN. For collagen II, T50 and T100 groups had 8.5-fold ($p < 0.05$) and 10-fold ($p < 0.01$) higher values compared to the negative control. DT50 (PHA + TGF-β3-50 + Peptide D-100 μM) and DT100 (PHA + TGF-β3-50 + Peptide D-100 μM) had 21-fold ($p < 0.0001$) and 18.5-fold ($p < 0.0001$) higher collagen II gene expression compared to the negative control. For SOX-9, T100 had 6-fold ($p < 0.0001$) higher gene expression compared to the negative control. DT25, DT50, and DT100 had 6-fold ($p < 0.0001$), 11-fold ($p < 0.0001$), and 10-fold ($p < 0.0001$) higher SOX-9 gene expression, respectively, compared to the negative control. DT50 and DT100 had 1.9-fold ($p < 0.0001$) and 1.7-fold ($p < 0.001$) higher SOX-9 gene expression compared to DT25.

We additionally evaluated the gene expression of osteogenic markers, collagen I, collagen X, Runx2, and BGLAP **(Figure 6.2B)**. For collagen I, PHA with soluble TGF-β3 had the lowest gene expression for collagen I compared to all groups including 66% lower ($p < 0.0001$) value compared to the negative control. T50 had 25% lower ($p < 0.05$) collagen I gene expression compared to negative control. DT25, DT50, and DT100 had

25% ($p < 0.05$), 44% ($p < 0.0001$), and 44% ($p < 0.0001$) lower collagen I gene expression compared to the negative control. DT100 had 34% lower ($p < 0.01$) collagen I gene expression compared to T100. For collagen X, PHA with soluble TGF-β3 had the highest gene expression of collagen X compared to all groups including 19-fold higher ($p < 0.0001$) value compared to the negative control. DT50 had 26% lower ($p < 0.05$) collagen X gene expression compared to T50, and DT100 had 27% lower ($p < 0.01$) value compared to T100. No significant difference was observed between T25 and DT25 for collagen X gene expression. For Runx2, no significant difference was observed between soluble TGF-β3 and the negative control. DT25, DT50, and DT100 had 2.7-fold ($p < 0.01$), 4.8-fold ($p < 0.0001$), and 2.5-fold ($p < 0.0001$) higher Runx2 gene expression compared to T25, T50, and T100, respectively. For BGLAP, no significant difference was observed between soluble TGF-β3 and the negative control. DT25, and DT50 had 2.7-fold ($p < 0.0001$), and 1.7-fold ($p < 0.0001$) higher BGLAP gene expression compared to T25, and T50, respectively. No significant difference was observed between DT100 and T100 groups.

For the GAG content **(Figure 6.3A)**, PHA + Peptide D + TGF-25 nM (DT25) had the highest DNA content, including 2.3-fold ($p < 0.05$) higher value compared to the negative control. No significant differences were observed for the remaining groups. PHA + TGF-25 nM (T25) had 2.3-fold ($p < 0.0001$) higher value compared to the negative control. No significant differences were observed among the PHA + TGF groups (T groups), or among the PHA + TGF +Peptide D (DT) groups. As for the GAGs/DNA content, T100 had 2-fold ($p < 0.01$) higher content compared to the negative control. We evaluated the viability of hBMSCs encapsulated in the hBMSCs on day 1, 3, 7, 14 and 21

(Figure 6.3B). On day 1 DT25, had the highest viability compared to all other groups, by day 3 no significant differences were observed among all groups, on day 7 and day 14 DT25 had the highest viability among groups, but by day 21 no significant differences were observed among groups. On day 21, we normalized the viability to DNA content **(Figure 6.3C)**, and only DT25 showed a significant decrease in viability compared to the negative control; however, no other significant differences were observed among the remaining groups.

We additionally evaluated Peptide D in the nM range **(Figure 6.4)**. No significant differences were observed among all groups without conjugated TGF-β3 for all genes. PHA + TGF-β3 had 555-fold higher ($p < 0.0001$) ACAN gene expression, and 6-fold higher ($p < 0.0001$) collagen II gene expression compared to the negative control. For collagen II, no significant differences were observed among groups with conjugated TGF-β3 and conjugated Peptide D. However, for SOX-9, for PHA groups with conjugated TGF-β3, the conjugation of Peptide D at 10, 100, and 1000 nM resulted in 2.9-fold ($p < 0.0001$), 3.3-fold ($p < 0.0001$), and 4.9-fold ($p < 0.0001$) higher values, respectively, compared to PHA with conjugated TGF-β3 only.

Discussion

This study presents a novel PHA hydrogel with conjugated Peptide D that enhanced the chondrogenic differentiation of encapsulated hBMSCs in the presence of TGF-β3. While the ultimate goal would be to synthesize a chondroinductive biomaterial without the need of growth factors, the fact that no continuous TGF-β3 supplementation was needed over a 21-day period is considered to be a major step toward the synthesis of an off-the-shelf chondroinductive biomaterial.

In the current study, Peptide D was first evaluated at 1, 10, and 100 μM in the presence or absence of soluble TGF-β3. Peptide D at 100 μM had the best outcome in terms of collagen II and SOX-9 gene expression in the presence or absence of soluble TGF-β3. However, the lack of ACAN gene expression in the absence of TGF-β3 prompted the inclusion of a conjugated TGF-β3 and Peptide D in the PHA hydrogel for subsequent experiments.

Next, we evaluated the chondroinductive potential of PHA + Peptide D (100 μM) with or without conjugated TGF-β3. Conjugated TGF-β3 was evaluated at 25, 50, and 100 nM, where the best performing concentration was determined to be 100 nM. Specifically, the 100 nM concentration resulted in significantly higher production of GAGs/DNA, in addition to higher gene expression of ACAN, collagen II, and SOX-9 compared to the negative control. Interestingly, PHA with soluble TGF-β3 resulted in significantly higher gene expression of ACAN and collagen II compared to PHA with conjugated TGF-β3; however, there was no significant differences between soluble TGF-β3 and conjugated TGF-β3 groups for GAGs/DNA production, which indicates that the

controlled release of TGF-β3 might have a temporal outcome on the gene expression of chondrogenic markers; however, the overall production of GAGs was similar. Importantly, a synergy between Peptide D and conjugated TGF-β3 (TGF-SH) was observed, as PHA + Peptide D + TGF-SH (50 and 100 nM) resulted in higher gene expression of collagen II and SOX-9 compared to the PHA+TGF-SH. Interestingly, the gene expression of ACAN did not increase, which warrants additional investigation at earlier timepoints to monitor the change in the longitudinal gene expression of ACAN by hBMSCs. For future *in vitro* and *in vivo* studies, we decided to move forward with 100 nM TGF-SH. Prior to proceeding to *in vivo* studies, we evaluated the chondroinductive potential at lower concentrations of Peptide D at 10, 100, and 1000 nM, together with conjugated TGF-β3 at 100 nM. Unfortunately, we observed a significant decrease in the gene expression of ACAN and collagen II with all 3 concentrations compared to PHA + TGF-SH. Therefore, we decided to move forward with PHA + TGF-SH (100 nM) + Peptide D (100 μM) for *in vivo* studies.

Future work exploring amino acid substitutions in the Peptide D sequence would be valuable to enhance the chondroinductive potential of Peptide D. Additionally, further characterization of TGF-β3 conjugation within the hydrogel by comparing the release kinetics of TGF-β3 from hydrogels with conjugated or mixed-in TGF-β3.

Chapter 7: Conclusion

The development of an acellular chondroinductive biomaterial would be advantageous for clinical translation as it would be offered as a cost-effective, off-the-shelf material that can be implanted in cartilage defects regardless of the size and location of the defect. Additionally, chondroinductive biomaterials may be implanted in a single surgery, thus avoiding the 2-step surgeries and the cell expansion required with currently approved ACI techniques. While the recent approval of Agili-C™ from CartiHeal, Inc. is encouraging for the field of biomaterials, the calcium carbonate-based biomaterial plug comes in a fixed size, requiring the adjustment of the defect size via removal of healthy cartilage to fit the implant. Moreover, it is not yet reported whether Agili-C™ has any chondroinductive potential that would lead to the regeneration of a hyaline-like cartilage tissue.

The development of a chondroinductive biomaterial requires two main components, the base material (e.g., hydrogel, extracellular matrix), and the chondroinductive factor to induce the chondrogenic differentiation of endogenous stem cells. The latter is the focus of this dissertation. My journey started with a literature review (Chapter 2) to identify already existing chondroinductive factors that I divided into two major classes, small compounds and peptides. Based on efficacy reported in the literature, I identified two compounds, kartogenin and SM04690, and two peptides, CM10 and CK2.1, as promising chondroinductive factors. One major takeaway from my review was that there was a lot of variability in the cell sources, culture conditions, *in vitro* models, and *in vivo* models used to evaluate the chondroinductive potential of peptides and compounds. Most importantly, there was a clear absence of appropriate controls from a

handful of studies. Hence, the following aims were drafted as a roadmap for this dissertation (1) Establish an *in vitro* model for chondrogenesis and evaluate the chondrogenic potential of equine, and human BMSCs (eBMSCs, and hBMSCs), and equine umbilical cord mesenchymal stromal cells (eUCMSCs) in spheroid culture. (2) Evaluate the chondrogenic potential of promising synthetic chondrogenic factors (CFs) and their combinations with hBMSCs. (3) Synthesize and evaluate the chondroinductive potential of a bioactive PHA hydrogel with conjugated TGF-β3 and synergistic Peptide D.

I started by developing a standardized *in vitro* model to evaluate chondrogenesis. I evaluated varying cell spheroid sizes, medium formulations, and culture conditions (i.e., hypoxia). Eventually, I established a spheroid culture model, and cultured the cells in hypoxia for 21 days prior to the evaluation of the gene expression of chondrogenic markers and/or GAG content. I successfully applied this model to rat BMSCs, equine BMSCs (eBMSCs), and human BMSCs (hBMSCs). Then, I directly evaluated and compared the chondroinductivity of the most promising peptides and compounds that I had identified from the literature (i.e., kartogenin, SM04690, CM10, and CK2.1) with equine (Chapter 3) and human BMSCs (Chapter 4). None of these evaluated peptides and compounds resulted in chondrogenesis (i.e., significantly higher gene expression of chondrogenic markers or higher GAG synthesis compared to the negative control), with both eBMSCs and hBMSCs. The results of chapters 3 and 4 emphasize that *in vitro* chondroinduction may depend on the cell source and culture conditions (e.g., medium composition, monolayer vs. pellet vs. 3D scaffold, hypoxia vs. normoxia), which highlights the importance of including a universal positive control such as TGF-β3 to allow proper and rigorous evaluation of results across different papers.

Given that none of the compounds from the previous literature turned out promising, I decided to move away from compounds and shift my focus to peptides, since peptides can be easily modified. Additionally, peptides are advantageous in biocompatibility as they are biodegradable into amino acids that naturally exist in our bodies. Moving away from peptides reported in the literature, I focused on newly discovered growth factor-derived peptides, that were identified by our team using a new peptide discovery approach. I evaluated 11 peptides in total (Peptides 1, 2, 4, 5, 6, 9 and peptides C1-C5); however, none of the evaluated peptides resulted in the chondrogenic differentiation of hBMSCs (Chapter 5). I mainly focused on peptides C1-C5 because they exhibited a higher binding affinity to human TGF-β receptors than Peptides 1-9. Interestingly, flow cytometry showed positive staining with C1, indicating that there was an interaction between Peptide C1 and hBMSCs; however, the interaction between Peptide C1 and hBMSCs did not translate into an upregulation of chondrogenic markers. I speculate that the peptide is binding to the receptor, but not initiating the necessary downstream signaling required to induce chondrogenesis. Future work may include running a PCR array for the human TGFβ pathway, to determine whether downstream signaling is activated. Additionally, running a competitive ELISA with the peptides and TGF-β3 may help determine whether the peptides are indeed binding to the TGF-β receptors in their native form. The evaluation of Peptide C1's interaction with hBMSCs using flow cytometry was a preliminary evaluation and I attempted both overnight incubation and 30 min incubation of the peptide with cells, and both staining methods resulted in a positive staining signal. However, it is not yet clear what the significance of a positive staining with C1 indicates, as it may imply that the peptide is simply interacting

with the cells (e.g., entrapped in the membrane, uptaken by the cell, non-specific binding) and not really binding to the TGF-β receptors. To better evaluate the specificity of the interaction, a scrambled peptide control may be utilized in flow. Alternatively, a more specific binding analysis method may be explored, Bio-Layer Interferometry (BLI), which is an alternative to Surface Plasmon Resonance (SPR). BLI allows the quantification of the specific binding of a soluble analyte to an immobilized receptor (K_D). Apart from chondrogenesis, identifying a peptide that can bind to TGF-β receptors is a desired application in the cancer field, as blocking the TGF-β pathway is a potential treatment for late-stage tumor progression.[196,197]

Up to that point, I had not discovered a chondroinductive peptide in my thesis work; therefore, the objective of my dissertation shifted to using a synergistic peptide that enhanced the chondrogenic differentiation of hBMSCs in the presence of TGF-β3. The peptide named D had recently been identified by our team using the same peptide discovery strategy used in chapter 5, and our team found that soluble Peptide D increased the gene expression of chondrogenic markers in combination with soluble TGF-β3 compared to TGF-β3 alone. Therefore, in chapter 6, my objective was to synthesize a biomaterial with conjugated Peptide D and conjugated TGF-β3. The base of the biomaterial was an existing biomaterial developed by our team, a pentenoate-functionalized hyaluronic acid hydrogel (PHA), which was chosen for its fast-crosslinking time of ~2 min and the paste-like consistency of the precursor solution[198] (the paste-like behavior allows the filling of defects of any shape or size). I synthesized PHA with conjugated Peptide D and conjugated TGF-β3 and evaluated the chondroinductivity of a range of concentrations for Peptide D and TGF-β3 on encapsulated hBMSCs. Eventually,

I successfully synthesized a chondroinductive biomaterial that enhanced the gene expression of chondrogenic markers (collagen II, and SOX-9). Future work may consider the use of a scrambled Peptide D to confirm that the synergy observed with TGF-β3 is specific to Peptide D. Furthermore, future work involving conjugated TGF-β3 warrants the investigation of the release kinetics of conjugated TGF-β3, and a comparison of conjugated versus mixed-in TGF-β3 in a PHA hydrogel (e.g., entrapped, controlled release from microspheres) to determine the most effective method to incorporate TGF-β3.

For the outcome analyses used throughout this dissertation, the *in vitro* screening method relied on the evaluation of the chondroinductive potential of the peptides after 21 days in culture; however, it was observed that ACAN gene expression was low or not detectable on day 21 for some experimental groups. Additionally, 21 days in culture is an extended period of time to wait for results and limits the throughput. The 21-day culture period is essential to evaluate the production of glycosaminoglycans (GAGs) by cells; however, for the evaluation of gene expression, earlier timepoints could be explored. Therefore, for future screening of peptides, it might be worth evaluating the gene expression of chondrogenic markers on day 7 or day 14 in an attempt to increase the throughput of the *in vitro* model, followed by 21-day studies for promising groups to evaluate GAG production. Another highly important point regarding qPCR as an outcome analysis was the choice of probes. Early on in the dissertation, a TaqMan collagen II probe from ThermoScientific was used, and we observed that only the positive control samples resulted in a quantifiable gene expression value, whereas no collagen II was detected with the negative controls or experimental groups. Later in the dissertation, we

switched to Bio-Rad probes, and we were able to detect collagen II with the negative and positive controls, and the experimental groups. While that does not change the outcome of the earlier studies as we quantify the relative change in the gene expression, the choice of gene expression probes can change the sensitivity of the assay. Most of the outcome analyses in this dissertation relied on gene expression of chondrogenic markers, which is a snapshot that captures the particular cell state at the timepoint. However, knowing that the gene expression of chondrogenic markers changes over the culture period, it is vital to look at the protein expression of chondrogenic markers such as collagen II, and hypertrophy marker (collagen I) using ELISA, to determine the accumulation of the measured protein over the culture period in order establish a better evaluation and comparison among different peptides and TGF-β3 positive control.

The culmination of all the *in vitro* work in this dissertation led to the initiation of a currently ongoing *in vivo* study to evaluate the chondroinductive potential of the synthesized PHA biomaterial with conjugated Peptide D and conjugated TGF-β3 in an osteochondral cartilage defect model in rabbits.

The identification of Peptide D is proof of concept that the peptide discovery method employed resulted in the identification of a peptide that interacts with and enhances the chondrogenic differentiation of hBMSCs. Looking at the future, in addition to pursuing more in-depth evaluation of PHA with conjugated Peptide D and conjugated TGF-β3, the objective to identify a peptide that is chondroinductive in the absence of growth factors remains an attainable target. Future peptide discovery methods could focus on designing peptides that mimic the critical binding domain of TGF-β3 to its

receptor maximizing the chances of peptide-receptor interaction. Additionally, new peptide discovery strategies could focus on capturing the importance of secondary and tertiary structure of the proteins when designing the peptides.

In the current dissertation, a chondroinductive biomaterial was synthesized that induced the chondrogenic differentiation of hBMSCs *in vitro*. This biomaterial represents a first step in the design of an off-the-shelf chondroinductive biomaterial poised to revolutionize cartilage therapies.

References

1. BMUS: The Burden of Musculoskeletal Diseases in the United States. *BMUS: The Burden of Musculoskeletal Diseases in the United States* https://www.boneandjointburden.org/.
2. Jones, I. A., Togashi, R., Wilson, M. L., Heckmann, N. & Vangsness, C. T. Intra-articular treatment options for knee osteoarthritis. *Nat Rev Rheumatol* **15**, 77–90 (2019).
3. Steadman, J. R., Rodkey, W. G. & Briggs, K. K. Microfracture: Its History and Experience of the Developing Surgeon. *Cartilage* **1**, 78–86 (2010).
4. Medvedeva, E. V. *et al.* Repair of Damaged Articular Cartilage: Current Approaches and Future Directions. *Int J Mol Sci* **19**, (2018).
5. Richter, D. L., Schenck, R. C., Wascher, D. C. & Treme, G. Knee Articular Cartilage Repair and Restoration Techniques. *Sports Health* **8**, 153–160 (2016).
6. Brittberg, M. *et al.* Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N. Engl. J. Med.* **331**, 889–895 (1994).
7. Brittberg, M. Clinical articular cartilage repair—an up to date review. *Annals of Joint* **3**, (2018).
8. Davies, R. L. & Kuiper, N. J. Regenerative Medicine: A Review of the Evolution of Autologous Chondrocyte Implantation (ACI) Therapy. *Bioengineering* **6**, 22 (2019).
9. Commissioner, O. of the. FDA approves first autologous cellularized scaffold for the repair of cartilage defects of the knee. *FDA* https://www.fda.gov/news-events/press-announcements/fda-approves-first-autologous-cellularized-scaffold-repair-cartilage-defects-knee (2020).
10. Na, Y. *et al.* Is implantation of autologous chondrocytes superior to microfracture for articular-cartilage defects of the knee? A systematic review of 5-year follow-up data. *Int J Surg* **68**, 56–62 (2019).

11. Słynarski, K. *et al.* Single-Stage Autologous Chondrocyte-Based Treatment for the Repair of Knee Cartilage Lesions: Two-Year Follow-up of a Prospective Single-Arm Multicenter Study. *The American Journal of Sports Medicine* **48**, 1327–1337 (2020).
12. Arshi, A., Petrigliano, F. A., Williams, R. J. & Jones, K. J. Stem Cell Treatment for Knee Articular Cartilage Defects and Osteoarthritis. *Curr Rev Musculoskelet Med* **13**, 20–27 (2020).
13. Zhang, S. *et al.* Articular cartilage regeneration: The role of endogenous mesenchymal stem/progenitor cell recruitment and migration. *Semin. Arthritis Rheum.* **50**, 198–208 (2020).
14. Doyle, E. C., Wragg, N. M. & Wilson, S. L. Intraarticular injection of bone marrow-derived mesenchymal stem cells enhances regeneration in knee osteoarthritis. *Knee Surg Sports Traumatol Arthrosc* (2020) doi:10.1007/s00167-020-05859-z.
15. Armiento, A. R., Stoddart, M. J., Alini, M. & Eglin, D. Biomaterials for articular cartilage tissue engineering: Learning from biology. *Acta Biomaterialia* **65**, 1–20 (2018).
16. Roseti, L., Desando, G., Cavallo, C., Petretta, M. & Grigolo, B. Articular Cartilage Regeneration in Osteoarthritis. *Cells* **8**, 1305 (2019).
17. Jessop, Z. M. *et al.* Tissue specific stem/progenitor cells for cartilage tissue engineering: A systematic review of the literature. *Applied Physics Reviews* **6**, 031301 (2019).
18. Graceffa, V. *et al.* Chasing Chimeras – The elusive stable chondrogenic phenotype. *Biomaterials* **192**, 199–225 (2019).
19. To, K., Zhang, B., Romain, K., Mak, C. & Khan, W. Synovium-Derived Mesenchymal Stem Cell Transplantation in Cartilage Regeneration: A PRISMA Review of in vivo Studies. *Front Bioeng Biotechnol* **7**, 314 (2019).
20. Francis, S. L., Di Bella, C., Wallace, G. G. & Choong, P. F. M. Cartilage Tissue Engineering Using Stem Cells and Bioprinting Technology—Barriers to Clinical Translation. *Front. Surg.* **5**, (2018).

21. Lo Monaco, M. *et al.* Stem Cells for Cartilage Repair: Preclinical Studies and Insights in Translational Animal Models and Outcome Measures. *Stem Cells Int* **2018**, (2018).
22. Kwan, H., Chisari, E. & Khan, W. S. Cell-Free Scaffolds as a Monotherapy for Focal Chondral Knee Defects. *Materials* **13**, 306 (2020).
23. Eftekhari, A. *et al.* The Use of Nanomaterials in Tissue Engineering for Cartilage Regeneration; Current Approaches and Future Perspectives. *Int J Mol Sci* **21**, (2020).
24. Del Bakhshayesh, A. R. *et al.* An overview of advanced biocompatible and biomimetic materials for creation of replacement structures in the musculoskeletal systems: focusing on cartilage tissue engineering. *J Biol Eng* **13**, 85 (2019).
25. Koh, R. H., Jin, Y., Kim, J. & Hwang, N. S. Inflammation-Modulating Hydrogels for Osteoarthritis Cartilage Tissue Engineering. *Cells* **9**, (2020).
26. Bao, W. *et al.* Advancements and Frontiers in the High Performance of Natural Hydrogels for Cartilage Tissue Engineering. *Front Chem* **8**, 53 (2020).
27. Li, J. *et al.* Advances of injectable hydrogel-based scaffolds for cartilage regeneration. *Regen Biomater* **6**, 129–140 (2019).
28. Walker, M., Luo, J., Pringle, E. W. & Cantini, M. ChondroGELesis: Hydrogels to harness the chondrogenic potential of stem cells. *Mater Sci Eng C Mater Biol Appl* **121**, 111822 (2021).
29. Morelli, X., Bourgeas, R. & Roche, P. Chemical and structural lessons from recent successes in protein-protein interaction inhibition (2P2I). *Curr Opin Chem Biol* **15**, 475–481 (2011).
30. Cesa, L. C., Mapp, A. K. & Gestwicki, J. E. Direct and Propagated Effects of Small Molecules on Protein–Protein Interaction Networks. *Front Bioeng Biotechnol* **3**, (2015).
31. Acar, H., Ting, J. M., Srivastava, S., LaBelle, J. L. & Tirrell, M. V. Molecular engineering solutions for therapeutic peptide delivery. *Chem Soc Rev* **46**, 6553–6569 (2017).

32. Li, T. *et al.* Small molecule compounds promote the proliferation of chondrocytes and chondrogenic differentiation of stem cells in cartilage tissue engineering. *Biomed Pharmacother* **131**, 110652 (2020).
33. Johnson, K. A. *et al.* Development of KA34 as a cartilage regenerative therapy for Osteoarthritis. *Osteoarthritis and Cartilage* **28**, S518 (2020).
34. Deshmukh, V. *et al.* A small-molecule inhibitor of the Wnt pathway (SM04690) as a potential disease modifying agent for the treatment of osteoarthritis of the knee. *Osteoarthr. Cartil.* **26**, 18–27 (2018).
35. Deshmukh, V. *et al.* Modulation of the Wnt pathway through inhibition of CLK2 and DYRK1A by lorecivivint as a novel, potentially disease-modifying approach for knee osteoarthritis treatment. *Osteoarthr. Cartil.* **27**, 1347–1360 (2019).
36. Yazici, Y. *et al.* A novel Wnt pathway inhibitor, SM04690, for the treatment of moderate to severe osteoarthritis of the knee: results of a 24-week, randomized, controlled, phase 1 study. *Osteoarthritis and Cartilage* **25**, 1598–1606 (2017).
37. Yazici, Y. *et al.* Results from a 52-week randomized, double-blind, placebo-controlled, phase 2 study of a novel, intra-articular wnt pathway inhibitor (SM04690) for the treatment of knee osteoarthritis. *Osteoarthritis and Cartilage* **26**, S293–S294 (2018).
38. Yazici, Y. *et al.* Lorecivivint, a Novel Intraarticular CDC-like Kinase 2 and Dual-Specificity Tyrosine Phosphorylation-Regulated Kinase 1A Inhibitor and Wnt Pathway Modulator for the Treatment of Knee Osteoarthritis: A Phase II Randomized Trial. *Arthritis & Rheumatology* **72**, 1694–1706 (2020).
39. Samumed LLC. *A Phase 3, 28-Week, Multicenter, Randomized, Double-Blind, Placebo-Controlled Study to Evaluate the Efficacy and Safety of a Single Injection of SM04690 Injected in the Target Knee Joint of Moderately to Severely Symptomatic Osteoarthritis Subjects*. https://clinicaltrials.gov/ct2/show/NCT04385303 (2020).

40. Liu, Q. *et al.* Functional peptides for cartilage repair and regeneration. *Am J Transl Res* **10**, 501–510 (2018).

41. Gonzalez-Fernandez, T., Sikorski, P. & Leach, J. K. Bio-instructive materials for musculoskeletal regeneration. *Acta Biomaterialia* **96**, 20–34 (2019).

42. Mahzoon, S. & Detamore, M. S. Chondroinductive Peptides: Drawing Inspirations from Cell-Matrix Interactions. *Tissue Eng Part B Rev* **25**, 249–257 (2019).

43. Akkiraju, H., Bonor, J. & Nohe, A. CK2.1, a novel peptide, induces articular cartilage formation in vivo. *Journal of Orthopaedic Research* **35**, 876–885 (2017).

44. Akkiraju, H. *et al.* CK2.1, a bone morphogenetic protein receptor type Ia mimetic peptide, repairs cartilage in mice with destabilized medial meniscus. *Stem Cell Res Ther* **8**, (2017).

45. Bragdon, B. *et al.* Casein Kinase 2 β-Subunit Is a Regulator of Bone Morphogenetic Protein 2 Signaling. *Biophys J* **99**, 897–904 (2010).

46. Shah, R. N. *et al.* Supramolecular design of self-assembling nanofibers for cartilage regeneration. *PNAS* **107**, 3293–3298 (2010).

47. Chen, J. *et al.* TGF-β1 affinity peptides incorporated within a chitosan sponge scaffold can significantly enhance cartilage regeneration. *J Mater Chem B* **6**, 675–687 (2018).

48. Patel, J. M., Wise, B. C., Bonnevie, E. D. & Mauck, R. L. A Systematic Review and Guide to Mechanical Testing for Articular Cartilage Tissue Engineering. *Tissue Eng Part C Methods* **25**, 593–608 (2019).

49. Blaschuk, O. W., Sullivan, R., David, S. & Pouliot, Y. Identification of a cadherin cell adhesion recognition sequence. *Dev. Biol.* **139**, 227–229 (1990).

50. Williams, E., Williams, G., Gour, B. J., Blaschuk, O. W. & Doherty, P. A novel family of cyclic peptide antagonists suggests that N-cadherin specificity is determined by amino acids that flank the HAV motif. *J. Biol. Chem.* **275**, 4007–4012 (2000).

51. Bian, L., Guvendiren, M., Mauck, R. L. & Burdick, J. A. Hydrogels that mimic developmentally relevant matrix and N-cadherin interactions enhance MSC chondrogenesis. *PNAS* **110**, 10117–10122 (2013).

52. Vega, S. L., Kwon, M., Mauck, R. L. & Burdick, J. A. Single Cell Imaging to Probe Mesenchymal Stem Cell N-Cadherin Mediated Signaling within Hydrogels. *Ann Biomed Eng* **44**, 1921–1930 (2016).

53. Kwon, M. Y. *et al.* Dose and Timing of N-Cadherin Mimetic Peptides Regulate MSC Chondrogenesis within Hydrogels. *Advanced Healthcare Materials* **7**, 1701199 (2018).

54. Eren Cimenci, C., Kurtulus, G. U., Caliskan, O. S., Guler, M. O. & Tekinay, A. B. N-Cadherin Mimetic Peptide Nanofiber System Induces Chondrogenic Differentiation of Mesenchymal Stem Cells. *Bioconjugate Chem.* **30**, 2417–2426 (2019).

55. Feng, X. *et al.* Enhanced regeneration of osteochondral defects by using an aggrecanase-1 responsively degradable and N-cadherin mimetic peptide-conjugated hydrogel loaded with BMSCs. *Biomaterials Science* **8**, 2212–2226 (2020).

56. Mohammed, M., Lai, T.-S. & Lin, H.-C. Substrate stiffness and sequence dependent bioactive peptide hydrogels influence the chondrogenic differentiation of human mesenchymal stem cells. *Journal of Materials Chemistry B* (2021) doi:10.1039/D0TB02008G.

57. Teng, B. *et al.* A chondrogenesis induction system based on a functionalized hyaluronic acid hydrogel sequentially promoting hMSC proliferation, condensation, differentiation, and matrix deposition. *Acta Biomater* (2021) doi:10.1016/j.actbio.2020.12.054.

58. Lam, H. J., Li, S., Lou, N., Chu, J. & Bhatnagar, R. S. Synthetic peptides cytomodulin-1 (CM-1) and cytomodulin-2 (CM-2) promote collagen synthesis and wound healing in vitro. *Conf Proc IEEE Eng Med Biol Soc* **2004**, 5028–5030 (2004).

59. Bhatnagar, R. S. & Qian, J. J. Peptide compositions with growth factor-like activity. (1997).

60. Bhatnagar, R. S. & Qian, J. J. Peptide compositions with growth factor-like activity. (1998).

61. Zhang, Z., Gupte, M. J., Jin, X. & Ma, P. X. Injectable Peptide Decorated Functional Nanofibrous Hollow Microspheres to Direct Stem Cell Differentiation and Tissue Regeneration. *Advanced Functional Materials* **25**, 350–360 (2015).

62. Park, S. H. *et al.* An injectable, click-crosslinked, cytomodulin-modified hyaluronic acid hydrogel for cartilage tissue engineering. *NPG Asia Materials* **11**, 1–16 (2019).

63. Lin, X., Zamora, P. O., Albright, S., Glass, J. D. & Peña, L. A. Multidomain synthetic peptide B2A2 synergistically enhances BMP-2 in vitro. *J. Bone Miner. Res.* **20**, 693–703 (2005).

64. Lin, X. *et al.* B2A peptide induces chondrogenic differentiation in vitro and enhances cartilage repair in rats. *J. Orthop. Res.* **30**, 1221–1228 (2012).

65. Mahzoon, S., Townsend, J. M., Lam, T. N., Sjoelund, V. & Detamore, M. S. Effects of a Bioactive SPPEPS Peptide on Chondrogenic Differentiation of Mesenchymal Stem Cells. *Ann Biomed Eng* (2019) doi:10.1007/s10439-019-02306-0.

66. Martin, H. & Dean, M. An N-terminal peptide from link protein is rapidly degraded by chondrocytes, monocytes and B cells. *Eur. J. Biochem.* **212**, 87–94 (1993).

67. Mwale, F. *et al.* A synthetic peptide of link protein stimulates the biosynthesis of collagens II, IX and proteoglycan by cells of the intervertebral disc. *Journal of Cellular Biochemistry* **88**, 1202–1213 (2003).

68. Wang, Z., Weitzmann, M. N., Sangadala, S., Hutton, W. C. & Yoon, S. T. Link protein N-terminal peptide binds to bone morphogenetic protein (BMP) type II receptor and drives matrix protein expression in rabbit intervertebral disc cells. *J. Biol. Chem.* **288**, 28243–28253 (2013).

69. He, R. *et al.* Link Protein N-Terminal Peptide as a Potential Stimulating Factor for Stem Cell-Based Cartilage Regeneration. *Stem Cells Int* **2018**, 3217895 (2018).

70. Knight, C. G. *et al.* Identification in collagen type I of an integrin alpha2 beta1-binding site containing an essential GER sequence. *J. Biol. Chem.* **273**, 33287–33294 (1998).

71. Knight, C. G. *et al.* The collagen-binding A-domains of integrins alpha(1)beta(1) and alpha(2)beta(1) recognize the same specific amino acid sequence, GFOGER, in native (triple-helical) collagens. *J. Biol. Chem.* **275**, 35–40 (2000).

72. Liu, S. Q. *et al.* Biomimetic hydrogels for chondrogenic differentiation of human mesenchymal stem cells to neocartilage. *Biomaterials* **31**, 7298–7307 (2010).

73. Mhanna, R. *et al.* GFOGER-Modified MMP-Sensitive Polyethylene Glycol Hydrogels Induce Chondrogenic Differentiation of Human Mesenchymal Stem Cells. *Tissue Eng Part A* **20**, 1165–1174 (2014).

74. Wojtowicz, A. M. *et al.* Coating of Biomaterial Scaffolds with the Collagen-Mimetic Peptide GFOGER for Bone Defect Repair. *Biomaterials* **31**, 2574 (2010).

75. Reyes, C. D., Petrie, T. A., Burns, K. L., Schwartz, Z. & García, A. J. Biomolecular surface coating to enhance orthopaedic tissue healing and integration. *Biomaterials* **28**, 3228–3235 (2007).

76. Clark, A. Y. *et al.* Integrin-specific hydrogels modulate transplanted human bone marrow-derived mesenchymal stem cell survival, engraftment, and reparative activities. *Nature Communications* **11**, 114 (2020).

77. Saito, A., Suzuki, Y., Ogata, S. ichi, Ohtsuki, C. & Tanihara, M. Activation of osteo-progenitor cells by a novel synthetic peptide derived from the bone morphogenetic protein-2 knuckle epitope. *Biochim. Biophys. Acta* **1651**, 60–67 (2003).

78. Renner, J. N., Kim, Y. & Liu, J. C. Bone Morphogenetic Protein-Derived Peptide Promotes Chondrogenic Differentiation of Human Mesenchymal Stem Cells. *Tissue Engineering Part A* **18**, 2581–2589 (2012).

79. Renner, J. N. & Liu, J. C. Investigating the effect of peptide agonists on the chondrogenic differentiation of human mesenchymal stem cells using design of experiments. *Biotechnol. Prog.* **29**, 1550–1557 (2013).

80. Goetinck, P. F., Stirpe, N. S., Tsonis, P. A. & Carlone, D. The tandemly repeated sequences of cartilage link protein contain the sites for interaction with hyaluronic acid. *J. Cell Biol.* **105**, 2403–2408 (1987).

81. Chow, L. W. *et al.* Peptide-directed spatial organization of biomolecules in dynamic gradient scaffolds. *Adv Healthc Mater* **3**, 1381–1386 (2014).

82. Butterfield, K. C., Conovaloff, A., Caplan, M. & Panitch, A. Chondroitin sulfate-binding peptides block chondroitin 6-sulfate inhibition of cortical neurite growth. *Neuroscience Letters* **478**, 82–87 (2010).

83. Parmar, P. A. *et al.* Collagen-mimetic peptide-modifiable hydrogels for articular cartilage regeneration. *Biomaterials* **54**, 213–225 (2015).

84. Pillutla, R. C. *et al.* Peptides identify the critical hotspots involved in the biological activation of the insulin receptor. *J. Biol. Chem.* **277**, 22590–22594 (2002).

85. Miller, R. E. *et al.* Effect of Self-assembling Peptide, Chondrogenic Factors, and Bone Marrow Derived Stromal Cells on Osteochondral Repair. *Osteoarthritis Cartilage* **18**, 1608–1619 (2010).

86. Kopesky, P. W., Vanderploeg, E. J., Sandy, J. S., Kurz, B. & Grodzinsky, A. J. Self-assembling peptide hydrogels modulate in vitro chondrogenesis of bovine bone marrow stromal cells. *Tissue Eng Part A* **16**, 465–477 (2010).

87. Kopesky, P. W. *et al.* Controlled delivery of transforming growth factor β1 by self-assembling peptide hydrogels induces chondrogenesis of bone marrow stromal cells and modulates Smad2/3 signaling. *Tissue Eng Part A* **17**, 83–92 (2011).

88. Kopesky, P. W. *et al.* Sustained delivery of bioactive TGF-β1 from self-assembling peptide hydrogels induces chondrogenesis of encapsulated bone marrow stromal cells. *J Biomed Mater Res A* **102**, 1275–1285 (2014).

89. Miller, R. E. *et al.* Effects of the Combination of Microfracture and Self-Assembling Peptide Filling on the Repair of a Clinically Relevant Trochlear Defect in an Equine Model. *J Bone Joint Surg Am* **96**, 1601–1609 (2014).

90. Kisiday, J. D., Colbath, A. C. & Tangtrongsup, S. Effect of culture duration on chondrogenic preconditioning of equine bone marrow mesenchymal stem cells in self-assembling peptide hydrogel. *J. Orthop. Res.* **37**, 1368–1375 (2019).

91. Lu, J. *et al.* Increased recruitment of endogenous stem cells and chondrogenic differentiation by a composite scaffold containing bone marrow homing peptide for cartilage regeneration. *Theranostics* **8**, 5039–5058 (2018).

92. Shao, Z. *et al.* Polycaprolactone electrospun mesh conjugated with an MSC affinity peptide for MSC homing in vivo. *Biomaterials* **33**, 3375–3387 (2012).

93. Huang, H. *et al.* A functional biphasic biomaterial homing mesenchymal stem cells for in vivo cartilage regeneration. *Biomaterials* **35**, 9608–9619 (2014).

94. Meng, Q. *et al.* A composite scaffold of MSC affinity peptide-modified demineralized bone matrix particles and chitosan hydrogel for cartilage regeneration. *Sci Rep* **5**, 17802 (2015).

95. Hesse, E. *et al.* Peptide-functionalized starPEG/heparin hydrogels direct mitogenicity, cell morphology and cartilage matrix distribution in vitro and in vivo. *J Tissue Eng Regen Med* **12**, 229–239 (2018).

96. Salinas, C. N. & Anseth, K. S. Decorin moieties tethered into PEG networks induce chondrogenesis of human mesenchymal stem cells. *Journal of Biomedical Materials Research Part A* **90A**, 456–464 (2009).

97. Zhang, J. *et al.* P15 peptide stimulates chondrogenic commitment and endochondral ossification. *International Orthopaedics (SICOT)* **41**, 1413–1422 (2017).

98. Tan, Q. *et al.* A novel FGFR1-binding peptide attenuates the degeneration of articular cartilage in adult mice. *Osteoarthr. Cartil.* **26**, 1733–1743 (2018).

99. Kuo, Y.-C. & Wang, C.-C. Surface modification with peptide for enhancing chondrocyte adhesion and cartilage regeneration in porous scaffolds. *Colloids Surf B Biointerfaces* **84**, 63–70 (2011).

100. Speer, J. E. *et al.* Development of a library of laminin-mimetic peptide hydrogels for control of nucleus pulposus cell behaviors. *J Tissue Eng* **12**, 20417314211021220 (2021).

101. Bridgen, D. T. *et al.* Regulation of human nucleus pulposus cells by peptide-coupled substrates. *Acta Biomaterialia* **55**, 100–108 (2017).

102. Barcellona, M. N. *et al.* Bioactive in situ crosslinkable polymer-peptide hydrogel for cell delivery to the intervertebral disc in a rat model. *Acta Biomaterialia* (2021) doi:10.1016/j.actbio.2021.06.045.

103. OrthoTrophix, Inc. *A Randomized, Double-Blind, Placebo-Controlled, Study Evaluating the Safety and Efficacy of a Second Course of Intra-Articular Injections of TPX-100 in Subjects Who Previously Received TPX-100 for Patellar Osteoarthritis Involving Both Knees*. https://clinicaltrials.gov/ct2/show/NCT02837900 (2017).

104. OrthoTrophix, Inc. *A Randomized, Double-Blind, Placebo-Controlled, Multi-Dose Phase 2 Study Evaluating the Safety and Efficacy of Intra-Articular Injections of TPX-100 in Subjects With Mild to Moderate Patello-Femoral Osteoarthritis Involving Both Knees*. https://clinicaltrials.gov/ct2/show/NCT01925261 (2017).

105. OrthoTrophix, Inc. *A Prospective Observational Study to Evaluate Long-Term Changes in Cartilage Morphology in Subjects Who Previously Received TPX-100 or Placebo in Study*

TPX-100-1 for Patellar Osteoarthritis Involving Both Knees. https://clinicaltrials.gov/ct2/show/NCT03125499 (2018).

106. McGuire, D. *et al.* TPX-100 leads to marked, sustained improvements in subjects with knee osteoarthritis: pre-clinical rationale and results of a controlled clinical trial. *Osteoarthritis and Cartilage* **26**, S243 (2018).
107. Ensol Bioscience. http://www.ensolbio.co.kr.
108. Acar, H. *et al.* Self-assembling peptide-based building blocks in medical applications. *Adv Drug Deliv Rev* **110–111**, 65–79 (2017).
109. van Weeren, P. R. & Back, W. Musculoskeletal disease in aged horses and its management. *Vet Clin North Am Equine Pract* **32**, 229–247 (2016).
110. McIlwraith, C. W., Frisbie, D. D. & Kawcak, C. E. The horse as a model of naturally occurring osteoarthritis. *Bone & Joint Research* **1**, 297–309 (2012).
111. Jacobs, C. C., Schnabel, L. V., McIlwraith, C. W. & Blikslager, A. T. Non-steroidal anti-inflammatory drugs in equine orthopaedics. *Equine Vet J* **54**, 636–648 (2022).
112. Baccarin, R. Y. A., Seidel, S. R. T., Michelacci, Y. M., Tokawa, P. K. A. & Oliveira, T. M. Osteoarthritis: a common disease that should be avoided in the athletic horse's life. *Anim Front* **12**, 25–36 (2022).
113. Monteiro, S. O., Bettencourt, E. V. & Lepage, O. M. Biologic strategies for intra-articular treatment and cartilage repair. *Journal of Equine Veterinary Science* **35**, 175–190 (2015).
114. Frisbie, D. D., Kawcak, C. E., Werpy, N. M., Park, R. D. & McIlwraith, C. W. Clinical, biochemical, and histologic effects of intra-articular administration of autologous conditioned serum in horses with experimentally induced osteoarthritis. *American Journal of Veterinary Research* **68**, 290–296 (2007).

115. Hraha, T. H., Doremus, K. M., McILWRAITH, C. W. & Frisbie, D. D. Autologous conditioned serum: The comparative cytokine profiles of two commercial methods (IRAP and IRAP II) using equine blood. *Equine Veterinary Journal* **43**, 516–521 (2011).
116. Camargo Garbin, L. & Morris, M. J. A comparative review of autologous conditioned serum and autologous protein solution for treatment of osteoarthritis in horses. *Frontiers in Veterinary Science* **8**, (2021).
117. Ortved, K. F. Equine autologous conditioned serum and autologous protein solution. *Veterinary Clinics: Equine Practice* **39**, 443–451 (2023).
118. Bertone, A. L. *et al.* Evaluation of a single intra-articular injection of autologous protein solution for treatment of osteoarthritis in horses. *American Journal of Veterinary Research* **75**, 141–151 (2014).
119. Mirza, M. H. *et al.* Gait changes vary among horses with naturally occurring osteoarthritis following intra-articular administration of autologous platelet-rich plasma. *Front Vet Sci* **3**, 29 (2016).
120. Bosch, G. *et al.* Effects of platelet-rich plasma on the quality of repair of mechanically induced core lesions in equine superficial digital flexor tendons: A placebo-controlled experimental study. *J Orthop Res* **28**, 211–217 (2010).
121. Camargo Garbin, L., Lopez, C. & Carmona, J. U. A critical overview of the use of platelet-rich plasma in equine medicine over the last decade. *Frontiers in Veterinary Science* **8**, (2021).
122. Colbath, A. C., Dow, S. W., Phillips, J. N., McIlwraith, C. W. & Goodrich, L. R. Autologous and Allogeneic Equine Mesenchymal Stem Cells Exhibit Equivalent Immunomodulatory Properties In Vitro. *Stem Cells and Development* **26**, 503–511 (2017).

123. Colbath, A. C., Dow, S. W., McIlwraith, C. W. & Goodrich, L. R. Mesenchymal stem cells for treatment of musculoskeletal disease in horses: Relative merits of allogeneic versus autologous stem cells. *Equine Vet J* **52**, 654–663 (2020).

124. Broeckx, S. Y. *et al.* The use of equine chondrogenic-induced mesenchymal stem cells as a treatment for osteoarthritis: A randomised, double-blinded, placebo-controlled proof-of-concept study. *Equine Vet J* **51**, 787–794 (2019).

125. McIlwraith, C. W. *et al.* Evaluation of intra-articular mesenchymal stem cells to augment healing of microfractured chondral defects. *Arthroscopy: The Journal of Arthroscopic & Related Surgery* **27**, 1552–1561 (2011).

126. Fugazzola, M. C. & van Weeren, P. R. Surgical osteochondral defect repair in the horse—a matter of form or function? *Equine Veterinary Journal* **52**, 489–499 (2020).

127. Johnson, S. A. & Frisbie, D. D. Cartilage Therapy and Repair in Equine Athletes. *Operative Techniques in Orthopaedics* **26**, 155–165 (2016).

128. Cokelaere, S., Malda, J. & van Weeren, R. Cartilage defect repair in horses: Current strategies and recent developments in regenerative medicine of the equine joint with emphasis on the surgical approach. *The Veterinary Journal* **214**, 61–71 (2016).

129. McIlwraith, C. W. & Frisbie, D. D. Microfracture: basic science studies in the horse. *Cartilage* **1**, 87–95 (2010).

130. Nixon, A. J. *et al.* Autologous chondrocyte implantation drives early chondrogenesis and organized repair in extensive full- and partial-thickness cartilage defects in an equine model. *Journal of Orthopaedic Research* **29**, 1121–1130 (2011).

131. Bodó, G., Vásárhelyi, G., Hangody, L. & Módis, L. Mosaic arthroplasty of the medial femoral condyle in horses — An experimental study. *Acta Veterinaria Hungarica* **62**, 155–168 (2013).

132. Lepage, S. I. M., Lee, O. J. & Koch, T. G. Equine Cord Blood Mesenchymal Stromal Cells Have Greater Differentiation and Similar Immunosuppressive Potential to Cord Tissue Mesenchymal Stromal Cells. *Stem Cells and Development* **28**, 227–237 (2019).

133. Rakic, R. *et al.* Differences in the intrinsic chondrogenic potential of equine umbilical cord matrix and cord blood mesenchymal stromal/stem cells for cartilage regeneration. *Sci Rep* **8**, 13799 (2018).

134. Lovati, A. B. *et al.* Comparison of equine bone marrow-, umbilical cord matrix and amniotic fluid-derived progenitor cells. *Vet Res Commun* **35**, 103–121 (2011).

135. Bailey, M. M., Wang, L., Bode, C. J., Mitchell, K. E. & Detamore, M. S. A comparison of human umbilical cord matrix stem cells and temporomandibular joint condylar chondrocytes for tissue engineering temporomandibular joint condylar cartilage. *Tissue Eng* **13**, 2003–2010 (2007).

136. Wang, L., Tran, I., Seshareddy, K., Weiss, M. L. & Detamore, M. S. A comparison of human bone marrow-derived mesenchymal stem cells and human umbilical cord-derived mesenchymal stromal cells for cartilage tissue engineering. *Tissue Eng Part A* **15**, 2259–2266 (2009).

137. Wang, L., Ott, L., Seshareddy, K., Weiss, M. L. & Detamore, M. S. Musculoskeletal tissue engineering with human umbilical cord mesenchymal stromal cells. *Regen Med* **6**, 95–109 (2011).

138. Ajeeb, B., Acar, H. & Detamore, M. S. Chondroinductive Peptides for Cartilage Regeneration. *Tissue Eng Part B Rev* (2021) doi:10.1089/ten.TEB.2021.0125.

139. Johnson, K. *et al.* A stem cell-based approach to cartilage repair. *Science* **336**, 717–721 (2012).

140. Kang, M. L., Ko, J.-Y., Kim, J. E. & Im, G.-I. Intra-articular delivery of kartogenin-conjugated chitosan nano/microparticles for cartilage regeneration. *Biomaterials* **35**, 9984–9994 (2014).

141. Liu, C., Ma, X., Li, T. & Zhang, Q. Kartogenin, transforming growth factor-β1 and bone morphogenetic protein-7 coordinately enhance lubricin accumulation in bone-derived mesenchymal stem cells. *Cell Biol Int* **39**, 1026–1035 (2015).

142. Ajeeb, B. & Detamore, M. Comparison of multiple synthetic chondroinductive factors in pellet culture against a TGF-β positive control. *Osteoarthritis and Cartilage Open* **5**, 100369 (2023).

143. Markway, B. D. *et al.* Enhanced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in low oxygen environment micropellet cultures. *Cell Transplant* **19**, 29–42 (2010).

144. Ranmuthu, C. K. I., Ranmuthu, C. D. S., Wijewardena, C. K., Seah, M. K. T. & Khan, W. S. Evaluating the Effect of Hypoxia on Human Adult Mesenchymal Stromal Cell Chondrogenesis In Vitro: A Systematic Review. *Int J Mol Sci* **23**, 15210 (2022).

145. Gale, A. L., Mammone, R. M., Dodson, M. E., Linardi, R. L. & Ortved, K. F. The effect of hypoxia on chondrogenesis of equine synovial membrane-derived and bone marrow-derived mesenchymal stem cells. *BMC Vet Res* **15**, 201 (2019).

146. Wang, L., Singh, M., Bonewald, L. F. & Detamore, M. S. Signalling strategies for osteogenic differentiation of human umbilical cord mesenchymal stromal cells for 3D bone tissue engineering. *J Tissue Eng Regen Med* **3**, 398–404 (2009).

147. De Schauwer, C. *et al.* In search for cross-reactivity to immunophenotype equine mesenchymal stromal cells by multicolor flow cytometry. *Cytometry A* **81**, 312–323 (2012).

148. Spaas, J. H. *et al.* Culture and characterisation of equine peripheral blood mesenchymal stromal cells. *Vet J* **195**, 107–113 (2013).

149. Hamilton, A. G., Townsend, J. M. & Detamore, M. S. Automated decellularization of musculoskeletal tissues with high extracellular matrix retention. *Tissue Engineering Part C: Methods* **28**, 137–147 (2022).

150. Uder, C., Brückner, S., Winkler, S., Tautenhahn, H.-M. & Christ, B. Mammalian MSC from selected species: Features and applications. *Cytometry A* **93**, 32–49 (2018).
151. Ahmed, N. *et al.* CD45-positive cells of haematopoietic origin enhance chondrogenic marker gene expression in rat marrow stromal cells. *International Journal of Molecular Medicine* **18**, 233–240 (2006).
152. Rojewski, M. T., Weber, B. M. & Schrezenmeier, H. Phenotypic characterization of mesenchymal stem cells from various tissues. *Transfus Med Hemother* **35**, 168–184 (2008).
153. Deschaseaux, F. *et al.* Direct selection of human bone marrow mesenchymal stem cells using an anti-CD49a antibody reveals their CD45med,low phenotype. *British Journal of Haematology* **122**, 506–517 (2003).
154. Sober, S. A., Darmani, H., Alhattab, D. & Awidi, A. Flow cytometric characterization of cell surface markers to differentiate between fibroblasts and mesenchymal stem cells of different origin. *Arch Med Sci* **19**, 1487–1496 (2023).
155. Soder, R. P. *et al.* A phase i study to evaluate two doses of wharton's jelly-derived mesenchymal stromal cells for the treatment of de novo high-risk or steroid-refractory acute graft versus host disease. *Stem Cell Rev Rep* **16**, 979–991 (2020).
156. Wang, L., Weiss, M. L. & Detamore, M. S. Recent Patents Pertaining to Immune Modulation and Musculoskeletal Regeneration with Wharton's Jelly Cells. *Recent Pat Regen Med* **3**, 182–192 (2013).
157. Weiss, M. L. *et al.* Immune properties of human umbilical cord Wharton's jelly-derived cells. *Stem Cells* **26**, 2865–2874 (2008).
158. Vonk, L. A., Roël, G., Hernigou, J., Kaps, C. & Hernigou, P. Role of matrix-associated autologous chondrocyte implantation with spheroids in the treatment of large chondral defects in the knee: a systematic review. *International Journal of Molecular Sciences* **22**, 7149 (2021).

159. Flanigan, D. C., Harris, J. D., Trinh, T. Q., Siston, R. A. & Brophy, R. H. Prevalence of chondral defects in athletes' knees: a systematic review. *Med Sci Sports Exerc* **42**, 1795–1801 (2010).

160. Osteoarthritis. *BMUS: The Burden of Musculoskeletal Diseases in the United States* https://www.boneandjointburden.org/fourth-edition/iiib10/osteoarthritis.

161. DeFroda, S. F., Bokshan, S. L., Yang, D. S., Daniels, A. H. & Owens, B. D. Trends in the Surgical Treatment of Articular Cartilage Lesions in the United States from 2007 to 2016. *J Knee Surg* **34**, 1609–1616 (2021).

162. Ye, W. *et al.* Articular cartilage reconstruction with TGF-β1-simulating self-assembling peptide hydrogel-based composite scaffold. *Acta Biomaterialia* **146**, 94–106 (2022).

163. Biosplice Therapeutics, Inc. *A Phase 3, 16-Week, Multicenter, Randomized, Double Blind, Placebo-Controlled Study to Evaluate the Efficacy and Safety of a Single Injection of Lorecivivint 0.07 Mg Dose in the Target Knee Joint of Subjects With Moderate to Severe Osteoarthritis Pain of the Knee*. https://clinicaltrials.gov/ct2/show/NCT05603754 (2022).

164. Boyette, L. B., Creasey, O. A., Guzik, L., Lozito, T. & Tuan, R. S. Human bone marrow-derived mesenchymal stem cells display enhanced clonogenicity but impaired differentiation with hypoxic preconditioning. *Stem Cells Transl Med* **3**, 241–254 (2014).

165. Gokce, A. *et al.* Synergistic Effect of TGF-β1 And BMP–7 on Chondrogenesis and Extracellular Matrix Synthesis: An In Vitro Study. *Open Orthop J* **6**, 406–413 (2012).

166. Huang, Y. *et al.* Synergistic interaction of hTGF-β3 with hBMP-6 promotes articular cartilage formation in chitosan scaffolds with hADSCs: implications for regenerative medicine. *BMC Biotechnol* **20**, 48 (2020).

167. Hamamoto, S. *et al.* Enhancement of chondrogenic differentiation supplemented by a novel small compound for chondrocyte-based tissue engineering. *Journal of Experimental Orthopaedics* **7**, 10 (2020).

168. Hoben, G. M., Willard, V. P. & Athanasiou, K. A. Fibrochondrogenesis of hESCs: Growth Factor Combinations and Cocultures. *Stem Cells Dev* **18**, 283–292 (2009).

169. Kim, M. *et al.* Donor Variation and Optimization of Human Mesenchymal Stem Cell Chondrogenesis in Hyaluronic Acid. *Tissue Eng Part A* **24**, 1693–1703 (2018).

170. Music, E., Klein, T. J., Lott, W. B. & Doran, M. R. Transforming growth factor-beta stimulates human bone marrow-derived mesenchymal stem/stromal cell chondrogenesis more so than kartogenin. *Sci Rep* **10**, 8340 (2020).

171. A Study Evaluating the Safety, Pharmacokinetics, and Pharmacodynamics of KA34 in Subjects With Knee Osteoarthritis - Full Text View - ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT03133676.

172. Basu, S. *et al.* Effect of Cytomodulin-10 (TGF-ß1 analogue) on wound healing by primary intention in a murine model. *International Journal of Surgery* **7**, 460–465 (2009).

173. Denker, A. E., Haas, A. R., Nicoll, S. B. & Tuan, R. S. Chondrogenic differentiation of murine C3H10T1/2 multipotential mesenchymal cells: I. Stimulation by bone morphogenetic protein-2 in high-density micromass cultures. *Differentiation* **64**, 67–76 (1999).

174. Carmona-Moran, C. A. & Wick, T. M. Transient Growth Factor Stimulation Improves Chondrogenesis in Static Culture and Under Dynamic Conditions in a Novel Shear and Perfusion Bioreactor. *Cel. Mol. Bioeng.* **8**, 267–277 (2015).

175. Kim, M., Erickson, I. E., Choudhury, M., Pleshko, N. & Mauck, R. L. Transient exposure to TGF-β3 improves the functional chondrogenesis of MSC-laden hyaluronic acid hydrogels. *Journal of the Mechanical Behavior of Biomedical Materials* **11**, 92–101 (2012).

176. Huang, A. H., Stein, A., Tuan, R. S. & Mauck, R. L. Transient exposure to transforming growth factor beta 3 improves the mechanical properties of mesenchymal stem cell-laden cartilage constructs in a density-dependent manner. *Tissue Eng Part A* **15**, 3461–3472 (2009).

177. Correa, D. *et al.* Sequential exposure to fibroblast growth factors (FGF) 2, 9 and 18 enhances hMSC chondrogenic differentiation. *Osteoarthr. Cartil.* **23**, 443–453 (2015).
178. Matsushita, T. *et al.* Surgical treatment of cartilage lesions in the knee: A narrative review. *Journal of Joint Surgery and Research* **1**, 70–79 (2023).
179. Dilley, J. E., Bello, M. A., Roman, N., McKinley, T. & Sankar, U. Post-traumatic osteoarthritis: A review of pathogenic mechanisms and novel targets for mitigation. *Bone Reports* **18**, 101658 (2023).
180. Muthu, S. *et al.* Failure of cartilage regeneration: emerging hypotheses and related therapeutic strategies. *Nat Rev Rheumatol* **19**, 403–416 (2023).
181. Huang, B. J., Hu, J. C. & Athanasiou, K. A. Cell-based tissue engineering strategies used in the clinical repair of articular cartilage. *Biomaterials* **98**, 1–22 (2016).
182. Ge, Z., Li, C., Heng, B. C., Cao, G. & Yang, Z. Functional biomaterials for cartilage regeneration. *Journal of Biomedical Materials Research Part A* **100A**, 2526–2536 (2012).
183. Kiyotake, E. A., Beck, E. C. & Detamore, M. S. Cartilage extracellular matrix as a biomaterial for cartilage regeneration. *Ann. N. Y. Acad. Sci.* **1383**, 139–159 (2016).
184. Ansari, M. & Eshghanmalek, M. Biomaterials for repair and regeneration of the cartilage tissue. *Bio-des. Manuf.* **2**, 41–49 (2019).
185. monimono. Agili-C™. *The Future of joint Repair* https://www.cartiheal.com/agili-c/.
186. Health, C. for D. and R. Agili-C – P210034. *FDA* (2022).
187. Nimni, M. E. Polypeptide growth factors: targeted delivery systems. *Biomaterials* **18**, 1201–1225 (1997).
188. Madry, H., Rey-Rico, A., Venkatesan, J. K., Johnstone, B. & Cucchiarini, M. Transforming growth factor Beta-releasing scaffolds for cartilage tissue engineering. *Tissue Eng Part B Rev* **20**, 106–125 (2014).

189. Hauptstein, J. *et al.* Tethered TGF-β1 in a Hyaluronic Acid-Based Bioink for Bioprinting Cartilaginous Tissues. *Int J Mol Sci* **23**, 924 (2022).

190. McCall, J. D., Luoma, J. E. & Anseth, K. S. Covalently tethered transforming growth factor beta in PEG hydrogels promotes chondrogenic differentiation of encapsulated human mesenchymal stem cells. *Drug Deliv. and Transl. Res.* **2**, 305–312 (2012).

191. Sridhar, B. V., Doyle, N. R., Randolph, M. A. & Anseth, K. S. Covalently tethered TGF-β1 with encapsulated chondrocytes in a PEG hydrogel system enhances extracellular matrix production. *J Biomed Mater Res A* **102**, 4464–4472 (2014).

192. Böck, T. *et al.* TGF-β1-Modified Hyaluronic Acid/Poly(glycidol) Hydrogels for Chondrogenic Differentiation of Human Mesenchymal Stromal Cells. *Macromolecular Bioscience* **18**, 1700390 (2018).

193. Schneider, M. C., Chu, S., Randolph, M. A. & Bryant, S. J. An In Vitro and In Vivo Comparison of Cartilage Growth in Chondrocyte-Laden Matrix Metalloproteinase-Sensitive Poly(Ethylene Glycol) Hydrogels with Localized Transforming Growth Factor β3. *Acta Biomater* **93**, 97–110 (2019).

194. Townsend, J. M. *et al.* Superior calvarial bone regeneration using pentenoate-functionalized hyaluronic acid hydrogels with devitalized tendon particles. *Acta Biomater* **71**, 148–155 (2018).

195. Kiyotake, E. A., Cheng, M. E., Thomas, E. E. & Detamore, M. S. The Rheology and Printability of Cartilage Matrix-Only Biomaterials. *Biomolecules* **12**, 846 (2022).

196. Zhou, C. *et al.* A targeted transforming growth factor-beta (TGF-β) blocker, TTB, inhibits tumor growth and metastasis. *Oncotarget* **9**, 23102–23113 (2018).

197. Kim, B.-G., Malek, E., Choi, S. H., Ignatz-Hoover, J. J. & Driscoll, J. J. Novel therapies emerging in oncology to target the TGF-β pathway. *Journal of Hematology & Oncology* **14**, 55 (2021).

198. Townsend, J. M., Sanders, M. E., Kiyotake, E. A. & Detamore, M. S. Independent control of molecular weight, concentration, and stiffness of hyaluronic acid hydrogels. *Biomed. Mater.* **17**, 065005 (2022).

Appendix A: Figures

Chapter 1: No Figures

Chapter 2: Figures 2.1-2.2

Chapter 3: Figures 3.1-3.10

Chapter 4: Figures 4.1-4.4

Chapter 5: Figures 5.1-5.15

Chapter 6: Figures 6.1-6.4

Chapter 7: No Figures

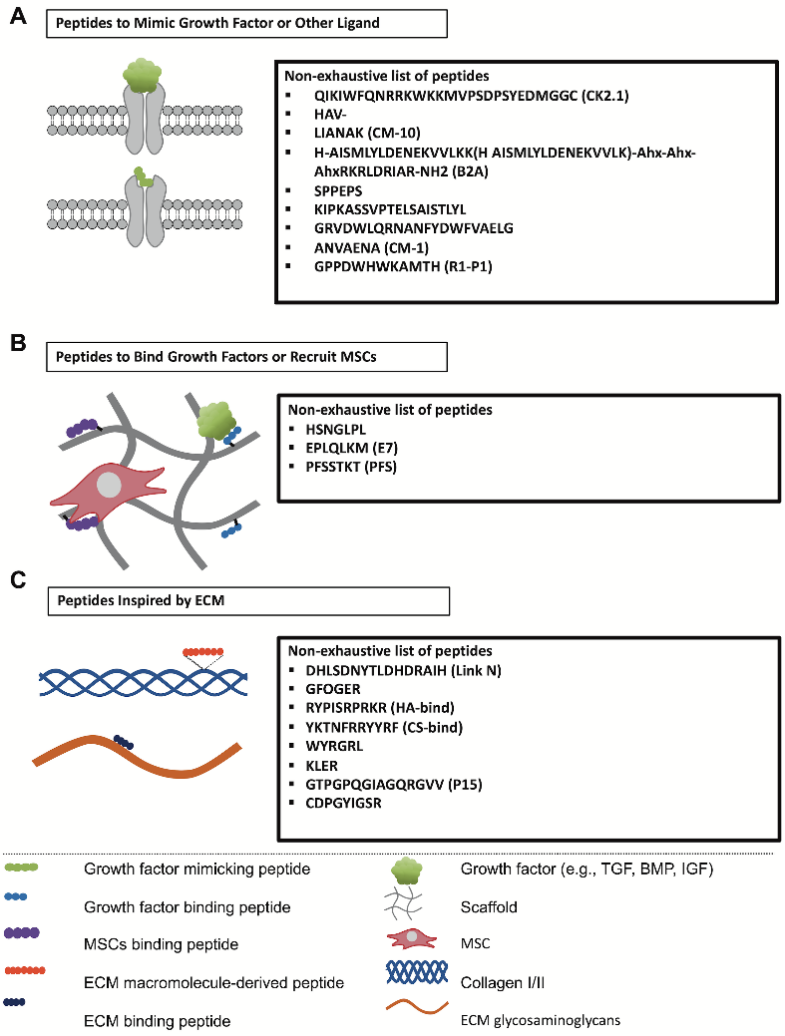

Figure 2.1: Different categories of peptides for cartilage regeneration with a non-exhaustive list of example peptides in each category.

A) Peptides derived from or inspired by growth factors that play a role in chondrogenesis or peptides, which are designed to bind growth factor receptors to activate chondroinductive-related pathways. B) Peptides designed to bind to growth factors or recruit MSCs, usually used in combination with scaffolds to induce chondrogenesis via endogenous cells and signals. C) Peptides derived from extracellular matrix components such as collagen I, II, Link N; or peptides that bind to glycosaminoglycans. Beyond these three general categories, self-assembling peptides represent a separate approach as a scaffolding material. Citations to individual peptides may be found in Tables 2.1, 2.2, 2.3, and 2.4. BMP: bone morphogenetic proteins; ECM: extracellular matrix; IGF: insulin-like growth factors; MSC: mesenchymal stem cell; TGF: transforming growth factors.

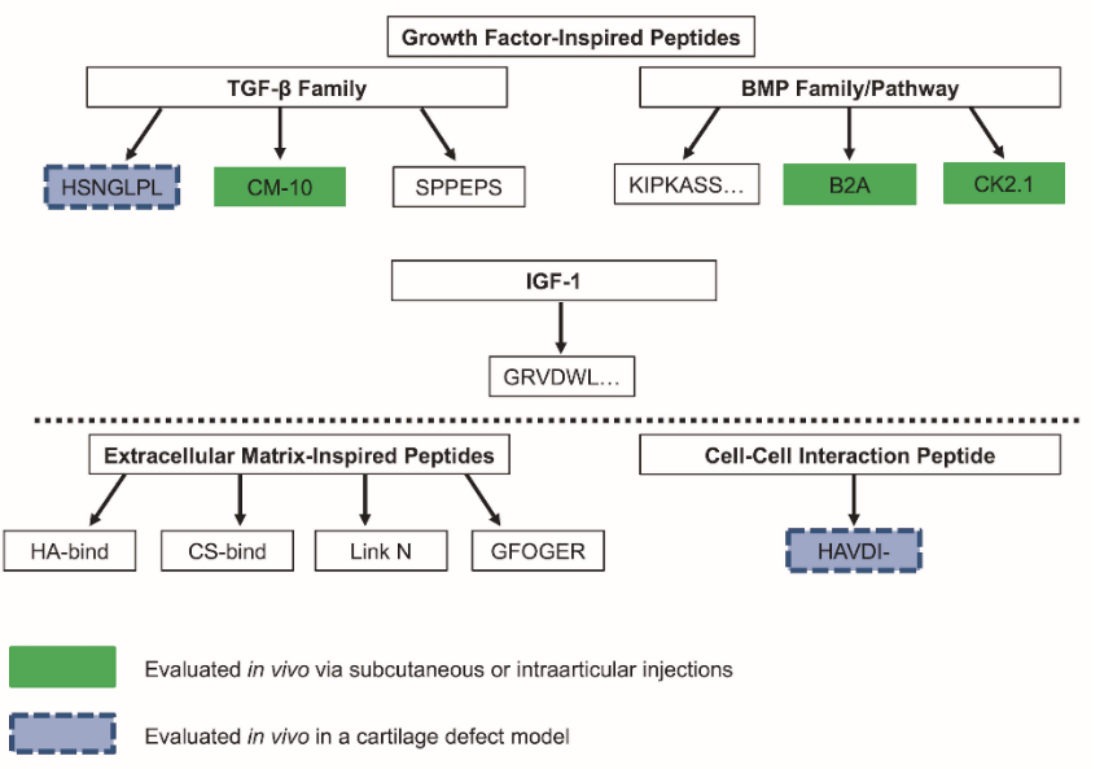

Figure 2.2: Categories of chondroinductive peptides that have been evaluated for cartilage regeneration without the addition of growth factors.

Example peptides are grouped based on their parent molecules or pathways. Peptides that have been evaluated *in vivo* are highlighted in green and blue. Peptides highlighted in green have to date been evaluated only subcutaneously or via intraarticular injections. Peptides highlighted in blue with a dashed outline were evaluated in cartilage defect models. Citations to individual peptides may be found in Tables 1 and 2. KIPKASS… = KIPKASSVPTELSAISTLYL; GRVDWL… = GRVDWLQRNANFYDWFVAELG

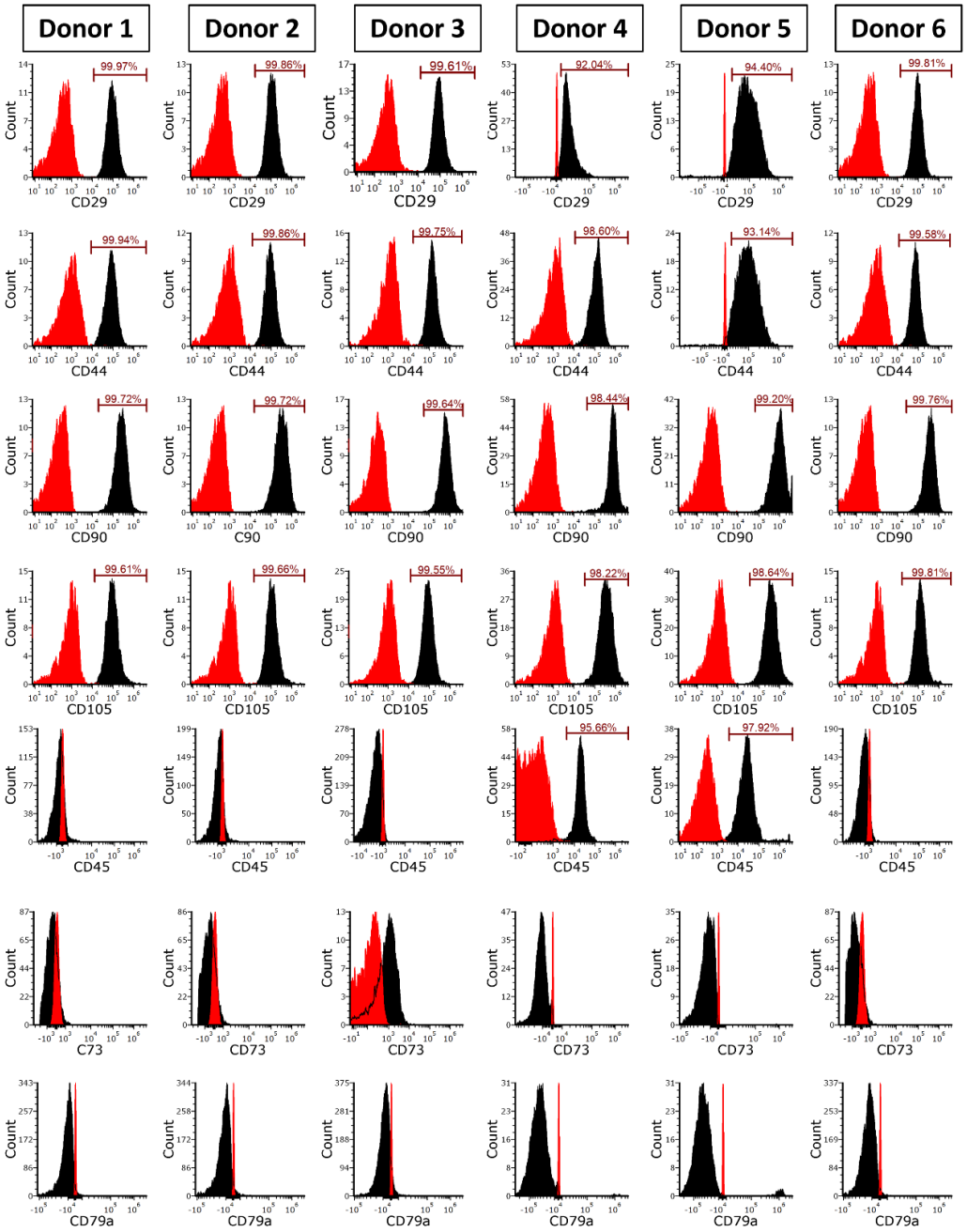

Figure 3.1: Flow cytometric histogram analyses of cell surface marker expressions of equine bone marrow-derived bone marrow-derived mesenchymal stem cells (eBMSCs) at Passage 4.

All eBMSC donors expressed CD29, CD44, CD90, and CD105 while being negative for CD73 and CD79a. Donors 1, 2, 3, and 6 were negative for CD45, whereas donors 4, and 5 were slightly positive for CD45. Red histograms represent unstained controls and black histogram represent stained samples.

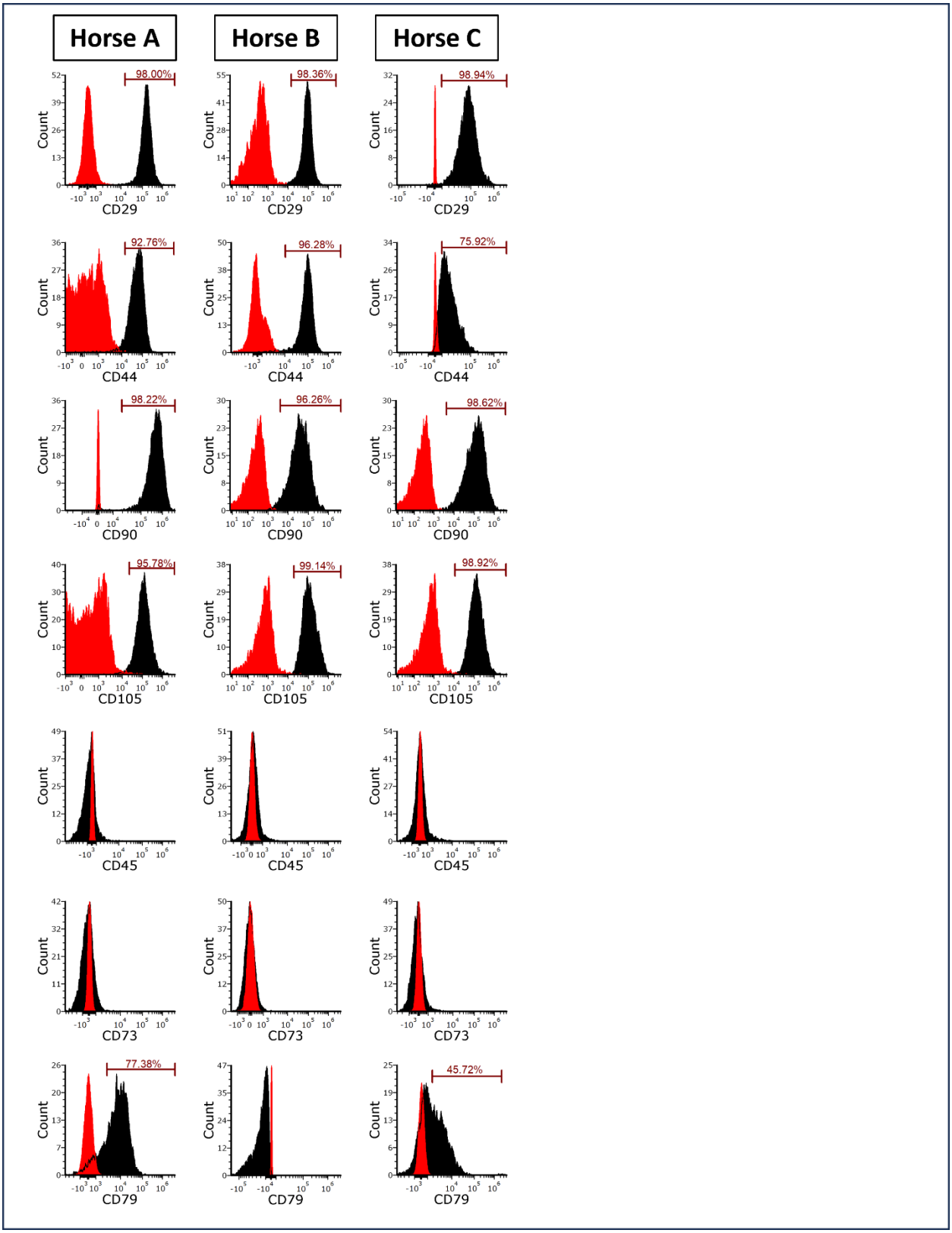
Horse A
Horse B
Horse C
98.00%
98.36%
98.94%
CD29
92.76%
96.28%
75.92%
CD44
98.22%
96.26%
98.62%
CD90
95.78%
99.14%
98.92%
CD105
CD45
CD73
77.38%
45.72%
CD79
Count

Figure 3.2: Flow cytometric histogram analyses of cell surface marker expressions of Equine umbilical cord-derived mesenchymal stromal cells (eUCMSCs) at Passage 4.

All eUCMSC donors expressed CD29, CD44, CD90, and CD105, while being negative for CD45, and CD73. Donors 1, and 3 were slightly positive for CD79A. Red histograms represent unstained controls and black histogram represent stained samples.

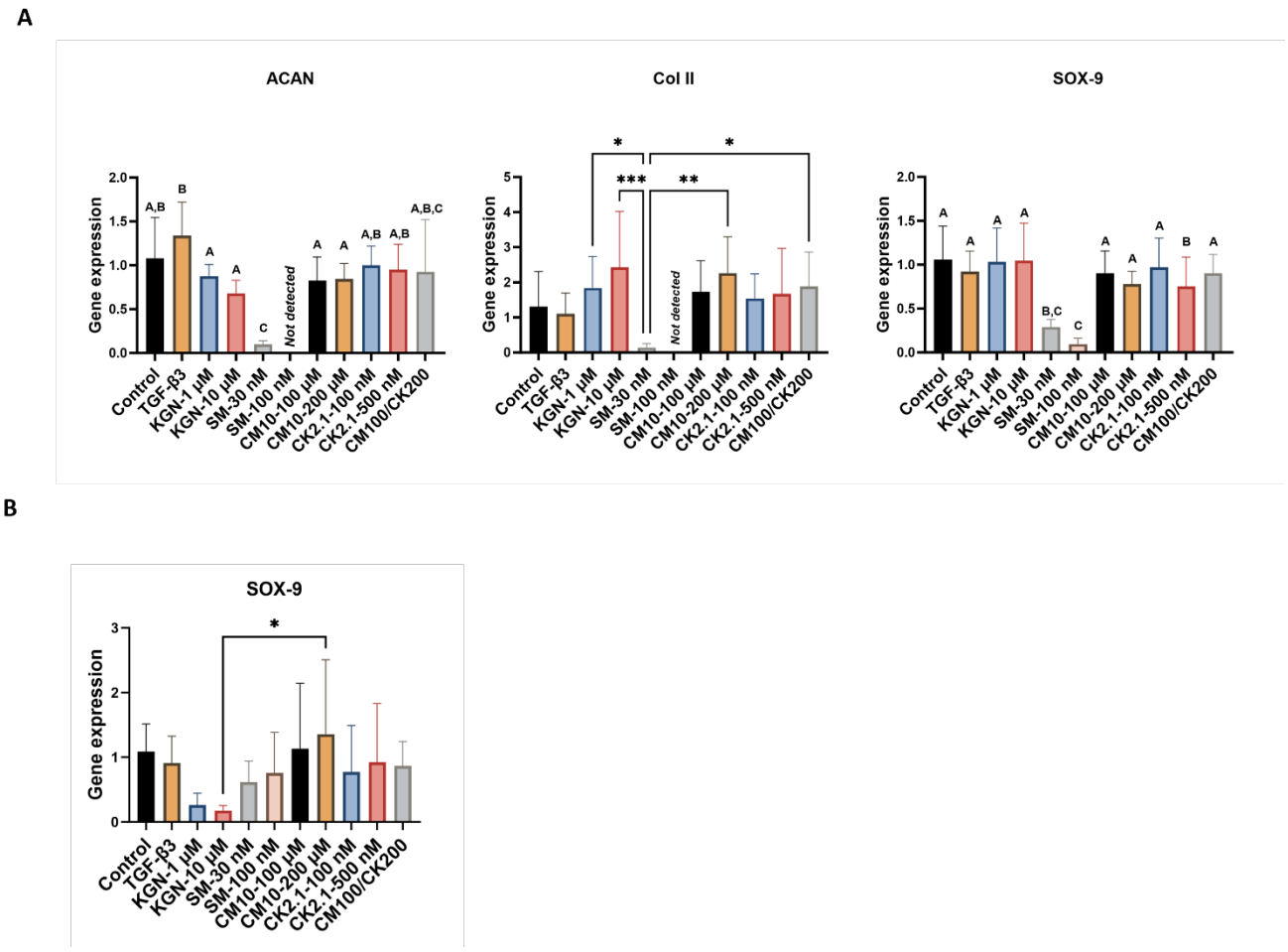

Figure 3.3: Gene expression after 21 days in spheroid culture under hypoxic conditions (5% O_2).

A) Equine bone marrow-derived mesenchymal stem cells (eBMSCs): no compound or peptide significantly increased chondrogenic gene expression of SOX-9, ACAN, or collagen II in eBMSCs compared to the control group. Interestingly, SM-30 nM and SM-100 nM *decreased* the gene expression of all three genes compared to the control and certain other groups. **B) Equine umbilical cord-derived mesenchymal stromal cells (eUCMSCs):** the eUCMSCs treated with CM10-200 µM had 7.6 times greater SOX-9 gene expression than that of the eUCMSCs treated with KGN-10 µM. No other differences were significant. There was no detectable ACAN gene expression except in some of the TGF-β3 samples (data not shown), and there was no detectable collagen II gene expression in any of the samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, each letter indicated significance from groups with other letters. n = 8 for Reported values are mean + standard deviation. SM = SM04690, KGN = Kartogenin, CM100/CK200 = CM10 at 200 µM and CK2.1 and 200 nM.

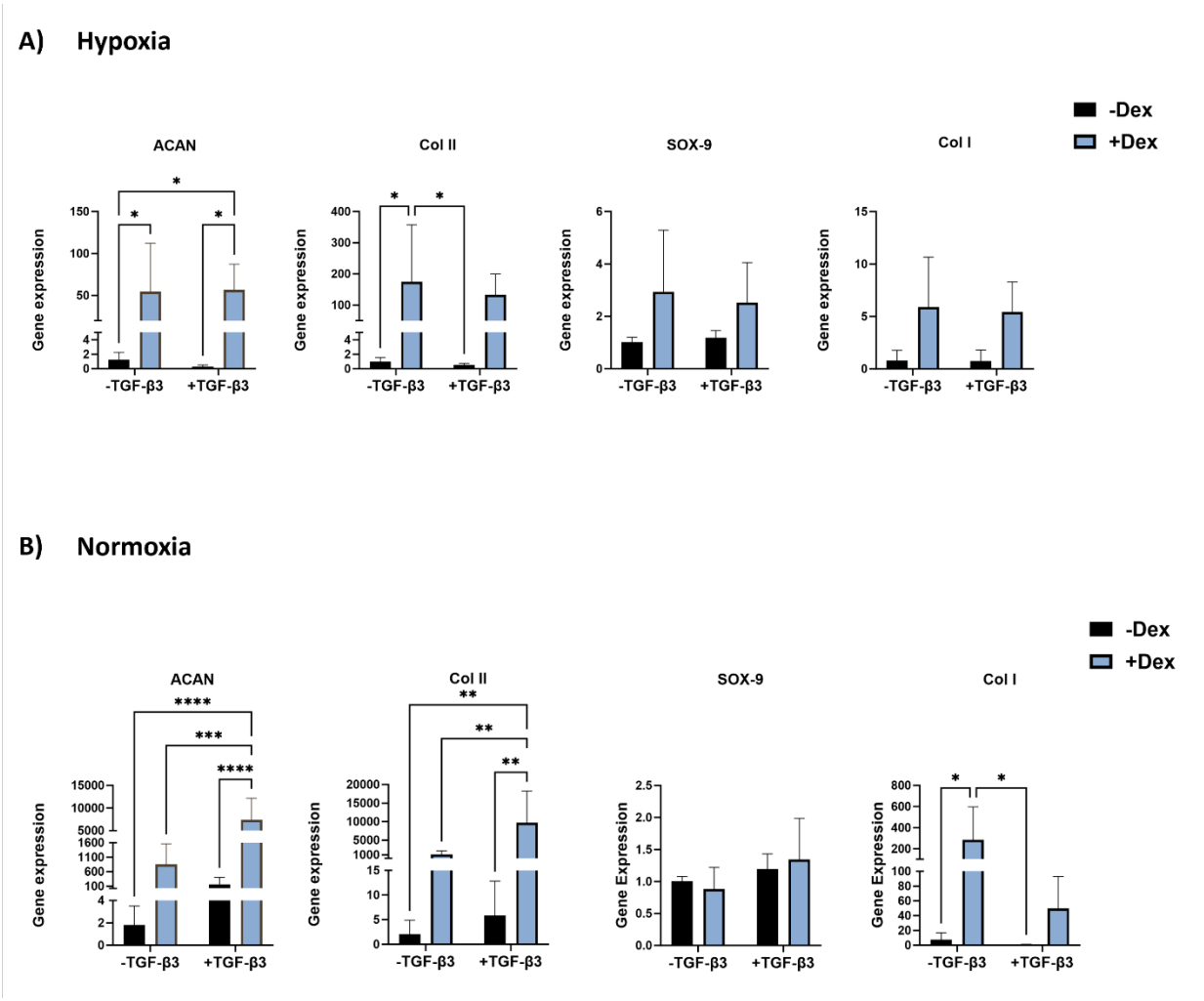

Figure 3.4: Gene expression of equine bone marrow-derived mesenchymal stem cells (eBMSCs) after 21 days in response to dexamethasone (Dex) in (A) hypoxia or (B) normoxia in spheroid culture.

A) In hypoxic conditions, eBMSCs treated with dexamethasone had 43-fold and 181-fold higher ACAN and collagen II gene expressions, respectively, compared to the negative control (i.e., no dexamethasone and no TGF-β3), whereas eBMSCs treated with both dexamethasone and TGF-β3 had 45-fold and 138-fold higher ACAN and collagen II gene expressions, respectively, compared to the negative control. No significant differences among groups were observed for SOX-9 or collagen I gene expression. B) In normoxia, eBMSCs treated with both dexamethasone and TGF-β3 had the highest gene expression for ACAN and collagen II, with 4108-fold and 4705-fold higher gene expressions, respectively, compared to the negative control. No significant differences were observed among groups for SOX-9. As for collagen I, eBMSCs treated with dexamethasone alone exhibited the highest gene expression with 38-fold higher gene expression compared to the negative control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n = 6-8. Reported values are mean + standard deviation.

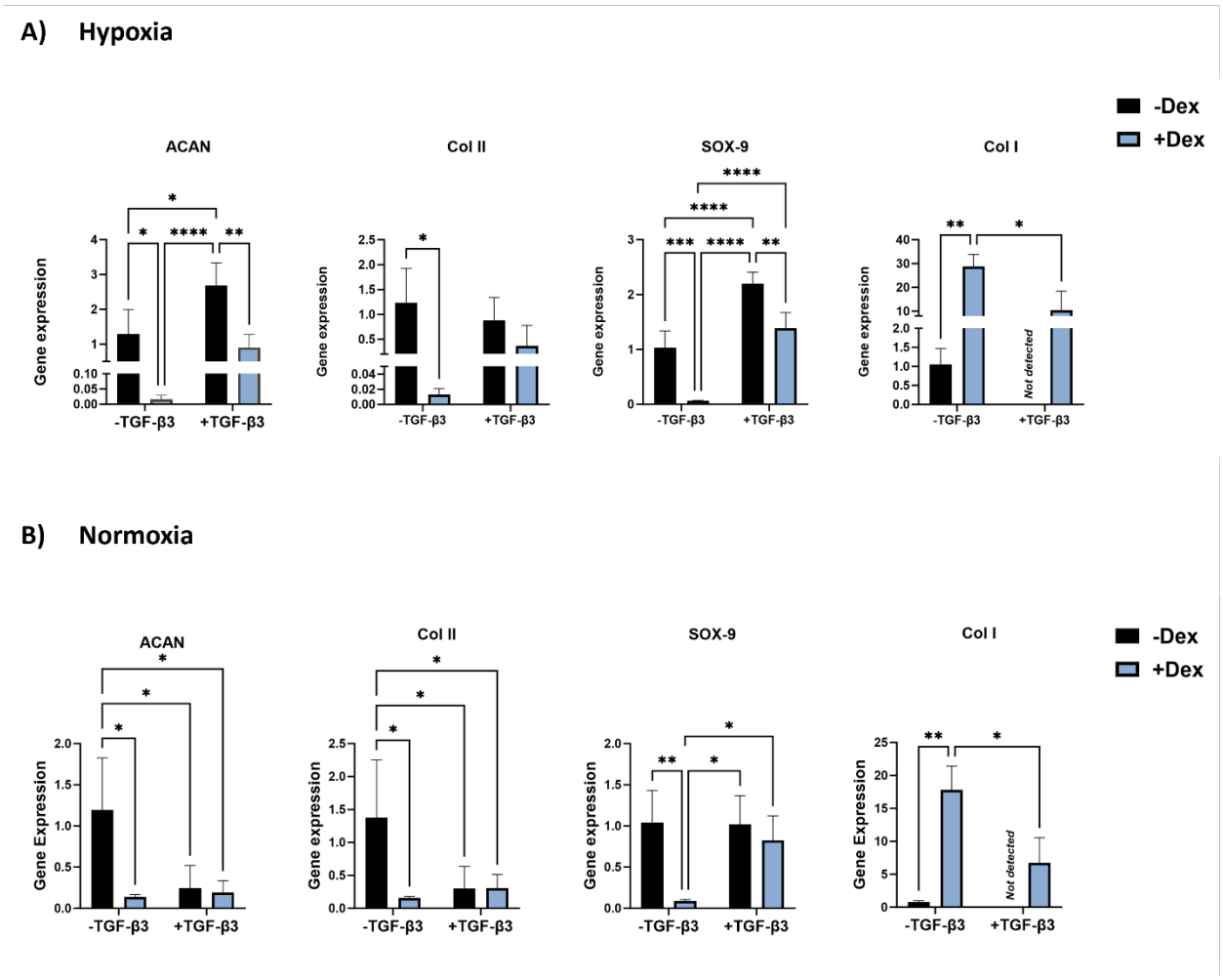

Figure 3.5: Gene expression of equine bone marrow-derived mesenchymal stem cells (eBMSCs) after 21 days in response to dexamethasone (Dex) in (A) hypoxia or (B) normoxia in monolayer culture.

A) In hypoxic conditions, eBMSCs treated with only dexamethasone resulted in significantly lower ACAN, collagen II, and SOX-9 gene expression as compared to the negative control; however, collagen I expression was 27-fold higher compared to the negative control. Treatment with TGF-β3 alone had 2-fold higher ACAN and SOX-9 gene expression, compared to the negative control. Treatment with both dexamethasone and TGF-β3 did not result in a significant change in the gene expression of ACAN, collagen II, SOX-9, or Col I. B) In normoxia, eBMSCs treated with dexamethasone, TGF-β3, or both resulted in significantly lower gene expression of ACAN and collagen II. Treatment with dexamethasone alone resulted in significantly lower SOX-9 gene expression and the highest gene expression of collagen I compared to the negative control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n = 3-4. Reported values are mean + standard deviation.

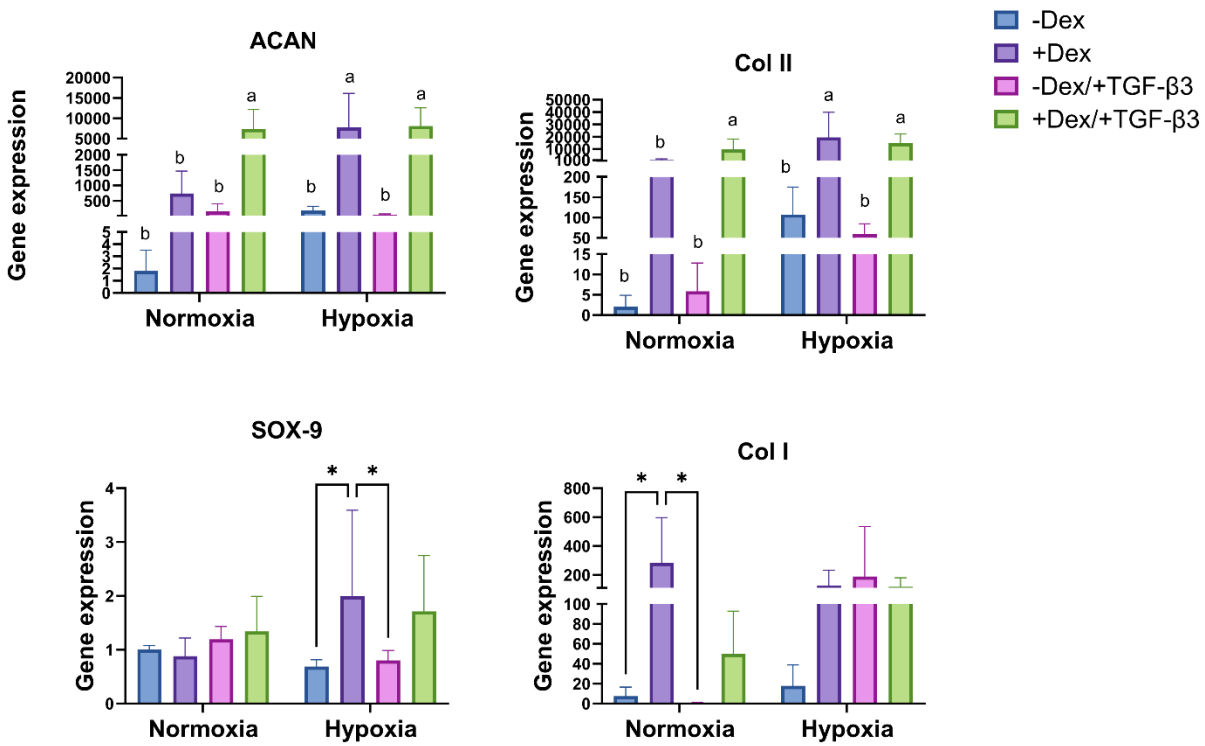

Figure 3.6: Gene expression of equine bone marrow-derived mesenchymal stem cells (eBMSCs) after 21 days in response to dexamethasone (Dex) in hypoxia or normoxia in spheroid culture.

In hypoxic conditions, eBMSCs treated with only dexamethasone resulted in 90.5% and 94.3% higher ACAN and collagen II gene expression, respectively, compared to eBMSCs treated with only dexamethasone in normoxia; For SOX-9 and collagen I, no significant differences were observed between hypoxia and normoxia for the remaining groups. * $p < 0.05$, each letter indicated significance from groups with other letters. n = 6-8. Reported values are mean + standard deviation.

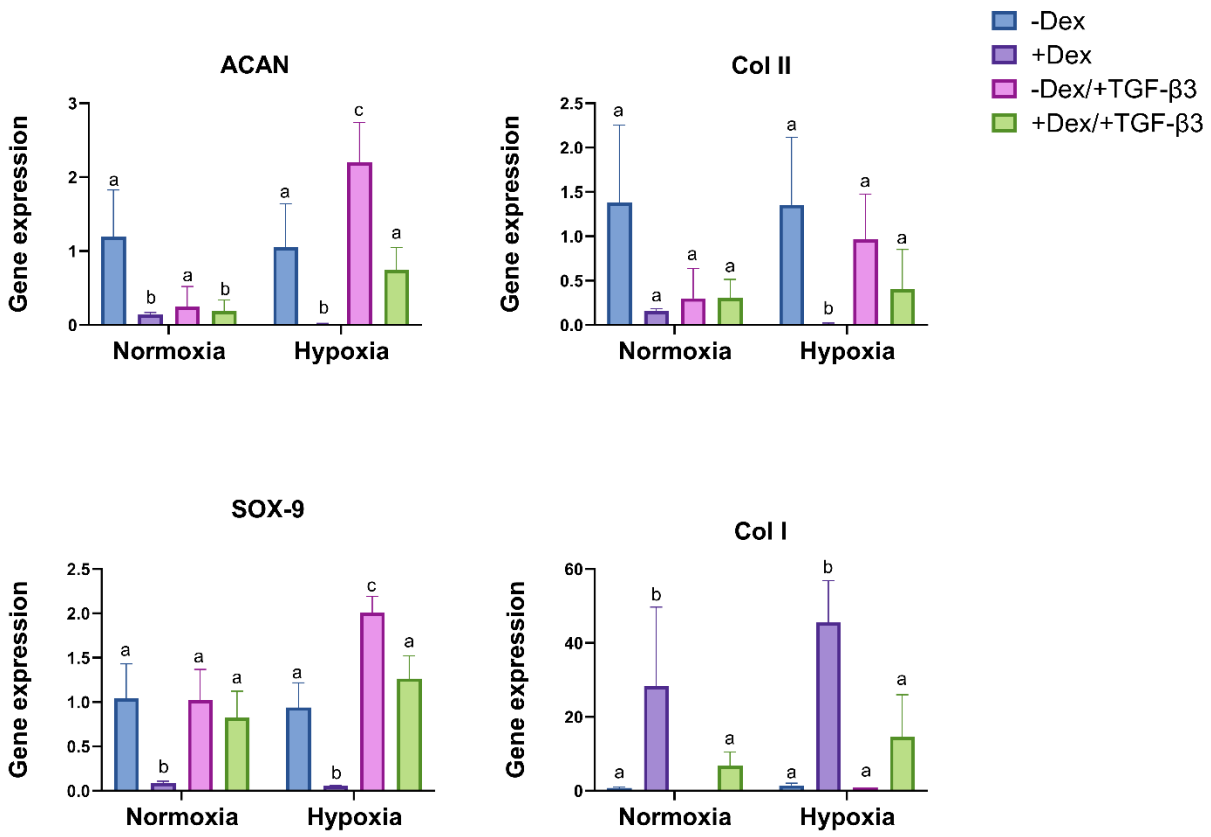

Figure 3.7: Gene expression of equine bone marrow-derived mesenchymal stem cells (eBMSCs) after 21 days in response to dexamethasone (Dex) in hypoxia or normoxia in monolayer culture.

In hypoxic conditions, eBMSCs treated with only TGF-β3 resulted in 89% and 49% higher ACAN and SOX-9 gene expression, respectively compared to eBMSCs treated with only TGF-β3 in normoxia. * $p < 0.05$, n = 3-4. Reported values are mean + standard deviation.

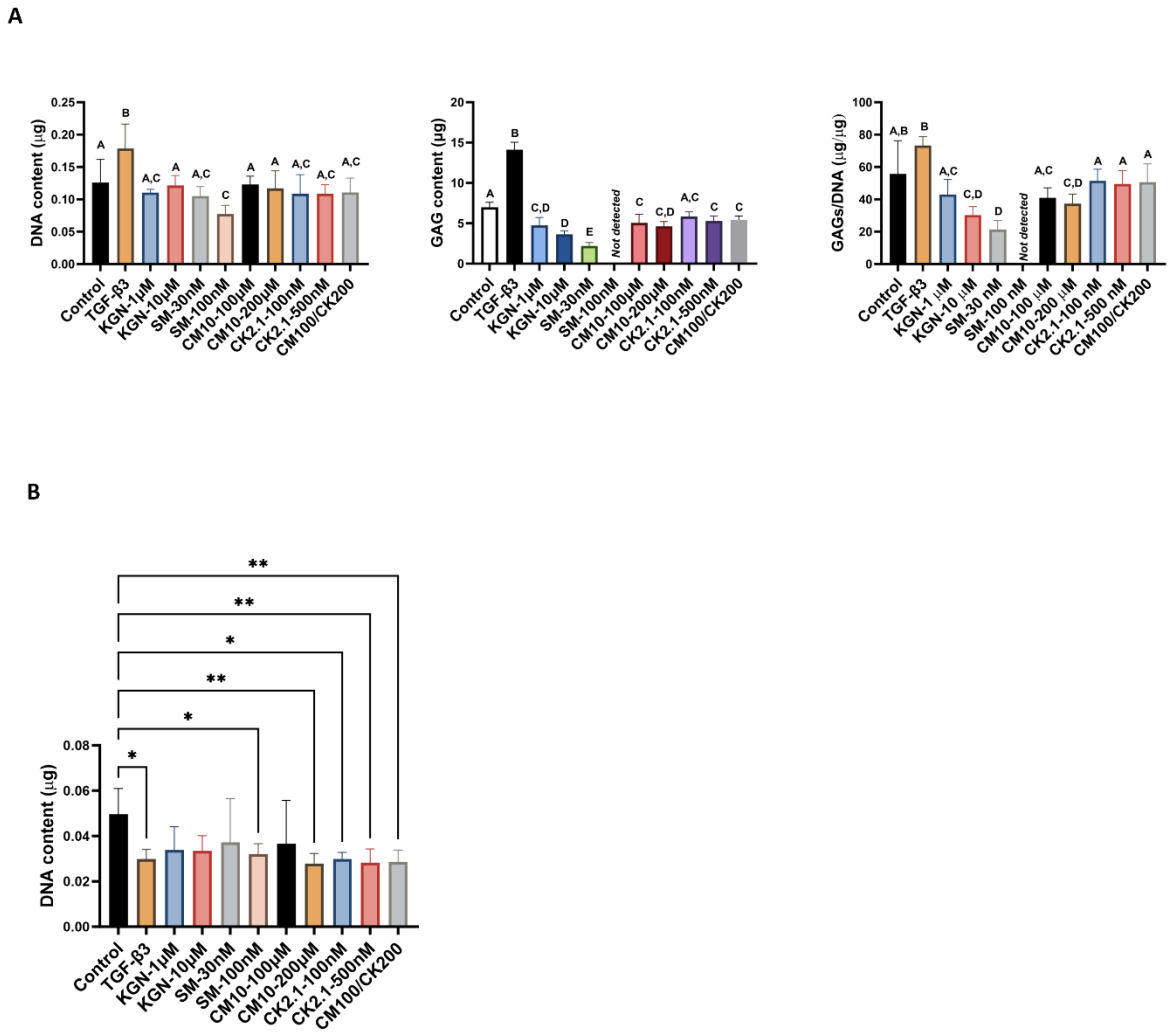

Figure 3.8: Glycosaminoglycan (GAG) synthesis after 21 days in spheroid culture.

A) Equine bone marrow-derived mesenchymal stem cells (eBMSCs): eBMSCs treated with TGF-β3 had the highest GAGs/DNA production with a 30% increase compared to the negative control. SM-30 nM had the lowest GAGs/DNA production, and no GAGs were detected with SM-100 nM. **B) Equine umbilical cord-derived mesenchymal stromal cells (eUCMSCs):** For eUCMSCs, GAG was not detected in any of the samples. * $p < 0.05$, ** $p < 0.01$, n = 4-8. Reported values are mean + standard deviation. SM = SM04690, KGN = Kartogenin, CM100/CK200 = CM10 at 200 µM and CK2.1 at 200 nM.

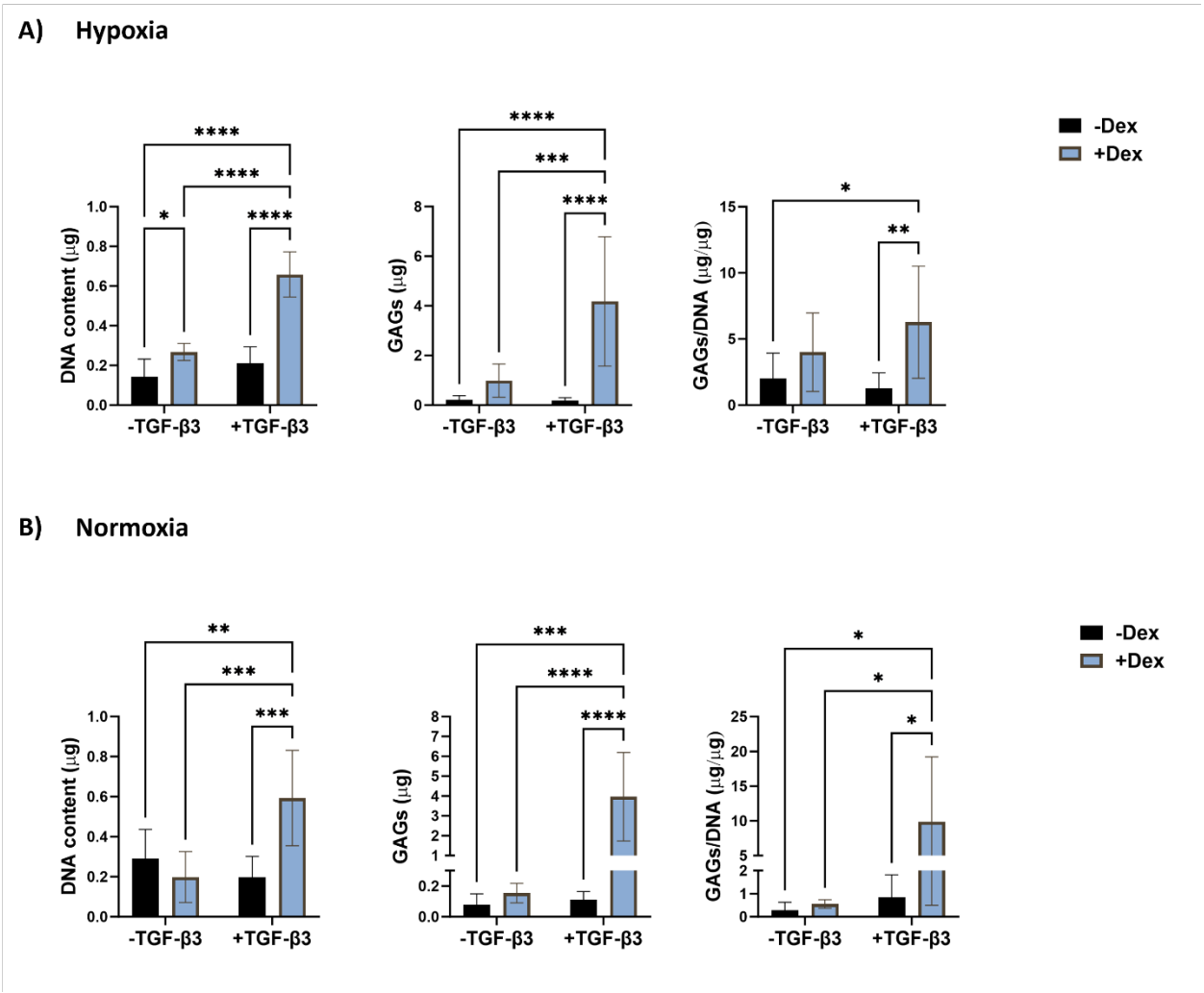

Figure 3.9: Glycosaminoglycan (GAG) synthesis of equine bone marrow-derived mesenchymal stem cells (eBMSCs) after 21 days in response to dexamethasone (Dex) in (A) hypoxia or (B) normoxia in spheroid culture.

A) In hypoxic conditions, eBMSCs treated with both dexamethasone and TGF-β3 resulted in 3-fold higher GAG/DNA content compared to the negative control. B) In normoxia, eBMSCs treated with both dexamethasone and TGF-β3 resulted in 35-fold higher GAG/DNA production compared to the negative control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n = 6-8. Reported values are mean ± standard deviation.

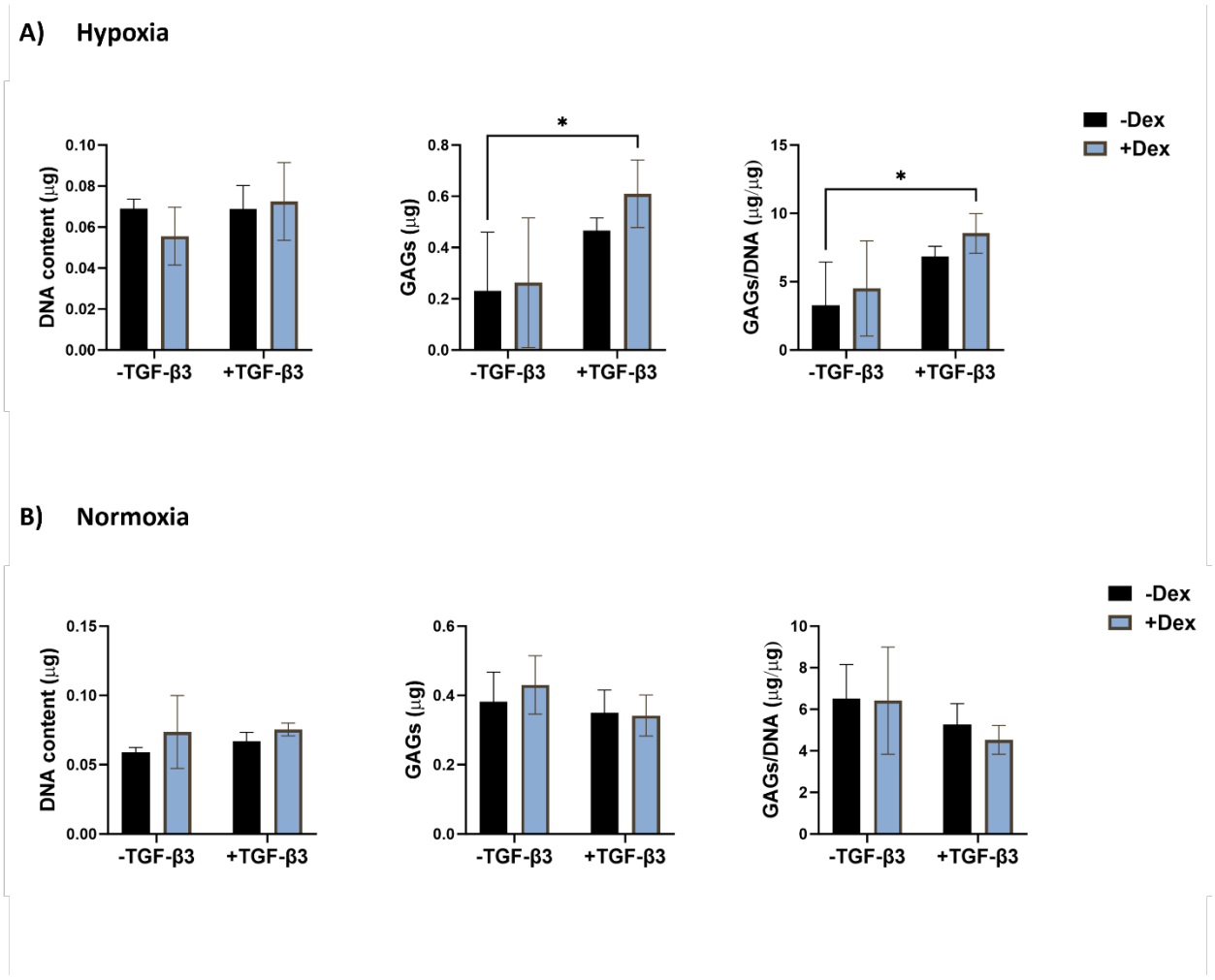

Figure 3.10: Glycosaminoglycan (GAG) synthesis of equine bone marrow-derived mesenchymal stem cells (eBMSCs) after 21 days in response to dexamethasone (Dex) in monolayer culture.

A) In hypoxic conditions, eBMSCs treated with dexamethasone and TGF-β3 resulted in 2.6-fold higher GAG production normalized to DNA content compared to the negative control. No significant differences were observed among the remaining groups. B) In normoxia, no significant differences were observed among the groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n = 3-4. Reported values are mean ± standard deviation.

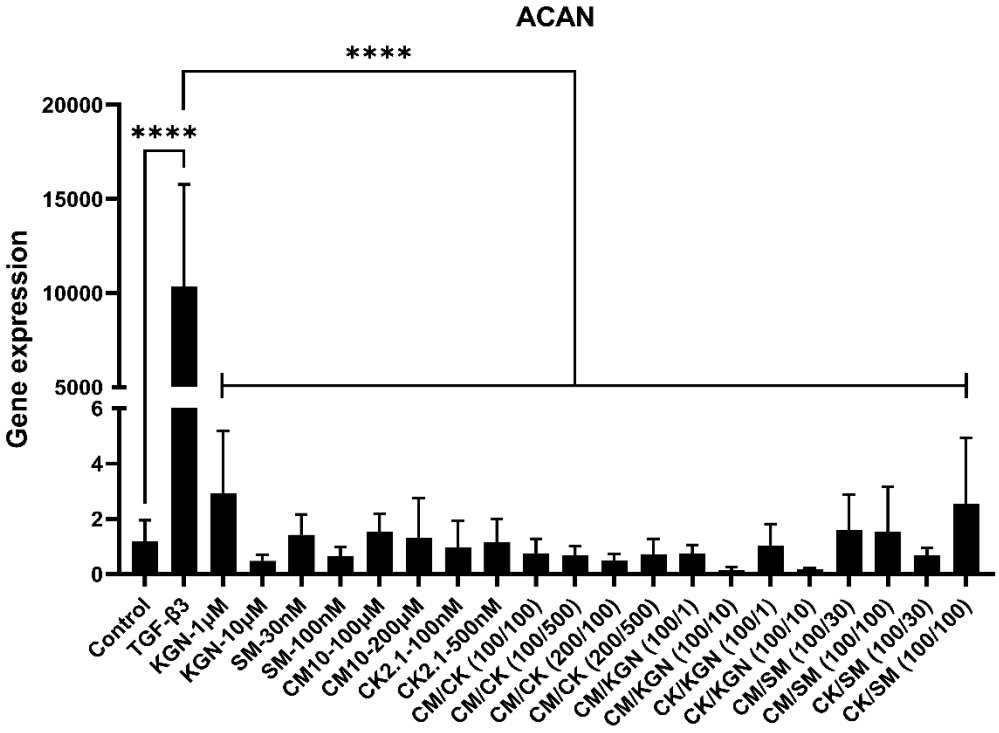

Figure 4.1: Chondrogenic gene expression of ACAN in hBMSCs spheroids after 21 days in culture.

No statistically significant differences were observed between groups except for TGF-β3, which had significantly higher aggrecan expression compared to all other groups, including a 10,000-fold larger value compared to the negative control. ****Refers to significant difference $p < 0.0001$, n = 6-8. Reported values are mean + standard deviation. SM = SM04690, CK = CK2.1, CM = CM10, CM/CK (100/100) = CM10 at 100 μM and CK2.1 and 100 nM, CM/CK(100/500) = CM10 at 100 μM and CK2.1 and 500 nM, CM/CK (200/100) = CM10 at 200 μM and CK2.1 and 100 nM, CM/CK(200/500) = CM10 at 200 μM and CK2.1 and 500 nM, CM/KGN (100/1) = CM10 at 100 μM and kartogenin at 1 μM, CM/KGN (100/10) = CM10 at 100 μM and kartogenin at 10 μM, CK/KGN (100/1) = CK2.1 at 100 nM and kartogenin at 1μM, CK/KGN (100/10) = CK2.1 at 100 nM and kartogenin at 10 μM, CM/SM (100/30) = CM10 at 100 μM and SM04690 at 30 nM, CM/SM (100/100) = CM10 at 100 μM and SM04690 at 100 nM, CK/SM (100/30) = CK2.1 at 100 nM and SM04690 at 30 nM, CK/SM (100/100) = CK2.1 at 100 nM and SM04690 at 100 nM.

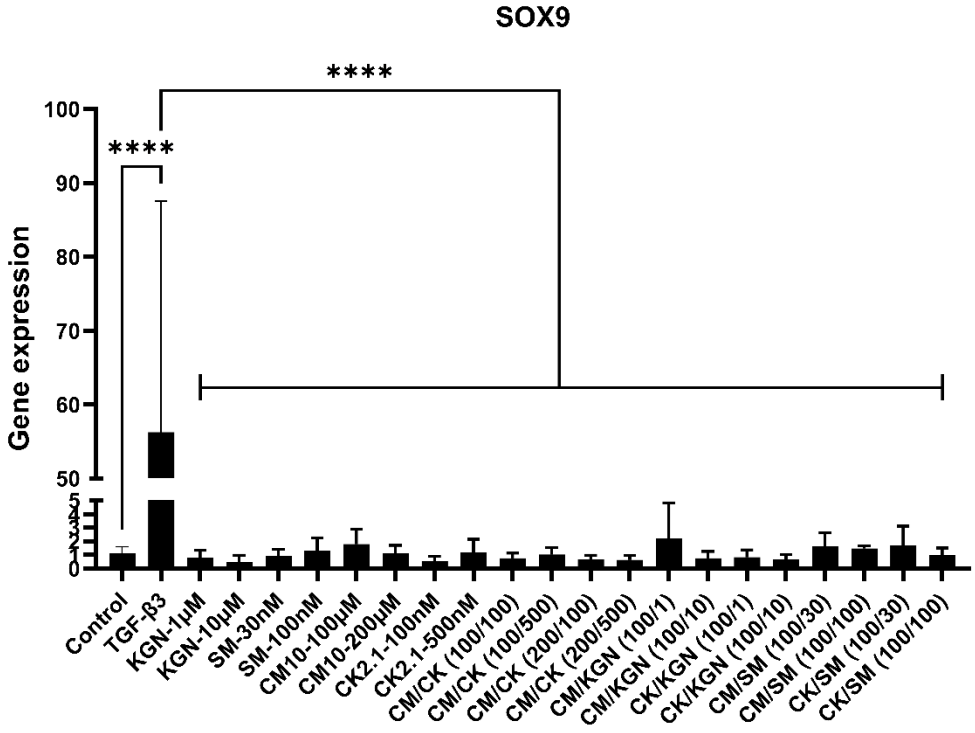

Figure 4.2: Chondrogenic gene expression of SOX-9 in hBMSCs spheroids after 21 days in culture.

No significance was observed between groups except for TGF-β3 with 50-fold higher gene expression as compared to the negative control. ****Refers to significant difference $p < 0.0001$, n=6-8. Reported values are mean + standard deviation. SM = SM04690, CK = CK2.1, CM = CM10, CM/CK (100/100) = CM10 at 100 μM and CK2.1 and 100 nM, CM/CK(100/500) = CM10 at 100 μM and CK2.1 and 500 nM, CM/CK (200/100) = CM10 at 200 μM and CK2.1 and 100 nM, CM/CK(200/500) = CM10 at 200 μM and CK2.1 and 500 nM, CM/KGN (100/1) = CM10 at 100 μM and kartogenin at 1 μM, CM/KGN (100/10) = CM10 at 100 μM and kartogenin at 10 μM, CK/KGN (100/1) = CK2.1 at 100 nM and kartogenin at 1μM, CK/KGN (100/10) = CK2.1 at 100 nM and kartogenin at 10 μM, CM/SM (100/30) = CM10 at 100 μM and SM04690 at 30 nM, CM/SM (100/100) = CM10 at 100 μM and SM04690 at 100 nM, CK/SM (100/30) = CK2.1 at 100 nM and SM04690 at 30 nM, CK/SM (100/100) = CK2.1 at 100 nM and SM04690 at 100 nM.

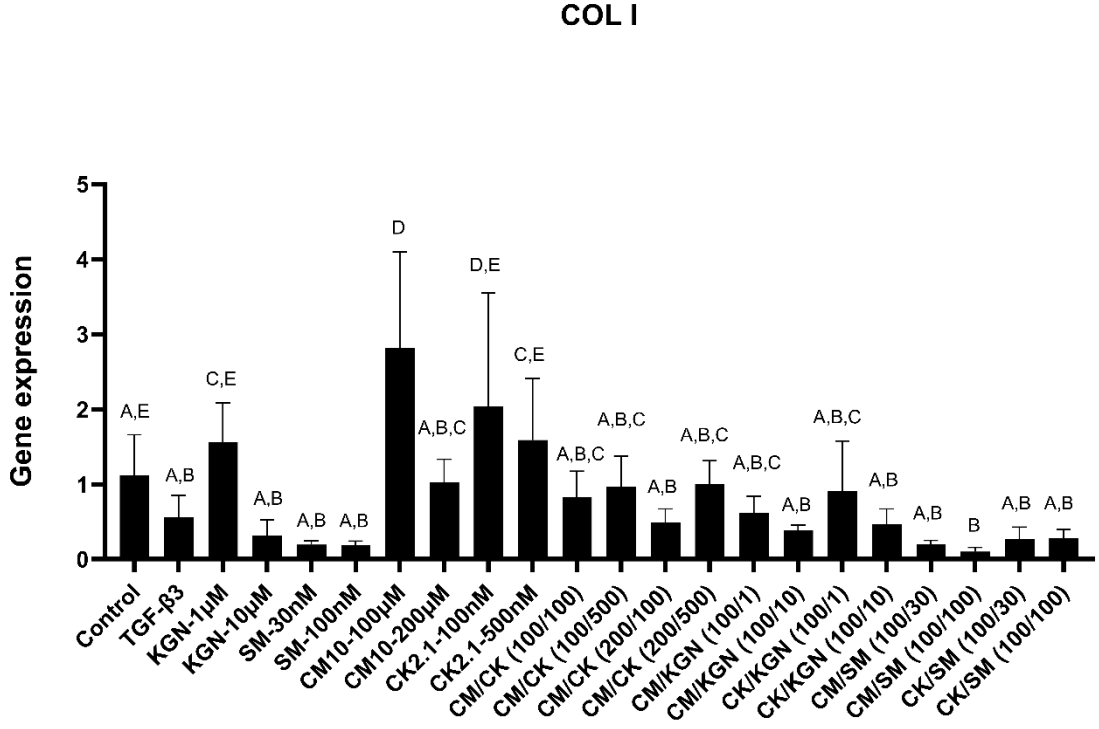

Figure 4.3: Gene expression of collagen I of hBMSCs spheroids after 21 days in culture.

A variation in the gene expression of collagen I by hBMSCs was observed in response to the evaluated compounds and peptides. CM10 (100 μM) had the highest gene expression with 2.5-fold higher expression compared to the control group. CK2.1 exhibited higher gene expression of collagen I by 1.8 folds and 1.4 folds at 100 nM and 500 nM, respectively. The combination of compounds and peptides did not induce a significant difference in collagen I gene expression as compared to the negative control group. Groups with the same letters indicate no significant difference, n=6-8. Reported values are mean + standard deviation. SM = SM04690, CK = CK2.1, CM = CM10, CM/CK (100/100) = CM10 at 100 μM and CK2.1 and 100 nM, CM/CK(100/500) = CM10 at 100 μM and CK2.1 and 500 nM, CM/CK (200/100) = CM10 at 200 μM and CK2.1 and 100 nM, CM/CK(200/500) = CM10 at 200 μM and CK2.1 and 500 nM, CM/KGN (100/1) = CM10 at 100 μM and kartogenin at 1 μM, CM/KGN (100/10) = CM10 at 100 μM and kartogenin at 10 μM, CK/KGN (100/1) = CK2.1 at 100 nM and kartogenin at 1μM, CK/KGN (100/10) = CK2.1 at 100 nM and kartogenin at 10 μM, CM/SM (100/30) = CM10 at 100 μM and SM04690 at 30 nM, CM/SM (100/100) = CM10 at 100 μM and SM04690 at 100 nM, CK/SM (100/30) = CK2.1 at 100 nM and SM04690 at 30 nM, CK/SM (100/100) = CK2.1 at 100 nM and SM04690 at 100 nM.

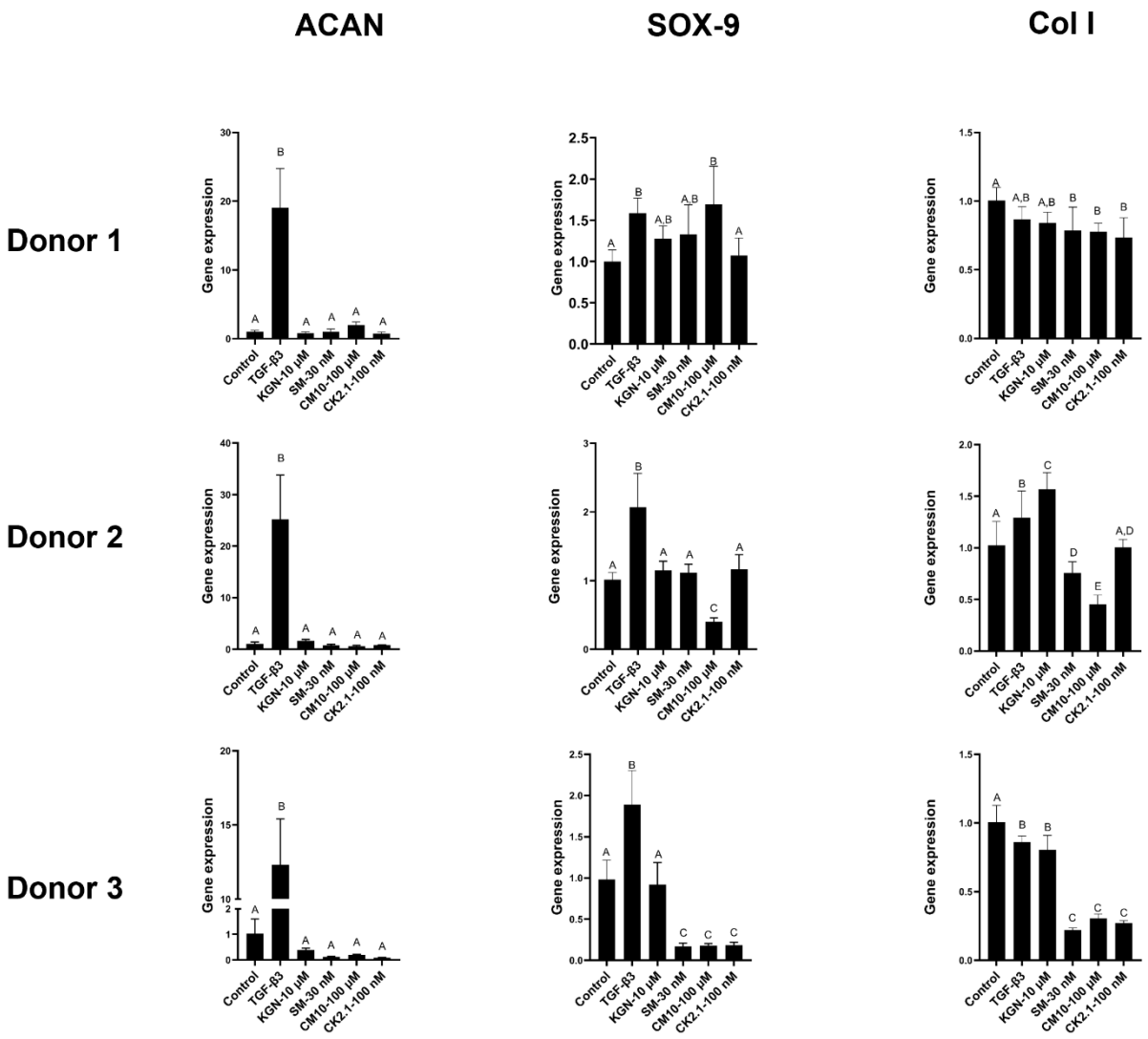

Figure 4.4: Gene expression of ACAN, SOX-9, and collagen I of hBMSCs spheroids from 3 different donors after 21 days in culture.

For ACAN, no statistically significant differences were observed between groups except for TGF-β3 with all donors. Donor-based variation in the gene expression of SOX-9 in response to experimental groups was observed. Notably, CM10 had a significantly higher gene expression of SOX-9 as compared to the negative control only with donor 1 (Female, 20 yrs). SM04690, CM10, and CK2.1 induced a significantly lower gene expression of collagen I as compared to the negative control with all donors, whereas KGN had variable outcomes based on the donor. Groups with the same letters indicate no significant difference, n=6-8. Reported values are mean + standard deviation. SM = SM04690, CK = CK2.1, CM = CM10.

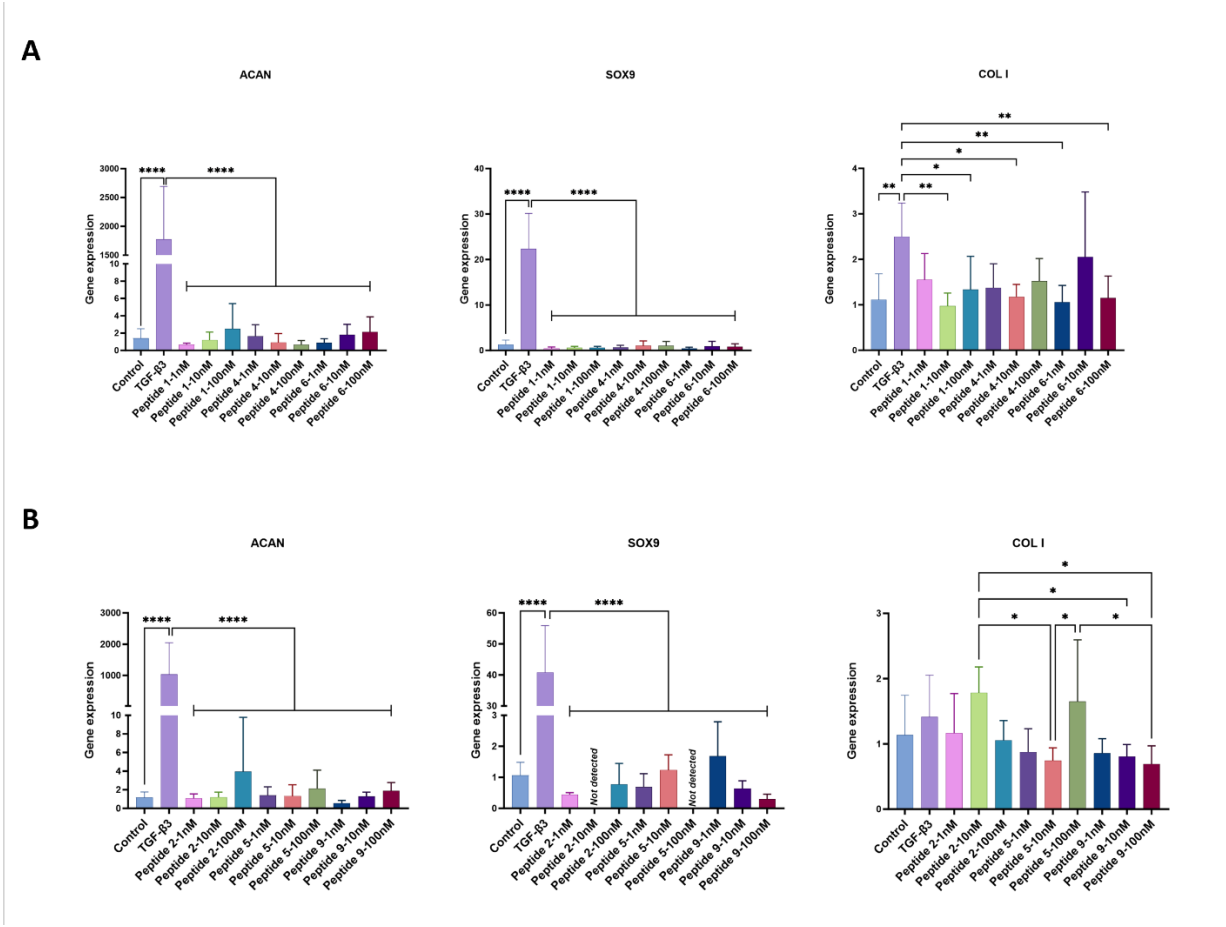

Figure 5.1: Gene expression of human bone marrow-derived mesenchymal stem cells (hBMSCs) after 21 days of treatment with A) Peptides 1, 4, or 6, or B) Peptides 2, or 5, or 9, in spheroid culture.

A) For ACAN, and SOX-9, no statistically significant differences were observed among groups except for TGF-β3, which had significantly higher ACAN and SOX-9 gene expression compared to all other groups, including 1269-fold higher value for ACAN and 17-fold higher value for SOX-9, compared to the negative control. As for collagen I, TGF-β3 had the highest gene expression with 2.3-fold higher value compared to the negative control. B) For ACAN and SOX-9, no statistically significant differences were observed among groups except for TGF-β3, which had significantly higher ACAN and SOX-9 gene expression compared to all groups, including 884-fold and 55-fold higher values compared to the negative control. As for collagen I, no significant differences were observed between the negative and positive controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n = 4-8. Reported values are mean + standard deviation.

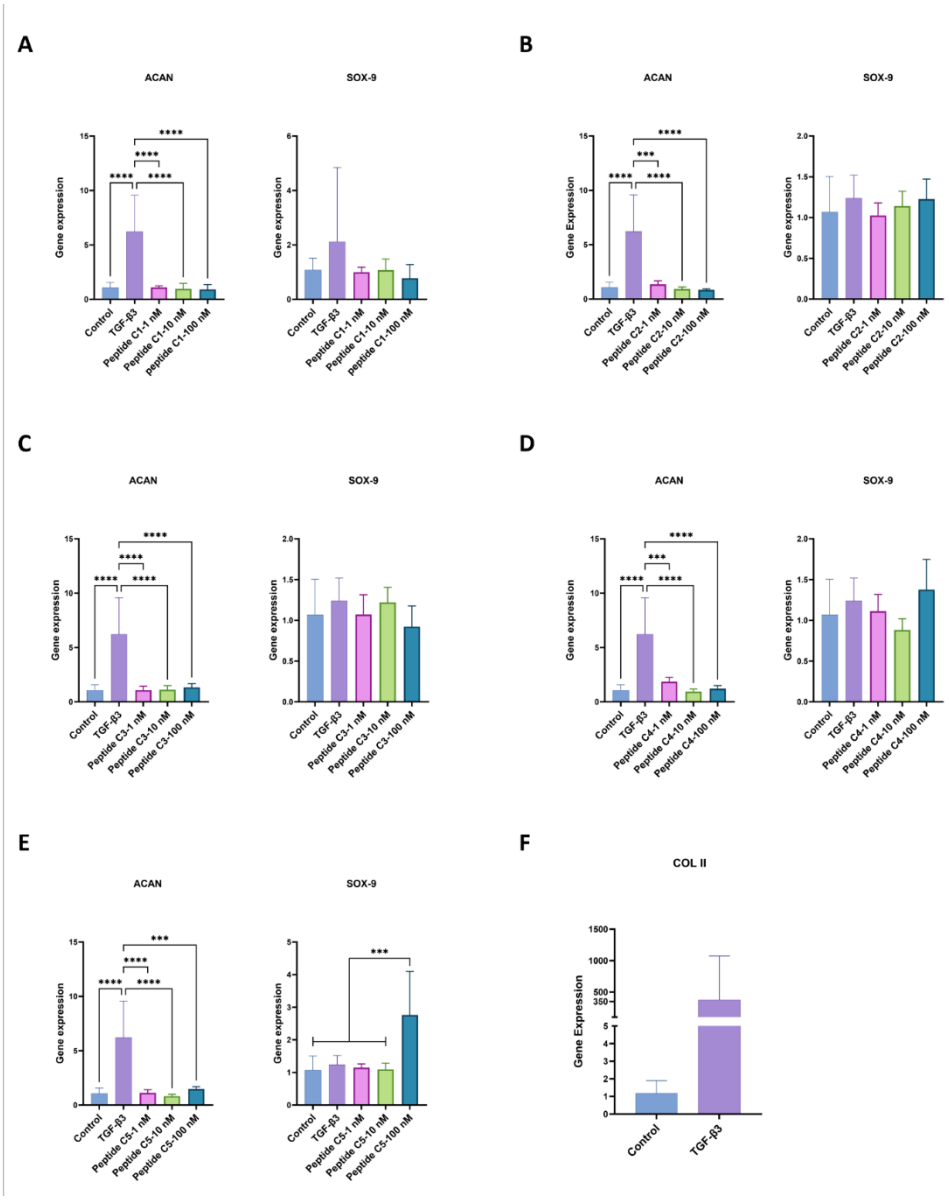

Figure 5.2: Gene expression of human bone marrow-derived mesenchymal stem cells (hBMSCs) after 21 days of treatment with nanomolar (nM) concentrations of A) Peptide C1, B) Peptide C2, C) Peptide C3, D) Peptide C4, E) Peptide C5, in spheroid culture. F) Collagen II gene expression in control groups.

For ACAN, for all peptides no statistically significant differences were observed among groups except for TGF-β3, which had significantly higher ACAN expression compared to all other groups, including 5.7-fold larger value compared to the negative control. For SOX-9, no significant differences were observed among groups for peptides C1, C2, C3, and C4. Peptide C5 at 100 nM had significantly higher SOX-9 gene expression compared to all other groups including 2.6-fold increase compared to the negative control. *** $p < 0.001$, **** $p < 0.0001$, $n = 6$-8. Reported values are mean + standard deviation.

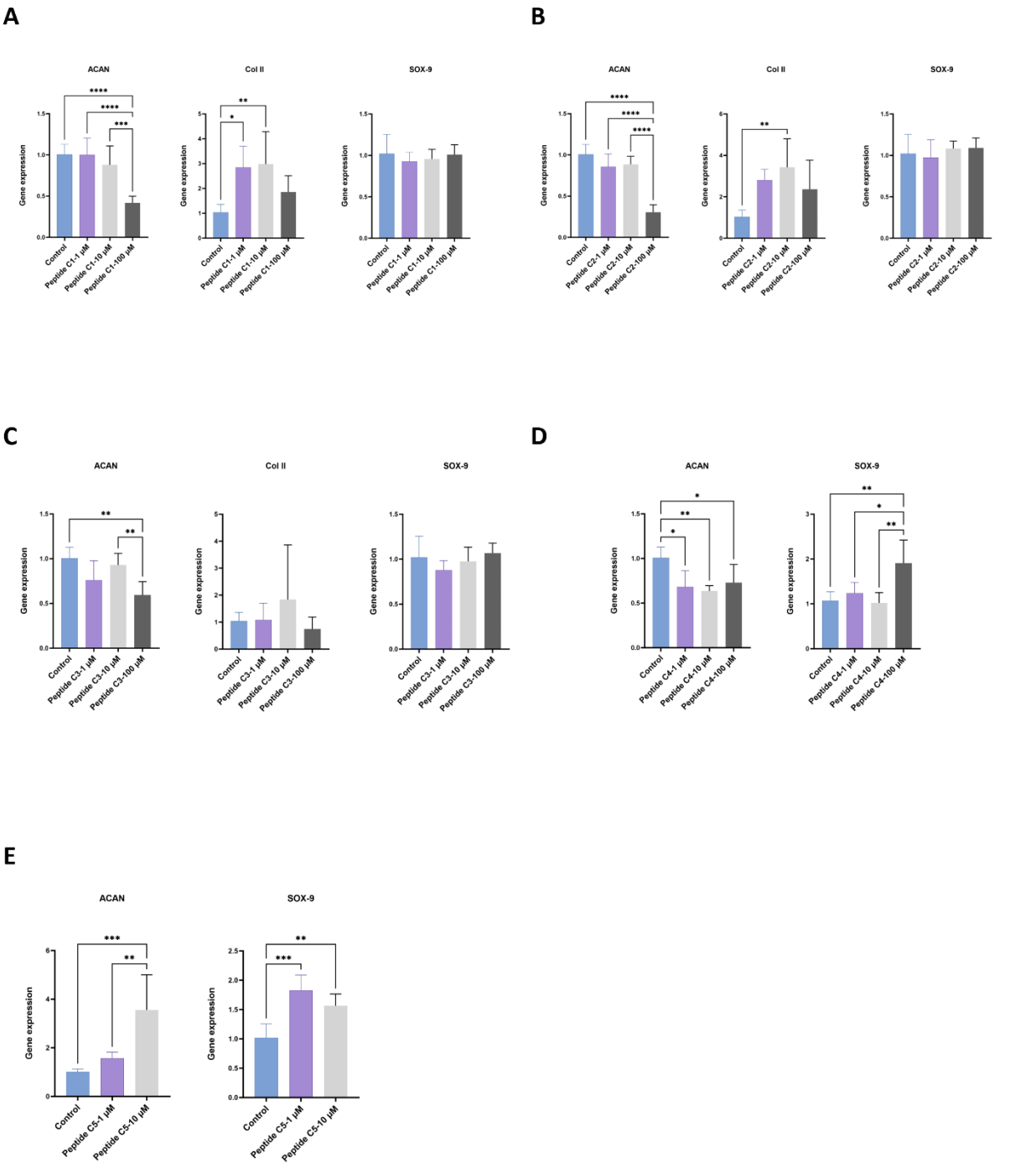

Figure 5.3: Gene expression of human bone marrow-derived mesenchymal stem cells (hBMSCs) after 21 days of treatment with micromolar (µM) concentrations of A) Peptide C1, B) Peptide C2, C) Peptide C3, D) Peptide C4, E) Peptide C5, in spheroid culture.

A) For ACAN, C1 at 100 μM had significantly lower ACAN expression compared to all other groups, including a 59% lower value compared to the negative control. C1 at 1 and 10 μM had 2.7-fold and 2.8-fold higher collagen II gene expression, respectively, compared to the negative control. B) For ACAN, C2 at 100 μM had significantly lower ACAN expression compared to all other groups, including a 70% lower value compared to the negative control. C2 at 10 μM had a 3.2-fold higher collagen II gene expression compared to the negative control. C) C3 at 100 μM had 42% lower ACAN expression compared to the control group. D) For ACAN, the control group had 1.5-fold, 1.6-fold, and 1.4-fold higher gene expressions compared to treatment with C4 at 1 μM, 10 μM and 100 μM, respectively. No collagen II gene expression was observed following treatment with Peptide C4. As for SOX-9, C4 at 100 μM had a significantly higher gene expression compared to all other groups including 2-fold higher value compared to the negative control. E) For ACAN, C5 at 10 μM had significantly higher gene expression compared to the control and C5-1 μM groups, including 3.5-fold higher gene expression compared to the control group. No collagen II gene expression was observed following treatment with Peptide C5. As for SOX-9, a significantly higher gene expression was observed with both the C5-1 μM and C5-10 μM groups, with 1.7-fold and 1.5-fold higher values, respectively, compared to the negative control. * Refers to significant difference $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 6$-8. Reported values are mean + standard deviation.

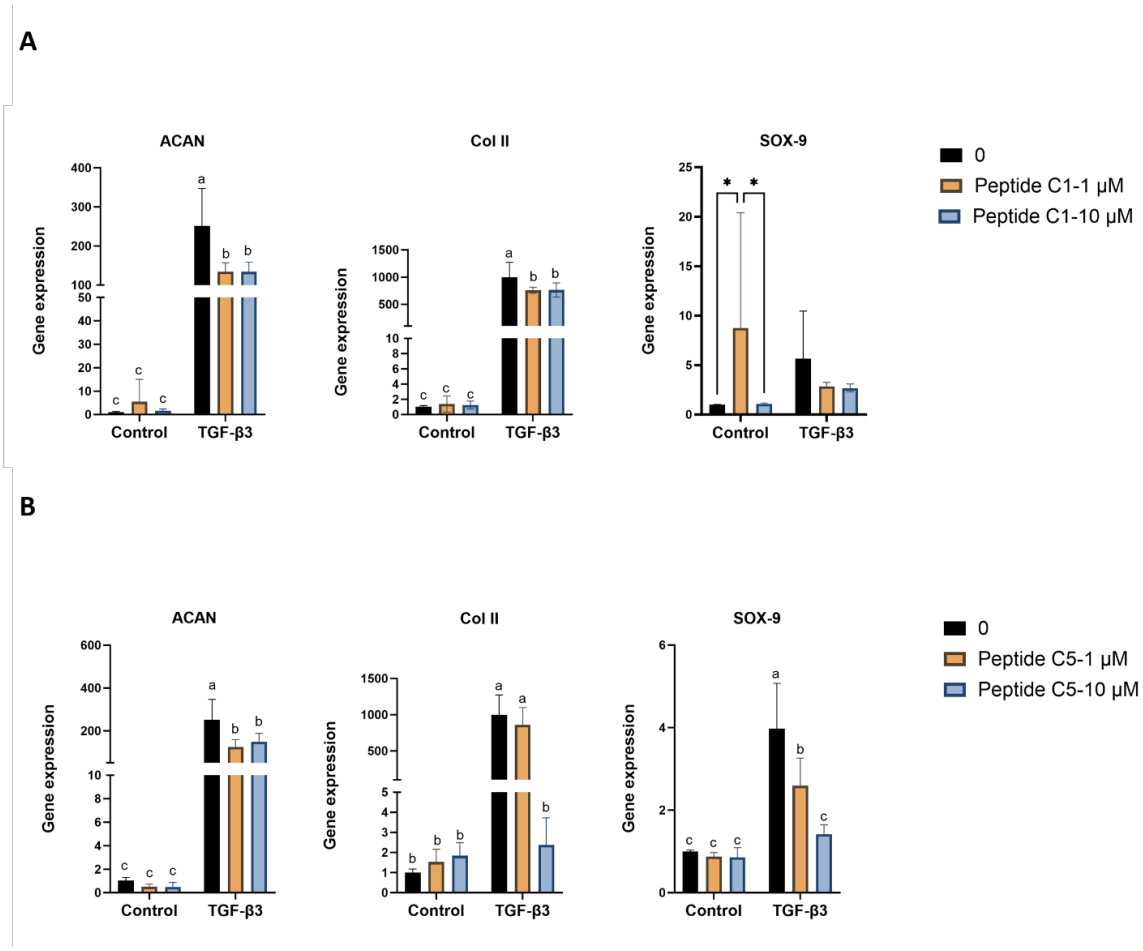

Figure 5.4: Gene expression of human bone marrow-derived mesenchymal stem cells (hBMSCs) after 21 days in spheroid culture with A) Peptide C1 (1 μM, 10 μM), B) Peptide C5 (1 μM, 10 μM) with and without TGF-β3.

A) TGF-β3 had the highest ACAN and collagen II gene expressions with a 243-fold and a 986-fold higher values, respectively, compared to the negative control. In TGF-β3 medium, C1 at 1 μM or 10 μM resulted in 47% lower gene expression compared to TGF-β3 alone. For collagen II, in TGF-β3 medium C1 at 1 μM or 10 μM resulted in 24% lower gene expression compared to TGF-β3 alone. For SOX-9, Peptide C1 at 1 μM had an 8.8-fold higher value compared to the negative control. B) TGF-β3 had the highest ACAN and SOX-9 gene expression with 243-fold higher and 4-fold higher values, respectively, compared to the negative control. C5 at 1 μM or 10 μM resulted in 50% and 41% lower ACAN gene expression compared to TGF-β3 alone. For collagen II, C5 at 1 μM had an 850-fold higher value compared to the negative control. In TGF-β3 medium, C5 at 10μM had 99.7% lower collagen II gene expression compared to TGF-β3 alone. For SOX-9, control. In TGF-β3 medium C5 at 1 μM and 10 μM had 35% and 64% lower SOX-9 gene expression compared to TGF-β3 alone. * Refers to significant difference $p < 0.05$, and groups with the same letters indicate no significant difference from each other, n=6-8. Reported values are mean + standard deviation.

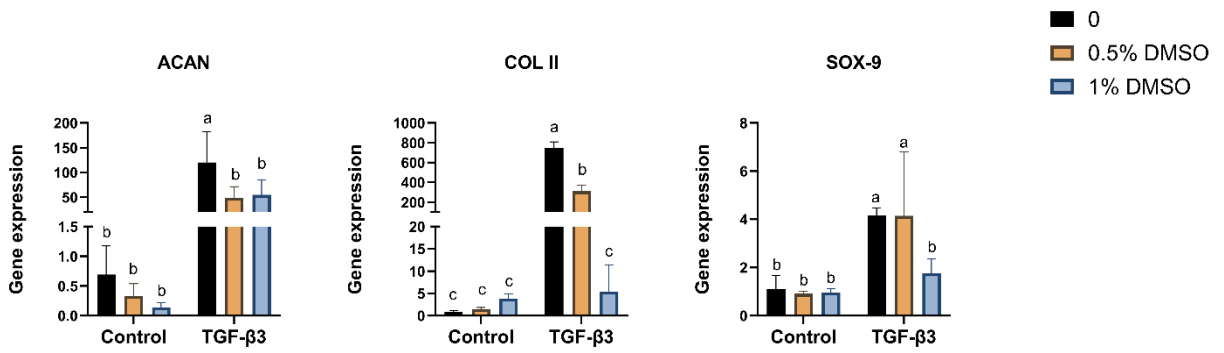

Figure 5.5: The effect of dimethyl sulfoxide (DMSO) on the chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells (hBMSCs) after 21 days in spheroid culture.

For ACAN, lower gene expression was observed with TGF-β3 + 0.5% DMSO and TGF-β3 + 1% DMSO compared to TGF-β3 alone with 59%, and 54% lower values compared to TGF-β3 alone. For collagen II lower gene expression was observed with TGF-β3 + 0.5% DMSO and TGF-β3 + 1% DMSO with 59% and 99% lower values compared to TGF-β3 alone. For SOX-9, significantly lower gene expression was observed with TGF-β3 + 1% DMSO with a 58% lower value compared to TGF-β3 alone. Groups with the same letters indicate no significant difference, n=5-6. Reported values are mean + standard deviation.

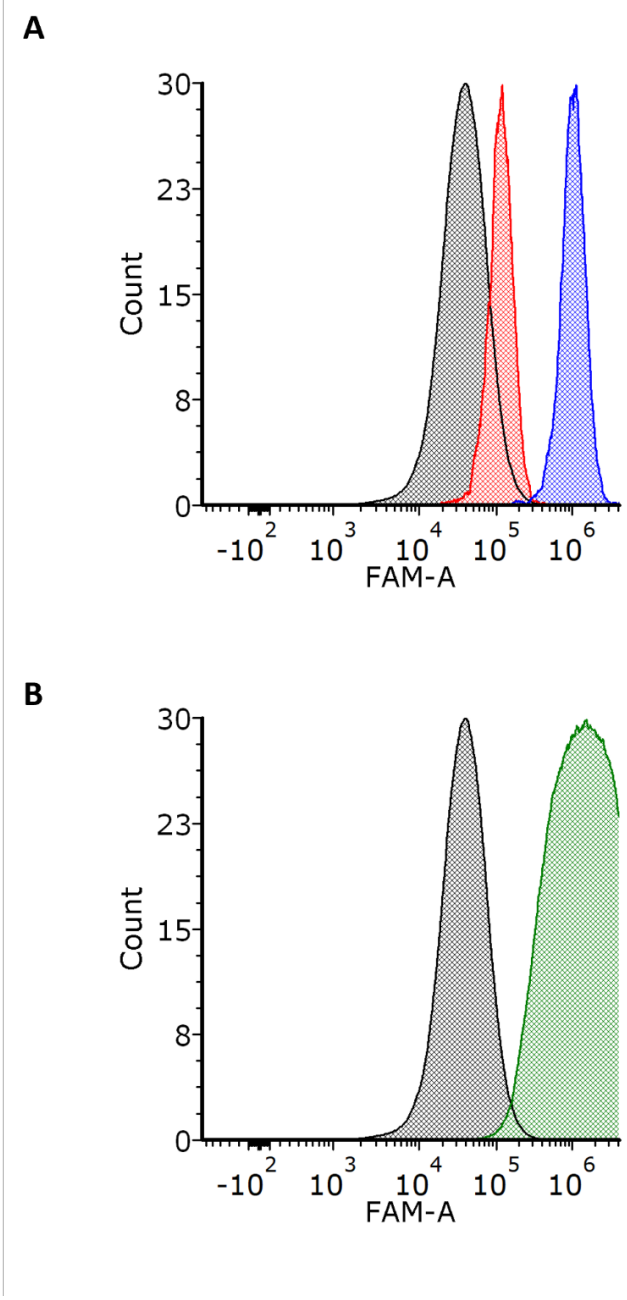

Figure 5.6: Flow cytometry to evaluate the interaction of Peptide C1-FAM with human bone marrow-derived mesenchymal stem cells (hBMSCs) following (A) overnight (C1-10 µM and C1-100 µM) or (B) same day staining (C1-10 µM).

A) Overnight treatment of hBMSCs with Peptide C1 at 10 µM (red histogram) or 100 µM (blue histogram) resulted in a positive staining compared to unstained hBMSCs (black histogram). B) Treatment of hBMSCs with 100 µM Peptide C1-FAM for 25 min, resulted in positive staining (green histogram) compared to unstained hBMSCs.

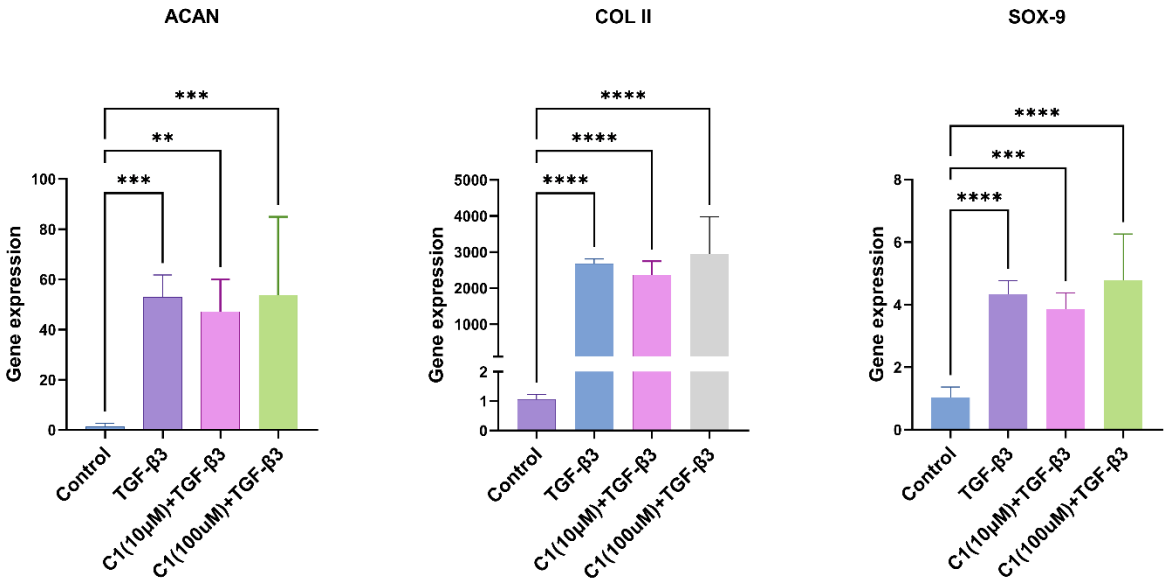

Figure 5.7: Gene expression of human bone marrow-derived mesenchymal stem cells (hBMSCs) after 21 days in spheroid culture with Peptide C1 (10 μM or 100 μM) and TGF-β3.

TGF-β3 resulted in significantly higher ACAN, collagen II, and SOX-9 gene expression compared to the negative control. The combination of TGF-β3 and Peptide C1 treatment did not result in any significant difference compared to the TGF-β3 group. * Refers to significant difference $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 6$. Reported values are mean + standard deviation.

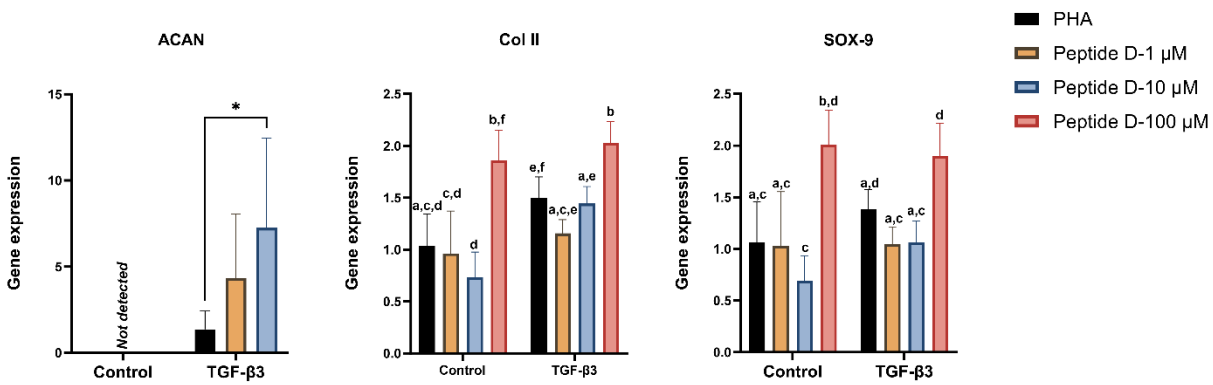

Figure 6.1: Gene expression of encapsulated human bone marrow-derived mesenchymal stem cells (hBMSCs) in pentenoate-functionalized hyaluronic acid (PHA) hydrogel with conjugated Peptide D (1 µM, 10 µM, 100 µM) with and without soluble TGF-β3.

For ACAN, in TGF-β3 medium, PHA with conjugated Peptide D at 10 µM exhibited the highest gene expression with 5-fold higher value compared to the negative control. For collagen II, PHA with conjugated Peptide D at 100 µM had the highest gene expression with both the control medium and TGF-β3 medium with 1.8-fold and 2-fold higher value, respectively, compared to PHA alone with control medium. For SOX-9, PHA with conjugated Peptide D at 100 µM had the highest gene expression with both the control medium and TGF-β3 medium with 1.9-fold and 1.8-fold higher value, respectively, compared PHA alone with control medium. * $p < 0.05$, groups with the same letters indicate no significant difference, n = 6-8. Reported values are mean + standard deviation.

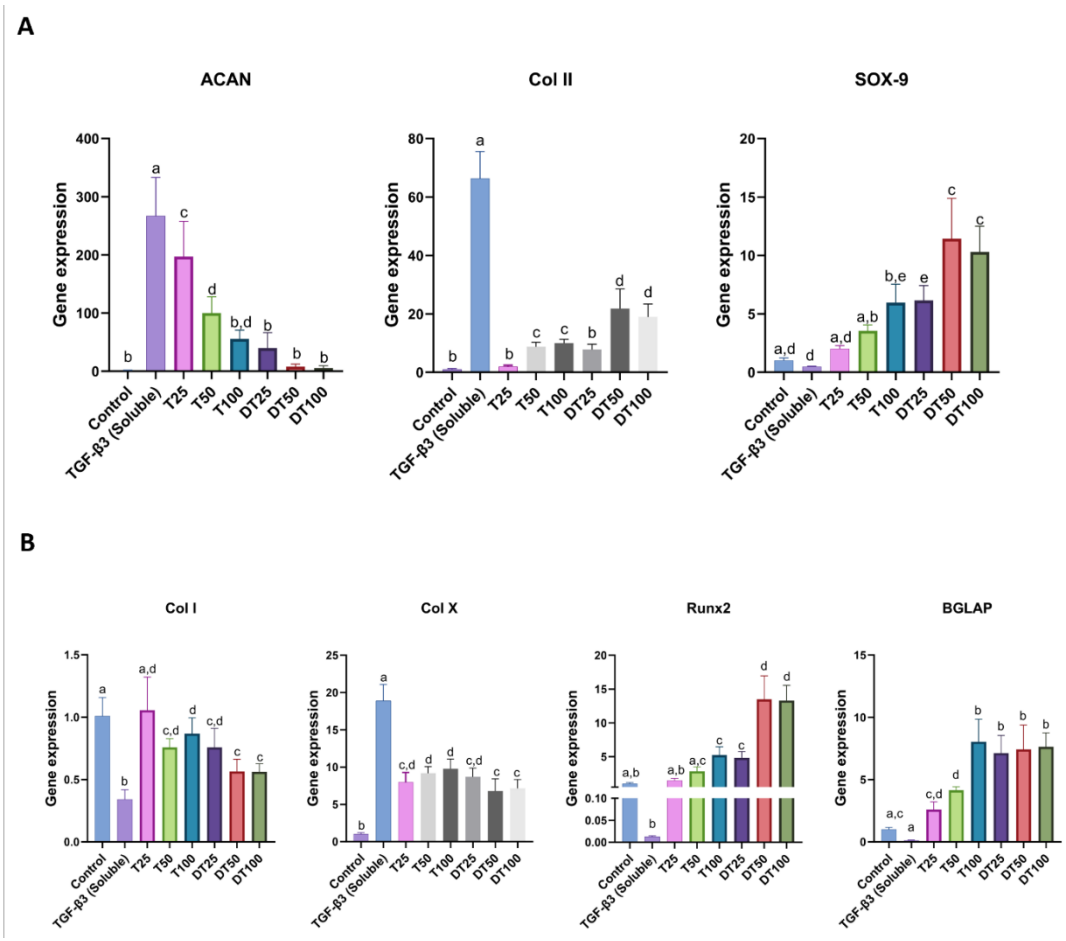

Figure 6.2: Chondrogenic (A) and osteogenic (B) gene expression of encapsulated human bone marrow-derived mesenchymal stem cells (hBMSCs) in pentenoate-functionalized hyaluronic acid (PHA) hydrogel with conjugated Peptide D at 100 µM and conjugated TGF-β3 (25 nM, 50 nM, 100 nM) after 21 days of culture.

A) PHA with soluble TGF-β3 had the highest gene expression for ACAN and collagen II with 230-fold and a 64-fold higher values, respectively, compared to the negative control. As for SOX-9, DT50 and DT100 groups had an 11-fold and 10-fold higher values, respectively, compared to the negative control. B) DT25, DT50, and DT100 had 25%, 44%, and 44% lower collagen I gene expression compared to the negative control. For collagen X, DT50 and DT100 had 26%, and 27% lower collagen X gene expression compared to T50, and T100, respectively. DT50, and DT100 had 4.8-fold, and 2.5-fold higher Runx2 gene expression compared to T50, and T100, respectively. For BGLAP, DT25, and DT50 had 2.7-fold, and 1.7-fold higher values compared to T25, and T50, respectively.* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. Groups with the same letters indicate no significant difference, n = 6-8. Reported values are mean + standard deviation. T25 = PHA + TGF-SH (25 nM), T50 = PHA + TGF-SH (50 nM), T100 = PHA + TGF-SH (100 nM), DT25 = PHA + Peptide D (100 µM) + TGF-SH (25 nM), DT50 = PHA + Peptide D (100 µM) + TGF-SH (50 nM), DT100 = PHA + Peptide D (100 µM) + TGF-SH (100 nM).

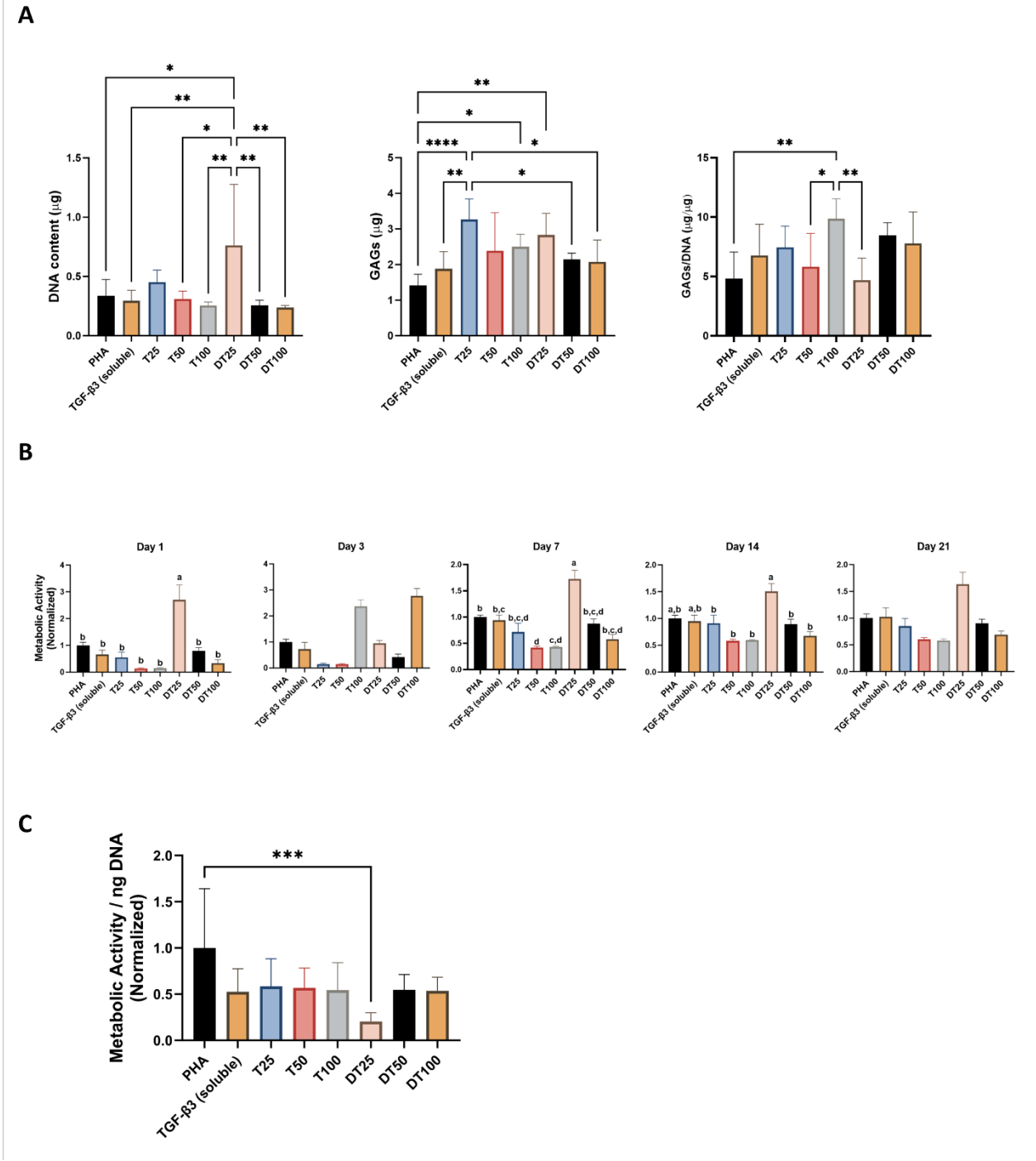

Figure 6.3: Biochemical content (A) and viability (B, C) of encapsulated human bone marrow-derived mesenchymal stem cells (hBMSCs) in pentenoate-functionalized hyaluronic acid (PHA) hydrogel after 21 days in culture.

A) DT25 had the highest DNA content, including 2.3-fold higher value compared to the negative control. No significant differences were observed for the remaining groups. T25 had 2.3-fold higher GAG content compared to the negative control. As for the GAGs/DNA content, T100 had 2-fold higher content compared to the negative control. B) The viability of encapsulated hBMSCs was evaluated on days 1, 3, 7, 14, and 21. Variable viability results were observed on different days, on day 1, DT25 had the highest viability compared to all remaining groups. On day 21, no significant differences were observed among groups. C) For viability normalized to DNA content on day 21, DT25 had a lower value compared to PHA alone, and no other significant differences were observed among the remaining groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Groups with the same letters indicate no significant difference, n = 6-8. Reported values are mean + standard deviation. T25 = PHA + TGF-SH (25 nM), T50 = PHA + TGF-SH (50 nM), T100 = PHA + TGF-SH (100 nM), DT25 = PHA + Peptide D (100 μM) + TGF-SH (25 nM), DT50 = PHA + Peptide D (100 μM) + TGF-SH (50 nM), DT100 = PHA + Peptide D (100 μM) + TGF-SH (100 nM).

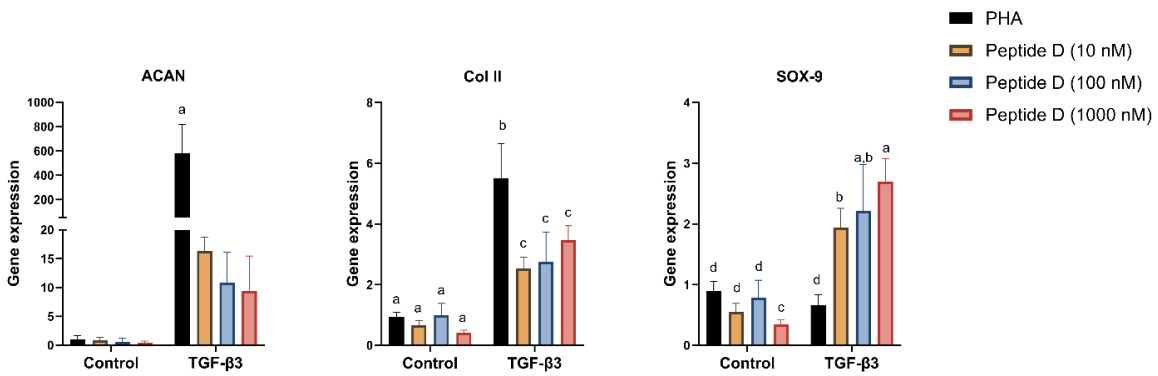

Figure 6.4: Gene expression of encapsulated human bone marrow-derived mesenchymal stem cells (hBMSCs) in pentenoate-functionalized hyaluronic acid (PHA) hydrogel with conjugated Peptide D (10 nM, 100 nM, 1000 nM) with and without conjugated TGF-β3.

PHA with conjugated TGF-β3 had 555-fold and 6-fold higher ACAN and collagen II gene expression, respectively, compared to the negative control. For SOX-9, for PHA groups with conjugated TGF-β3, the conjugation of Peptide D at 10, 100, and 1000 nM resulted in 2.9-fold ($p < 0.0001$), 3.3-fold ($p < 0.0001$), and 4.9-fold ($p < 0.0001$) higher values, respectively, compared to PHA with conjugated TGF-β3 only. Groups with the same letters indicate no significant difference, n = 6-8. Reported values are mean + standard deviation. T25 = PHA + TGF-SH (25 nM), T50 = PHA + TGF-SH (50 nM), T100 = PHA + TGF-SH (100 nM), DT25 = PHA + Peptide D (100 μM) + TGF-SH (25 nM), DT50 = PHA + Peptide D (100 μM) + TGF-SH (50 nM), DT100 = PHA + Peptide D (100 μM) + TGF-SH (100 nM).

Appendix B: Tables

Chapter 1: No Tables

Chapter 2: Tables 2.1-2.4

Chapter 3: Table 3.1

Chapter 4: Tables 4.1-4.2

Chapter 5: Table 5.1-5.2

Chapter 6: Table 6.1-6.2

Table 2.1: Summary of *in vivo* studies performed with chondroinductive peptides.

Peptide	Parent/Target Molecule	Reference	Sham	Negative /Material control	Positive control	Growth factors added	Animal Model	Defect type and size	Cells included	Injection method	Evidence of Chondroinduction
CK2.1	**Inhibits the binding of CK2 to BMPRIa**	**Akkiraju 2017a**[43]	None	PBS injection	BMP-2	None	C57BL/6J mice	N/A	None	Systemic	Enhanced articular cartilage formation based on Saf-O/fast green staining and col-II, IX immunostaining (N, P).
		Akkiraju 2017b[44]	Yes	PBS injection	None	None	C57BL/6J mice	DMM	None	Intra-articular	Increased immunostaining of col-II and IX along with a low immunostaining of col-X and osteocalcin (M, N).
HSNGLPL	**TGF-β1 affinity**	**Shah 2010**[46]	None	None	1) rhTGF-β1 2) TGFBPA +rhTGF-β1	None	Rabbits	Two 2 mm diameter full-thickness chondral defects followed by microfracture were created in the femoral trochlear groove	None	Self-assembly supramolecular nanofibers (TGFBPA) fitted into defects	Significant enhancement of the regenerative potential of microfracture-treated chondral defects based on gross morphology, col-II immunostaining, and GAG staining by Saf-O (M, P).
		Chen 2018[47]	Yes	Chitosan scaffold	None	None	Rabbits	4 mm diameter osteochondral defects in the middle of the femoral trochlear groove	None	Sponges press-fit into defects	Cartilage surface of peptide treated groups was smooth and continuous with higher GAG and col-II staining (Sh, M)

Table 2.1: continued

Peptide	Parent/Target Molecule	Reference	Sham	Negative /Material control	Positive control	Growth factors added	Animal Model	Defect type and size	Cells included	Injection method	Evidence of Chondroinduction
HAVDI-	**N-Cadherin**	**Bian 2013**[51]	None	MeHA hydrogel group and scrambled group	None	TGF-β3 loaded microspheres	Nude mice	N/A	hMSCs	Subcutaneous injection	Peptide-containing implants exhibited a higher content of GAGs and collagen, and more intense staining of col-II and CS in the presence of hMSCs and TGF-β3 loaded microspheres (M, S).
		Feng 2020[55]	Yes	Aggrecanase 1 degradable hydrogel	None	None	Rabbits	4 mm diameter osteochondral defects in the patellar groove	Rabbit BMSCs	Hydrogels implanted into defects	HAV-conjugated hydrogels exhibited more intense staining of GAGs and col-II and no col-I staining (M, S).
		Teng 2021[57]	None	MeHA hydrogel	None	None	Nude mice	N/A	hBMSCs	Subcutaneous injection	MeHA+HAV+RGD hydrogels exhibited enhanced GAG and collagen content (M). MeHA+HAV+RGD hydrogels with encapsulated kartogenin exhibited a high and significant increase in GAG and collagen content as compared to MeHA+HAV+RGD.

Table 2.1: continued

Peptide	Parent/Target Molecule	Reference	Sham	Negative /Material control	Positive control	Growth factors added	Animal Model	Defect type and size	Cells included	Injection method	Evidence of Chondroinduction
CM10 (LIANAK)	**TGF-β1**	**Zhang 201561**	None	Microspheres	None	None	Mice	N/A	Rabbit BMSCs	Subcutaneous injection	Ectopic cartilage formation was observed as demonstrated by intense Saf-O staining of GAGs and col-II immunostaining, with no apparent mineralization (M).
		Park 201962	None	Hydrogel alone	None	None	Mice	N/A	+/- hPLSCs	Subcutaneous injection	CM10 resulted in significant chondrogenic differentiation with increased staining of col-II and GAGs. Increased gene expression of SOX9, ACAN, and col-II (M).
B2A	**BMP-2 receptors**	**Lin 201264**	None	Saline injection	Normal	None	Rats	Chemically induced OA by MIA	None	Synovial space injection	Significant repair of articular cartilage in B2A treated group based on H&E and Saf-O staining (N).

Sh = As compared to sham; M = As compared to material control; N = As compared to negative control; S = As compared to scrambled group; P = As compared to positive control

Abbrev: ACAN = Aggrecan; BMSCs = Bone marrow stem cells; CK2 = Casein Kinase 2; CS = Chondroitin sulfate; DMM = Destabilized medial meniscus; GAG = Glycosaminoglycans; hMSCs = human mesenchymal stem cells; hPLSCs = human periodontal ligament stem cells; MeHA = Methacrylated hyaluronic acid; MIA = Monoiodoacetate; OA = Osteoarthritis; Saf-O = Safranin O

Table 2.2 Summary of *in vitro* studies performed with chondroinductive peptides.

Peptide	Parent Molecule	References	Format	Cells	Negative/ Material control	Positive control	Growth factors added	Evidence of Chondroinduction
CK2.1	**Inhibits the binding of CK2 to BMPRIa**	**Akkiraju 2017a**[44]	Soluble peptide	C3H10T 1/2 stem cells	Peptide-free medium	BMP-2	None	CK2.1 stimulated an increase in proteoglycan and col-II synthesis, along with lower gene expression of col-X and osteocalcin (N, P).
HSNGLPL	**TGF-β1**	**Shah 2010**[46]	PA gels	Human MSCs	Filler PA	Filler PA+TGF-β1	TGF-β1	HSNGLPL treatment resulted in a higher ACAN gene expression level after 4 wks of culture but not at previous time points (M). After three weeks, there was no difference in GAG production between peptide and control groups (M).
		Chen 2018[47]	Chitosan scaffold (CHI)	Pig MSCs	Chitosan scaffold	CHI+TGF-β1	Pre-loaded with TGF-β1	Scaffolds with 10:3 chitosan to peptide ratio exhibited the highest gene expression levels of SOX9, col-II, and ACAN, whereas col-X was not upregulated (M, P)
HAVDI-	**N-Cadherin**	**Bian 2013**[51]	MeHA hydrogel+peptide	Human MSCs	MeHA Hydrogel & scrambled	None	TGF-β3	HAVDING significantly increased the gene expression of col-II, ACAN, and SOX9 on days 1 and 3; however, by Day 7, no significant difference was observed between groups. Following 28-day culture with hMSCs, HAVDING increased GAG and collagen content (M, S).
		Kwon 2018[53]	MeHA hydrogel + HAV motif with ADAM10-cleavable domain	Human MSCs	MeHA Hydrogel group & scrambled peptide group	None	TGF-β3	HAVDING treatment resulted in a dose-dependent increase in col-II gene expression during the first seven days of culture but not at 14 days. At 56 days, increased levels of sGAGs and col-II staining was observed (M, S).
		Cimenci 2019[54]	Amphiphilic peptide nanofiber system+HAV motif	Rat BMSCs	Amphiphilic peptide nanofiber system+nonbioactive motif & TCP	None	StemPro Chondrogenesis Differentiation Kit	Increased GAG accumulation was observed in rBMSCs cultured on HAV-nanofibers (M). Increased gene expression of col-II, ACAN, and SOX-9 was observed at day 3, 7, and 14 in rBMSCs cultured on HAV-nanofibers (M).
		Teng 2021[57]	Crosslinked with MeHA hydrogel	Human BMSCs	MeHA hydrogel	None	TGF-β3	MeHA + HAV + RGD significantly upregulated the gene expression of ACAN, and col-II as compared to MeHA and MeHA + HAV. The encapsulation of kartogenin in MeHA + HAV + RGD resulted in the highest gene expression of ACAN, col-II, and SOX-9 as compared to MeHA + RGD + HAV.

Table 2.2 continued

Peptide	Parent Molecule	References	Format	Cells	Negative/ Material control	Positive control	Growth factors added	Evidence of Chondroinduction
SPPEPS	**Aggrecan and TGF-β3**	**Mahzoon 2019**[65]	Soluble or with PHA hydrogel	Rat BMSCs	Peptide-free group	TGF-β3 (proteomic analysis only)	None	SPPEPS in soluble form enhanced the expression of SOX9 and col-II after 3 days of culture (M). Proteomic analysis showed 60 proteins common between the SPPEPS and positive control groups on day 7. At day 14, 26 proteins were shared between the SPPEPS group and the positive control group.
CM10 (LIANAK)	**TGF-β1**	**Zhang 2015**[61]	Nanofibrous microspheres + CM10	Rabbit BMSCs	Nanofibrous microspheres	None	None	Positive Saf-O staining, appearance of round differentiated cells encased in lacunae (M).
		Park 2019[62]	Soluble or crosslinked with HA hydrogel	Human PLSCs	Peptide and growth factor-free	TGF-β	None	CM10 increased the staining of col-II and GAGs (N). CM10 increased gene expression of SOX9, ACAN, and col-II at a comparable fold increase to TGF-β group (N, P).
LINK N	**Hyaluronate and aggrecan**	**He 2018**[69]	Soluble peptide	CSPCs	Peptide and growth factor-free	TGF-β3	None	Increased concentrations of Link N protein resulted in increased gene expression of SOX9, col-II, and ACAN with no increase in Runx2 nor col-X expression. Link N stimulated the chondrogenic differentiation of CSPCs in 3D culture, based on col-II, SOX9 immunostaining. The best performance was for the peptide + TGF-β3 group (N, P).
GFOGER (CMP)	**Binds α2β1 integrin**	**Liu 2010**[72]	PEG hydrogel + peptide	Human MSCs	Peptide-free hydrogel	TGF-β3	± TGF-β3	CMP enhanced GAG, col-II, and ACAN staining in the presence of TGF-β3 (M). SOX9 gene upregulated, col-X gene downregulated (M).
		Mhanna 2014[73]	PEG hydrogel with MMP sensitive motifs + peptide	Human MSCs	Peptide-free hydrogel	None	TGF-β3	GFOGER increased GAG and DNA synthesis. Gene expression of col-II was highest in GFOGER-modified degradable gels (M).
B2A	**BMP-2 receptors**	**Lin 2012**[63]	Soluble peptide	C3H10T1/2 Human BMSCs Human NACs	Peptide-free culture medium	Only in the col-II experiment	None	B2A upregulated gene expression of Fgfr1, Fgfr2, Fgf1, Smad1, Smad4, and Twist1 (N). Matrix genes col-I and col-II were upregulated (N). B2A stimulated the production of GAGs and collagen II in hBMSC and human chondrocytes in micromass culture (N).

Table 2.2 continued

Peptide	Parent Molecule	References	Format	Cells	Negative/Material control	Positive control	Growth factors added	Evidence of Chondroinduction
KIPKASSVPTELS AISTLYL	**BMP-2**	**Renner 2012**[78]	Soluble peptide	Human MSCs	Peptide-free culture medium	BMP-2; TGF-β3	None	BMP peptide increased the production of GAGs (N, P). Significant increase in total collagen. No increase in AP activity or col-I deposition (P). Upregulated gene expression of col-II and SOX9 at week 1 (N). Col-I and X gene expression increased as well (N), but at reduced values as compared to BMP-2 treated cells.
RYPISRPRKR	**HA-bind**	**Parmar 2015**[83]	MMP7-Scl2	Human MSCs	Peptide-free hydrogel; scrambled peptide hydrogel	None	TGF-β3	HAbind and CSbind enhanced the gene expression of col-II, ACAN, and SOX9 in hMSCs (M). The highest gene upregulation of chondrogenic markers was for HAbind at 10% functionalization group. Col-I and X gene expression were significantly lower in HAbind and CSbind hydrogels (M). Total collagen, sGAG, and DNA content were highest for the HAbind (10%) groups (N, P).
YKTNFRRYYRF	**CS-bind**							
GRVDWLQRNANF YDWFVAELG	**IGF-1**	**Renner 2013**[79]	Soluble peptide	Human MSCs	Peptide and growth factor free medium	TGF-β3	Insulin and TGF-β3	In the presence of Insulin and TGF-β3, the insulin peptide treatment resulted in an increased production of GAG as compared to the TGF-β3 positive control

M = As compared to material control; N = As compared to negative control; S = As compared to scrambled group; P = As compared to positive control Abbrev: ACAN = Aggrecan; BMSCs = Bone marrow stem cells; CSPCs = Cartilage stem/progenitor cells; GAG = Glycosaminoglycans; HA = Hyaluronic acid; MSCs = Mesenchymal stem cells (tissue source not specified); NAC = articular chondrocytes; PLSCs = Periodontal ligament stem cells; MeHA = Methacrylated hyaluronic acid; MMP = Matrix metalloproteinase; PA = Peptide amphiphile; PEG = Polyethylene glycol; PHA = Pentenoate functionalized hyaluronic acid; Saf-O = Safranin O; Scl2 = Recombinant streptococcal collagen-like protein; TCP = Tissue culture plate

Table 2.3. Summary of *in vivo* studies for peptides reported to be used for cartilage regeneration.

Peptide	Parent/Target Molecule	Reference	Sham	Negative /Material control	Positive control	Growth factors added	Animal Model	Defect type and size	Cells included	Injection method	Evidence of Chondroinduction
WYRGRL	**Col-II**	**Hesse 2018**[95]	None	StarPEG/heparin hydrogels alone	None	None	Mice	N/A	Chondrocytes	Subcutaneous injection	Not attractive to induce collagen deposition (M).
KLER	**Decorin**	**Hesse 2018**[95]	None	StarPEG/heparin hydrogels	None	None	Mice	N/A	Chondrocytes	Subcutaneous injection	Not attractive to induce collagen deposition (M).
EPLQLKM (E7)	**MSC affinity peptide**	**Huang 2014**[93]	None	Microfracture / DBM+Chitosan scaffold	None	None	Rabbits	Full-thickness osteochondral defects (4 mm diameter, 2.5 mm deep) were created on the trochlear groove	None	DBM (4 mm diameter, 2 mm height) was implanted into the osteochondral defects followed by injection of pre-prepared chitosan solution	At 24 weeks, E7-containing scaffolds showed superior cartilage repair quality and quantity based on IHC, toluidine blue staining, and did not exhibit hypertrophic cartilage remodeling (M, N).
		Meng 2015[94]	None	DBM +Chitosan scaffold/Chitosan scaffold	None	scaffolds pre-cultured in chondrogenic medium	Mice	N/A	BMSCs	Injection into fossa iliaca subcutaneous region	E7-containing scaffold exhibited translucent and superior cartilage-like structures, based on gross observation, H&E, toluidine blue and col-II staining (M).

Table 2.3 continued

Peptide	Parent/Target Molecule	Reference	Sham	Negative /Material control	Positive control	Growth factors added	Animal Model	Defect type and size	Cells included	Injection method	Evidence of Chondroinduction
KLD	**Self-assembling peptide**	**Miller 2010**[85]	Yes-contralateral untreated control	None	None	None	Rabbits	Full-thickness, critically sized defect (3 mm-diam × 2 mm-deep) in the central region of the femoral trochlear groove	+/- BMSCs	Peptide solution directly added to defects followed by Lactated Ringer's Solution to polymerize into the hydrogel	KLD hydrogel alone resulted in a significantly higher Saf-O, col-II immunostaining, and cumulative histology scores as compared to KLD hydrogels containing chondrogenic factors or BMSCs (Sh).
		Miller 2014[89]	Yes	+/- MFX	None	None	Horses	15-mm-diameter defect in the medial trochlear ridge	None	Peptide solution directly added to defects followed by Lactated Ringer's Solution to polymerize into the hydrogel	KLD-treated defects exhibited decreased ACAN and Col-II staining in the proximal side of the repair tissue (Sh). The combination of MFX+KLD presented no additional improvement compared to MFX or KLD-only treatment, and it resulted in decreased col-II staining compared with MFX-only treatment.
(RADA16-I)/PFS (PFSSTKT)	**Self-assembling peptide/Bone marrow homing peptide**	**Lu 2018**[91]	Microfracture	1) RAD alone 2) ACM-RAD	None	None	Rabbits	Cylindrical full-thickness defect (3 mm in diameter × 1.5 mm depth) in the center of the trochlear groove	None	Implants were placed flush with the surface of the surrounding cartilage	ACM-KLD/PFS exhibited an increased gene expression of aggrecan, SOX9, and col-II, with the highest increase being for col-II (Sh, M).

Sh = As compared to sham; M = As compared to material control; N = As compared to negative control; S = As compared to scrambled group; P = As compared to positive control

Abbrev: ACM = acellular cartilage matrix; BMSCs = Bone marrow stem cells; DBM = demineralized bone matrix; MFX = Microfracture; Saf-O = Safranin O

Table 2.4. Summary of *in vitro* studies for peptides reported to be used for cartilage regeneration.

Peptide	Parent Molecule	References	Format	Cells	Negative/Material control	Positive control	Growth factors added	Outcome
ANVAENA (CM-1)	**TGF-β1**	**Renner 2013**[79]	Soluble	Human MSCs	Peptide and growth factor free medium	TGF-β3	None	ANVAENA treatment resulted in much lower GAG content (P). No significant increase as compared to the negative control.
GPPDWHWKAMTH (R1-P1)	**FGFR1**	**Tan 2018**[98]	Soluble	ATDC5& chondrocytes	Peptide-free medium	PD173074, FGFR1 inhibitor	None	Intra-articular injection of R1-P1 was reported to significantly decreased the cartilage destruction and IL-1β-induced proteoglycan loss in an OA mouse model.
WYRGRL	**Col-II**	**Hesse 2018**[95]	StarPEG/Heparin hydrogels functionalized with CWYRGRL	Human MSCs	Peptide-free hydrogels	None	TGF-β1	StarPEG/Heparin hydrogels functionalized with CWYRGRL induced a significant decline of MSC proliferation and chondrogenic markers (SOX9, BGN, col-II) gene expression (N).
KLER	**Decorin**	**Salinas and Anseth 2009**[96]	PEG scaffold functionalized with RGD and KLER	Human MSCs	Scrambled peptide group	None	± TGF-β1	KLER promotes chondrogenesis of MSCs when combined with RGD in PEG scaffold in chondrogenic medium.
		Hesse 2018[95]	StarPEG/Heparin hydrogels functionalized with KLER	Human MSCs	Peptide-free hydrogels	None	TGF-β1	StarPEG/Heparin hydrogels functionalized with KLER did not promote advanced col-II deposition (N).
GTPGPQGIAGQRGVV (P15)	**Col-I**	**Zhang 2017**[97]	Coated on culture dishes	C3H10T1/2	Uncoated dishes	None	TGF-β (not specified if 1 or 3)	P15 enhanced cell commitment to the chondrocyte lineage. P15 may serve to increase the attachment of mesenchymal cells to the matrix and then, in response to BMP-2/growth factors, leads to increased chondrogenic differentiation and an earlier start to endochondral Bone formation.
CDPGYIGSR	**Laminin**	**Kuo and Wang 2011**[99]	CDPGYIGSR-modified scaffolds PEO and chitosan	BKCs	Peptide-free scaffolds	None	None	CDPGYIGSR modified scaffolds supported the proliferation of BKCs. Increased content of glycosaminoglycans (GAGs), and collagen was reported (M).

Table 2.4: continued

Peptide	Parent Molecule	References	Format	Cells	Negative/Material control	Positive control	Growth factors added	Outcome
EPLQLKM (E7)	**MSC affinity peptide**	**Meng 2015**[94]	E7-modified DBM particles and Chitosan scaffold	Rat BMSCs	1) Chitosan scaffold 2) Chitosan/DBM scaffold	None	None	E7 increased the gene expression of col-II and ACAN. Increased staining of GAGs was observed (M).
RADA	**N/A**	**Kopesky 2010**[86]	Self-assembling peptide	BMSCs	1) Agarose hydrogel 2) TGF-β1-free medium	None	± TGF-β1	RADA hydrogels upregulated the gene expression of ACAN, SOX9, and col-II along with no upregulation of col-I in the presence of TGF-β1 (M). A higher cell number was observed with RAD16-I and KLD12 peptide hydrogels as compared to agarose.
KLD	**N/A**							

M = As compared to material control; N = As compared to negative control; S = As compared to scrambled group; P = As compared to positive controlAbbrev: ACAN = Aggrecan; BKCs = Bovine knee chondrocytes; BMSCs = Bone marrow stem cells; DBM = demineralized bone matrix; GAG = Glycosaminoglycans; MSCs = Mesenchymal stem cells (tissue source not specified); MMP = Matrix metalloproteinase; OA = Osteoarthritis; PEG = Polyethylene glycol; PEO = containing polyethylene oxide; Saf-O = Safranin O

Table 3.1: Antibodies used for flow cytometric analysis of cell surface markers of eBMSCs and eUCMSCs.

Marker	Antibody	Supplier	Part number	References
CD29	Anti-human CD29 Antibody-clone TS2/16-Reacts with horse	BioLegend	303003	De Schauwer et al.2012
CD44	Rat anti-mouse-CD44 Antibody-clone IM7-reacts with horse	Thermo Scientific	MA1-10229	
CD45	Mouse anti-human CD45 antibody-clone F10-89-4-Reacts with horse	Biorad	MCA87A700T	Spaas, J.H. *et al.* (2013)
CD73	Mouse anti-Human/Equine CD73 antibody-clone 606112	R&D	FAB5795S	
CD79	Mouse anti Human CD79a- clone HM57-Reacts with Horse	Biorad	MCA2538A647T	De Schauwer, C. *et al.* (2012) Spaas, J.H. *et al.* (2013)
CD90	CD90 Monoclonal Antibody-clone 5E10-Reacts with horse	Thermo Scientific	A15726	
CD105	Mouse anti-human CD105 antibody-clone SN6-Reacts with horse	Biorad	MCA1557SBV440	De Schauwer, C. *et al.* (2012)

Table 4.1: List of experimental groups and concentrations evaluated

Experimental groups	Concentrations
Kartogenin (KGN)	1 µM, 10 µM
SM04690 (SM)	30 nM, 100 nM
CM10	100 µM, 200 µM
CK2.1	100 nM, 500 nM
Combinations	
CM10/CK2.1	100 µM /100 nM; 100 µM /500 nM 200 µM /100 nM; 200 µM /500 nM
CM10/KGN	100 µM /1 µM; 100 µM /10 µM
CM10/SM04690	100 µM /30 nM; 100µM /100 nM
CK2.1/KGN	100 nM /1 µM; 100 nM /10 µM
CK2.1/SM04690	100 nM /30 nM; 100 nM /100 nM

Table 4.2: TaqMan probes information

Target gene	Assay ID	Interrogated sequence	Probe context sequence
GAPDH	Hs02786624_g1	NM_001256799.2	CGCTGCCAAGGCTGTGGGCAAGGTC
ACAN	Hs00153936_m1	NM_001135.3	CCGCTGCCAGGGATCCTTCCTACTT
Collagen I	Hs00164004_m1	NM_000088.3	AAGACGAAGACATCCCACCAATCAC
Collagen II	Hs00264051_m1	NM_001844.4	TGGTCTTGGTGGAAACTTTGCTGCC
SOX-9	Hs00165814_m1	NM_000346.3	GAGCACTCGGGGCAATCCCAGGGCC

Table 5.1: TaqMan probes information

Target gene	Assay ID	Interrogated sequence	Probe context sequence
GAPDH	Hs02786624_g1	NM_001256799.2	CGCTGCCAAGGCTGTGGGCAAGGTC
ACAN	Hs00153936_m1	NM_001135.3	CCGCTGCCAGGGATCCTTCCTACTT
Collagen I	Hs00164004_m1	NM_000088.3	AAGACGAAGACATCCCACCAATCAC
Collagen II	Hs00264051_m1	NM_001844.4	TGGTCTTGGTGGAAACTTTGCTGCC
SOX-9	Hs00165814_m1	NM_000346.3	GAGCACTCGGGGCAATCCCAGGGCC

Table 5.2: Bio-Rad probes information

Target gene	Assay ID	Amplicon context sequence
GAPDH	qHsaCEP0041396	GTATGACAACGAATTTGGCTACAGCAACAGGGTGGTG GACCTCATGGCCCACATGGCCTCCAAGGAGTAAGACC CCTGGACCACCAGCCCCAGCAAGAGCACAAGAGGAA GAGAGAGACCCTCACTGCTGGGGAGTCCCTGCCACA C
Collagen II	qHsaCEP0023931	TGTTCTTGCAGTGGTAGGTGATGTTCTGGGAGCCTTC CGTGGACAGCAGGCGTAGGAAGGTCATCTGGACGTT GGCAGTGTTGGGAGCCAGATTGTCATCTCCATAGC TGAAATGGAAGCCACCATTGATGGT
SOX-9	qHsaCEP0051345	AACCTTGGCTAAATGGAGCAGCGAAATCAACGAGAAA CTGGACTTTTTAAACCCTCTTCAGAGCAAGCGTGGAG GATGATGGAGAATCGTGTGATCAGTGTGCTAAATC TCTCTGCCTGTTTGGACTTTGTAATTAT

Table 6.1: TaqMan probes information

Target gene	Assay ID	Interrogated sequence	Probe context sequence
GAPDH	Hs02786624_g1	NM_001256799.2	CGCTGCCAAGGCTGTGGGCAAGGTC
ACAN	Hs00153936_m1	NM_001135.3	CCGCTGCCAGGGATCCTTCCTACTT
Collagen I	Hs00164004_m1	NM_000088.3	AAGACGAAGACATCCCACCAATCAC
Collagen II	Hs00264051_m1	NM_001844.4	TGGTCTTGGTGGAAACTTTGCTGCC
SOX-9	Hs00165814_m1	NM_000346.3	GAGCACTCGGGGCAATCCCAGGGCC

Table 6.2: Bio-Rad probes information

Target gene	Assay ID	Amplicon context sequence
GAPDH	qHsaCEP0041396	GTATGACAACGAATTTGGCTACAGCAACAGGGTGGT GGACCTCATGGCCCACATGGCCTCCAAGGAGTAAGA CCCCTGGACCACCAGCCCCAGCAAGAGCACAAGAG GAAGAGAGAGACCCTCACTGCTGGGGAGTCCCTGCC ACAC
Collagen II	qHsaCEP0023931	TGTTCTTGCAGTGGTAGGTGATGTTCTGGGAGCCTTC CGTGGACAGCAGGCGTAGGAAGGTCATCTGGACGTT GGCAGTGTTGGGAGCCAGATTGTCATCTCCATAGC TGAAATGGAAGCCACCATTGATGGT
SOX-9	qHsaCEP0051345	AACCTTGGCTAAATGGAGCAGCGAAATCAACGAGAA ACTGGACTTTTTAAACCCTCTTCAGAGCAAGCGTGGA GGATGATGGAGAATCGTGTGATCAGTGTGCTAAATC TCTCTGCCTGTTTGGACTTTGTAATTAT