ENGINEERING A CHONDROGENIC MICROENVIRONMENT TO PROMOTE MSC CHONDROGENESIS

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ENGINEERING A CHONDROGENIC MICROENVIRONMENT TO PROMOTE MSC CHONDROGENESIS

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LIST OF SYMBOLS AND ABBREVIATIONS

BMP	Bone morphogenetic protein
DMMB	Dimethylmethylene Blue
ECM	Extracellular Matrix
GAGs	Glycosaminoglycans
H&E	Hematoxylin and Eosin
MSC	Mesenchymal Stem Cells
OA	Osteoarthiritis
SafO	Safranin O
TGF-β	Transforming Growth Factor Beta

SUMMARY

Osteoarthritis (OA) is characterized by the degradation of articular cartilage and affects 27 million people in the US. Mesenchymal stem cells (MSCs) are a promising cell source for OA therapies because of their immunomodulatory properties and ability to be differentiated along a chondrogenic lineage. Traditional chondrogenic differentiation of MSCs relies on using growth factors such as TGF-βs, but cells rapidly undergo hypertrophy and are not able to withstand the same mechanical load as healthy hyaline cartilage. Decellularized cartilage contains important growth factors and extracellular matrix (ECM) proteins to support chondrogenesis at physiologically relevant concentrations and may be an alternative or additive to improve chondrogenic differentiation.

The objective of this study was to investigate whether digested cartilage ECM incorporation into MSC pellets could improve chondrogenic differentiation alone or in combination with exogenous growth factors such as TGF-β1. Porcine articular cartilage was decellularized and then digested in pepsin to form an ECM digest. The ECM digest was incorporated into 250,000 cell pellets at various concentrations to determine an appropriate dose. The ECM digest was then subsequently incorporated into MSCs with and without the addition of TGF-β1. The chondrogenic TGF-β1 treated control with no additional ECM was negative for glycosaminoglycan (GAG) staining after 21 days in culture, so subsequent experiments investigated the role of donor-to-donor variability, passage number, and media composition in affecting MSC chondrogenic differentiation. Chondrogenic differentiation of MSC pellets had better glycosaminoglycan (GAG) content with TGF-β3 induction compared to TGF-β1, but this differentiation was greatly limited in multiple donors with high (>p4) passage number. Future studies will compare ECM addition with chondrogenic induction of MSCs from earlier passages.

CHAPTER 1

INTRODUCTION

Osteoarthritis (OA) is the degradation of articular cartilage and affects 26.9 million people in the US [1]. Healthy articular cartilage is a largely avascular tissue with low cellular density and is mainly composed of hyaline cartilage [2]. Hyaline cartilage is characterized by its complex extracellular matrix (ECM) of proteins, proteoglycans, and collagens (primarily collagen type II) [2]. Collagen is involved in structural support while the negatively charged glycosaminoglycans (GAGs) attract water molecules and allow for its absorption and release, giving the tissue its compressive mechanical properties. When the mechanical integrity of cartilage is damaged, it is no longer able to appropriately shield stress, causing the nerve endings in bone and other tissues in the joint capsule to transmit pain. Additionally, inflammatory mediators and local inflammation in the synovium (synovitis) are also major driving factors for OA-related pain [3].

Chondrocytes are the specialized cells in cartilage that produce and maintain the extracellular matrix. They originate from mesenchymal condensations *in vivo* and a differentiation process known as chondrogenesis that is mediated by multiple transcription and growth factors [2]. One of the current treatments for cartilage defects, autologous cartilage implantation, involves harvesting chondrocytes from a non-load-bearing area, expanding the chondrocytes, then re-implanting the expanded cells into the defect and sealing with the periosteal flap. Chondrocytes can lose their phenotype during proliferation *in vitro*, however, and undergo dedifferentiation, marked by increased secretion of collagen type I (indicative of fibrocartilage formation and inferior mechanical properties) [3]. Additionally, the use of the periosteal flap to prevent leakage of grafted chondrocytes often stimulates osteophyte formation [4]. This inappropriate tissue formation often necessitates total knee arthroplasty [5].

Mesenchymal stem cells (MSCs) may be a promising alternative cell source because they can be isolated from adult tissues, have been shown to have immunomodulatory properties [6], and can be differentiated along a chondrogenic lineage [7]. Traditional chondrogenic differentiation, however, relies on growth factors (TGF-βs and/or BMPs) for induction and unfortunately, growth factor mediated chondrogenesis with single or multiple growth factors is unable to mimic the complex microenvironment experienced *in vivo.* Thus, MSCs chondrogenically differentiated *in vitro* rapidly undergo hypertrophy and/or osteogenic differentiation, which can lead to the formation of calcified cartilage or bone *in vivo* rather than healthy hyaline cartilage [8]. While growth factors play an important role in mediating chondrogenesis *in vivo,* chondrogenesis is a complex process, involving multiple growth factors with specific concentration profiles and multiple ECM components that bind to and release growth factors in a concentration dependent manner [9]. There is also a lack of standardization of differentiation protocols for MSCs, perhaps because chondrogenesis can be affected by donor-to-donor variability [10], cell passage number [11], as well as media composition [12].

3-D culture formats can improve chondrogenic yield. Hydrogels can be composed of multiple biologic or synthetic materials, but many lack the mechanical properties necessary for use *in vivo* [13]*,* or those with appropriate mechanical properties have limited cellular compatibility or infiltration [14]. Clustering of cells in micromass and pellet culture with high cell-cell contact is believed to mimic the microenvironment experienced by MSCs undergoing mesenchymal condensation during embryonic development [15]. Pellet culture has been shown to reduce the rate of hypertrophy and increase chondrogenic ECM production with exogenous growth factors, but MSC pellets still undergo hypertrophy (typically after 21 to 28 days) [15].

Alternatively, decellularized cartilage, cartilage ECM where the cellular components have been removed to avoid an immune response, may more closely mimic the natural cartilage microenvironment as it is multifactorial, containing multiple growth factors and ECM proteins to support chondrogenesis [9]. A previous study found that milled decellularized cartilage ECM (20-30 nm) incorporation in MSC pellets can upregulate chondrogenic markers (Collagen Type II and SOX9), but did not compare ECM incorporation to traditional growth factor mediated chondrogenesis or determine if the cells still underwent hypertrophic differentiation [16]. Another study found that decellularized cartilage scaffolds (8 mm x 1 mm) supported cell viability and proliferation of chondrocytes on the surface of the scaffold, but there was limited cellular infiltration and abnormal cell densities due to the low porosity of the scaffold [17].

Therefore, the objective of this study was to investigate whether digested cartilage ECM incorporation into MSC pellets could enhance MSC chondrogenesis and decrease hypertrophy alone or in combination with exogenous growth factor(s). Dosing in pellet culture was determined using ATDC5 cells, a cell line derived from mouse teratocarcinoma cells and well characterized as a chondrogenic cell line that mimics mesenchymal condensation and chondrogenic and osteogenic differentiation [18]. Additionally, this study also compares multiple chondrogenic differentiation protocols with varying media compositions, donors, and at multiple passage numbers to determine appropriate controls to compare for standard chondrogenic differentiation. Our hypothesis is that incorporating decellularized cartilage ECM will improve MSC chondrogenic differentiation and GAG production and result in less hypertrophic and osteogenic differentiation in comparison to traditional exogenous growth factor mediated differentiation alone.

CHAPTER 2

METHODS

Decellularization of cartilage and ECM preparation*:* Cartilage was isolated from porcine articular cartilage and decellularized in a series of chemical and enzymatic washes. The protocol is outlined in Table 1 below where we used TrypLE Express, a recombinant form of trypsin and a DNase and RNase wash. Decellularization was validated through lack of visible nuclei with Hoescht and hematoxylin and eosin (H&E) staining and DNA was quantified using PicoGreen Assay (Invitrogen). The isolated cartilage was milled using a Mini-Wiley Mill and then sieved to select decellularized pieces between sieve sizes 180 microns and 400 microns. Milled cartilage pieces were digested in 10 mg/ml pepsin in 0.01M hydrochloric acid for 48 hours on a stir plate at room temperature. After digestion, the ECM degradation products were frozen at -20°C until further use. For subsequent studies, ECM degradation products were thawed on ice, pH was adjusted to 7.3, and ECM concentration was determined through a BCA protein macro assay (Thermo Fisher Scientific).

Table 1: Protocol for Decellularization

	Chemical	Time
1	TryplE Express	6 hours
2	Type I water	15 min, 9x
3	70% ethanol	15 hours
4	3% hydrogen peroxide	15 min
5	Type I water	15 min, 4x
6	1% Triton X-100 in EDTA/Trizma	6 hours, 6x
7	Type I water	15 min, 9x
8	DNase/RNase Solution	24 hours
9	Type I water	15 min, 4x
10	0.1% PAA/4% ethanol	2 hours
11	Sterile d-PBS	15 min, 9x

Seeding and culture of ATDC5 cells and pellet culture ECM: ATDC5 cells were seeded at a density of 65,000 cells/cm^2 and expanded in growth media (Dulbecco's modified eagle medium/F-12 (DMEM/F-12) with L-glutamine (Invitrogen) with 1% antibiotic/antimycoctic, 5% fetal bovine serum, 10 µl/ml transferrin (Invitrogen), and 3×10^{-8} M sodium selenite (Sigma)). ECM degradation products (0, 10, 20, 30 µg/mL) were added to 2.5×10^5 ATDC5 cells in 500 µl of ATDC5 growth media and then centrifuged at 200g for 5 minutes to form MSC-ECM pellets. The pellets were then cultured in 15mL conical tubes with partially open caps in an incubator to promote gas exchange for 7 days and media was changed every 2-3 days. Pellets were then fixed in 10% neutral buffered formalin before manual processing for histology.

Seeding and culture for MSCs with ECM: Human mesenchymal stem cells pooled from two donors (Texas A&M) were seeded at a density of 65 cells/cm^2 and expanded in MSC growth media [Minimum Essential Medium alpha (MEMα) containing 16.3% fetal bovine serum (Atlanta Biologicals), 1% antibiotic/antimyoctic and 1% L-glutamine]. 2.5×10^5 MSCs with or without 20 µg/mL ECM degradation products were centrifuged at 200g for 5 minutes to create MSC pellets in 500 µl of Mesenchymal Stem Cell Growth Media. The pellets were then cultured in 15mL conical tubes with partially open caps in the incubator to promote gas exchange for up to 21 days and media was changed every 2-3 days. Some treatment groups also contained 10ng/ml TGF-β1 (Peprotech), an exogenous growth factor commonly used for chondrogenic induction [8]. A description of the treatment groups and subsequent methods of analysis is shown in Table 2. After 21 days, pellets were fixed in 10% neutral buffered formalin before manual processing for histology.

Table 2: Experimental Design for ECM Incorporation in MSC Pellets

Group	Exogenous Factors	ECM Digest	Evaluation
MSCs + TGF-β1 (Positive control)	10ng/mL TGF-β1	-	PicoGreen (n=6) Histology (n=3)
MSCs Alone (Negative control)	-	-	
MSCs + ECM	-	20 µg/mL	
MSCs + TGF-β1+ ECM	10ng/mL TGF-β1	20 µg/mL	

Comparison of different chondrogenic differentiation protocols: Mesenchymal stem cells from three different donors (lot #s 0049, 0055 and 0081) from RoosterBio at two different passages (population doubling numbers ~12 and ~15) were each seeded at a density of 3,333 cells/cm^2 and expanded for 1 week in RoosterBio High Performance Growth Media (KT-001) for a 10 fold expansion (to passage doubling numbers ~15 and ~18). 2.5 × 10^5 MSCs were then centrifuged at 200g in 15 mL conical tubes for 5 minutes in 500 µl of chondrogenic media (three different compositions shown in Table 3) to create MSC pellets. The pellets were then cultured for 21 days at 37°C and 5% CO_2 with partially open caps to promote gas exchange and media was changed every 2-3 days. Pellets were fixed in 10% neutral buffered formalin before manual processing for histology.

Table 3: Chondrogenic Media Formulations

DMEM with TGF-β1	DMEM with TGF-β3	RoosterBio with TGF-β1
• DMEM (Dulbecco's modified Eagle Medium) • 1% non essential amino acids (NEAA) • 1% antibiotic • 1% ITS+ • 50 µg/ml Ascorbic acid 2-phosphate (AA2P) • 100 nM dexamethasone • 10 ng/ml TGF-β1	• DMEM (Dulbecco's modified Eagle Medium) • 4.7 µg/ml Linoleic-oleic acid • 1% antibiotic • 1% ITS+ • 50 µg/ml Ascorbic acid 2-phosphate (AA2P) • 100 nM dexamathasone • 10 ng/ml TGF-β3	• RoosterBio Chondrogenic Differentiation Media • 100 nM dexamethasone • 10 ng/ml TGF-β3

Chondrogenic Differentiation: Histological stains for Safranin-O (SafO), Alcian blue, and dimethylmethylene blue (DMMB) assay of the pellets was performed to quantify glycosaminoglycan (GAG) content.

Statistical Analysis: Student's t-test was used for DNA quantification through PicoGreen® analysis to elucidate differences between native and decellularized cartilage.

CHAPTER 3

RESULTS AND DISCUSSION

Decellularization Quantification

DNA quantification with a PicoGreen® Assay (Figure 1) indicates effective DNA removal with less than 1 ng DNA/mg dry wt. in the decellularized cartilage which is in accordance with commercially available ECM scaffolds to decrease the risks of an immune response *in vivo* [19]. Histological analysis from hematoxylin and eosin (H&E) and Hoescht staining also indicate decellularization through to the lack of visible nuclei, but GAGs were also removed after decellularization, as shown by SafO staining (Figure 2).

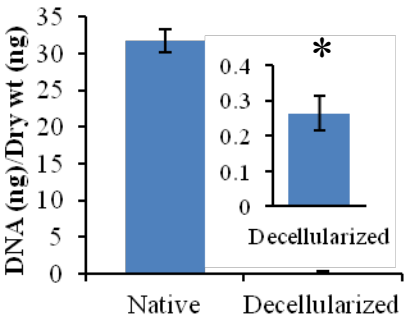


Figure 1. DNA removal in decellularized cartilage. DNA is effectively removed after decellularization in comparison to native articular cartilage as determined by Picogreen analysis (*p<0.05)

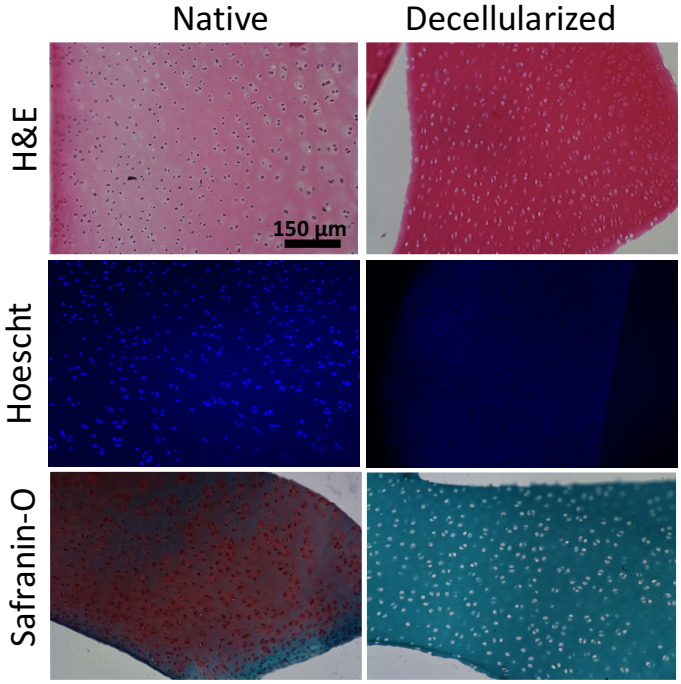


Figure 2. Histological staining of shows effective decellularization and GAG removal after decellularization. H&E and Hoescht staining indicate effective decellularization as seen by a lack of visible nuclei in the decellularized samples in comparison to the native cartilage controls. Safranin-O staining indicates GAG removal with decellularization through lack of positive (red) staining in the decellularized sample.

ECM dosing with ATDC5 cells

Decellularized cartilage ECM was incorporated in 250,000 ATDC5 cell pellets in a preliminary experiment in order to determine an appropriate dosage that allowed for both cell-cell and cell-matrix contact. Pellets with 30µg or more of ECM did not tightly compact during centrifugation and pellets fell apart in culture. 20 µg of ECM allowed for cell-cell as well as cell-ECM interactions and seemed to incorporate well in the pellets and was used for subsequent hMSC pellet studies.

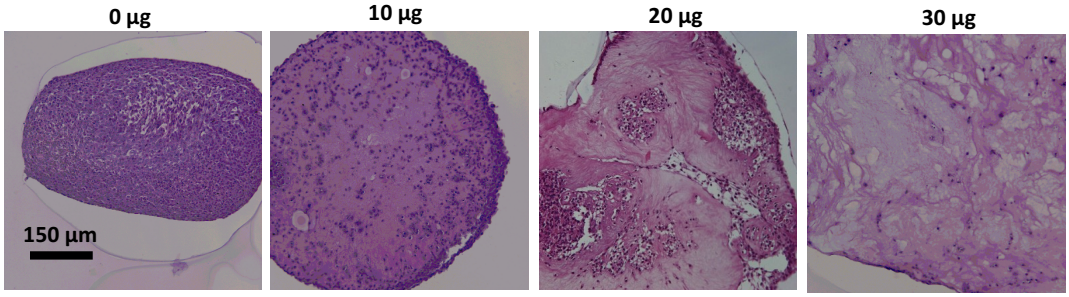


Figure 3. Decellularized cartilage ECM incorporation in ATDC5 cell pellets. 250,000 ATDC5 cells were centrifuged with varying amounts decellularized cartilage ECM digest (0 µg, 10 µg, 20 µg, 30 µg) in 15 ml conical tubes and cultured for 4 days in growth media before histological processing and staining with H&E.

Chondrogenesis with ECM degradation products

To determine whether cartilage ECM could enhance chondrogenic differentiation, 20 μg of ECM degradation products was incorporated in different groups of MSC pellets. Histological staining for Safranin O was negative as shown in Figure 4 (all data not shown), which is inconsistent with other groups that have shown positive SafO staining after chondrogenic differentiation with TGF-β1 [20]. Hematoxylin and Eosin staining was also done for tissue structure and morphology. Additionally, Alcian Blue (stains blue for acidic polysaccharides including GAGs) staining was performed to qualitatively assess GAG production. As shown in Figure 5, the MSCs alone had lighter Alcian Blue staining after 21 days of culture compared to the other groups, indicative of less GAG production, but all groups had very little positive staining. To investigate whether this inconsistency was due to donor variability, passage number, or media formulation, we performed as subsequent study comparing the effect of these factors on chondrogenic differentiation of MSCs in pellet culture.

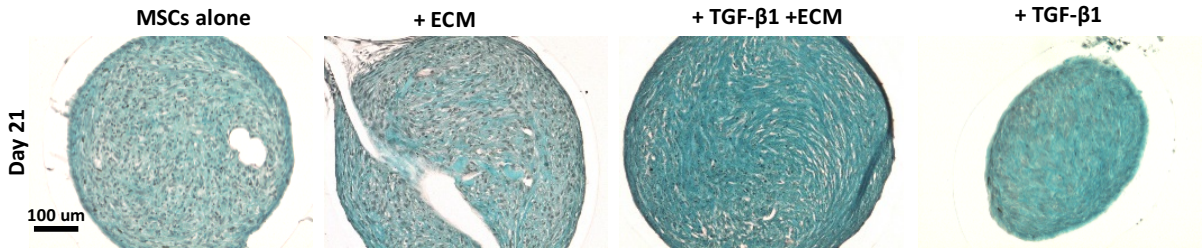


Figure 4. Limited GAG staining in MSC pellets with cartilage ECM incorporation and/or exogenous growth factors. 250,000 Human MSCs were centrifuged with or without 20 μg ECM digest to form MSC pellets. MSCs were cultured up to 21 days with exogeneous chondrogenic factor TGF-β1 (10 ng/mL) in growth media and then hstained with Safranin-O, which selectively stains sulfated glycosaminogly cans (sGAGs).

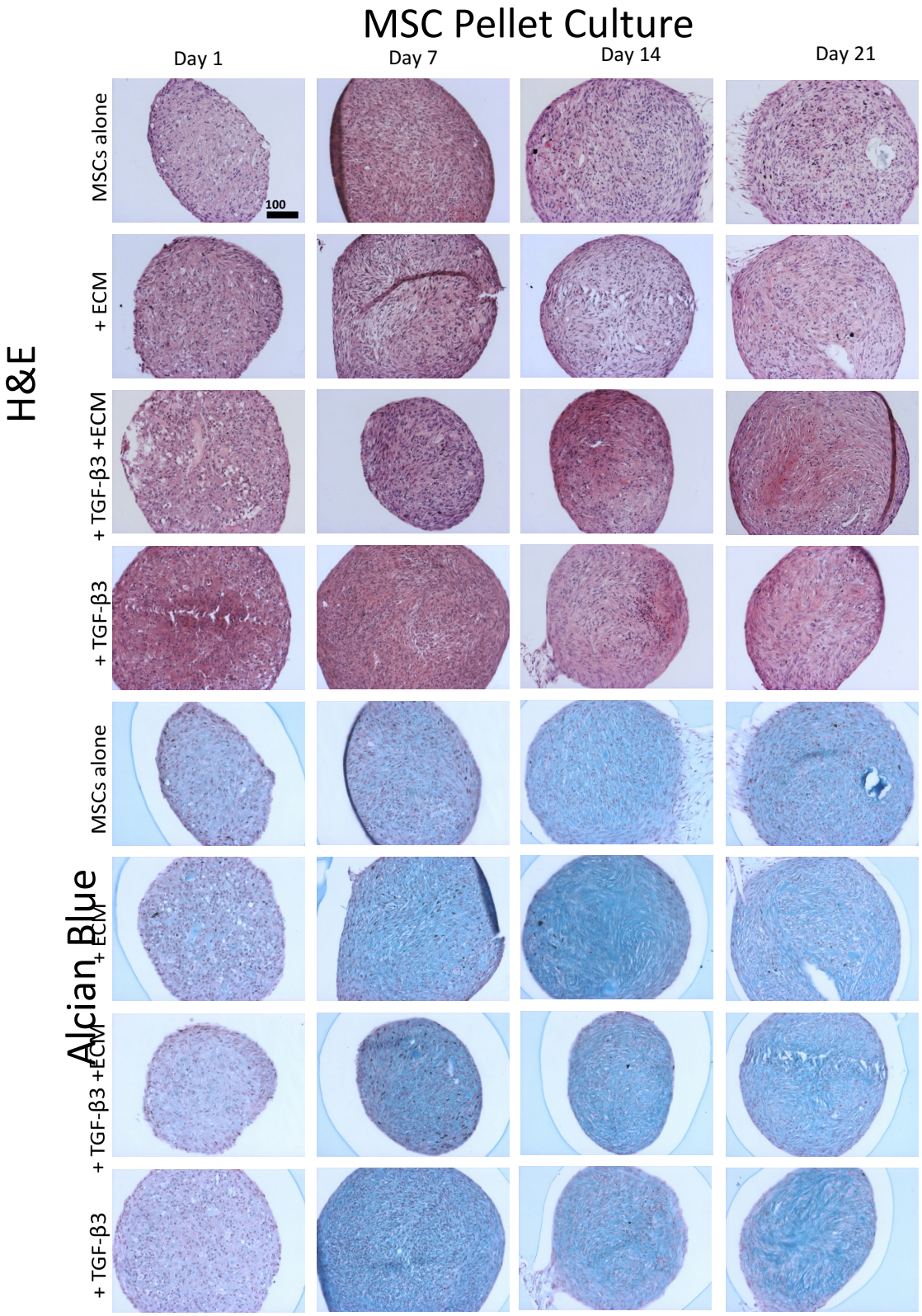


Figure 5. Histological staining of MSC pellets cultured with ECM degradation products and/or exogenous growth factors for 21 days. 250,000 Human MSCs were centrifuged with or without 20 µg ECM digest to form MSC pellets. MSCs were cultured up to 21 days with exogeneous chondrogenic factor TGF-β1 (10 ng/mL) in growth media. Histological samples were stained with hematoxylin & eosin (top) and alcian blue (bottom), which stains acidic polysaccharides including sulfated glycosaminoglycans (sGAGs).

Comparison of Chondrogenic Differentiation

To determine appropriate controls to compare for standard chondrogenic differentiation, multiple chondrogenic differentiation protocols with varying media compositions, donors, and population doubling levels were compared. Histological staining for Safranin-O revealed no entirely positive pellets in any of the experimental groups but control groups with native porcine cartilage in Figure 6 below indicate positive staining for GAGs (Figure 6). Groups cultured with serum free media containing TGF-β3 and cells from donor lot #0049 had greater GAG production and some places with positive staining whereas groups treated with serum containing media (RoosterBio) had the lowest GAG production (Figure 7).

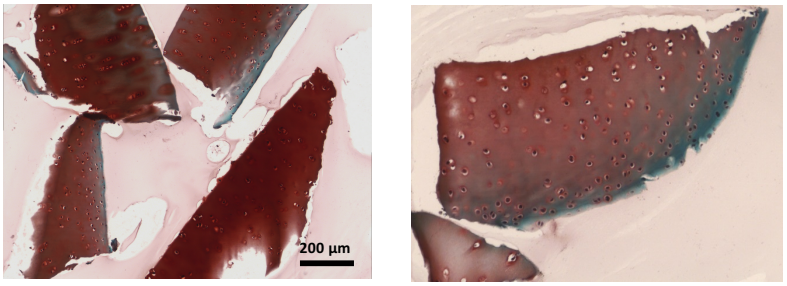
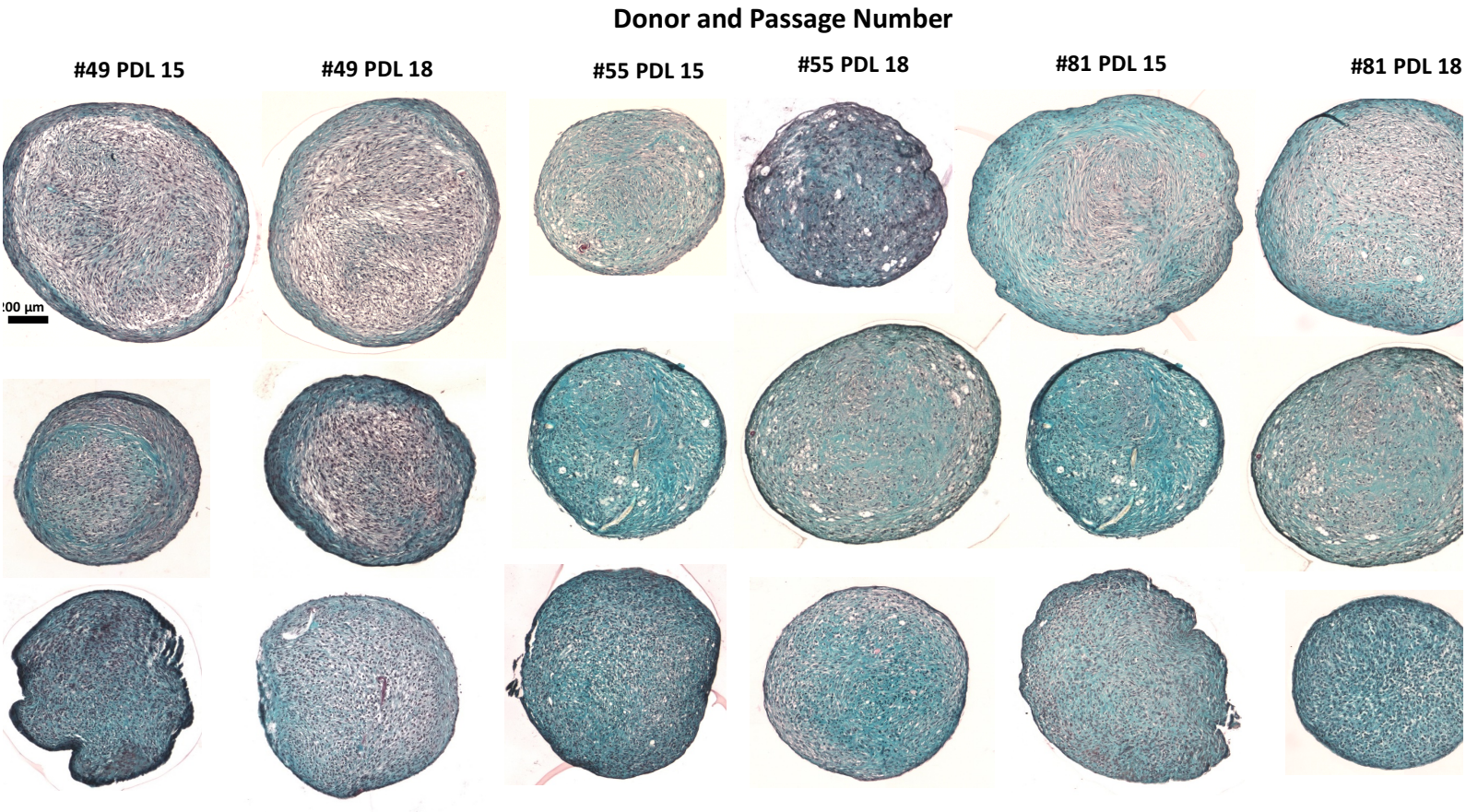


Figure 6. Positive staining for GAGs in native porcine cartilage. Positive controls for SafO staining of experimental MSC groups were native porcine cartilage pieces.



7. **Comparison of traditional chondrogenesis protocols with varying media compositions, donors and population doubling levels.** 250,000 H from various donors with different passage numbers were centrifuged with to form MSC pellets. MSCs were cultured up to 21 days with three types containing exogeneous chondrogenic factor TGF-β1 or TGF-β3 (10 ng/mL) in growth media supplemented with or without serum. Histological samp tained with Safranin-O, which selectively stains sulfated glycosaminoglycans (sGAGs).

Effect of passaging on MSCs

No apparent difference in GAG production between passages or population doubling levels was seen as the staining seen in Figure 5 was comparable for both tested population doubling levels (15 and 18). Extent of chondrogenic differentiation has been shown to decrease with increasing passage numbers and lost around the 6^{th} passage [21, 22]. Additionally, MSC markers (CD105 and Oct3/4) have also been shown to decrease with increasing passage number [21]. MSCs also show characteristics of cellular aging such as actin accumulation [11] as well as reduced hematopoietic cytokine synthesis (G-CSF, LIF and SCF) at late passages [11]. Chondrogenic differentiation potential is therefore significantly affected with increasing passage number and at late passages (>4) can affect the multipotent properties of MSCs [21]. Our results suggest chondrogenic differentiation potential is adversely affected after as little at 15 passage doublings, as none of the groups tested had true positive staining for GAGs.

Donor to donor variability

All the donors (RoosterBio lot #0049, #0055, #0081) used in this experiment were male and between 18-30 years old with >90% expression of MSC surface markers (CD166, CD105, CD90, CD73) to indicate stemness. Although the expression of surface markers was similar, there was still variability in the chondrogenic potential from each donor. MSCs from lot #0049 produced more ECM during expansion, proliferated more slowly, and took longer to trypsinize. This donor also had more positive staining for GAGs after 21 days of pellet culture. These differences in differentiation potential could result from inherent biological differences between donors. Literature has shown that even with similar donor age and expression of biomarkers, there can still be dramatic changes in differentiation capacity [10]. Additionally, at late passage numbers (>4), these differences can be emphasized and exhibit higher variability in gene expression [10].

Media composition

Although serum containing media can promote growth, it is inherently ill-defined as it has unidentified components at varying concentrations [23]. Additionally, serum-containing media also has a higher risk of contamination, contains xenogeneic proteins that could elicit an immune response in patients [23]. Commonly used serum such as fetal bovine serum (FBS) has been shown to decrease chondrogenesis and contains unknown inhibitory components [24]. Defined cell culture medium can help circumvent some of these drawbacks and has less batch to batch variation. Our results indicated that a serum free media had greater GAG production, which is consistent with literature that shows serum free media can better conserve chondrogenic differentiation potential in comparison to MSCs cultured with serum containing media [12].

TGF-β signaling is an important pathway in traditional chondrogenesis protocols as it promotes the production of type II collagen, a major component of cartilage specific ECM. The most common isoforms of TGF-β used for chondrogenic differentiation are TGF-β1 and TGF-β3 [25]. Both isoforms are >75% identical and are structurally similar but have distinct temporal and spatial expression *in vivo*. However, using TGF-β3 in the cell culture media has been proven to deposit more GAGs in pellet culture [20]. Our findings are consistent with these previous findings as the MSC pellets treated with TGF-β3 had more positive staining with Safranin-O (Figure 6).

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTION

The objective of this study was to enhance MSC chondrogenic differentiation using a cartilage ECM degradation products in pellet culture. Porcine articular cartilage was effectively decellularized and used to make ECM degradation products. A dosing analysis for ECM in pellets using ATDC5 cells indicated 20 μg/ml to be adequate for cell-cell and cell-ECM interactions. Subsequent MSC pellet culture to test ECM addition used chondrogenic growth factor TGF-β1 treated MSCs as a control. Our control group was negative for GAG staining after 21 days in culture, so subsequent experiments investigated traditional chondrogenic protocols with a focus on the role of donor-to-donor variability, passage number, and media composition. Chondrogenic differentiation of MSC pellets had better GAG content with TGF-β3 induction compared to TGF-β1, but this differentiation was greatly limited in multiple donors with high (>p4) passage number.

Future studies will compare chondrogenic induction with TGF-β3 and/or cartilage ECM of human MSCs at earlier passages with more quantitative analysis with DMMB, PCR and ELISAs. We will also tag the ECM products with a fluorescent dye to differentiate between the ECM degradation products and ECM produced by cells.

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