Indirect Co-culture of Human Adipose-Derived Stem Cells and Nucleus Pulposus Cells for Intervertebral Disc Regeneration Using Peptide-Conjugated Hydrogels

by

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Abstract

Intervertebral disc degeneration (IVDD) is a main cause of chronic low back pain, which results in significant economic loss to society and compromised life quality of patients. Traditional treatments of IVDD include surgical procedures, such as discectomy, and spinal fusion. However, these methods cannot preserve the biological or biomechanical functions of the intervertebral disc (IVD). Moreover, they decrease the mobility of the associated spinal motion segment and increase the load and stress on contiguous discs. Currently, artificial IVD replacements have gained interest. Although these implants are capable of preserving disc motion and disc height, they are unable to sustain compressive forces applied on the IVD due to their lack of elasticity. Therefore, there is a great need for an alternative therapeutic strategy for the treatment of IVDD. For mild or early stages of IVDD, regeneration by injecting pre-differentiated adipose-derived stem cells (ASCs) into the nucleus pulposus (NP) has been considered. This study aimed to investigate whether NP cells could direct human adipose-derived stem cells (hASCs) to differentiate into an NP cell phenotype. These cells could then serve as an alternative cell source for the treatment of intervertebral disc degeneration in the clinical setting. Interactions of ASCs and NP cells were studied via an indirect co-culture system using N-cadherin conjugated hyaluronic acid (HA) embedded within calcium alginate gels as scaffolds by evaluating the changing profiles of extracellular matrix (ECM) components, including glycosaminoglycans (GAGs) and type II collagen. NP cells showed the potential ability to induce hASC differentiation in terms of enhanced ECM production, such as collagen and GAG in an indirect co-culture system.

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List of Abbreviations

ABC	Avidin/Biotinylated enzyme Complex
AF	Annulus Fibrosus
ASC	Adipose-derived Stem Cell
BSA	Bovine Serum Albumin
CD 44	Cluster of Differentiation 44
CD 168	Cluster of Differentiation 168
DMEM	Dulbecco's Modified Eagle's Medium
3D	Three-dimensional
DAB	3, 3-Diaminobenzidine
DEX	Dexamethasone
DMMB	Dimethylmethylene Blue
ECM	Extracellular Matrix
EBSS	Earle's Buffered Saline Solution
FasL	Fas Ligand
FBS	Fetal Bovine Serum
GAGs	Glycosaminoglycans
HA	Hyaluronic Acid
HAV	His-Ala-Val
IHC	Immunohistochemical
IVD	Intervertebral Disc
IVDD	Intervertebral Disc Degeneration
KRB	Kreb's Ringer Buffer

MMPs	Matix Metalloproteinases
MSC	Mesenchymal Stem Cell
MWCO	Molecular Weight Cut-off
NMR	Nuclear Magnetic Resonance
NP	Nucleus Pulposus
OH	Hydroxyproline
P1	Passage 1
P2	Passage 2
PBS	Phosphate Buffered Saline
PBS-T	0.2% TWEEN in Phosphate Buffered Saline
PGs	Proteoglycans
TBS	Tris-buffered saline
TE	Tris-EDTA
TGF-β3	Transforming Growth Factor β3
VEP	Vertebral Endplate

Chapter 1

Introduction

1.1 Intervertebral Disc (IVD) and IVD Degeneration (IVDD)

Intervertebral disc degeneration is the most common feature in patients who suffer from low back pain, which affects 80% of the adult population, leading to a $90 billion cost each year in terms of treatment and post-surgical recovery [1]. There are three distinct and structurally different compositions in the intervertebral disc, which are the nucleus pulposus (NP), the annulus fibrosus (AF) and the vertebral endplate (VEP) [1]. Cells isolated from different part of the discs also exhibit different characteristics in terms of extracellular matrix (ECM) composition and cell morphology [2]. The nucleus pulposus cells display a chondrocyte-like and round morphology and produce primarily type II collagen and glycosaminoglycans (GAGs), which support the disc height and functions. The annulus fibrosus cells show a more fibroblastic and elongated morphology, and produce a larger amount of type I collagen in comparison to NP cells [3].

The intervertebral discs develop from both the notochord (nucleus pulposus) and the mesenchyme (annulus fibrosus) [4]. In some species, such as rodents and cats, the notochordal cells remain in the NP region of the disc throughout their entire life. However, in other species, such as horses, the notochordal cells vanish after the time of birth. In humans, the notochordral cells disappear within the first few years of life [4][5].

Although the role of notochordal cells in the NP is unclear, it is interesting to notice that the loss of these cells is considered as the early sign of disc degeneration [6].

As humans age, their disc start degenerating. It is believed by some researchers that IVD degeneration results from a natural aging process, which may be a balance to maintain homeostasis [1] . However, other researchers consider that some other factors, such as the limited transport of nutrition and removal of waste in the IVD, the presence of degenerative enzymes, and/or undue mechanical loading on the discs may be responsible for IVD degeneration [7]. Vertebral endplate calcification may contribute to disc degeneration, since these deposits present in the VEPs increase as the discs age, preventing nutrient diffusion, including O_2 [8]. IVD degeneration is characterized by NP cell morphology changing from chondrocyte-like to more fibrotic, disc tissue loss and destruction over time, which decreases disc height and water content in the NP, and ultimately sacrifices biomechanical properties of the IVD [7].

1.2 Current treatments for IVD degeneration

Currently, for mild IVD degeneration, most patients are treated with rest, exercises and medicine to temporarily alleviate low back pain. When these treatments are ineffective, surgery is necessary. There are two common surgical treatments used in the clinic for patients with IVD degeneration, which are discectomy and spinal fusion. Discectomy is a

procedure in which patients undergo a surgery to remove the degenerative or damaged portion of the discs, which can temporarily alleviate the pain. However, the surgery will cause further IVD degeneration and compromise the biomechanical stability of the disc since the structure of the disc has been changed [1]. Due to the limited success of discectomy to treat IVD degeneration and its disadvantages compared to other therapies, spinal fusion, which is a process to fuse two adjacent vertebral bodies together, has received more interest. The major purpose of spinal fusion is to immobilize the degenerated IVD segment, thereby reducing the perceived pain. However, problems after spinal fusion include possible degeneration of spinal segments and failure to entirely immobilize the degenerated part of the discs [1].

Discectomy and spinal fusion always lead to further degeneration of the IVD and loss of the IVD motion and functions [9]. IVD replacements, including partial IVD replacement and total IVD replacement, have some advantages over discectomy and spinal fusion in terms of not only just decreasing the pain, but also restoring the IVD height and structure and therefore helping restore the biological and biomechanical functions of degenerated IVD to some extent. The artificial implants for IVD replacements should be biocompatible, nontoxic, durable and most importantly, contain a high amount of water, which would facilitate the nutrient delivery and waste removal [10].

IVD replacement allows some motion, but has limited ability to promote IVD tissue remodeling and therefore restoring a functional IVD tissue. IVD tissue engineering, which encompasses the use of biomaterials, stem cells and growth factors, offers an advantageous and attractive alternative and are being developed at a research level for possible future application. This approach could restore the biological, chemical and biomechanical properties of the IVD via regeneration of native IVD tissue. These biomaterials should not trigger a negative or chronic immune response, and should have a similar structure to native IVD tissue, and show similar biomechanical properties as the ECM of normal IVD and also should be biocompatible and biodegradable [11].

When considering the combination of biomaterials and stem cells as a treatment for IVD regeneration, it is important to choose the porous geometries, which can promote diffusion of the nutrients and waste and also facilitate cell attachment and proliferation and later ECM production. Biomaterials that can act as scaffolds or carriers are important since they provide cells with a three-dimensional (3D) environment to attach, proliferate and communicate and therefore can guide cell behavior. The success of a tissue engineered IVD is evaluated by the ability of the new tissue to increase and maintain the disc height and water content in the disc [1] . These properties are provided by collagen I and II and GAG content of the newly produced ECM, which should approximate those of native IVD tissue.

Recently, an increasing number of studies have been focusing on adipose-derived stem cell (ASC) therapy for disc tissue regeneration due to the relative abundance of ASCs and because they are easily harvested from fat tissue obtained by simple liposuction. ASCs have a remarkable ability of multi-lineage differentiation, and have the ability to produce a large amount of NP-specific ECM, including collagen and GAGs, which makes them a promising stem cell source for the disc regeneration [12] [13].

We propose that injection of ASCs that have been pre-differentiated into NP cells within a suitable polymer matrix is a possible solution for IVD regeneration. In detail, the cell-based therapy being developed includes (i) the indirect co-culture of ASCs and allogeneic NP cells for ASC differentiation within a peptide-conjugated alginate-based gel, (ii) harvesting the NP-like ASCs by converting the gel to a solution, and then (iii) implantation of the NP-like ASCs into the IVD within an appropriate carrier. The focus of my work was on the first step in this process, which involves exploring the differentiation of ASCs into an NP cell phenotype using an indirect co-culture system.

Chapter 2

Literature Review

2.1 Biology of the Intervertebral Disc (IVD) and pathology of IVD degeneration

Low back pain has an effect on 80% of the adult population, resulting in $90 billion in annual costs for treatment and a decline in patients' life quality in the world [7]. One major cause of low back pain is IVD degeneration, during which a remarkable loss in proteoglycans (PGs), water and type II collagen in the NP portion of disc occurs. The underlying mechanisms of IVD degeneration are not well known, but many complicating factors, including the natural aging process, chemical and mechanical factors, and genetic background, have been indicated to play a vital role [14][15].

There are three discrete but interdependent components of the IVD: the nucleus pulposus (NP), the annulus fibrosus (AF), and the vertebral end plates (VEP) (Figure 1). The nucleus pulposus is the inner region of the IVD, which is characterized by a low cell density and a high abundance of ECM components. The ECM of the NP contains a high concentration of negatively charged PGs, which makes the NP retain water and maintain its swelling pressure so that it can bear an exterior load. The ECM also contains type II collagen, which is the predominant collagen in the NP and aids in maintaining the disc height and regulating cell behavior through cell-ECM interactions. The annulus fibrosus is the outer layer of the IVD and has a lamellar structure that is composed of collagen

type I and II fibrils. The AF helps retain the tensile properties of the IVD. The vertebral endplates are cartilaginous plates that are cross-linked with the AF at the disc-vertebrae interface and supply nutrients to the discs [7].

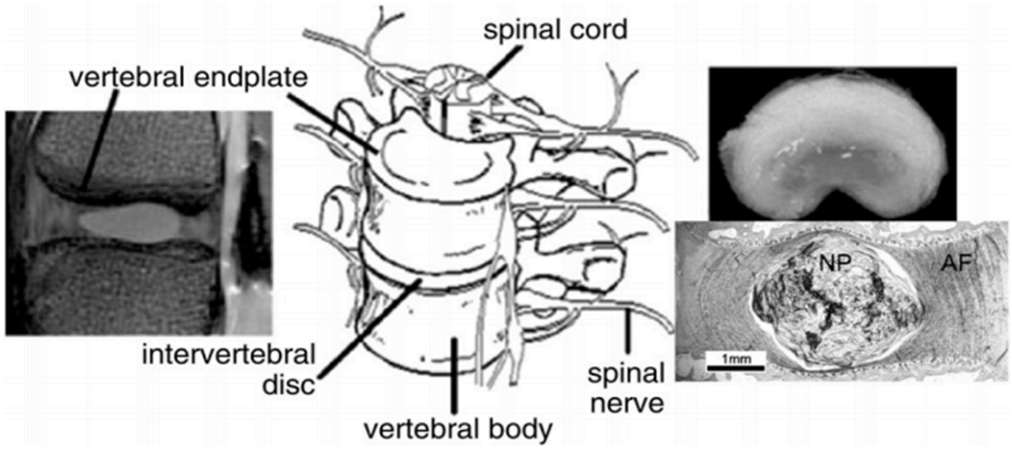

Figure 1. Magnetic resonance imaging (MRI) of an IVD showing the NP and AF in distinct regions (left). Schematic of the spinal column (middle). Anatomy of a normal disc with histological stain (right) [7].

IVD is the largest avascular tissue in the human body, and due to its avascular character, the nutrient supplement and waste removal processes mostly rely on diffusion through the VEP and within the ECM [16]. The NP contains roughly 4×10^6 cells/mL, which are responsible for constantly secreting the ECM components to maintain the stability of the NP tissue and help the NP function properly [17].

The IVD degeneration is characterized by disc tissue loss and destruction over time, which decreases disc height and water content in the NP, and ultimately reduces

biomechanical properties of the IVD. Tissue turnover is common in the normal disc. However, during disc degeneration, the ECM is both altered and degraded via a series of biochemical processes. The ECM components, including aggrecan and type II collagen are degraded by serine proteinases and matrix metalloproteinases (MMPs) [19]. Compression of the peripheral nerves of the discs as a result of decreased disc height is thought to be the reason for the patient's pain. As degeneration progresses, NP cell morphology may alter from a chondrocyte-like phenotype to a more fibrotic phenotype [20].

2.2 Cell-based therapy for IVD regeneration

Cell transplantation into the NP is a promising approach for IVD regeneration due to the structure of the IVD, in which the NP tissue is surrounded by the AF and the VEP, which inhibits cell migration and allows space for transplanted cells to settle down [20]. NP cells have been reported to express Fas ligand (FasL), a specific protein expressed in immune-privileged organs [21]. These anatomical and molecular structures indicate that the NP region may accept implanted cells from other parts of the body or other cell sources. The cell-based strategies that have been or are currently being investigated can be classified into three types based on the use of different cell sources, which are NP cells, and mesenchymal stem cells.

Using active and healthy NP cells for disc regeneration is impractical due to the limited NP cell sources and low cell density of active NP cells in a single disc. Moreover,

harvesting NP cells from healthy discs may cause degeneration in the donor discs. Several groups have studied disc regeneration using donor NP cells. Gruber *et al*. [22] implanted autologous disc NP cells in a sand rat model, and indicated those autologous disc NP cells can successfully survive and integrate into the disc. Ganey *et al*. [23] studied a canine model, in which disc degeneration was induced by partial discectomy. Autologous disc cells expanded *in vitro* were injected into the NP region without a delivery system. The discs in the dogs receiving transplanted NP cells maintained disc height and structure significantly better than in the control animals.

Recently, there are an increasing number of studies focusing on stem cell based therapies for IVD regeneration, especially using mesenchymal stem cells (MSCs). MSCs can be harvested from sites such as the bone marrow and fat without significant donor site morbidity, and can also be expanded *in vitro* to a sufficient number for transplantation purposes, making them promising cell sources for disc regeneration. Nevertheless, several studies have confirmed the effectiveness and feasibility of MSC transplantation for the treatment of IVDD in animal models. Zhang *et al*. [24] implanted autologous bone marrow MSCs containing the marker gene LacZ into rabbit intervertebral discs to determine the potential of this cell-based therapy. They reported that transplanted MSCs could not only survive in the discs but also increase the synthesis of proteoglycans. Crevensten *et al*. [25] demonstrated that rat bone marrow MSCs injected into the pressurized rat discs using a hyaluronic acid gel as a scaffold remained viable and

proliferated and disc height was maintained during the 28-day study period due to the ECM synthesis by the viable MSCs.

Recently, several studies have indicated that adipose-derived MSCs can be directed into an NP cell phenotype by co-culture with NP cells, employing either direct cell-to-cell contacts, or a cell and scaffold combination [26][27][28]. Adipose-derived stem cells (ASCs), which possess many advantages over other stem cells, including ease of harvesting by non-invasive procedures, high stem cell yields compared to bone marrow-derived MSCs, and multi-lineage differentiation potential, have been identified as another potential cell source for IVD regeneration [29][30][31]. Gaetain *et al*. [26] studied a three dimensional (3D) co-culture of human ASCs (hASCs) and NP cells in alginate capsules, and indicated that co-culture of NP cells and ASCs improved the property of the reconstructed tissue in vitro in term of ECM production and cell organization. Lu *et al*. [27] studied the culture configuration effect of NP-induced ASC differentiation, and demonstrated that only when both cells were micromass-cultured, could ASCs be directed to an NP cell phenotype by soluble factors produced by the NP cells. Choi *et al*. [32] showed that an indirect co-culture system using porous membranes could also induce ASC differentiation into NP cells without the need for adding chondrogenic supplements.

In this study, the potential differentiation by indirect co-culture of ASCs in the presence of NP cells was investigated. Specifically, human ASCs were encapsulated in peptide-conjugated hyaluronic acid (HA)-alginate hydrogels in cell culture inserts (pore size: 0.4 μm) with NP cells isolated from bovine caudal discs cultured in the well bottom. Differentiation was assessed by evaluating ECM productions, including aggrecan and type II collagen. As the differentiated ASCs are needed to be kept separate and to be readily harvested for direct implantation into the IVD, we chose indirect co-culture as opposed to direct co-culture. Bovine NP cells were used as a proof of principle, because they are easily obtained, as opposed to human NP cells, and because the bovine NP cells were from young cows and therefore would be more active than NP cells from human donor tissue, which is typically from older patients.

2.3 Hydrogels as the basis of the differentiation of hASCs

Hydrogels can possess a large amount of water, which aids the transport of nutrition and waste products, and can also serve as a temporary scaffold to facilitate encapsulated cell growth. Bio-functionalized injectable hydrogel scaffolds are increasingly being investigated as a treatment strategy for IVD regeneration [11]. These scaffolds serve as an artificial ECM to direct cells into a three-dimensional structure and to present stimuli, which guide cell adhesion, proliferation, differentiation and eventually to form a functional tissue. Scaffold design and materials selection for this purpose depend on various design criteria including physical properties (mechanics, gel formation, bio-

degradation), mass transport properties (diffusion), and biological properties (cell adhesion, proliferation, differentiation, biocompatibility) [11].

Hydrogels as the scaffolds for cell culture and further differentiation must be capable of gelation without damaging cells, and must be nontoxic or minimally toxic to cells before and after gelling, and must allow the proper diffusion of nutrients and metabolic products into and out of the encapsulated cells. Further, the gels must possess the appropriate biomechanical properties, such as stiffness, as a stimuli to facilitate cell attachment, proliferation and differentiation [11]. For the current project, another criteria is that the hydrogels must have the ability to facilitate the easy retrieval of the differentiated cells. In this study hASCs were encapsulated in N-cadherin conjugated hyaluronic acid (HA) embedded within calcium alginate gels as scaffolds to investigate hASC differentiation profile induced by co-culture with NP cells in an indirect co-culture system. The approach features the capability of mild gel-sol transition of calcium alginate to sodium alginate that allows the cells to be released with high viability after successful *in vitro* differentiation.

Alginate Hydrogels

Alginate is a natural linear polysaccharide copolymer of (1-4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) units, and is derived mainly from brown seaweed and bacteria [33][34]. It has been utilized in a variety of biomedical applications,

including drug delivery, cell encapsulation, and wound dressings due to its relatively mild gel-sol transmission condition to allow cells with high viability, low toxicity, and simple modifications with peptide sequences to fulfill new biological functions [35]. The molecular weight of alginate highly influences the viscosity of the alginate hydrogels [36]. An alginate solution formed with high molecular weight alginate tends to become more viscous, which could damage the cells by the high shear forces generated during mixing [37].

Due to their ability to undergo gelation under mild conditions that do not cause cell damage, alginate gels are increasingly being investigated as scaffolds for 3D cell culture *in vitro* [35]. However, because of its inherent inability to bind to mammalian cell surface receptors to facilitate cell adhesion due to its hydrophilic nature, alginate hydrogels are commonly functionalized with cell-adhesive peptides, such as RGD (Arg-Gly-Asp), DGEA (Asp-Gly-Glu-Ala) [38] and YIGSR (Tyr-Ilr-Gly-Ser-Arg) [39] sequences, which can be chemically coupled to the backbone of alginate by water-soluble carbodiimide chemistry [40].

Alginate hydrogels can be formed by ionic cross-linking, in which divalent cations such Ca^{2+}, Sr^{2+} or Ba^{2+} cooperatively interact with blocks of G monomers to form an ionic bridge between different chains of alginate [41]. In particularly, ionically cross-linked

alginate gels have been widely investigated for IVD regeneration [42]. Studies showed that NP cells cultured in alginate gel scaffolds maintained a rounded, NP-like morphology [10]. One potential disadvantage of this method is the limited stability of the formed gels. These gels can be converted back to solution by releasing the bivalent cations through competition for the G-block binding sites with monovalent cations, such as Na^+ [35].

For successful cell differentiation within a hydrogel, it is important to consider the appropriate transport of small molecules, such as soluble signals, oxygen, and vitamins, as well as higher molecular weight molecules, including albumin, myoglobin, and fibrinogen, which are not able to diffuse freely in the hydrogel scaffolds since the supply of oxygen and nutrients and the removal of waste products is vital for the survival of the encapsulated cells in the scaffolds [11]. In a hydrogel, the diffusion rate of a certain molecule is dependent on the properties of hydrogel and diffusing molecule as well as the interaction between them [35]. The hydrogel structure is determined by the polymer fraction, polymer size and crosslinking concentration. Therefore, the diffusion rate is influenced by the molecular weight and size of the diffusion molecules. The diffusion rate through ionically cross-linked alginate hydrogel will decrease as the alginate and Ca^{2+} concentration increase [43]. Bron *et al*. investigated the relation between alginate concentration and scaffold stiffness and optimized the conditions where the viscoelastic

behavior mimics that of the NP. They found that alginate hydrogels formed by 2% freeze dry alginate (LVCR sodium alginate, Monsanto, San Diego, CA) in solution closely mimic the viscoelastic properties of the NP and the phenotype of native NP cells is preserved in alginate, regardless of the alginate concentration [42].

Hyaluronic Acid

HA is a commonly used biomaterial for IVD regeneration strategies because HA is a component of the ECM of NP and also due to its high water absorption capability, which increases the disc load bearing capacity. HA is a linear polysaccharide, consisting of a repeating disaccharide of (1-3) and (1-4)-linked β-D-glucuronic acid and N-acetyl-beta-D-glucosamine units [44].

HA has also been incorporated with other polymers, such as alginate and collagen to yield hydrogels possessing better biological and biomechanical properties for application in IVD regeneration [45][46][47][48] . HA has the potential ability to provide the biological cues such as CD44 and CD168 interactions due to the role of HA in cellular signaling and can promote cell functions, such as adhesion, proliferation, and differentiation, and eventually tissue formation [49].

Peptide modification with N-cadherin

N-cadherin is one of the cell adhesion molecules that facilitates cell-cell interaction during stem cell condensation, a process mediated by surface contact that leads to aggregation of progenitor cells [50]. The His-Ala-Val (HAV) motif in the first extracellular domain of N-cadherin plays a critical role in mediating cell-cell adhesion [51]. Optimized synthetic peptides with the HAV motif have been proved to possess the N-cadherin-like binding activities. Bian *et al*. conjugated HA hydrogels with N-cadherin peptide sequence and evaluated their roles in regulating chondrogenesis and cartilage specific matrix production, and they found that the functionalized HA based hydrogels enhanced both early chondrogenensis and cartilage matrix deposition in culture, compared to controls [52]. Because NP cells are phenotypically similar to chondrocytes, the differentiation markers used for ASC differentiation towards chondrocytes might be feasible to be used for ASC differentiation towards NP cells [7].

2.4 Indirect co-culture

Indirect cell co-culture was chosen as part of the overall strategy. In an indirect co-culture system, a permeable membrane is placed between two cell populations. The cells can communicate through paracrine signaling, which is a signaling pathway simulated by soluble factors, such as proteins, growth factors, and hormones, that are secreted by cells and released to extracellular environment to affect adjacent cells [53]. Indirect co-culture is commonly conducted by placing cell culture inserts, which are small cylindrical

containers with a permeable membrane on the bottom to facilitate cell-cell interaction through the membrane, in a matched cell culture plate. However, this indirect co-culture system doesn't allow cells to communicate through gap junction and other types of direct cell-cell interactions by eliminating the contact effect between cells. The indirect co-culture system is beneficial for our purpose because the differentiated ASCs can be easily retrieved following their differentiation and can be used for further cell delivery to NP portion of the discs.

2.5 Research Objectives

The objective of this study was to investigate whether NP cells can direct ASCs to differentiate into an NP phenotype in an indirect co-culture system, in which the ASCs were encapsulated in N-cadherin conjugated-HA embedded within calcium alginate gels. The gel environment was chosen to mimic the natural pericelluar environment of NP cells by using HA conjugated with N-cadherin peptides (His-Ala-Val) and embedded within calcium alginate gels to mimic the native cell-matrix and cell-cell interactions during development. Moreover, due to the mild gel-sol transition of calcium alginate to sodium alginate, the gels should allow for the easy retrieval of the differentiated ASCs with high viability following their differentiation.

The long-term objective of this program is to provide an alternative cell source for surgical procedures employing injection of differentiated ASCs into the NP portion of the

disc, which is a promising cell-based therapy for intervertebral disc regeneration in the clinical setting.

2.6 Scope

The thesis was carried out by conducting the following separate, but related three studies.

(1) Indirect co-culture model of hASC pellets with NP cells for 2 weeks and 4 weeks

(2) Indirect co-culture model of hASCs in alginate gels with NP cells in micromass culture for 2 weeks and 4 weeks

(3) Indirect co-culture model of hASCs in N-cadherin peptide (His-Ala-Val)-conjugated HA embedded within calcium alginate gels with NP cells in micromass culture for 4 weeks

Chapter 3

Materials and Methods

3.1 Indirect co-culture of hASCs pellets and nucleus pulposus cells

3.1.1 Materials

Bovine tails were obtained from local slaughterhouses (Quinn's Meat or Wallace's Beef, Kingston, ON, Canada) in Kingston, ON, Canada. Human adipose tissue was collected from patients undergoing breast reduction at the Hotel Dieu Hospital or Kingston General Hospital in Kingston, ON, Canada, with Research Ethics Board Approval from Queen's University (REB # CHEM-002-07).

3.1.2 Human ASC isolation and culture

ASCs were isolated from human adipose tissues following the lab protocol of Flynn et al. [54]. Briefly, the adipose tissue samples transported to the lab in iced transport solution (20 mg/mL Bovine Serum Albumin in PBS, cat #: A7979, Sigma) were minced into fine pieces (1 mm^3). The minced tissue samples were then digested for 45 min at 37˚C under agitation at 100 rpm in Kreb's Ringer Buffer (KRB) Solution containing 3 mM glucose (Sigma), 25 mM HEPES (Sigma), 2 mg/mL type I collagenase (Worthington) and 35% (w/v) bovine serum albumin (BSA, Sigma). The digested samples were filtered through a

1 mm pore pre-filter and 250 μm pore stainless steel filter to remove any undigested tissue segments. The floating adipocytes in the upper layer were aspirated off after 5 min of gravity separation. An equal volume of complete medium (Dulbecco's modified Eagle's medium nutrient mixture:Ham's F-12 Nutrient mixture with 10% fetal bovine serum (FBS), Gibco; 10,000 units/mL penicillin and 10,000 μg/mL streptomycin, cat#: SV30010, HyClone) was added to the filtrate to inactivate the collagenase. The samples were then centrifuged at 1200 g for 5 min, and the cell pellets were suspended in 20 mL of erythrocyte lysing buffer and agitated gently for 10 min at room temperature. The cell suspension was filtered through a 100 μm nylon mesh (Fisher) after centrifuging and re-suspending in 20 mL complete medium. The cells were then washed with complete medium twice and plated in T-75 tissue culture flasks (Cat #: 130190, Thermo). After 24 hours to allow cell attachment, the cells were washed repeatedly with phosphate buffered saline (PBS, HyClone) to remove any non-adherent cells or fragments. The cells were given fresh medium at least three times per week and incubated at 37˚C with 5% CO_2. Cells at 80% confluence were passaged by washing with PBS and releasing with 0.25% trypsin-EDTA (Gibco) and re-plated in new flasks. Passage 2 (P2) human ASCs were used for co-culture experiments.

3.1.3 Bovine NP cell isolation and culture

Bovine tails obtained from local slaughterhouse were washed and sterilized with 10% bleach for 1 h, followed by sterilizing with 70% ethanol for 1 h. NP cells were isolated from nucleus pulposus tissues that were obtained from bovine caudal discs within 3 h of slaughter. The connective tissues between each vertebral body were cut along the disc-vertebrae interface to preserve as much NP tissue as possible. NP tissues were removed from 5-6 bovine caudal discs and placed in sterile Earle's Buffered Saline Solution (EBSS, Sigma). NP tissues were minced into small pieces with scissors, washed with PBS and then centrifuged. NP tissues were then pre-digested with 2 mg/mL pronase (Cat#: 10165921001, Roche) in serum-free low glucose Dulbecco's modified eagle medium (DMEM, Sigma) containing 10,000 units/mL penicillin and 10,000 μg/mL streptomycin with constant agitation (37˚C, 100 rpm). After 1 h pre-digestion, the samples were subjected to a 14 h digestion with 0.5 mg/ml collagenase II (Worthington) in serum-free low glucose DMEM containing 1% penicillin/streptomycin in an incubator. After this 14 h incubation, the cell suspension was filtered through 100 μm nylon mesh and then centrifuged. The cell pellets were then washed with low glucose DMEM containing 10,000 units/mL penicillin and 10,000 μg/mL streptomycin and 10% FBS. NP cells were plated at a cell density of 250,000 NP cells per T-175 flask and passage 1 (P1) NP cells were used for further co-culture with ASCs.

3.1.4 Indirect co-culture of hASC pellets and NP Cells

Indirect co-culture of hASCs and NP cells (referred to hereafter as co-culture) was performed by growing NP cells (0.6 x 10^6 cells/insert) in cell culture inserts (Cat #: 662641, Greinerbio-one) coated with collagen type II (Cat #: C9301, Sigma, 0.5 mg/mL), and plating ASC pellets (1 x 10^6 cells /pellet) in 24-well plate (Cat#: 930186, Thermo) coated with 2% agarose (type VII, Cat #: BP165-25, Fisher) in DMEM: Ham's F-12 to minimize cell attachment and spreading on the membrane, as shown in Figure 2. hASCs pellets cultured alone in complete medium (referred to hereafter as hASCs-alone negative) and hASCs pellets cultured in inductive medium (referred to hereafter as hASCs-alone positive), comprised of DMEM: Ham's F12 complete medium, supplemented with 100 nM dexamethasone (DEX, Sigma), 6.25 μg/mL insulin (Cat#: I9278, Sigma), 50 μg/mL Ascorbate-2-phosphate (A2P, Sigma), and 10 ng/mL transforming growth factor β3 (TGF-β3, Peprotech), were included in parallel as control groups. Triplicate samples were included in every study. After co-culture with NP cells for 14 and 28 days, the ASC pellets were harvested for qualitative and quantitative ECM component evaluation.

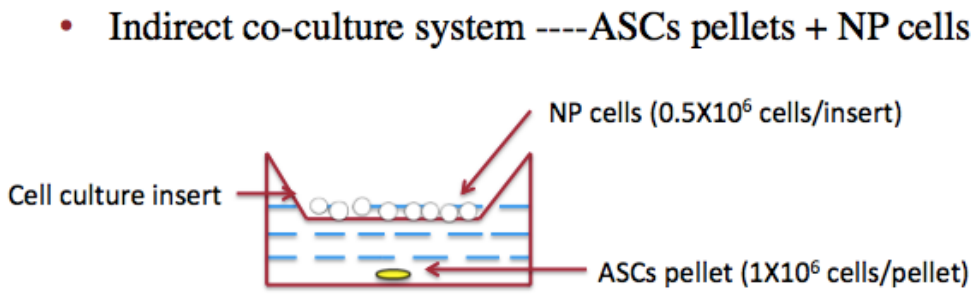

Figure 2. Indirect co-culture of ASCs pellets and NP cells. The NP cells were grown in the cell culture inserts, while the ASCs pellets were cultured on the 24-well plates.

In detail, three-dimensional hASC pellets were prepared in 15 mL vented conical tubes (CELLTREAT) by centrifugation at 300 xg for 10 min and *in situ* cultured in the 15 mL conical tubes for 72 h to generate stable and packed cell pellets to facilitate cell-cell interaction, and then carefully transferred to 24-well plates coated with 2% agarose (type VII, Sigma). The NP cells were collected and seeded in the 0.5 mg/mL collagen type II (Sigma) coated cell culture inserts (0.4 μm pores) and separately cultured for 48 h to let the cells naturally condense at the bottom of the inserts via gravity, and then combined in the agarose-coated plates with hASC pellets for 4 weeks. Media were refreshed every 3 days and hASC pellets and NP cells were harvested at day 14 and day 28 for differentiation analyses.

3.1.5 Analyses of hASCs differentiation in co-culture system

In order to analyze the differentiation of the ASCs pellets in co-culture and ASCs-alone both positive and negative controls, immunohistochemical (IHC) staining was used for detecting collagen types I and II, and toluidine blue staining was used to detect GAGs. Quantitative analysis of sulfated GAG was conducted by the dimethylmethylene blue (DMMB) assay. PicoGreen assay was used to measure total DNA of ASCs. All assays were run after 14 days and 28 days of the initiation of the co-culture.

Immunohistochemical (IHC) Staining for Collagen Type I and II:

For IHC staining, triplicate hASC pellets (n=3) were harvested after 14 days and 28 days culture, and fixed in 4% paraformaldehyde (ASROC, New Jersey, USA) overnight. The cell pellets were wrapped with wax paper to protect them from washing away in the tissue-processing machine (Botterell Hall, Queen's University) before embedding and sectioning (5 μm sections). After de-paraffinization and rehydration, sections were incubated in 0.1% typsin-EDTA solution (Cat#:1697785, Gibco) at 37˚C for 30 min for antigen retrieval. Treated sections were then washed with PBS three times and incubated with primary antibodies overnight at either a 1:100 dilution of mouse anti-collagen I (Cat #: ab90395, Abcam) or a 1:200 dilution of rabbit anti-collagen II (Cat#: ab34712, Abcam). The following day, sections were PBS-washed 3 times, followed by 1h incubation with secondary antibodies, at either a 1:200 dilution of goat anti-mouse (Cat #: ab150077, Abcam) or a 1:400 dilution of goat anti-rabbit (Cat #: ab150115, Abcam).

After incubation with secondary antibodies, the sections were washed with PBS 3 times and then counter-stained with DAPI (Abcam) for 20 min. Negative controls without primary antibodies were included to detect for non-specific binding. Sections were imaged using a fluorescent microscope (Imager.M1, Zeiss). Bovine cartilage samples were used as collagen Type II staining controls and rat kidneys were used as collagen Type I staining controls.

Toluidine blue staining:

For toluidine blue staining, triplicate hASC pellets that had been cultured for 14 and 28 days were fixed with 4% paraformaldehyde overnight, embedded and sectioned as described for IHC staining. Sections were de-paraffinizated and rehydrated with distilled water and then stained with toluidine blue working solution (Sigma, pH 2.3- 2.5) for 2-3 min. Stained sections were then washed in distilled water 3 times, and dehydrated twice quickly through 95% and 100% ethanol, and cleaned in xylene twice. After mounting onto glass slides with Permount (Sigma), the sections were imaged using a bright-field microscope (WILOVERT S, Fisher Scientific).

Dimethylmethylene blue (DMMB) Assay:

After being co-cultured with NP cells for 14 and 28 days, triplicate ASC pellets were washed with PBS and digested in papain digestion solution (Sigma-P-3125, 40 μg/mL) at 65˚C for 72 h until completely digested. Triplicate ASCs-alone positive and negative control pellets were also included in this assay using an identical method. After 72 h

digestion, the papain-digested samples were aliquoted to100 µL and stored at -20 ˚C for the DMMB assay. Briefly, GAG standard stock solution (chondroitin sulfate sodium salt Sigma C-6737, 10 mg/mL) was diluted 1:10 to 1 mg/mL to make a series of standards with 1% BSA in PBS. The 40 µL of diluted samples and standards were loaded in each well and 125 µL DMMB dye solution was added. The samples were mixed briefly and absorbance was measured at 595 nm using a multimode plate reader (EnSpire, PerkinElmer). For the calculation, the measurements of all samples and standards were subtracted from the optical density (OD) of blank samples and normalized to total DNA.

The DMMB assay, as described above, was also used to evaluate how much GAG was secreted into the culture medium by measuring the medium samples collected after medium changes.

PicoGreen Assay:

A PicoGreen assay kit (ThermoFisher) was used for the measurement of the total DNA content of the papain-digested ASC pellet samples. Briefly, the diluted papain digested samples were sonicated and spun down to remove cell debris, with the DNA remaining in suspension. The Lamda DNA stock (100 µg/mL) was diluted 1:50 to 2 µg/mL to make a series of standards with 1X TE (Tris-EDTA) buffer. 100 µL diluted samples and standards were added to each well of a 96-well plate and mixed with 100 µL of PicoGreen reagent made by diluting the Quanti-iT Picogreen reagent stock 1:200 with 1X TE buffer. The samples were then incubated for 5 min in the dark and mixed briefly. The

fluorescence was measured at 480 nm using a multimode plate reader (EnSpire, PerkinElmer). For calculations, all measurements of samples and standards were subtracted from the fluorescence value of blank samples. The concentration of all samples was calculated using the standard curve equation.

3.2 Indirect co-culture of hASC with/ without N-cadherin conjugated HA alginate gels with NP cell micromass

3.2.1 N-cadherin HA synthesis

Hyaluronic acid carbohydrate polymer (100 kg/mol, Lifecore Biomedical) was linked at various ratios with N-[β-maleimidopropionic acid] hydrazide (BMPH) to yield maleimide-HA, which was then further reacted with free-thiol N-cadherin peptide (Ac-HAVDIGGGC) to give HA-Cadherin. This work was done by Dale Marecak of the Amsden lab. Briefly, HA (142 mg, 0.35 mmol COOH) was mixed with either 39.7 mg or 18.8 mg BMPH in a 50 mL conical tube. These were fully dissolved in 18mL of 200 mM BisTris buffer (pH 4.47) over 2 h. Carbodiimide (EDC-HCl) was dissolved in water (17 mg/2mL) quickly (<50 s), then immediately dispensed and mixed with the buffered HA/BMPH solution (0.80 mL (0.035 mmol EDC) to 39.7mg BMPH tube = "10%" and 0.20 mL (0.0088 mmol EDC) to 18.8 mg BMPH tube = "2%"). After 4 h tumbling at 37^{o}C, the reaction pHs were adjusted from the initially measured 3.6 to a final of 5.0 using 1 M NaOH, and allowed to continue tumble-reacting for 48 h. Exhaustive dialysis

was carried out in 6-8K molecular weight cut-off (MWCO) regenerated cellulose membranes for 2 days through 3 baths of 5 L milli-Q water each.

HA-maleimide in pH 8 200 mM sodium phosphate buffer at 6-15 mg/mL was degassed and argon-purged using house vacuum (55 cm Hg vacuum). Solid N-cadherin peptide (Ac-HAVDIGGGC) was added in 1.5 molar equivalents to maleimide, calculated from the actual EDC added in the first step and assuming 100% reaction. After 3 days of reaction the materials were exhaustively dialyzed, frozen in liquid nitrogen and lyophilized. The synthesized N-cadherin HA material, which had N-cadherin degrees of substitution (DOS) of 8% determined using nuclear magnetic resonance (NMR), were stored in 50 mL tubes at 4 ^{o}C until used.

3.2.2 Seeding hASCs in alginate gels

(a) Seeding hASCs in alginate gels without N-cadherin HA

A 2% sodium alginate solution was made by dissolving the alginate powder (Cat#: A0682-100G, low viscosity, Sigma) in 0.9% NaCl with 20mM HEPES. P2 ASCs (0.4 x10^6 cells/ insert, 13.2 μL) were suspended in 52.8 μL alginate solution per insert before being ionically cross-linked with 0.1 M $CaCl_2$ with 10mM HEPES for 5-10 min. The $CaCl_2$ solution was then replaced with complete medium.

(b) Seeding hASCs in N-cadherin conjugated HA alginate gels

For the N-cadherin conjugated HA alginate gels, the prepared N-cadherin conjugate HA materials were exposed to UV light for disinfection for 30 min and dissolved in 2% alginate solutions in 0.9% NaCl with 20 mM HEPES (pH 7.4) to make 0.5% and 1% N-cadherin HA solutions (DOS 8%) respectively. After being completely dissolved, the N-cadherin HA/alginate solutions were sterile-filtered through a 0.22 μm pore size filter. The P2 ASCs (0.4 $x10^6$ cells/ insert, 13.2 μL) were then seeded in 52.8 μL of these solutions per insert before being ionically cross-linked using 0.1 M $CaCl_2$ with 10 mM HEPES for 5-10 min. The $CaCl_2$ solution was then replaced with complete medium.

3.2.3 NP cells seeding in agarose ring molds

The 1.5% (w/v) agarose (type VII, low melting temperature, Fisher, New Jersey, USA) solution was made in a DMEM: Ham's F12 medium by heating in a microwave periodically for 3-9 seconds. After sterile-filtered with a 0.2 μm filter, 300 μL aliquots of this solution were placed in each well of 24-well plates to coat the whole area of the wells and allowed to sit for 3-5 min to solidify by cooling. A 5 mm diameter biopsy punches (Miltex, Inc. York, USA) was used to create holes within the agarose, as shown in Figure 3. The rings thus formed were washed with PBS twice before seeding with 10 μL NP cell suspension ($0.5x10^6$ cells/well). After being seeded in the agarose rings, the NP cells were incubated for 4 h in order to settle to the bottom of the wells. The 50 μL of complete

media were then added to each well to prevent the cells from drying out before being incubated overnight. The following day, the 200 μL of complete media were added to each well to continue the co-culture study.

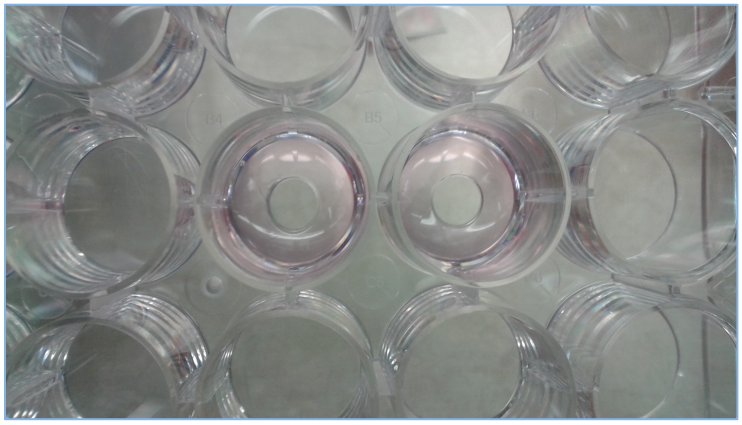

Figure 3. Agarose ring molds for NP cells micromass culture. Agarose rings were made in 24-well plates.

3.2.4 Indirect co-culture of hASC in gels with NP cells

In the first trial, indirect co-culture of hASCs encapsulated in 2% alginate gels and NP cells in agarose rings (referred to as co-culture) was performed by seeding ASCs in suspension (0.4 x 10^6 cells /insert) in sterile alginate gels in cell culture inserts and growing NP cells (0.5 x 10^6 cells/well) in the centre of the agarose ring to form an NP cell micromass and therefore, maintain the NP cell phenotype [27], as shown in Figure 4. For the co-culture studies, four groups, which were ASCs co-cultured with NP cells in complete medium, ASCs cultured alone in complete medium as a negative control, ASCs cultured alone in chondrogenic-induced medium as a positive control, and NP cells

cultured alone in agarose rings as a negative control, were included. The co-culture studies were conducted for 28 days and the samples were harvested on days 14 and 28 for proteins and GAG expression analyses. The medium was refreshed every two days.

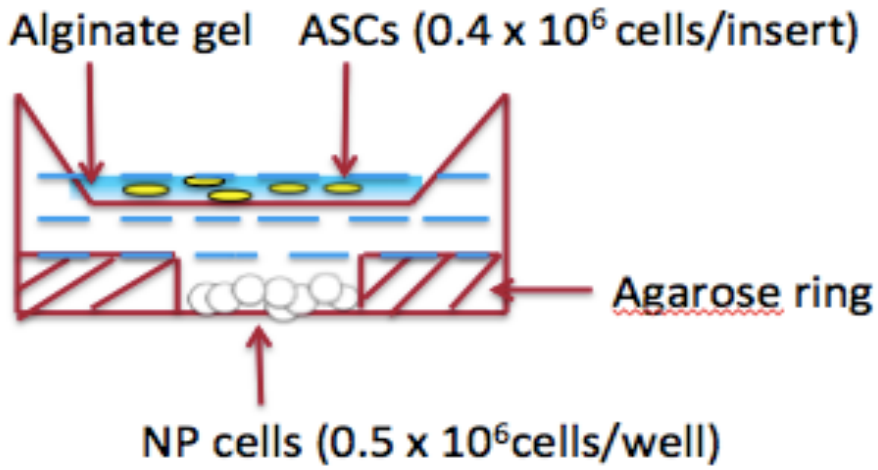

Figure 4. Indirect co-culture of ASCs in alginate gels with NP cells micromass culture. Blue block represents alginate gel, and red blocks represent the agarose ring.

In the second trail, indirect co-culture of hASCs encapsulated in 1% N-cadherin conjugated-HA/alginate hydrogels and NP cells in agarose rings was conducted by encapsulating ASCs (0.4 x10^6 cells /insert) in N-cadherin HA alginate gels formed in cell culture inserts and growing NP cells (0.5 x 10^6 cells/well) in agarose ring as described above. For these studies, seven groups were included, as follows:

1. NP cells co-cultured with ASCs in 2% (w/v) alginate gels cultured in non-inductive medium (complete medium)

2. NP cells co-cultured with ASCs in 0.5% (w/v) N-cadherin HA alginate gels cultured in non-inductive medium (complete medium)

3. NP cells co-cultured with ASCs in 0.5% (w/v) N-cadherin HA alginate gels cultured in inductive medium

4. NP cells co-cultured with ASCs in 1% (w/v) N-cadherin HA alginate gels cultured in non-inductive medium (complete medium)

5. NP cells co-cultured with ASCs in 1% (w/v) N-cadherin HA alginate gels cultured in inductive medium

6. NP cells cultured alone in non-inductive medium (complete medium)

7. NP cells cultured alone in inductive medium

The co-culture studies (n=3) with N-cadherin HA present in the calcium alginate hydrogels were conducted for 28 days and the samples were harvested on day 28 for protein and GAG expression analyses. The medium was refreshed every two days.

3.2.5 Analyses of hASC differentiation

In order to analyze the differentiation of hASCs encapsulated in N-cadherin conjugated HA/calcium alginate gel, immunohistochemical staining was used for detecting the presence of collagen II, and colorimetric staining was used to detect the presence of collagen I. Quantitative analysis of sulfated GAG was conducted by the DMMB assay

and the hydroxyproline (OH) assay was utilized to assess the total content of collagen. Quantifluor dsDNA assay was used to measure the total DNA of ASCs and NP cells. All assays were run after 28 days of the initiation of the co-culture.

Immunohistochemical (IHC) Staining for Collagen Type I (fluorescent staining):

Triplicate hASC gel samples from different conditions (n=3) and NP cells samples as controls were harvested after 28 days culture, and fixed in 4% paraformaldehyde with $BaCl_2$ (pH 7.4) to irreversibly crosslink the alginate matrix overnight. The hASCs gels were wrapped with wax paper to protect them from washing away in the tissue-processing machine (Botterell Hall, Queen's University) before embedding and sectioning (5 μm sections). After de-paraffinization and rehydration, sections were incubated in 0.15% typsine (Gibco) in PBS solution at 37˚C for 10 min to retrieve antigen availability. Treated sections were then washed with PBS-T (0.2% TWEEN in PBS) three times and then blocked with 10% goat serum (G9023, Sigma) in PBS for one hour at room temperature, followed by incubation with primary antibodies 1:200 dilution of rabbit anti-collagen II (Abcam, cat #: ab34712) overnight. The following day, sections were PBS-T washed three times, followed by one hour incubation with secondary antibodies, 1:400 dilution of goat anti-rabbit (Abcam, cat #: ab150115). After incubation with secondary antibodies, the sections were washed with PBS-T three times and then counter-stained with DAPI for 20 min. Negative controls without primary antibodies were included to detect non-specific binding. Sections were imaged by fluorescent

microscope. Bovine cartilage samples and bovine NP tissue were used as collagen Type II staining controls.

Immunohistochemical (IHC) Staining for Collagen Type I (colorimetric staining):

Triplicate hASC gel samples from different conditions (n=3) and NP cells samples as controls were harvested after 28 days culture, and fixed in 4% paraformaldehyde with $BaCl_2$ (pH 7.4) to irreversibly crosslink the alginate matrix overnight. The hASCs gels were wrapped with wax paper to protect them from washing away in the tissue-processing machine (Botterell Hall, Queen's University) before embedding and sectioning (5 μm sections). After de-paraffinization and rehydration, sections were washed with Tris-buffered saline (TBS) with 0.025% Triton X-100 two times. Washed sections were then blocked with 10% horse serum (Sigma) with 1% BSA in TBS in PBS for 2 h at room temperature. Blocked samples were then incubated with primary antibodies, using a 1:100 dilution of rat anti-collagen I (Abcam, cat #: ab90395) in TBS with 1% BSA overnight. The following day, sections were washed with TBS with 0.025% Triton X-100 twice, followed by 15min incubation in 0.3% H_2O_2 in TBS to damage the epitopes the primary antibody binds with. Treated samples were then incubated with secondary antibodies, using a 1:500 dilution of biotinylated anti-mouse/anti-rabbit IgG (BA-1400, Vector labs) in TBS with 1% BSA for one hour at room temperature. After incubation with secondary antibodies, the sections were washed with TBS with 0.025% Triton X-100 three times and then incubated with

avidin/biotinylated enzyme complex (ABC) solution (PK-4000, Vector labs) for 1 h at room temperature. After washing three times with TBS, the sections were treated with 3, 3-diaminobenzidine (DAB) solution (SK-4100, Vector labs) for 6min. The samples were then quickly rinsed with TBS three times, and then imaged on a bright-field microscope (WILOVERT S, Fisher Scientific). Negative controls without primary antibodies were included to detect non-specific binding. Rat kidney samples and bovine NP tissue were used as collagen Type I staining positive controls.

Hydroxyproline (OH) Assay:

The total collagen content was determined by measuring the hydroxyproline content in the samples. After hASC gel samples (n=3) were completely digested in papain solution as described in the DMMB assay section above, 100 µL papain digested aliquots were hydrolyzed with 100 µL 6 N HCl in glass test tubes with Teflon lined-screw caps at 110 ˚C for 18 h and neutralized by adding 100 µL of 5.7 N NaOH. The hydrolysates were then reacted with 0.05 N Chloramine-T (Sigma) for 20min, 3.15 N Perchloric acid for 5min, and 0.2 g/mL Ehrlich's regent for 20 min at 60 ˚C. The samples were then cooled for 5 min at 4 ˚C, brought to room temperature, and allowed to sit for at least 30 min to stabilize the color. The plate was read in a multimode plate reader (EnSpire, PerkinElmer) at 560 nm. The method that was used for the DMMB assay was as described in section 3.1.5.

QuantiFluor dsDNA Assay:

A QuantiFluor assay kit (Promega) was used for the measurement of total DNA content of papain-digested ASCs and NP cell samples. Briefly, the dsDNA standard stock (100 µg/mL dsDNA) was diluted 1:50 to 2 ng/µL to make a series of standards with 1xTE (Tris-EDTA) buffer. 100 µL diluted samples and standards were added to each well of a black flat-bottom plate and dyed with 100 µL QuantiFluor dsDNA dye working solution made by diluting the dye stock 1:200 with 1xTE buffer. The samples were incubated for 5 min in dark and mixed briefly. The fluorescence was measured at $504nm_{Ex}/531nm_{Em}$. For calculations, all measurements of samples and standards were subtracted from the fluorescence of blank samples. The concentration of all samples was calculated using the standard curve equation.

3.3 Statistical Analyses

The results are represented as the mean ± standard deviation. One-way or two-way ANOVA (Bonferroni correction) were used to determine statistically significant differences ($P < 0.05$) between groups. All statistical calculations were performed using GraphPad Prism 6. In each experiment, triplicate samples (n=3) were included.

Chapter 4

Results and Discussion

4.1 Results

4.1.1 Bovine NP cell isolation and culture

Sterilized Bovine tail was shown in Figure 5 A. NP tissues (Figure 5 B) were removed from each disc. After seeding in the T75 flasks for 24 h, most of the NP cells attached and started to spread (Figure 5 C). By day 3, the NP cells started elongating and became more flat and close to each other (Figure 5 D).

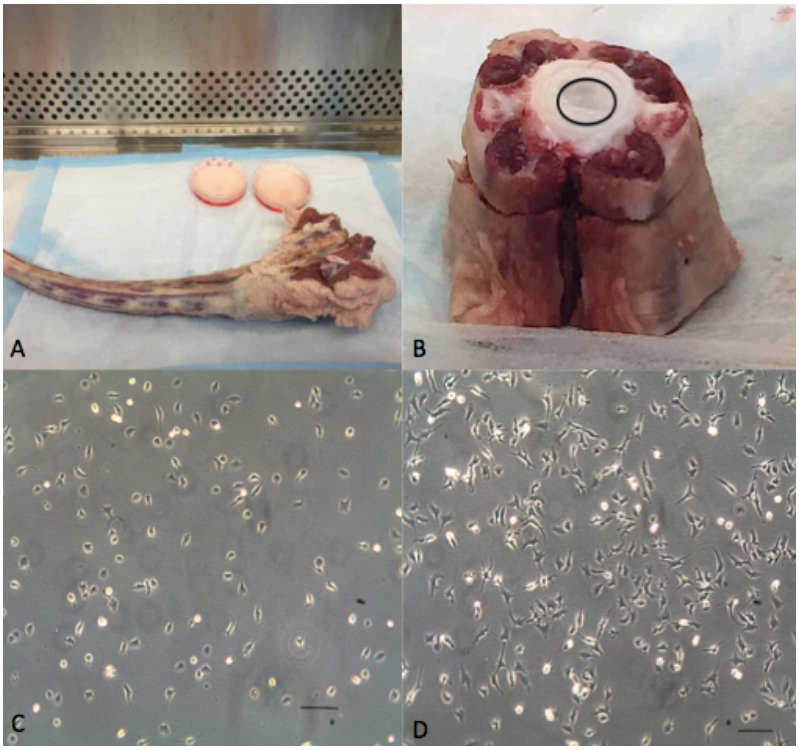

Figure 5. (A) Bovine tail after sterilizing with 10% bleach and 70% ethanol. (B) The circle shows where the NP tissues were harvested from the caudal discs. (C) NP cells 24 h after seeding (D) NP cells 72 h after seeding. Scale bar = 100 µm.

4.1.2 ASC pellet differentiation induced by co-culture with NP cells

Triplicate ASC pellet samples were harvested at day 14 and day 28 and were analyzed for the level of accumulated GAG and collagen type I and type II. Fluorescent IHC staining results showed that by day 14, in contrast to ASC pellets grown in non-inductive medium (Figure 6 A) as a negative control, there was qualitatively more accumulated collagen II present in the ASC pellets co-cultured with NP cells (Figure 6 B). Moreover, a similar

amount of collagen II production was observed in the ASC pellet co-cultured group in comparison with ASC pellets cultured in inductive medium (Figure 6 C) as a positive control. Similarly, the ASC pellets cultured with NP cells for 28 days (Figure 6 E) produced more collagen II in comparison with both the positive (Figure 6 D) and negative controls (Figure 6 F). Bovine cartilage tissue was used as positive staining control (Figure 6 G).

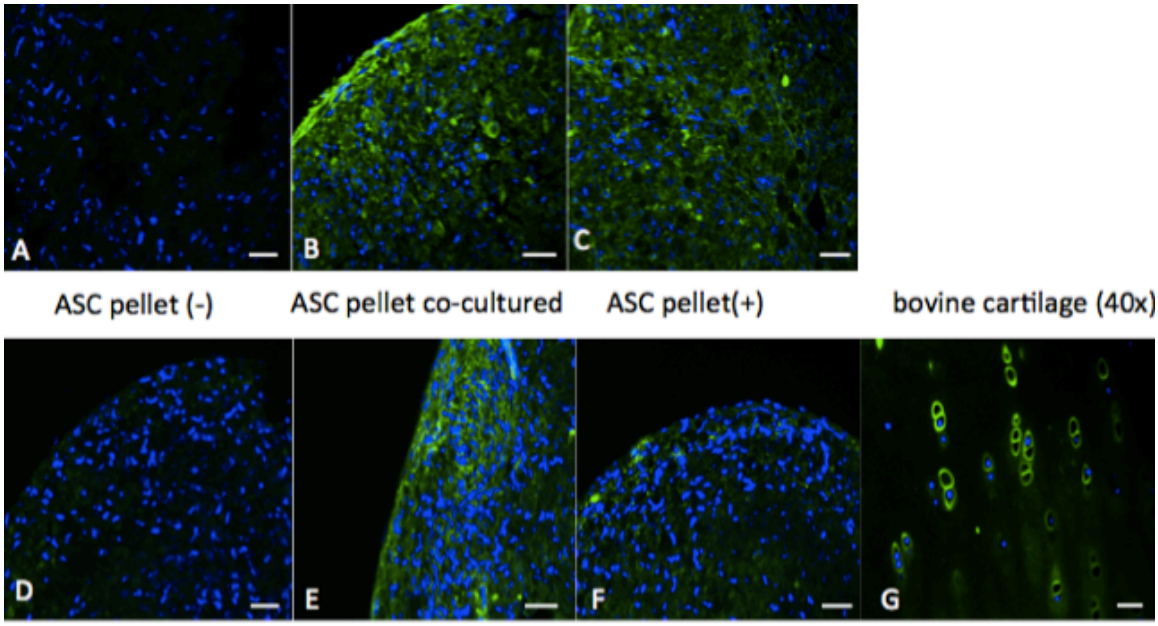

Figure 6. IHC staining for Collagen II (green) with DAPI (blue) counterstaining. (A-C) cell pellets cultured after 14 days. (D-F) cell pellets cultured for 28 days. (G) Bovine cartilage was used as staining positive control. ASC pellet (-) means ASC pellets cultured in non-inductive medium; ASC pellet co-cultured means ASC pellets co-cultured with NP cells in non-inductive medium; ASC pellet (+) means ASC pellets cultured in inductive medium. Scale bar = 100 µm (n=3). Negative controls of each sample were attached in Appendix A (Figure 18).

ASC pellets were also stained for collagen type I, which is the typical collagen that is higher expressed in AF tissue of the intervertebral discs and is a negative marker for differentiation towards NP cells [27]. The expression of collagen type I in NP tissue is qualitatively much lower than that of collagen II, which is the predominant collagen in NP tissue [28]. The IHC staining results for collagen I indicated that on day 14, in comparison to the ASC pellets cultured in non-inductive medium (Figure 7 A), there was qualitatively a higher amount of collagen I accumulation in the ASC pellets co-cultured with NP cells group (Figure 7 B) and ASC pellets cultured in inductive medium (Figure 7 C). The same trend was seen in the day 28 samples (Figure 7 D-F). It was also observed that on day 28, there was qualitatively more collagen I accumulated in the ASC pellets (Figure 7 D-F) compared to that on day 14 (Figure 7 A-C). Rat kidney tissue was used as positive staining control (Figure 7 G).

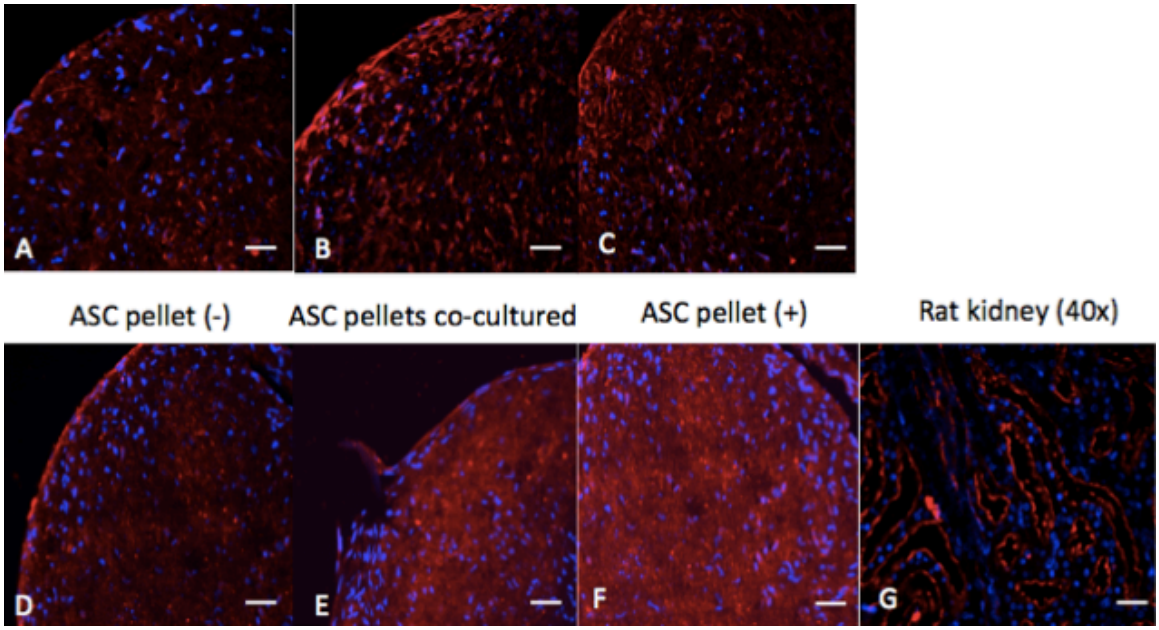

Figure 7. IHC staining for collagen I (red) with DAPI (blue) counterstaining. (A-C) cell pellets cultured after 14 days. (D-F) cell pellets cultured for 28 days. (G) Rat kidney tissue was used as staining positive control. ASC pellet (-) means ASC pellets cultured in non-inductive medium; ASC pellet co-cultured means ASC pellets co-cultured with NP cells in non-inductive medium; ASC pellet (+) means ASC pellets cultured in inductive medium. Scale bar = 100 µm (n=3). Negative controls of each sample were attached in Appendix A (Figure 19).

In order to determine the level of GAG production in the induced ASC pellets, ASC pellets of each group obtained on day 14 and day 28 were stained with toluidine blue to localize GAG accumulation, and also the DMMB assay was conducted to characterize GAG production quantitatively and further confirm the staining results. As shown in Figure 8, the ASC pellets co-cultured with NP cells produced qualitatively a higher

amount of GAGs compared to ASC pellets cultured in non-inductive medium, and lower amount of GAGs in comparison to ASC pellets cultured in inductive medium on day 14. Moreover, similar trend was observed on day 28 samples to day 14 samples. In general, there was qualitatively a greater amount of GAG accumulation on day 28 than seen by day 14. Bovine cartilage sample was used as positive staining control.

These qualitative results were confirmed using the DMMB assay (Figure 9). All data was normalized to total DNA measured using a Picogreen assay. Results showed that ASC pellets were induced by co-culture with NP cells on day 14, and the GAG accumulation was three times higher in ASC pellet samples co-cultured with NP cells on day 28 than that on day 14. All groups were significantly differently from each other ($P< 0.05$). Both qualitative and quantitative results demonstrated that ASCs were induced by co-culture with NP cells to produce more GAGs.

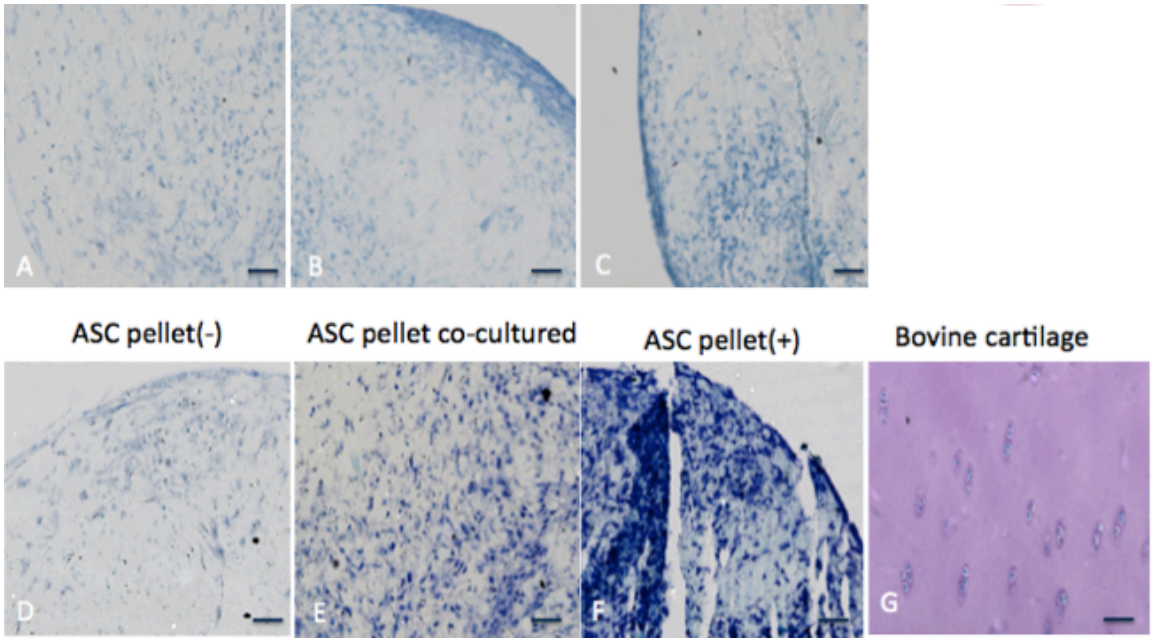

Figure 8. Toluidine Blue staining for GAG accumulation. (A-C) cell pellets cultured after 14 days. (D-F) cell pellets cultured for 28 days. (G) Bovine cartilage tissue was used as staining positive control. ASC pellet (-) means ASC pellets cultured in non-inductive medium; ASC pellet co-cultured means ASC pellets co-cultured with NP cells in non-inductive medium; ASC pellet (+) means ASC pellets cultured in inductive medium. Scale bar = 100 µm. (n=3)

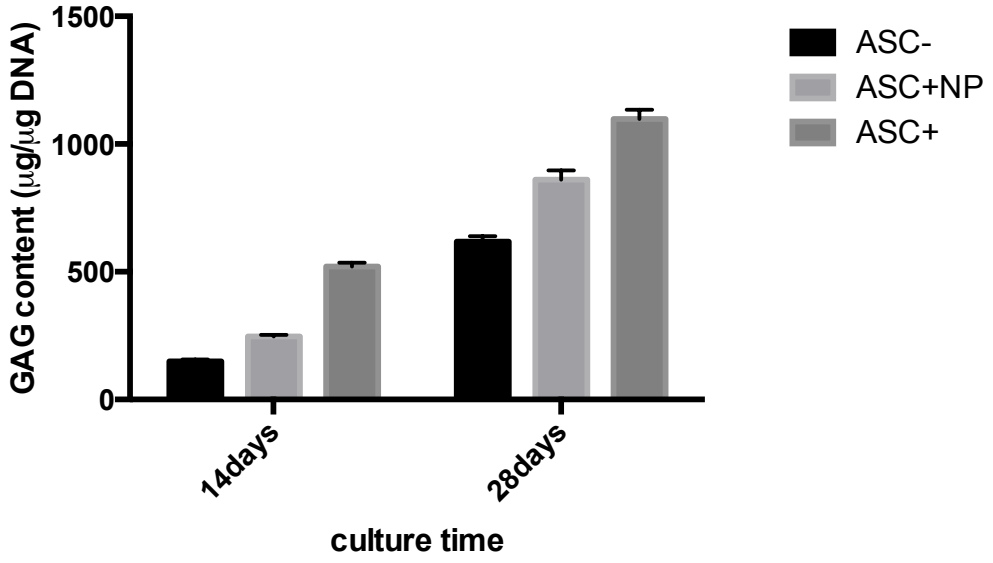

Figure 9. DMMB assay for determine of total GAG content in ASC pellets. All data was normalized to total DNA. ASC- means ASC pellets cultured in non-inductive medium. ASC+NP means ASC pellets co-cultured with NP cells. ASC+ means ASC pellets cultured in inductive medium. All groups are significantly different from each other (n=3, $P < 0.05$). Error bars represent the standard deviations.

4.1.3 Differentiation of ASCs encapsulated in alginate gels and co-cultured with NP cells

In order to investigate the feasibility of using hASCs as one of the potential stem cell sources for IVD regeneration, P2 ASCs encapsulated in alginate without N-cadherin conjugated HA were co-cultured with P1 NP cells seeded in agarose rings to determine the ASC differentiation profile over 4 weeks in culture.

As shown in Figure 10, there was more qualitatively intense staining for collagen II in all samples at day 28 compared to that of the samples at day 14, which means there was more collagen II synthesis in samples at day 28 than that of samples at day 14. There was no difference observed between the ASCs groups on day 14 and between the ASCs groups on day 28, and ASCs samples co-cultured with NP cells both on day 14 and day 28 displayed less intense staining than NP cells cultured in non-inductive medium, which indicates that either the NP cells had limited effect on inducing ASCs differentiation in gels or the collagen II produced by the ASCs did not remain in the gels.

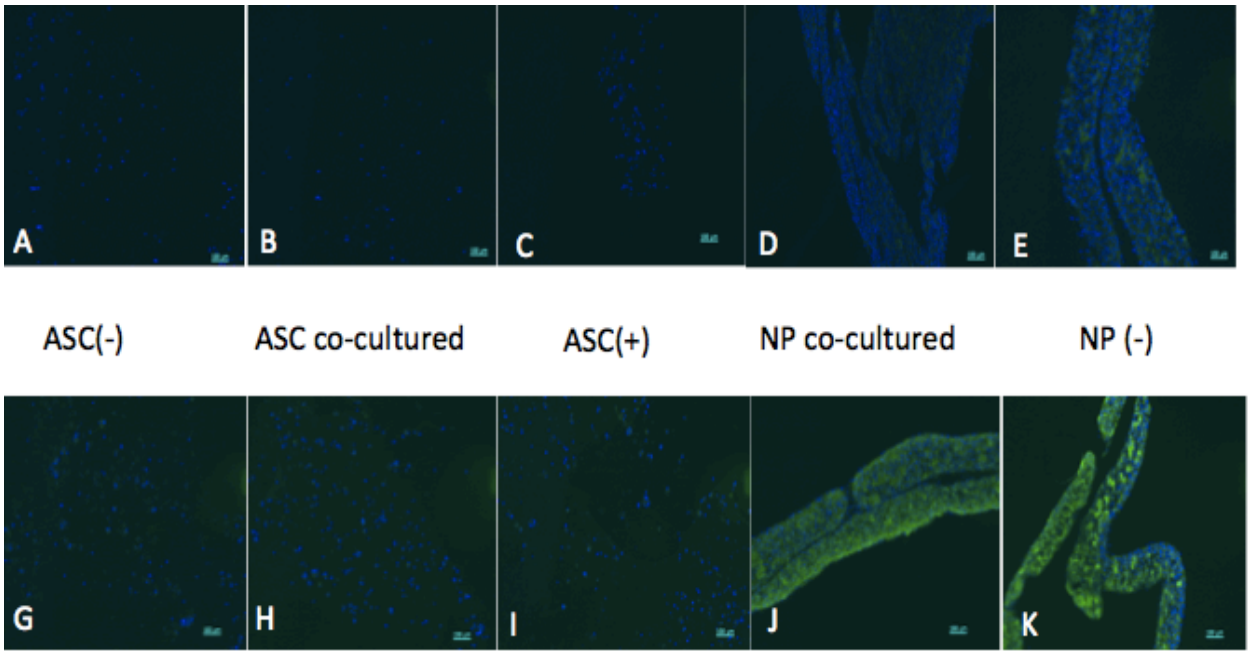

Figure 10. IHC staining for collagen II (green) with DAPI (blue) counterstaining. (A-E) samples cultured for 14 days. (G-K) samples cultured for 28 days. ASC (-) represents ASCs encapsulated in gels in non-inductive medium as negative control. ASC co-cultured represents ASCs encapsulated in gels co-cultured with NP cells in non-inductive medium.

ASC (+) represents ASCs in gels in inductive medium as positive control. NP co-cultured represents NP cells co-cultured with ASCs. NP (-) represents NP cells cultured alone in non-inductive medium. Scale bars = 100 µm (n=3). Negative controls of each sample were attached in Appendix A (Figure 20).

GAG concentrations in both the gels (Figure 11) and in the culture medium (Figure 12) were assessed using the DMMB assay. GAG concentration in the gels was normalized to total DNA of the ASCs using a QuantiFluor assay kit (Promega). In general, there were significantly higher amounts of GAG accumulation in the ASC-containing gels on day 28 as compared to that in the ASC-containing gels on day 14. Moreover, the ASCs co-cultured with NP cells on day 28 produced 2.5 times higher amount of GAG compared to ASCs co-cultured with NP cells on day 14, which could suggest that the ASCs in gels were further induced by co-culturing with NP cells over time. The GAG content in the gels containing ASCs co-cultured with NP cells on day 28 was significantly higher than that of the ASCs cultured in non-inductive medium and ASCs cultured in inductive medium on day 28, which suggests that the ASCs were induced to differentiate by co-culture with NP cells. However, the GAG content in the NP cells was very low compared to the ASC samples and there were no significant differences observed between NP cells co-cultured with ASCs in non-inductive medium and NP cells cultured in inductive medium. A possible explanation for this is that the GAG secreted by the NP cells did not remain *in situ,* but was released into the medium.

In order to evaluate how much GAG was secreted into the culture medium, the medium samples were collected after medium changes and pooled together. As shown in Figure 12, there was a significant amount of GAG present in the medium. The accumulated amount of GAGs in the medium was normalized to total DNA, which included the DNA of both the ASCs and NP cells. There were no significant differences observed between the groups.

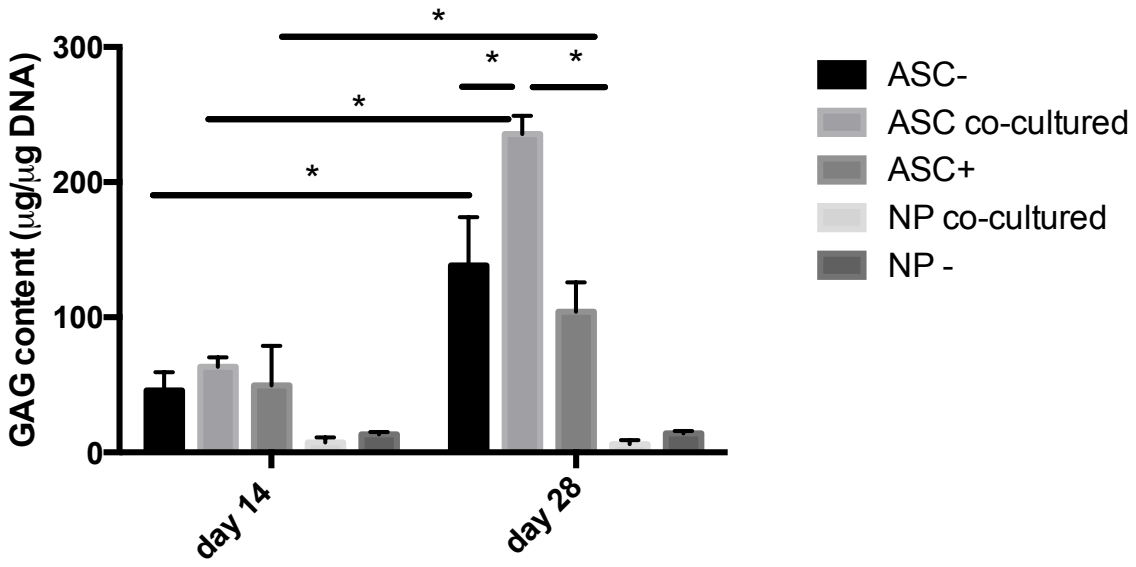

Figure 11. Accumulated GAG within the ASC containing gels or the NP cell mass. ASC (-) represents ASCs encapsulated in gels in non-inductive medium as negative control. ASC co-cultured represents ASCs encapsulated in gels co-cultured with NP cells in non-inductive medium. ASC (+) represents ASCs in gels in inductive medium as positive control. NP co-cultured represents NP cells co-cultured with ASCs. NP (-) represents NP cells cultured alone in non-inductive medium. ASCs data was normalized to total DNA of ASCs and NP cell mass data was normalized to total DNA of NP cells. (n=3, *p< 0.05)

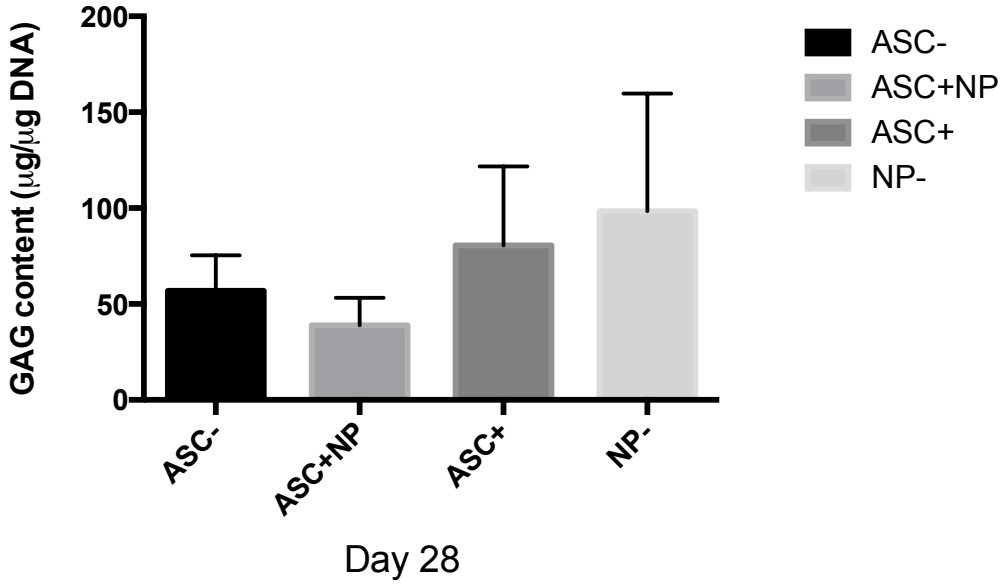

Figure 12. Accumulated soluble GAG content in the medium (28 days). ASC - represents ASCs encapsulated in gels in non-inductive medium as negative control. ASC+NP represents ASCs encapsulated in gels co-cultured with NP cells in non-inductive medium. ASC + represents ASCs in gels in inductive medium as positive control. NP - represents NP cells cultured alone in non-inductive medium. All data was normalized to total DNA (ASCs +NP cells) (n=3).

4.1.4 Differentiation of ASCs encapsulated in N-cadherin HA alginate gels with NP cells

In order to investigate the effect of addition of N-cadherin HA to alginate gels on ASCs differentiation in the indirect co-culture system with NP cells, P2 ASCs encapsulated in N-cadherin conjugated HA alginate hydrogels were co-cultured with P1 NP cells seeded in agarose rings for 4 weeks.

GAG concentrations in both the gels (Figure 13) and in the culture medium (Figure 14) were assessed using the DMMB assay. GAG content obtained from gels was normalized to total DNA of ASCs by using a QuantiFluor assay kit (Promega). As shown in Figure 13, there was no significant difference in the GAG content observed between the groups. Interestingly, for the co-culture study without N-cadherin HA in alginate gels, the ASCs co-cultured with NP cells (ASC co-cultured, Figure 11) produced a lower amount of GAG in the alginate gels compared to ASCs co-cultured with NP cells as negative control (0, Figure 13) in the co-culture study with N-cadherin HA in alginate gels. The possible reason for the inconsistent results could be explained by donor-to-donor variability of ASCs. We believe that primary cells isolated from different donors behave differently and ASCs obtained from different donors exhibit high level of donor-to-donor variability due to the differences in age, gender, height, and weight of donors. Moreover, ASCs obtained from different excision sites, such as abdomen or breast could also generate differences in ASCs differentiation induced by co-culture with NP cells.

Furthermore, the accumulated soluble GAGs in the medium were also assessed by DMMB assay and the data was normalized to total DNA, which included the DNA of ASCs and NP cells (Figure 14). In general, there was a substantial amount of GAG that had been secreted into the medium compared to that remaining in the gels. ASCs encapsulated in 1.0% N-cadherin HA with NP cells in inductive medium (co 1 +) produced significantly higher amount of GAGs in the culture medium as compared to

ASCs encapsulated in 1.0% N-cadherin HA with NP cells in non-inductive medium (co 1), which indicates that the inductive medium had a positive effect on ASCs differentiation. However, there were no significant differences between any groups and the co 0 group, which suggested that addition of 0.5% and 1.0% of N-cadherin HA to alginate gels did not promote GAG production. Moreover, there were no significant differences observed among co 0, co 0.5 and co 1.0 groups, as well as co 0, co 0.5+ and co 1.0 + groups, which illustrated that the concentrations of N-cadherin HA in the alginate gels did not affect the ASC differentiation or the increase in concentration from 0.5% to 1.0% was not large enough to cause any significant differences in ASC differentiation.

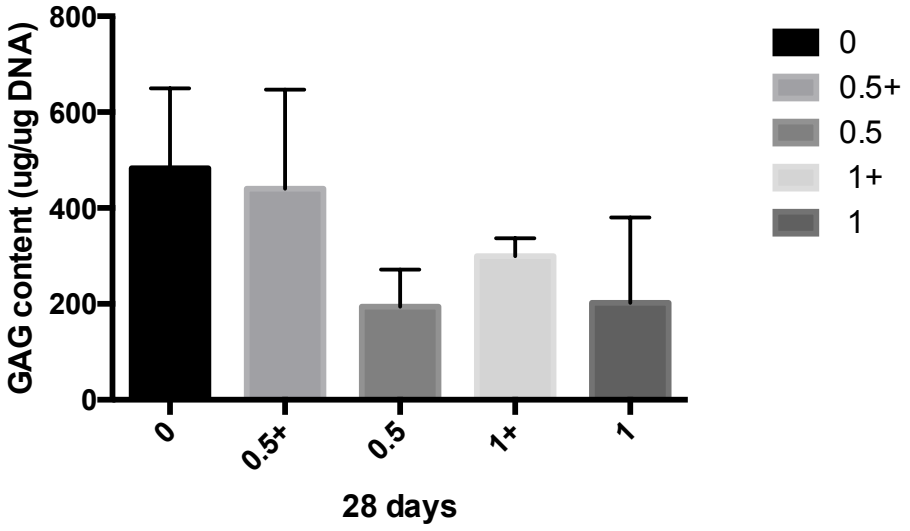

Figure 13. GAG content of ASCs in gels (normalized to DNA of ASCs). 0 indicates ASCs encapsulated in alginate gels without N-cadherin HA were co-cultured with NP cells in non-inductive medium and are under the same condition as ASC+NP group in previous study. 0.5 + and 0.5 indicate that ASCs encapsulated in 0.5% N-cadherin HA in alginate gels were

co-cultured with NP cells in either inductive "+" or non-inductive "-" medium. 1 + and 1 indicate that ASCs encapsulated in 1.0% N-cadherin HA in alginate gels were co-cultured with NP cells in either inductive "+" or non-inductive "-" medium (n=3).

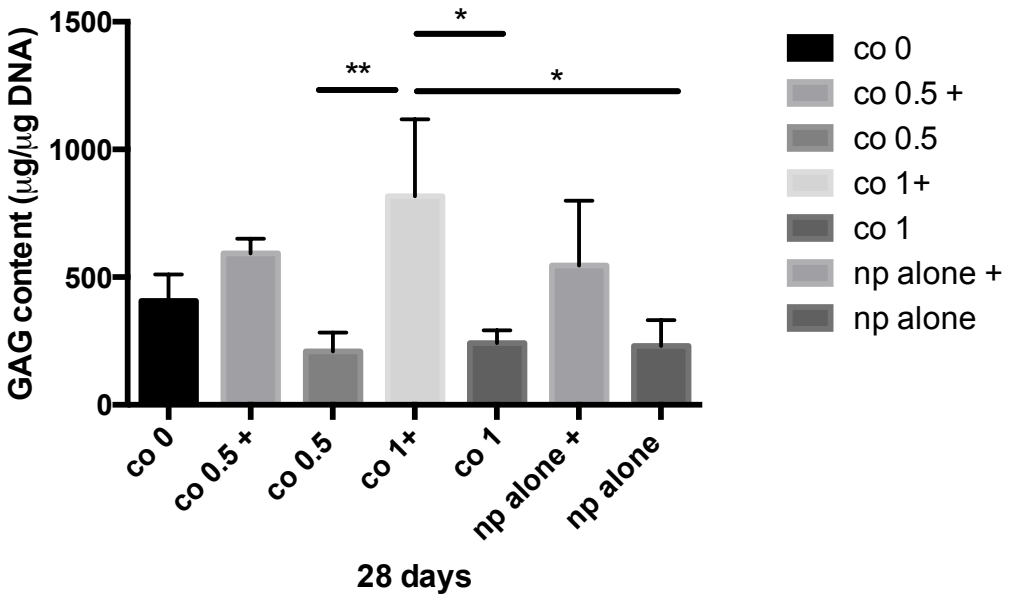

Figure 14. Accumulated soluble GAG content in medium (Normalized to total DNA of ASCs and NP cells). "co 0" indicates ASCs encapsulated in alginate gels without N-cadherin HA were co-cultured with NP cells in non-inductive medium and are under the same condition as ASC+NP group in previous study. "co 0.5 + "and "co 0.5" indicate that ASCs encapsulated in 0.5% N-cadherin HA in alginate gels were co-cultured with NP cells in either inductive "+" or non-inductive "-" medium. "co 1 +"and "co 1" indicate that ASCs encapsulated in 1.0% N-cadherin HA in alginate gels were co-cultured with NP cells in either inductive "+" or non-inductive "-" medium. "np alone +" indicates that np cells cultured alone in inductive medium and "np alone" means np cells cultured alone in non-inductive medium. (n=3, *P< 0.05)

The total collagen content produced by the ASCs in the gels was evaluated by the OH assay. As shown in Figure 15, the “co 1 +” and “co 0.5 +” groups produced higher amount of total collagen compared to “co 0” group, which indicates that the conjugation of N-cadherin HA to alginate gels enhanced the ASCs induced differentiation by co-culture with NP cells in inductive medium. Moreover, the OH assay result showed that “co 1 +” group synthesized significant higher amount of total collagen compared to “ co 0.5 +” and “co 0” groups, which demonstrates that increase in concentration of N-cadherin HA in alginate gels resulted in enhanced total collagen production in inductive medium. Furthermore, the “co 0.5 +” and “co 1 +” groups produced significantly higher amounts of total collagen as compared to the “co 0.5” and “co 1” groups, which indicates that the inductive medium had a positive effect on ASCs collagen production.

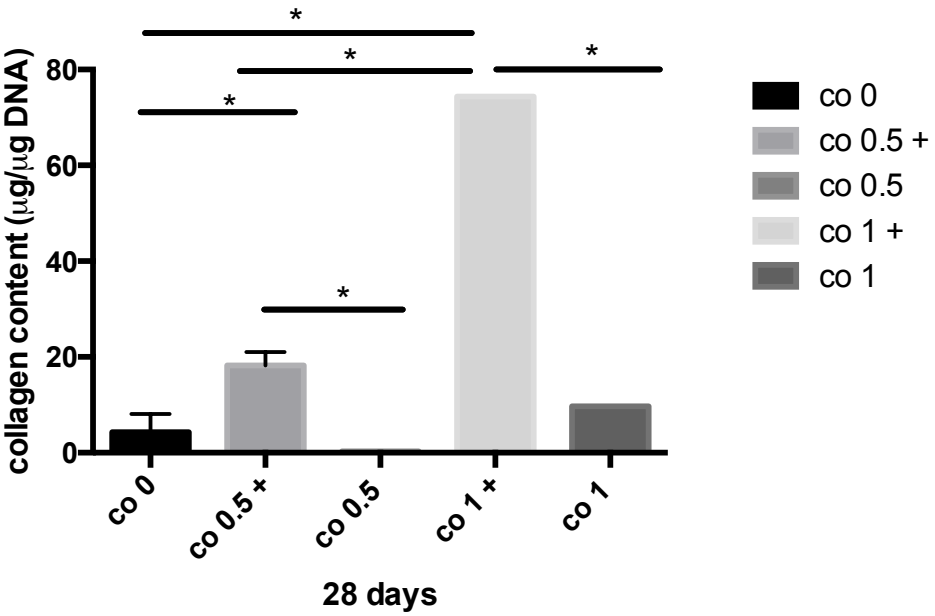

Figure 15. Total collagen content of ASCs in gels. "co 0" indicates ASCs encapsulated in alginate gels without N-cadherin HA were co-cultured with NP cells in non-inductive medium are under the same condition as ASC+NP group in previous study. "co 0.5 +" and "co 0.5 " indicate that ASCs encapsulated in 0.5% N-cadherin HA in alginate gels were co-cultured with NP cells in either inductive "+" or non-inductive "-" medium. "co 1 +" and "co 1" indicate that ASCs encapsulated in 1.0% N-cadherin HA in alginate gels were co-cultured with NP cells in either inductive "+" or non-inductive "-" medium. (n=3 for "co 0", n=2 for other groups, *p<0.05)

Furthermore, IHC staining and colorimetric staining were performed to localize collagen II and collagen I production in ASC-containing gels after 28 days co-culture with NP cells in different conditions. Based on the IHC staining results (Figure 16), after 28 days co-culture with NP cells, ASCs from all groups produced a similar amount of collagen II to NP cell groups with no qualitative difference observed between groups, which

indicated that ASCs co-cultured under different conditions were all induced by co-culture with NP cells. However, the increase of N-cadherin-HA concentration in alginate gels did not appear to have an effect on qualitative levels of collagen II in the ASCs in gels. Moreover, both the ASCs samples and NP cells cultured in inductive medium did not qualitatively produce more collagen II in comparison to those cultured in the non-inductive medium.

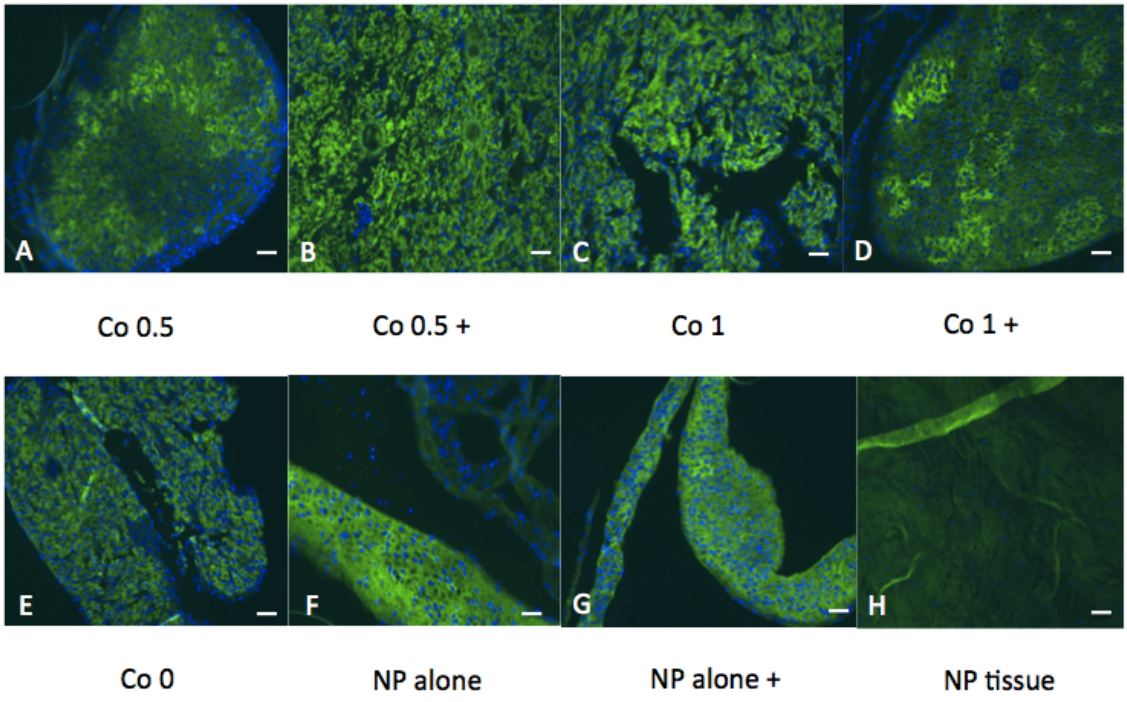

Figure 16. IHC staining for Collagen II (green) of ASCs in gels with DAPI counterstaining (blue) 28 days. "co 0" (A) indicates ASCs encapsulated in alginate gels without N-cadherin HA were co-cultured with NP cells in non-inductive medium. "co 0.5 +" and "co 0.5" (B, C) indicate that ASCs encapsulated in 0.5% N-cadherin HA in alginate gels were co-cultured with NP cells in either inductive "+" or non-inductive "-" medium. "co 1 + "and "co 1" (D,

E) indicate that ASCs encapsulated in 1.0% N-cadherin HA in alginate gels were co-cultured with NP cells in either inductive "+" or non-inductive "-" medium. "NP alone +" (F) indicates that NP cells cultured alone in inductive medium and "NP alone" (E) means NP cells cultured alone in non-inductive medium. NP tissue (H) was used as positive staining control (n=3). Negative controls of each sample were attached in Appendix A (Figure 21).

Colorimetric staining of Collagen I for the ASCs in gels after 28 days co-culture with NP cells showed that " co 0.5 +" and "co 1 +" groups displayed more intense staining compared to "co 0.5" and "co 1" groups, which indicates that induced medium applied to co-culture resulted in higher amount of collagen I production. Additionally, compared to "co 0" group, other ASCs co-cultured groups showed more intense staining, which suggests that modified alginate gels with N-cadherin HA promoted collagen I accumulation. However, since there were similar levels of staining observed between the "co 0.5" and "co 1" groups, as well as " co 0.5 +" and "co 1 +" groups, the concentration increase in N-cadherin HA in alginate gels from 0.5% to 1.0% did not qualitatively affect the collagen I production of the ASCs in gels (as shown in Figure 17).

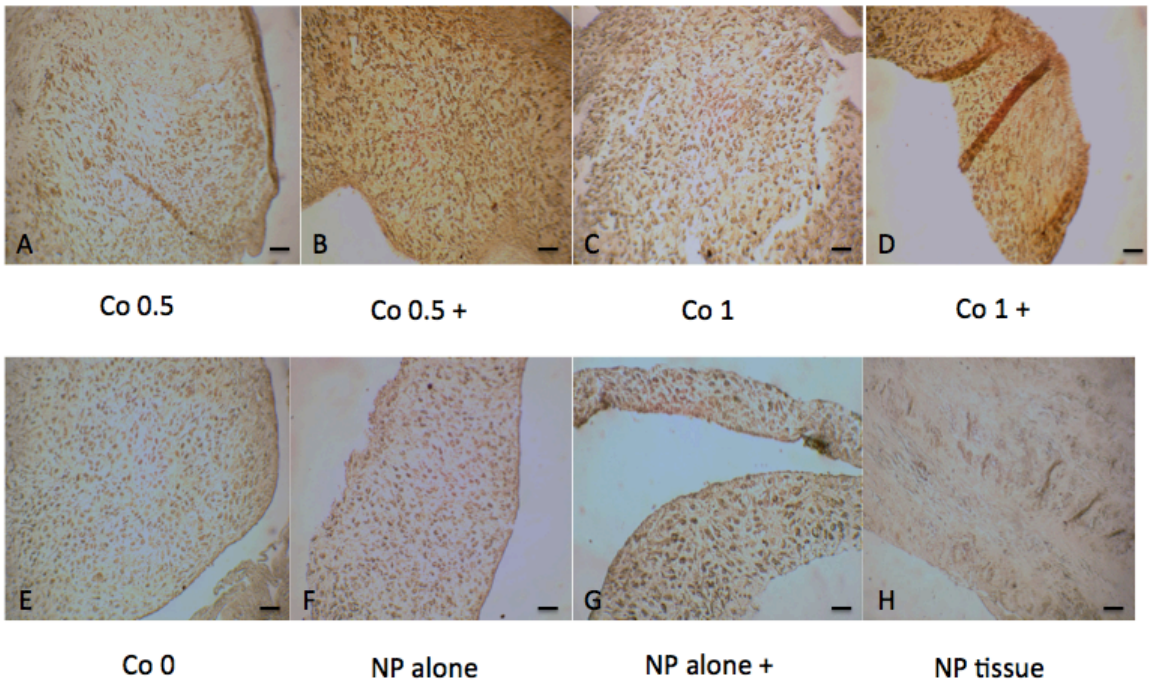

Figure 17. Colorimetric staining for Collagen I (brown) of ASCs in gels (28 days). Collagen I was stained brown and cell nuclei were stained black. "co 0" (A) indicates ASCs encapsulated in alginate gels without N-cadherin HA were co-cultured with NP cells in non-inductive medium. "co 0.5 +" (B) and "co 0.5" (C) indicate that ASCs encapsulated in 0.5% N-cadherin HA in alginate gels were co-cultured with NP cells in either inductive "+" or non-inductive "-" medium. "co 1 + " (D) and "co 1" (E) indicate that ASCs encapsulated in 1.0% N-cadherin HA in alginate gels were co-cultured with NP cells in either inductive "+" or non-inductive "-" medium. "NP alone +" (F) indicates that NP cells cultured alone in inductive medium and "NP alone" (G) means NP cells cultured alone in non-inductive medium. NP tissue (H) was used as positive staining control (n=3). Negative controls of each sample were attached in Appendix A (Figure 22).

4.2 General Discussion

Recent *in vitro* studies have demonstrated successful differentiation of ASCs induced by co-culture with NP cells towards an NP cell phenotype in different settings [26][27][32][28]. However, it still remains unknown whether ASC differentiation can be promoted by co-culture of ASCs with NP cells in a more similar environment to the native ECM of NP cells, since it is well-known that ECM plays a critical role in guiding stem cell differentiation [28]. In this study, we demonstrated that ASC pellets could be induced to produce more collagen II and GAG by co-culture with NP cells. Co-culture of ASCs encapsulated in alginate gels with NP cells promoted ECM production of ASCs.

However, modified alginate gels with N-cadherin HA peptide did not show an overall positive effect on ASC differentiation. Hydroxproline assay results showed that the addition of N-cadherin HA to alginate gels enhanced the total collagen production of ASCs in gels and the increase in N-cadherin HA concentration from 0.5% (w/v) to 1.0% (w/v) in alginate gels did promote total collagen production of the ASCs encapsulated in the gels. However, IHC staining for collagen and DMMB assay results showed that the increase in N-cadherin HA concentration from 0.5% (w/v) to 1.0% (w/v) in the alginate gels did not enhance the production of collagen II and GAG. Interestingly, the colorimetric staining results showed that the addition of N-cadherin HA promoted the collagen I production of ASCs in the gels. The possible reason for the non-concomitant

results obtained from different assays could be that the amount of N-cadherin HA added to the alginate gels was too low to make any differences in terms of proteins or GAG production in the ASC-containing gels. Also, the N-cadherin HA concentration increase from 0.5% to 1.0% in the alginate gels might have been too slight to make any consistent and detectable results occur. There is only one paper reported that the conjugation of 1.0% N-cadherin peptides to HA hydrogels promoted both earlier chondrogenesis of MSCs and cartilage-specific matrix production with culture in comparison with unmodified controls or the insert of a scrambled peptide [52]. Addition of N-cadherin HA to alginate hydrogels might not have a substantial effect on the ASCs differentiation when co-cultured with NP cells.

Additionally, a loss of alginate gel structural integrity was noted after 14 days of co-culture, which could explain the reason that there was ECM production, such as GAG and collagen, that could not be fully detected within the gels. The ASC-containing alginate gels remained intact for around 14 days of co-culture, but started to become softer and more fragile afterwards. One possible reason could be the force applied to the gels during medium changes. Medium changes for the co-culture study were performed every other day; the frequent medium changes may have resulted in more potential damage to the gels, and therefore would negatively affect the structural integrity of the gels. Since higher viscosity alginate gel could potentially damage encapsulated cells, lower viscosity alginate was used for this study [35]. The other possible reason could be

the loss of the bivalent cation Ca^{2+} in the alginate gels after crosslinking over time with in culture. After 5-10 min crosslinking, the 0.1 M $CaCl_2$ with 10 mM HEPES was replaced with complete medium, since it is reported that long-term exposure of cells in calcium solution could result in significant loss of cell viability [55]. However, there was no calcium supplement added to the medium during 4 weeks of the co-culture study, and cells are taking up calcium for proliferation and differentiation with culture. The gradual loss of calcium supply could cause a failure in the alginate structural integrity. It is reported that elevated calcium concentration (8 mM) in extracellular environment could induce osteogenesis and inhibit chondrogenesis of hASCs compared to basal calcium concentration (1.8 mM) [56]. Since NP cells are phenotypically similar to chondrocytes, what works for ASC differentiation towards chondrocytes might work for ASC differentiation towards NP cells [7]. Therefore, for future studies, it might be beneficial to use culture medium supplemented with basal calcium (1.8 mM concentration) to maintain the alginate structural integrity and also to keep the NP differentiation potential of hASCs. Alginate is composed of (1-4) linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) units, and the relative ratio of G to M units varies depending on different species of algae [10]. During ionic crosslinking, carboxyl functional groups of the G units of adjacent chains are ionically linked to form the alginate gels. As ionic crosslinking is dependent on the number of available G units, alginate with a higher percentage of G units produces stiffer and more stable gels compared to a higher M units

alginate after crosslinking [57]. In future studies, it is highly recommended to use higher G block content alginate.

Chapter 5

Conclusions

This work indicated that ASC pellets could be induced to produce more collagen II and GAG by co-culture with NP cells. Co-culture of ASCs encapsulated in alginate gels without N-cadherin-HA with NP cell mass promoted ASCs differentiation in terms of GAG production compared to ASCs cultured in both non-inductive medium and inductive medium by day 28. ASCs encapsulated in 0.5% and 1.0% N-cadherin HA in alginate gels were induced by co-culture with NP cell mass to produce similar amount of GAG in the culture medium, and collagen II to NP cells. However, the addition of N-cadherin HA to the alginate gels and the increase of N-cadherin HA concentration from 0.5% to 1.0% in alginate gels did not show a significant effect on ASC differentiation induced by NP cells in indirect co-culture in terms of GAG production in the culture medium and collagen II production. Interestingly, indirect co-culture of ASCs encapsulated in 0.5% and 1.0% N-cadherin HA in alginate gels promoted greater total collagen production compared to ASCs encapsulated in pure alginate gels and the increase in N-cadherin HA concentration in alginate gels from 0.5% to 1.0% enhanced the total collagen production.

The NP cells showed the potential ability to induce hASC differentiation in terms of enhanced ECM production, such as collagen and GAG in an indirect co-culture system. The work also suggested that regeneration by injection of pre-differentiated ASCs into the NP could be a promising strategy for disc regeneration.

Chapter 6

Future Work

The focus of my work was exploring the differentiation of ASCs into an NP cell phenotype using an indirect co-culture system. The NP cells showed the potential ability to induce hASC differentiation in terms of enhanced ECM production, such as collagen and GAG in an indirect co-culture system. Additional characterization could be performed to more fully understand the ASC differentiation profile and investigate whether ASCs could be induced to differentiate towards an NP cell phenotype. Real time quantitative polymerase chain reaction (PCR) could be used to characterize NP cell specific gene markers, such as paired box 1 (PAX 1), forkhead box F1 (FOXF 1), which are novel NP-positive gene markers and are highly expressed in NP cells in comparison with cartilage chondrocytes, and intergrin-binding sialoprotein (IBSP) and cytokine-like 1 (CYTL1), which are novel NP-negative gene markers and are highly expressed in cartilage chondrocytes in comparison with NP cells [58].

Moreover, the isolated NP cells could be characterized by running real time quantitative PCR based on the novel NP specific genes mentioned above before performing the co-culture studies. Due to the special structure of IVD, the obtained NP tissue from IVD dissection could potentially contain some AF tissue, and therefore the isolated NP cells could potentially contain some AF cells, which are fibrotic and possess different

phenotype compared to NP cells [7]. The impurity of NP cell source could potentially affect the ASC differentiation induced by NP cells, in terms of NP specific gene expression and ECM production, such as collagen I and II, and GAG.

The adipose-derived stem cell-based therapy for IVD regeneration involves the injection of ASCs that have been pre-differentiated into an NP cell phenotype within a suitable polymer matrix into the NP portion of the degenerated discs. My work focused on the first step, which involved of performing an indirect co-culture of ASCs and allogeneic NP cells within a peptide-conjugated alginate-based gel to investigate ASC differentiation profile. Future work will be harvesting the NP-like ASCs by converting the gel to a solution, and then implantation of the NP-like ASCs into the IVD within an appropriate carrier.

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Appendix A

Staining Negative Controls

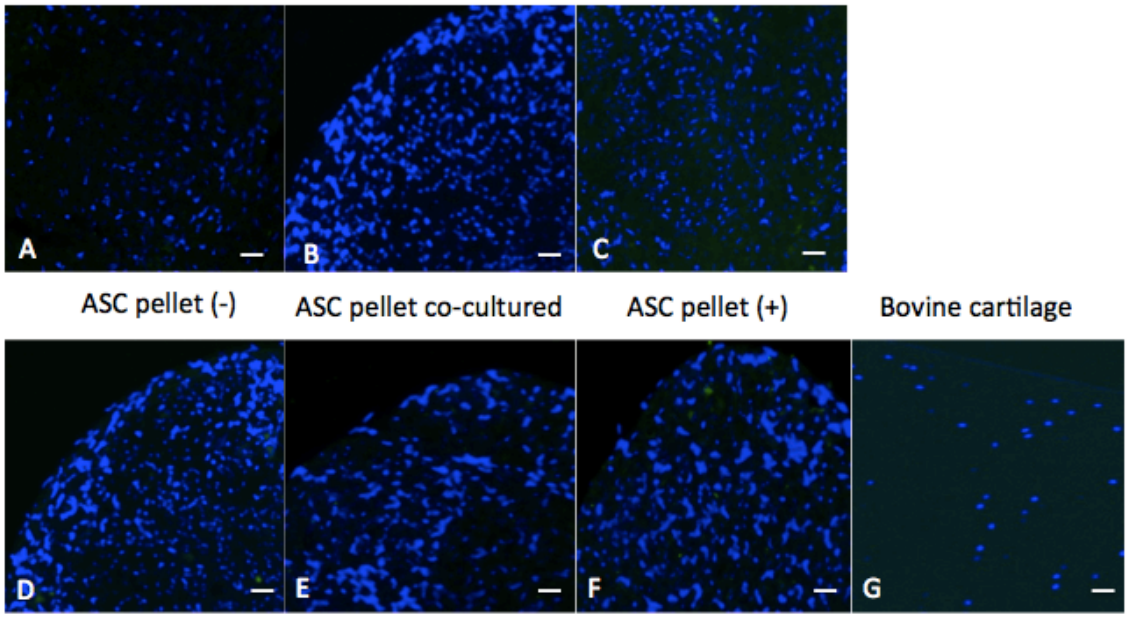

Figure 18. Negative controls of IHC staining for Collagen II (Green) with DAPI (blue) counterstaining. (A-C) cell (A-C) cell pellets cultured after 14 days. (D-F) cell pellets cultured for 28 days. (G) Bovine cartilage was used as staining positive control. ASC pellet (-) means ASC pellets cultured in non-inductive medium; ASC pellet co-cultured means ASC pellets co-cultured with NP cells in non-inductive medium; ASC pellet (+) means ASC pellets cultured in inductive medium. Scale bar = 100 µm (n=3).

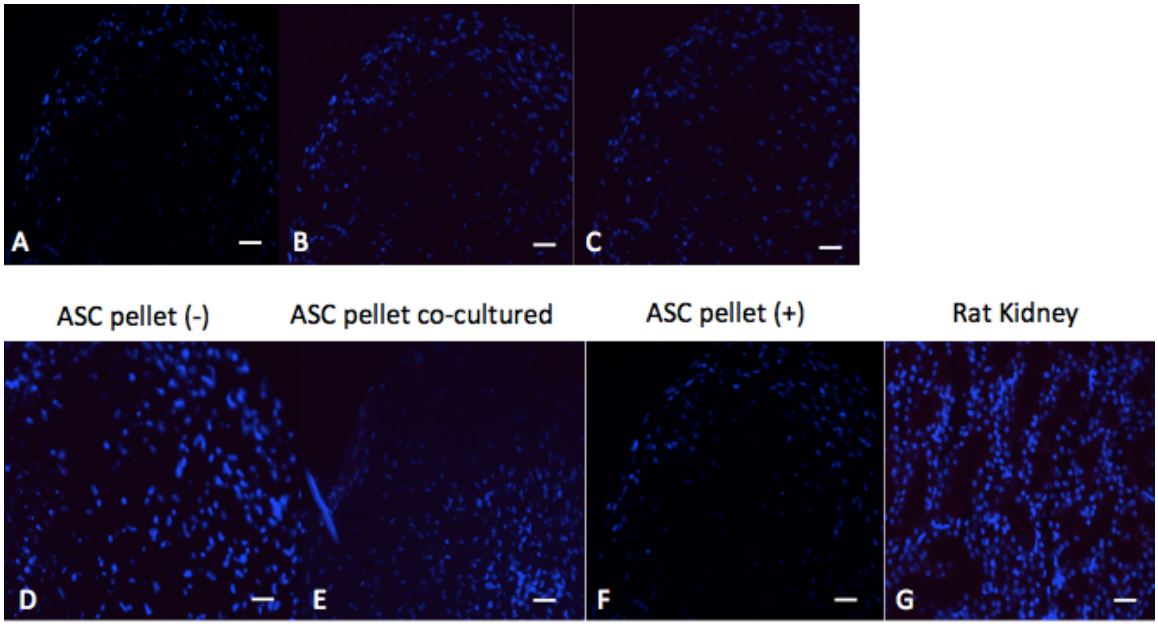

Figure 19. Negative controls of IHC staining for collagen I (red) with DAPI (blue) counterstaining. (A-C) cell pellets cultured after 14 days. (D-E) cell pellets cultured for 28 days. (G) Rat kidney tissue was used as staining positive control. ASC pellet (-) means ASC pellets cultured in non-inductive medium; ASC pellet co-cultured means ASC pellets co-cultured with NP cells in non-inductive medium; ASC pellet (+) means ASC pellets cultured in inductive medium. Scale bar = 100 µm (n=3).

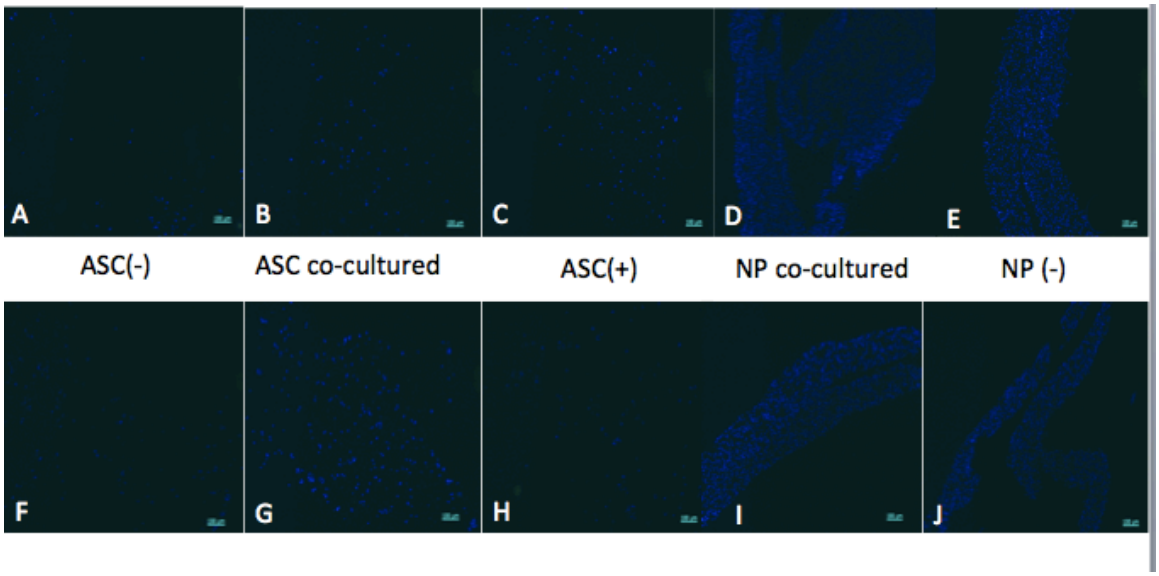

Figure 20. Negative controls of IHC staining for collagen II (green) with DAPI (blue) counterstaining. (A-E) samples cultured for 14 days. (G-K) samples cultured for 28 days. ASC (-) represents ASCs encapsulated in gels in non-inductive medium as negative control. ASC co-cultured represents ASCs encapsulated in gels co-cultured with NP cells in non-inductive medium. ASC (+) represents ASCs in gels in inductive medium as positive control. NP co-cultured represents NP cells co-cultured with ASCs. NP (-) represents NP cells cultured alone in non-inductive medium. Scale bars = 100 µm (n=3).

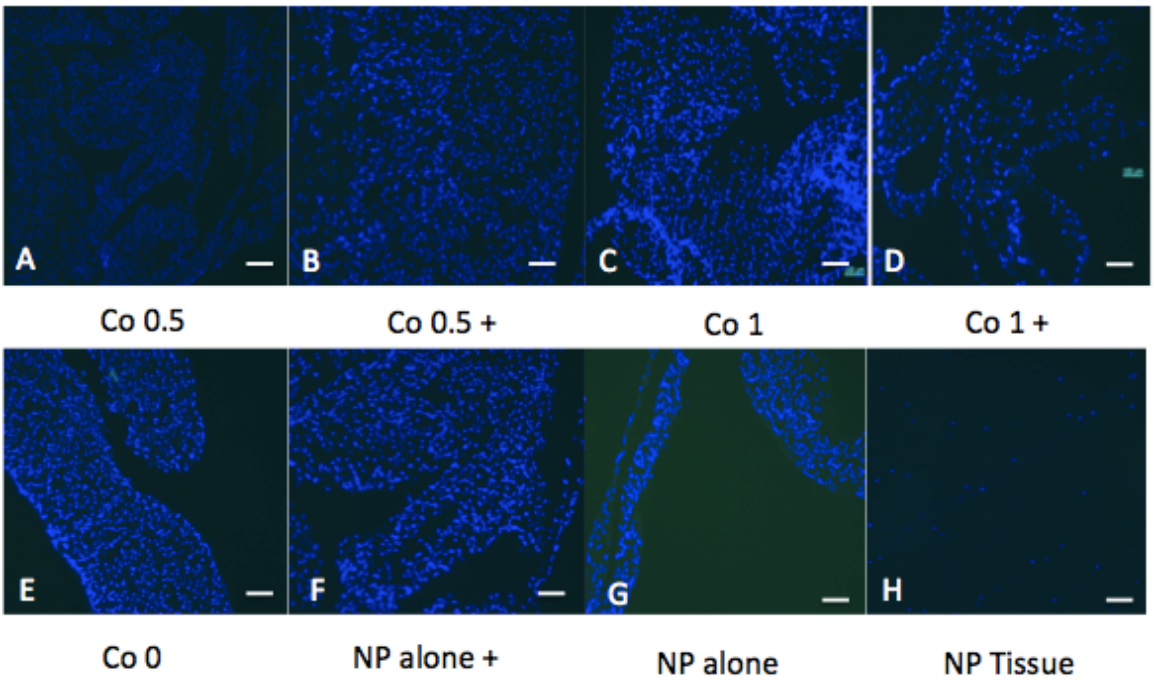

Figure 21. Negative controls of IHC staining for Collagen II (green) of ASCs in gels with DAPI counterstaining (blue) 28 days. "co 0" (A) indicates ASCs encapsulated in alginate gels without N-cadherin HA were co-cultured with NP cells in non-inductive medium. "co 0.5 +" and "co 0.5" (B, C) indicate that ASCs encapsulated in 0.5% N-cadherin HA in alginate gels were co-cultured with NP cells in either inductive "+" or non-inductive "-" medium. "co 1 + "and "co 1" (D, E) indicate that ASCs encapsulated in 1.0% N-cadherin HA in alginate gels were co-cultured with NP cells in either inductive "+" or non-inductive "-" medium. "NP alone +" (F) indicates that NP cells cultured alone in inductive medium and "NP alone" (E) means NP cells cultured alone in non-inductive medium. NP tissue (H) was used as positive staining control (n=3).

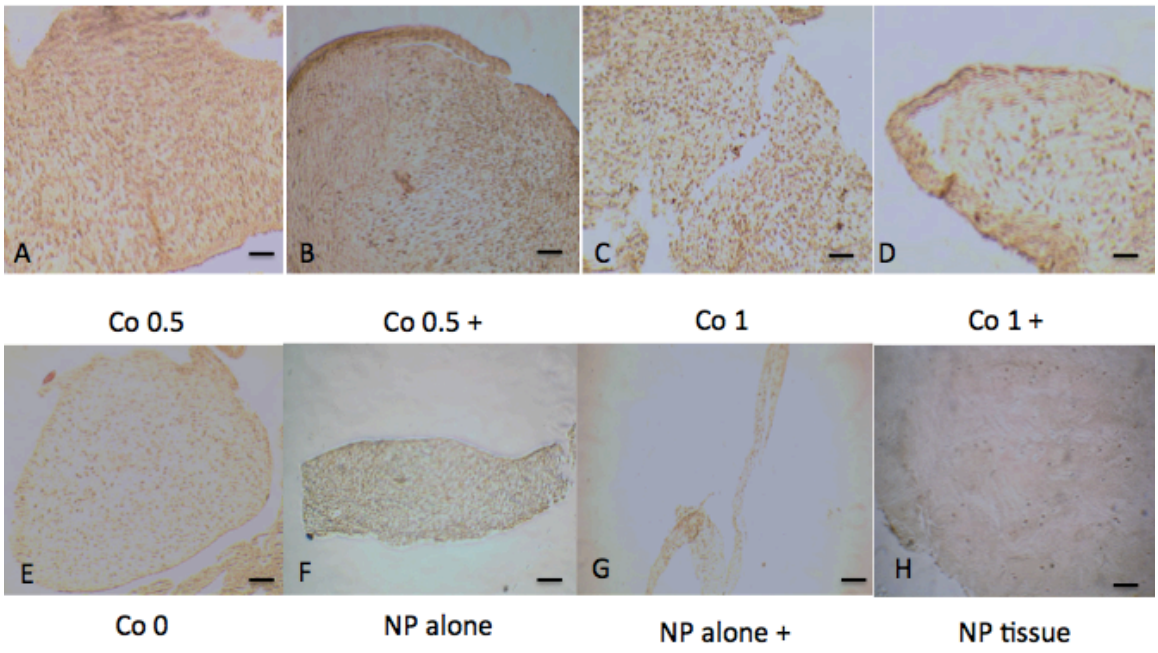

Figure 22. Negative controls of colorimetric staining for collagen I (brown) of ASCs in gels (28 days). Collagen I was stained brown and cell nuclei were stained black. "co 0" (A) indicates ASCs encapsulated in alginate gels without N-cadherin HA were co-cultured with NP cells in non-inductive medium. "co 0.5 +" (B) and "co 0.5" (C) indicate that ASCs encapsulated in 0.5% N-cadherin HA in alginate gels were co-cultured with NP cells in either inductive "+" or non-inductive "-" medium. "co 1 + " (D) and "co 1" (E) indicate that ASCs encapsulated in 1.0% N-cadherin HA in alginate gels were co-cultured with NP cells in either inductive "+" or non-inductive "-" medium. "NP alone +" (F) indicates that NP cells cultured alone in inductive medium and "NP alone" (G) means NP cells cultured alone in non-inductive medium. NP tissue (H) was used as positive staining control (n=3).