Co-Cultures of Articular Chondrocytes and Mesenchymal Stem Cells for Cartilage Tissue Engineering

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Abstract

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Articular cartilage lines the surfaces of synovial joints to protect underlying bone and provide a smooth surface for articulation. Damage to articular cartilage typically leads to long-term pain and disability, as current treatments are unable to fully restore the functional tissue. Thus, tissue engineers seek to develop technologies to enhance cartilage repair. This thesis investigated two strategies for cartilage engineering: flow perfusion bioreactor culture and co-cultures of chondrocytes with mesenchymal stem cells (MSCs). First, we designed a novel bioreactor and then investigated the effect of flow perfusion on chondrocytes when combined with chondrogenic stimuli, including hypoxia and transforming growth factor-β3 (TGF-β3). We demonstrated that the combination of flow perfusion and hypoxic conditions enhanced proliferation, cartilage-like extracellular matrix production, and chondrogenic gene expression compared to perfusion alone. However, these results also demonstrated the need for a more potent chondrogenic stimulus, and thus the effect of perfusion with TGF-β3 was investigated on both chondrocytes and co-cultures of chondrocytes and MSCs. Here, we described the advantages of using exogenous growth factors in flow perfusion cultures, and the utility
of flow perfusion for creating large tissue-engineered constructs. The second part of this thesis investigated co-cultures of chondrocytes and MSCs having the potential to reduce the demand for chondrocytes, which overcomes a significant challenge to current approaches toward cartilage repair. We first investigated the sensitivity of this cell population to TGF-β3 and then investigated the stability of the cell phenotype resulting from growth factor supplementation. The results demonstrated that co-cultures of chondrocytes and MSCs enable a reduced concentration and duration of TGF-β3 exposure to achieve an equivalent level of chondrogenesis compared to chondrocyte or MSC monocultures. Thus, the present work implicates that the promise of co-cultures for cartilage engineering is enhanced by their robust phenotype and heightened sensitivity to TGF-β3. The final section of this thesis investigated the ability of such co-cultures to repair cartilage in a rat osteochondral defect model. Here, it was demonstrated that co-cultures achieved equivalent cartilage repair compared to the chondrocytes, thus demonstrating the potential use of co-cultures of articular chondrocytes and MSCs for the \textit{in vivo} repair of cartilage defects.
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Chapter 1. Objective

The work in this thesis is divided into three specific aims.

Specific Aim 1: In the first part of this work, we aimed to design a high-throughput flow perfusion bioreactor for cartilage engineering. We then aimed to use this perfusion bioreactor to investigate the effect of flow perfusion on chondrogenic cultures. Specifically, we investigated the effect of flow perfusion in combination with hypoxia on the culture of articular chondrocytes for cartilage engineering. We then investigated the effects of transforming growth factor-β3 (TGF-β3), in combination with flow perfusion on the chondrogenic phenotype of articular chondrocytes in monoculture and in co-culture with mesenchymal stem cells (MSCs).

Specific Aim 2: In the second part of this work, and in portions of work presented in the Appendix, we investigated the use of co-cultures of articular chondrocytes and MSCs as a potential cell source for cartilage engineering. In the work presented in this thesis, we investigated the sensitivity of such co-cultures to the chondrogenic stimulus, TGF-β3, and investigated the stability of the established phenotype after the removal of the growth factor.

Specific Aim 3: In the final portion of this work, we investigated the ability of co-cultures of articular chondrocytes and MSCs to repair articular cartilage defects in vivo. Namely, we investigated the ability of the cell population seeded on electrospun polymer scaffolds to repair articular cartilage in a rat trochlear groove defect.
Chapter 2. Polymeric Nanofibers in Tissue Engineering

ABSTRACT

Polymeric nanofibers can be produced using methods such as electrospinning, phase separation, and self-assembly, and the fiber composition, diameter, alignment, degradation, and mechanical properties can be tailored to the intended application. Nanofibers possess unique advantages for tissue engineering. The small diameter closely matches that of extracellular matrix fibers, and the relatively large surface area is beneficial for cell attachment and bioactive factor loading. This review will update the reader on the aspects of nanofiber fabrication and characterization important to tissue engineering, including control of porous structure, cell infiltration, and fiber degradation. Bioactive factor loading will be discussed with specific relevance to tissue engineering. Finally, applications of polymeric nanofibers in the fields of bone, cartilage, ligament and tendon, cardiovascular, and neural tissue engineering will be reviewed.

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INTRODUCTION

Tissue engineering approaches typically involve three key elements: scaffolds, cells and biochemical and/or mechanical stimuli. Scaffolds generally serve as the foundation for many strategies to promote tissue formation. Although a wide range of scaffold materials are available, polymeric scaffolds are commonly employed to support tissue growth and to serve as carriers for bioactive factor delivery. Since polymeric nanofibers are well-suited for such applications, they are gaining popularity in tissue engineering and have been used in attempts to regenerate a variety of tissues. The popularity of nanofibers is demonstrated by the number of reviews focusing on their production [1-3], application [1, 2, 4] and interaction with cells [5]. This review discusses information not previously reviewed with regard to the fabrication of polymeric nanofibers in the context of its effect on the physical properties of nanofibrous scaffolds, relevant to tissue engineering, including fiber degradation and control of pore structure and cell infiltration. Finally, bioactive factor loading and applications of polymeric nanofibers in the fields of bone, cartilage, ligament and tendon, cardiovascular and neural tissue engineering will be presented to demonstrate the utility of nanofibers in tissue engineering.

The unique properties of polymeric nanofibers make them a valuable tool to tissue engineers. In this field, the term “nanofiber” is typically used to describe fibers with diameters ranging from 1 to 1000 nanometers [4]. The small diameter of nanofibers closely matches the size scale of extracellular matrix fibers, allowing them to be used as biomimetic scaffolds [6-8], and the high surface area to volume ratio is ideal for cell attachment [9] and drug loading [3, 10]. Compared to macroscale surfaces, nanofibers
have shown higher rates of protein adsorption, a key mediator in cell attachment to a biomaterial surface. For example, poly(L-lactic acid) (PLLA) fibers with diameters ranging from 50-500 nm were shown to have four times higher rates of protein adsorption than porous PLLA constructs with macroscale features. Additionally, the nanofibrous constructs were found to selectively enhance the adsorption of specific proteins, such as fibronectin and vitronectin [11], which is significant as fibronectin is one protein known to mediate cell adhesion and bind many growth factors [12].

Furthermore, polymeric nanofibers have been shown to display unique mechanical properties. Specifically, the tensile modulus [13-16], tensile strength [15] and shear modulus [17] have been shown to increase as fiber diameter decreases. While this occurrence is not fully understood, one explanation is that the decrease in fiber diameter leads to an increase in macromolecular chain alignment within the fibers [17-19], with nanofibers of a smaller diameter having a higher degree of crystallinity [20]. This might especially be true of electrospun fibers, where flow-induced crystallization is thought to occur during spinning [21]. These unique mechanical properties are useful for modulating cell behavior as well as providing adequate tension and strength to resist the forces from the cell cytoskeleton [15].

**Production Methods**

When producing polymeric nanofibers for tissue engineering, three production methods are commonly used: electrospinning, phase separation and self-assembly. Each technique has inherent advantages and disadvantages. Although these production
methods have been reviewed by other authors [1, 2], they will be discussed briefly here to highlight the influence each technique has on key scaffold properties.

**Electrospinning**

Electrospinning is a time and cost efficient technique to produce polymer fibers and is the most commonly used method to produce fiber meshes in tissue engineering [1, 3]. The technique is capable of producing long, continuous fibers ranging from 3 nm to 10 µm in diameter [3, 22]. The process is relatively simple, and relies on the electrostatic repulsion of a polymer solution to form polymer fibers. As shown in Figure 2.1A, the polymer solution is extruded, typically from a syringe and needle aimed at a collecting plate. Surface tension holds the polymer to the needle tip, but with the application of an electric field, a repulsive charge builds within the polymer. Once this repulsive force overcomes the attractive force of surface tension, a jet of polymer solution forms, directed towards the grounded collecting plate. The solvent evaporates from the polymer jet before it reaches the plate, creating a fibrous polymer mesh on the plate. There are several variables in this procedure that can be adjusted to control the fiber diameter. The main variables include the concentration of polymer dissolved in solution, polymer solution flow rate, magnitude of applied voltage and distance from the needle to the collecting plate [1-3]. By collecting fibers on a rotating mandrel, rather than a flat plate, the fiber orientation can be directed [23-27]. Other variations include coaxial spinning [28, 29], which can create hollow tube nanofibers or fibers with an inner core composed of one material and an outer layer composed of a second material [30], which can be used to alter the chemical [31] or mechanical [31, 32] properties of the fibers. Advantages of
this technique include the efficiency and simplicity of the procedure, the inexpensive set up and the ability to control many factors, such as the fiber diameter, orientation and composition; disadvantages include the use of organic solvents and the limited control of pore structures [1-3].

A variety of natural and synthetic polymers have been electrospun into nanofibers. Typically, synthetic polymers are considerably easier than natural polymers to electrospin [33], which is reflected by the larger number of synthetic polymers that have been electrospun into nanofibers. Natural polymers are often blended with synthetic polymers or salts to increase the solution viscosity and consistency in electrospinning [34]. For example to electrospin alginate/chitosan composite fibers, poly(ethylene oxide) (PEO) has been added to increase the chain entanglements and decrease the conductivity of the charged polysaccharide solution. PEO can be leached from the fibers with water following electrospinning [35]. Other natural polymers include gelatin [33] and collagen, which was spun with diameters as small as 100 nm and displayed structural properties similar to native collagen [6]. Synthetic polymers include poly(ε-caprolactone) (PCL) [36, 37], poly(L-lactic acid) (PLLA) [24], polyurethane (PU) [26], copolymers of poly(ethylene glycol) (PEG) and PCL [38], poly(L-lactic acid-co-ε-caprolactone) (P(LLL-CL)) [23, 39], and poly (D,L-lactic–co–glycolic acid) (PLGA) [40, 41]. Additionally, composite fibers can be created, such as chitosan-poly(vinyl alcohol) (PVA) nanofibers [42] and silk, PEO and hydroxyapatite nanoparticle composites [43]. Fibers containing a blend of collagen and elastin were electrospun with diameters ranging from 220 to 600 nm [34]. Similarly, polymer/nanotube composites can be created; for
example, poly(methyl methacrylate) (PMMA)/nanotube composite nanofibers were made in which the nanotubes assemble along the axis of the fiber, enhancing the mechanical properties of the fibers [44]. Furthermore, nanofibers made of different P(LLA-CL) compositions, ranging from 70/30 to 30/70 PLLA to PCL, exhibited tunable fiber mechanical properties [9]. These few examples illustrate the tremendous versatility of electrospinning.

**Phase Separation**

Phase separation is a method that has long been used to create porous polymer membranes and scaffolds by inducing the separation of a polymer solution into a polymer-poor phase and a polymer-rich phase [45, 46]. More recently, the method has been used to produce polymeric nanofibrous constructs from aliphatic polyesters. The matrices were shown to have up to 98.5% porosity and fiber diameters ranging from 50 to 500 nm. To produce the matrices (Fig. 2.1B) a polymer, such as PLLA, is dissolved in a solvent, such as tetrahydrofuran, and rapidly cooled to induce phase separation. Afterwards the solvent is exchanged with water, and the construct is freeze-dried. Nanofibers can be achieved by selecting the appropriate gelling temperature. Higher gelling temperatures were shown to lead to microfiber formation, but with lower gelling temperatures the diameter was reduced to nanofiber dimensions [47]. However, adjustments of the gelling temperature within the temperature range capable of creating nanofibers, was shown to not significantly affect fiber diameter [7]. Likewise it has been shown that fiber diameter is not influenced by polymer concentration [7]. Increasing the polymer concentration has been found to increase the tensile modulus and tensile strength
of the constructs, allowing the mechanical properties to be tailored to the specific application without affecting fiber diameter [7]. Variations of this technique can be used to create nanofibrous constructs with controlled and carefully designed macroporous architectures [48]. Originally the technique was demonstrated with PLLA or PLGA, but more recently the technique has been performed with polyhydroxyalkanoate [49], chitosan [47], gelatin [50] and gelatin/apatite composites [51]. The advantages of this method are that it does not require specialized equipment, and there is little variation between batches. Additionally, constructs can be produced in a mold to achieve a specific geometry. However, this process can only be performed with a limited number of polymers and would be difficult to scale-up to a commercial setting [1, 7].

**Self-Assembly**

Self-assembly is a bottom-up approach to nanofiber fabrication that relies on weak noncovalent interactions to build nanofibers from small molecules, proteins, peptides, and nucleic acids [8, 52, 53]. Several approaches have been demonstrated [8], but all rely on intermolecular forces to assemble small units into fibers with diameters of approximately 10 nm, arranged into networks with a very high water content (>99.5%) [8, 54]. The building blocks can be naturally occurring or designed for the intended application [2, 8, 54], [55]. Furthermore, this approach could be used to assemble the nanofibers *in vivo* to create an injectable scaffold for tissue repair [56]; however competition with natural amphiphiles present *in vivo* could complicate this application. While this approach creates nanofibers of the smallest scale (5-8 nm), the fabrication
process is a challenging technique, limited to a few polymers, and can only create short fibers with lengths of one to several μm [1, 57].

Peptide-amphiphiles (PAs) are a common building block for self-assembled nanofibers (Fig. 2.1C) and have been used for over a decade [58]. PAs consist of a hydrophobic aliphatic tail attached by an amide bond to a hydrophilic peptide sequence of four or more amino acids [54, 59]. Initiation of PA assembly can be controlled by adjusting the ion content of the PA solution, which results in a gel-like structure. When the electrostatic repulsion between molecules is neutralized, the peptides will spontaneously assemble into cylindrical, micelle-like structures with the hydrophobic tails clustered in the core. This structure leaves the hydrophilic regions positioned on the exterior of the fiber and available to interact with cells. The four amino acids closest to the core have been shown to be responsible for the formation of beta-sheet hydrogen bonds oriented down the z-axis of the fiber, and the disruption of these bonds will lead to the formation of a spherical nanostructure [59]. The mechanical properties of the gels can be controlled by the structure and concentration of PAs; however, the ability to control mechanical properties by modifying the interactions between fibers has also been demonstrated [60].

**Pore Structure and Cellular Infiltration**

Control of pore structure is an important aspect of scaffold fabrication, as it directly affects cell infiltration. Of the three production methods discussed, phase separation allows for the greatest control of pore structure [48]. The limited control of pore structure is a significant disadvantage of the electrospinning technique. The pore
size of electrospun scaffolds is dependent on the fiber diameter, with smaller diameter fibers leading to smaller average pore sizes, which in turn leads to decreased cellular infiltration. In some cases infiltration can be limited to a very thin layer of cells on top of the nanofibrous scaffold. This occurrence limits the potential benefits of the nanofibers for certain tissue engineering applications. The cell-nanofiber interaction is reduced to the outer regions of the scaffold, which while beneficial reduces the advantages of three-dimensional tissue culture. The importance of pore structure can be seen in comparisons of microfibrous and nanofibrous constructs. In some cases, the larger pore size of microfibrous scaffolds has been shown to promote higher levels stem cell differentiation, in addition to improved cell infiltration [61].

The importance of pore structures has led to the development of strategies aimed at increasing the pore size of the electrospun constructs, while maintaining nanoscale features. These include salt leaching techniques, where salt crystals are mixed with the fibers during fabrication and leached after spinning [62]. Similarly, researchers have induced the formation of ice crystals on the collecting plate, which leads to larger pores in the construct after melting the ice crystals [63]. A dual electrospinning setup has been created with the additional stream of polymer serving to create a sacrificial fiber that is eluted after spinning, increasing the void space in the construct [64]. While these strategies have successfully increased scaffold pore size, the mechanical strength of the constructs was reduced [62]. Recently, it was shown that by using a spherical collecting dish with metallic pegs dispersed throughout, an uncompressed, cotton ball-like mesh of nanofibers can be electrospun. These meshes have much larger pores and have shown
improved cell infiltration compared to nanofiber meshes spun onto flat collecting plates [65]. Other approaches include dispersing nanofibers in a microfiber framework. The microfibers serve to increase the pore size in the construct, while the nanofibers are dispersed to allow cell contact with nanofibers [36]. In one example, nanofibers were electrospun on top of microfibers created by a fiber bonding process. Here the MSCs were able to interact with the nanofibers, which contributed to a change in morphology compared to MSCs cultured on microfibers alone [66]. Similarly, collagen type I nanofibers have been electrospun onto starch based microfibers, which were present to provide macroscale support to the scaffold [67]. In another example, PCL nanofibers, 600 nm in diameter, were co-electrospun with fibers 5 μm in diameter and seeded with MSCs cultured in osteogenic media. Here the nanofibers did increase cell spreading but still limited the cell infiltration into the scaffold [36]. Alternatively, electrospun microfibers with nanoporous features have been created and shown to lead to increased spreading of human MSCs, compared to smooth fibers, while maintaining the same the pore size [68]. Other methods include the use of mechanical force to aid cells in infiltrating small pores. For example, flow perfusion bioreactor culture was shown to increase cell infiltration in multilayer nanofiber/microfiber scaffolds; however infiltration through the nanofiber layers was still limited [36]. Finally, alignment of nanofibers has been shown to increase cell infiltration both in vivo and in vitro, and addition of grafted collagen [69], heparin [70] and cationized gelatin [71] to the surface polymer nanofibers was shown to increase cell infiltration in vivo and in vitro. Thus, while efforts have been aimed at increasing cell infiltration in nanofibrous constructs, infiltration remains a challenge and is a major
drawback of nanofibrous constructs. These factors must be considered when such constructs are being designed for cell cultures.

**Bioactive Factor Loading**

In addition to serving as scaffolds to support cell infiltration and tissue formation, nanofibrous constructs can be designed to serve as bioactive factor delivery vehicles to induce a desired cellular or tissue response. Toward this end, nanofibrous constructs can be with loaded bioactive factors via several methods (Fig. 2.2). Regardless of the chosen method, a significant burst release is often observed, which can be undesirable in tissue engineering, but may be desirable in cases requiring rapid delivery. A burst release will provide an initial high delivery rate and a much lower release rate over an extended period of time, limiting the overall time course of drug release at effective levels [29]. In the case of polymeric nanofibers, the degree of burst release varies depending on the method of loading and the steps taken to control the delivery. For example, altering the polymer composition or coating the surface of the fibers with a polymer [72] can be performed to reduce the degree of initial burst and extend the time course of release.

Bioactive factors can be directly loaded into nanofibers produced via electrospinning. Blending the bioactive factor into the polymer solution during electrospinning has been used to load proteins [15, 40, 43, 73], small molecules [15, 74] and nucleic acids [75-77] for release. Depending on the relative properties of the polymer and bioactive factors, this method can lead to the factors being homogenously dispersed in the fibers [15], randomly dispersed in aggregates throughout the fibers [15, 73] or localized to the surface of the fibers [10, 75]. Additionally, this technique has been used
to create a controlled gradient of factors throughout the thickness of the construct to spatially control the differentiation of cells [78]. This approach is relatively straightforward, yet several disadvantages are inherent to this process. For example, a significant burst release is reported when using this approach alone [29, 72]. Consequently, approaches have been taken to alter the release profiles. Varying the polymer composition of the fibers has been shown to alter the release profile, and in some cases significantly extend the release. For example, release from poly(ethylene-co-vinyl acetate) (PEVA) microfibers or a 50/50 blend of PEVA and PLA was shown to exhibit a smoother and longer release than from PLA microfibers [79]. Additionally, coating the surface of the fibers with a polymer has been shown to delay release and reduce the degree of burst release [72]. Furthermore, while some factors could be easily blended with polymers in organic or aqueous solvents [43], others such as those that are highly charged and large, are difficult to homogenously dissolve in organic solvents typically used in electrospinning [10], and some simply cannot be electrospun using this method [40]. Additionally, phase separation between proteins and the polymer solution can occur [15, 73, 80], reducing the encapsulation efficiency [73, 80] and leading to weak, brittle fibers [15]. In some instances a surfactant can be added to prevent phase separation [80]. Additionally, the exposure of bioactive factors to organic solvents should be limited in order to prevent a reduction in activity following release [10, 80]. Thus, while the simplicity of this approach is advantageous, there are several key challenges that must be overcome for its practical implementation in sustained bioactive factor delivery for tissue engineering.
Coaxial electrospinning is one method that can be used to overcome some of these challenges. Here, two solutions are simultaneously pumped through concentric needles or cylinders and electrospun to create a fiber with a core and sheath of varied composition [29]. Typically, the two components have different solubility in aqueous and organic solvents, which prevents the mixing of the two phases during the electrospinning process. A hydrophilic core is beneficial to load factors and preserve their bioactivity as they are protected from organic solvents used to dissolve the outer polymer, while a hydrophobic sheath promotes fiber formation [28, 29]. In this case, the factors will be restricted to the central core of the fibers, creating a reservoir for factor release. Factors contained in the inner core can be released through pores in the outer sheath or over time as the sheath degrades. The core-sheath strategy has similar encapsulation efficiency as blending but reduces the burst release of the compounds and extends the time course of release [29]. Coaxial production has been shown to extend the release of basic fibroblast growth factor (bFGF) from PLGA nanofibers from one week to two weeks [40]. Similarly, a fluorescently labeled bovine serum albumin (BSA) protein loaded into a core of PEG with a PCL sheath was shown to be released continuously for more than 5 months. Furthermore, the release rate was dependent on the fiber diameter, with smaller fibers leading to faster release rates, and the system was shown to limit the burst release with an efficiency that was dependent on BSA loading [29].

Following nanofiber fabrication, proteins can be immobilized or adsorbed onto the surface of fibers. Techniques of surface modification, such as plasma or wet chemical treatment, can be used to treat the fibers prior to factor immobilization. Alternatively,
factors can be immobilized using techniques such as surface graft polymerization or chemical immobilization. A unique method of surface modification involves the treatment of the hydrophobic surface of PCL nanofibers with hydrophobins, which self-assemble on the fiber surface due to their hydrophobic regions, but leave hydrophilic regions of the protein exposed. Antibodies were then immobilized to the surface through protein-protein interactions. Some advantages of this technique are that it does not require chemical agents for cross-linking and will not compromise the mechanical integrity of the fibers [81]. Further details of immobilization and adsorption techniques have been reviewed by other authors [10] and are similar to those used with other biomaterials regardless of the structure, so the advantages and disadvantages of methods will be only briefly described here. Immobilization leads to low levels of release from nanofibers [80] until degradation of the polymer occurs. In contrast, adsorbed proteins can be released by competitive adsorption of proteins with a higher affinity for the polymer surface. While the simplicity of adsorbing proteins onto the surface of fibers can be favorable, immobilization has several advantages. The two methods were compared in a study evaluating the immobilization and adsorption of bFGF and epidermal growth factor (EGF) on PLLA nanofibers. Simply adsorbing the growth factors to the surface of the fibers was found to have a low efficiency with bFGF, and while the EGF successfully adsorbed to the surface, it was found to have little effect on human ESCs, indicating a loss of bioactivity. However, immobilization with a heparin linkage had high efficiency with both bFGF and EGF, and both growth factors appeared to maintain bioactivity [82]. Furthermore, immobilization of growth factors can lead to a different signaling effect
compared to a soluble form of the same factor, as the immobilization can reduce the endocytosis of growth factor receptors [12]. Human EGF chemically conjugated to the surface of nanofibers electrospun from copolymer PCL-PEG/PCL was found to be an effective tool in the treatment for diabetic ulcers. *In vitro* results found the immobilized EGF led to upregulation of keratinocytic genes in human primary keratinocytes, and *in vivo* results demonstrated improved wound closure in diabetic mice [38]. Finally, immobilization or adsorption could be used in combination with another approach, such as coaxial loading, to create a biphasic release of factors, which has been demonstrated with BSA protein and PCL-PEG nanofibers [80].

Self-assembled peptide nanofiber hydrogels can also be used as carriers, with release profiles dependent on the structure of the nanofiber hydrogel and factor to be released. Specifically, it has been shown that the release kinetics of molecules from self-assembled peptide hydrogels can be controlled by the hydrogel peptide concentrations. The apparent diffusivities of various dyes in such hydrogels were measured, and it was determined that the diffusivities of the dyes decreased with increasing hydrogel peptide concentrations [83]. Additional studies used single-molecule fluorescence correlation spectroscopy to determine the diffusion coefficient and release kinetics of proteins in acetyl-(Arg-Ala-Asp-Ala)$_4$-CONH$_2$[Ac-(RADA)$_4$-CONH$_2$] peptide hydrogels. Here, it was found that in addition to nanofiber density, the release depended on the size of the protein to be released. Additionally, the system was found to have an initial burst release over the first hour, which was likely due to proteins being released from the surface of the gel and through the larger pores in the construct. Finally, the secondary and tertiary
structures of the proteins were found to remain intact at the conclusion of the processing, potentially maintaining bioactivity [84]. These studies demonstrated the feasibility of using self-assembled nanofiber hydrogel networks as a reservoir for drug release and revealed key parameters governing the release.

**CHARACTERIZATION OF NANOFIBERS**

A variety of methods are used to characterize nanofibers and nanofibrous constructs after production and drug loading. Most of these methods of characterization are not unique to nanofibers; however in many cases special consideration must be taken due to the small size of the fibers.

**Morphology**

Fiber diameter, alignment and geometry are commonly determined with scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM) [40, 52]. SEM is probably the most commonly used due to availability and ease of use, but the electron beam can damage fibers with diameters less than 200 nm, reducing the accuracy. Additionally, non-conductive samples must be coated with a thin layer of a conductive metal such as gold, which can lead to questionable accuracy for very thin fibers. For these reasons, TEM or AFM are better suited for characterizing the morphology of especially small fibers [52].

The pore size of fibrous materials is a crucial parameter in tissue engineering as it directly affects the ability of cells to infiltrate the material. Pore characterization includes determination of the porosity of the construct, as well as the pore size and distribution. SEM can be used to characterize surface pore structure, but is unable to evaluate the
interior of the construct. More in depth pore characterization is commonly performed using mercury porosimetry \[2, 9, 14, 26, 36, 52\]. The technique can be complicated by nanofibrous scaffolds with small pores \[36\] or very thin samples \[52\].

**Drug and Protein Distribution**

The distribution of drugs and proteins loaded in polymer fibers can be determined by loading fluorescently tagged proteins, such as FITC-conjugated BSA, in the constructs and visualizing with fluorescent microscopy \[15, 40\] or with attenuated total reflectance-fourier transform infrared spectroscopy (ATR-FTIR) \[40, 43\].

**Molecular Structure**

X-ray diffraction (XRD) is commonly used to determine the crystal structure of polymer fibers \[15, 20, 42, 52\]. Often after chemical modification or the attachment of functional groups, the molecular structure is determined using FTIR \[42, 43, 85, 86\] or Nuclear Magnetic Resonance (NMR) analysis \[52\].

**Mechanical Characterization**

Mechanical properties are a key parameter in tissue scaffold design. The mechanical strength of a construct is essential for *in vivo* applications, where the construct often must withstand repeated mechanical loading. Mechanical characterization of the bulk nanofibrous construct can be performed using traditional methods such as tensile testing \[57\], and is commonly performed. In addition to the bulk scaffold properties, the mechanical properties of individual fibers can affect cell and tissue growth. As such, the mechanical properties of nanofibers themselves are evaluated. Specialized methods of testing individual fibers have been developed. Other authors have
provided a more in depth review of mechanical characterization of individual nanofibers [87], but the methods will be briefly described here in order to demonstrate the inherent difficulty.

Mechanical testing of individual nanofibers is complicated by their small size [57, 87]. Obtaining and handling individual fibers are just a few of the difficulties. Furthermore, specialized equipment is needed, such as a force transducer with high sensitivity and accuracy and an actuator with resolution large enough to precisely apply force [87]. Due to these complications, mechanical testing of individual fibers is rarely performed. Despite these challenges, methods for tensile testing, bending and nanoindentation of individual fibers have been reported. Several systems have been used to perform tensile testing of single fibers, most of which are custom-made. One such method uses a piezoresistive AFM cantilever to grip one end of a single fiber, which is connected on the opposite end to a movable, optical microscope stage. The stage is used to apply tensile force to the fiber, and a microscope and camera are used to observe the fiber during testing[87, 88]. A few commercial nanotensile testers have also been produced. One such system uses a cardboard frame to grip the nanofiber. The fiber is produced directly on the frame, which is then cut on both sides to allow uninhibited stretching of the fiber [87]. Three-point bending tests can be used to determine the tensile modulus and fracture strength of individual fibers [87]. Polymeric nanofibers are typically laid across a groove or hole in the testing surface and deflected with an AFM cantilever tip. Often the fibers are produced directly on the testing surface. Using beam bending theory and measuring the applied force and fiber deflection, the tensile modulus
of the fiber can be estimated [14, 87]. Nanoindentation can be used to determine the elastic modulus [87]. In these tests, an AFM probe is commonly used to indent the nanofiber, but many factors must be controlled, such as the humidity, the underlying surface and the fiber surface roughness [13].

Due to the technical difficulties of mechanical testing of individual nanofibers, models that could accurately predict the mechanical properties of fibers based on the fiber diameter, structure, material composition and processing techniques would have much utility in the field of tissue engineering. Such models are underdeveloped but would allow more knowledge to be gained from how the mechanical properties of individual fibers influence fiber-cell interactions. This knowledge would allow for more intelligent design of nanofibrous scaffolds in tissue engineering.

**Degradation Characteristics**

Degradable biomaterials are often used in tissue engineering to support the tissue during regeneration and are designed to degrade when the support is no longer necessary. In this case, the optimal degradation rate would match the rate of tissue growth. For this reason, the degradation characteristics of polymeric nanofibers are an important factor to understand for successful implementation in tissue engineering. The rate of polymer degradation depends on several parameters, including the material composition and structure.

Few studies have evaluated the influence of nanofiber structure on scaffold degradation rate. However, it has been seen that rapid degradation of nanofibrous constructs can adversely affect the ability of the scaffolds to support tissue growth, as it
was shown that cell viability on PGA scaffolds was significantly reduced after 5 days due to degradation of the nanofibrous construct [89]. Furthermore, rapidly degrading materials are generally not suitable for long-term cell culture but, depending on the specific application, may be well suited for rapid delivery of drugs.

Most studies of nanofiber degradation have emphasized the influence of material selection; however the nanofiber structure is thought to play a significant role in degradation characteristics. In one study, poly(glycolic acid) (PGA), poly(D,L-lactic acid) (PDLLA), PLLA, PLGA and PCL nanofibrous constructs were immersed in aqueous medium for six weeks. This study found that all constructs, except those composed of PLLA and PCL, were significantly degraded over the course of the study [2]. The structure of polymeric nanofibers is thought to affect their degradation in several ways. The large surface area to volume ratio of nanofibers makes them especially vulnerable to hydrolytic degradation, which could lead to an increased rate of degradation compared to microfibers [2, 90, 91]. Alternatively, an increase in crystallinity and chain orientation in small diameter fibers could lead to a reduction in the rate of degradation, as crystallinity in polymers is known to reduce the rate of degradation [89]. Finally, reduction in fiber diameter and increase in surface area increases the rate of diffusion of degradation byproducts from the fibers, which could decrease the rate of autocatalytic degradation [89-92]. It is likely that all three of these factors play a role in polymeric nanofiber degradation and should be taken into consideration when designing nanofibrous constructs for tissue engineering. Furthermore, the material composition of polymeric nanofibers should be tailored to the intended application to support tissue
growth or the release of bioactive factors over the proper time course. The following sections highlight examples of nanofibrous polymeric scaffolds developed for the engineering of specific tissues within the context of the unique requirements for each application.

**Specific Applications of Nanofibrous Polymeric Scaffolds in Tissue Engineering**

Polymeric nanofibers have been applied to many areas of tissue engineering as both cell scaffolds and carriers for bioactive factors. Although they have been used in many other fields, such as skin [93, 94] or kidney [95] tissue engineering, this section will focus on a sample of recent applications in the fields of bone, cartilage, tendon and ligament, neural and cardiovascular tissue engineering, which are summarized in Tables 2.1-5.

**Bone Tissue Engineering**

Collagen composes about 90% of the organic bone matrix, and 95% of this collagen is in the form of collagen type I fibrils. In bone, the native fibers are approximately 50 nm in diameter [96] and can be organized in aligned or irregular patterns, which lead to the distinguishing characteristics of lamellar and woven bone [97]. In bone engineering, polymeric nanofibers can be uniquely designed to approximate the size scale and fibrous nature of bone extracellular matrix.

The ability of polymeric nanofibers to serve as scaffolds for bone engineering both *in vitro* and *in vivo* has been demonstrated by several studies. Electrospun PCL scaffolds with fibers ranging from 20 nm to 5 μm in diameter have been shown to support mineralization and differentiation of bone marrow-derived rat MSCs *in vitro* [98], and
PCL scaffolds with average fiber diameter of approximately 370 nm were shown to support the adhesion and proliferation of MSCs and contribute to higher levels of alkaline phosphatase activity, mineralization and osteocalcin and osteopontin production compared to two-dimensional control surfaces [99]. The effectiveness of polymeric nanofibers in vivo was evaluated using nanofibrous PCL scaffolds seeded with MSCs and implanted in rat omenta for 4 weeks. Cells were shown to differentiate and infiltrate the scaffolds, and extracellular matrix production, including collagen type I, and mineralization were evident throughout the scaffold [100]. Furthermore, self-assembled PAs designed to promote cell binding and mineralization, have been formed by including the amino acid sequence Arg-Gly-Asp (RGD) and phosphoserine. The fibers were able to reversibly crosslink and were shown to promote mineralization with the alignment of hydroxyapatite crystals in a manner that replicates their orientation in natural bone [54].

Several approaches have been investigated in bone engineering with scaffolds composed of a combination of nano- and microfibers. The microfibers serve to increase the pore size in the construct, while the nanofibers are dispersed to allow cell contact with nanofibers [36]. In one example, nanofibers were electrospun on top of microfibers created by a fiber bonding process. The presence of nanofibers was shown to contribute to MSCs having a stretched morphology compared to microfibers alone, which resulted in a more rounded morphology [66]. In another example, PCL nanofibers, 600 nm in diameter, were electrospun with fibers 5 μm in diameter and seeded with MSCs cultured in osteogenic media. Here the nanofibers were found to limit the cell infiltration into the scaffold, and while the presence of nanofibers did not enhance cell attachment, it did
enhance cell spreading. The degree of cell spreading could affect the cell proliferation and differentiation [36].

Composite fibers containing osteoinductive factors have also been created. Fibers consisting of hydroxyapatite, collagen and chitosan, have been electrospun to create scaffolds with a mean fiber diameter of 180 nm and were shown to enhance osteoblast activity in vitro [101]. Similarly, composite nanofibers were electrospun containing silk, PEO, hydroxyapatite nanoparticles and BMP-2. The silk/PEO fibers were shown to support the osteogenic differentiation of hMSCs, and the presence of BMP-2 and hydroxyapatite nanoparticles were shown to greatly enhance bone formation in vitro as determined by calcium content and the transcription of bone-specific markers [43].

**Cartilage Tissue Engineering**

Articular cartilage lines the surfaces of articulating joints to provide lubrication and protect the underlying bone. The tissue consists of chondrocytes, collagen (primarily type II), proteoglycans and water. Much of the collagen is in the form of fibrils, which form a mesh structure, that provide the tensile properties of the tissue and trap other molecules in the network [102]. This fibrous structure of articular cartilage makes nanofibrous scaffolds an ideal option for engineering articular cartilage. For this reason, many researchers have investigated the influence of nanofibers in cartilage tissue engineering.

As the primary cell in cartilage tissue, chondrocytes are a common cell source in cartilage engineering; however because these cells are present in low numbers in native cartilage, cell expansion is typically required. This poses a challenge, as maintaining the
chondrocytic phenotype is difficult when culturing chondrocytes in vitro [103]. When expanded in monolayer culture, chondrocytes will dedifferentiate and halt the production of key molecules such as cartilage proteoglycans and collagen type II [104]. Several methods have been used to prevent the dedifferentiation and to promote the redifferentiation of chondrocytes, including the use of nanofibrous scaffolds. PCL nanofibrous scaffolds with an average diameter of 700 nm were evaluated for their ability to support chondrocyte expansion. Fetal bovine chondrocytes cultured in chondrogenic growth media on nanofibrous scaffolds proliferated and expressed higher levels of cartilage-associated genes compared to controls cultured on tissue culture polystyrene [103]. Additional studies compared the influence of fiber diameter on chondrocyte morphology. Primary bovine chondrocytes were seeded on PLLA electrospun scaffolds. Microfiber scaffolds contained fibers 15 μm in diameter, while the fiber diameters ranged from 500 nm to 900 nm in the nanofiber scaffolds. Both scaffolds supported cell proliferation, however a higher proliferation rate was seen in the nanofibrous scaffolds. Furthermore, the cell morphology varied between the scaffold types. Cells grown on microfibers appeared well-spread, while cells on nanofibrous scaffolds had higher rates of proliferation and maintained a rounded morphology, which is characteristic of the chondrocyte phenotype [105]. Similarly, self-assembled peptide hydrogels have also been shown to maintain the chondrocytic phenotype and promote chondrogenic ECM deposition. Bovine chondrocytes suspended in self-assembled peptide hydrogels were shown to maintain a rounded morphology and produce cartilage-like ECM with enhanced mechanical properties [106]. Additionally, the ability of chondrocytes to infiltrate
nanofiber scaffolds has been demonstrated in vitro with bovine articular chondrocytes seeded on PCL meshes with fiber diameters ranging from 400 to 1400 nm [107], further supporting the potential of nanofibrous constructs as scaffolds for the support of chondrocytes in vitro or in vivo.

Nanofibrous scaffolds have also been used to support the chondrogenesis of progenitor cells. Electrospun PCL scaffolds with fiber diameters of approximately 700 nm were shown to support multilineage differentiation of bone marrow-derived human mesenchymal stem cells (hMSCs) [108]. The level of chondrogenesis on the nanofiber scaffolds was shown to be equivalent and in some cases higher than the gold standard pellet cultures. However, nanofiber scaffolds have improved mechanical properties making them an option for in vivo transplantation [37]. Implantation of hMSCs on nanofibrous PCL scaffolds in a swine model led to the formation of hyaline-like cartilage with a smooth cartilage surface. Additionally, the nanofibrous scaffolds were found to be easily fixed to the surrounding tissue with sutures and did not require a periosteal covering, which reduced the morbidity associated with the procedure [109]. While researchers have found higher levels of chondrogenesis with nanofiber scaffolds compared to microfiber scaffolds [110], it should be noted that some groups have found higher levels of chondrogenic gene expression in progenitor cells grown on microfiber scaffolds compared to nanofiber scaffolds [61]. This occurrence could be due to the larger pore sizes in microfiber constructs.
**Tendon and Ligament Tissue Engineering**

The mechanical loading of tendons and ligaments is restricted to one direction. For this reason, there is a high degree of ECM fiber alignment that leads to highly anisotropic mechanical properties. The tensile properties of the tissues can be 200 to 500 times higher in the direction of fiber alignment than in the opposite direction [111]. Consequently, cells, commonly MSCs and fibroblasts [27], are often cultured on aligned fibers to engineer a similarly anisotropic structure.

Aligned nanofibers have been shown to be a promising scaffold for the engineering of ligaments and tendons, as the structure mimics the anisotropy if the native tissue [26]. Human tendon progenitor cells seeded onto aligned PLLA nanofibers oriented themselves along the direction of the fibers and expressed higher levels of tendon specific genes than cells seeded on randomly oriented fibers [27].

Braided fabrics are commonly used as scaffolds for ligament and tendon engineering; however these constructs have poor mass transfer, cell seeding, cell infiltration and mechanical strength. Knitted microfibers have been more effective for this application, yet cell seeding is complicated. As an alternative, knitted microfibers were used to provide mechanical strength, and nanofibers were added to increase the surface area for cell attachment [41]. PLGA nanofibers were spun on top of a PLGA microfiber scaffold and shown to promote cell seeding, proliferation, and function for use as a scaffold for the engineering of ligaments and tendons [41].

**Neural Tissue Engineering**

In the field of neural tissue engineering, a significant effort is placed on developing effective neural guidance conduits aimed at bridging gaps in damaged
peripheral or central neurons. These conduits are implanted into the tissue with the role of directing axonal sprouting, preventing the growth of fibrous tissue into the defect and promoting the diffusion of neurotrophic factors. Nanofibers are well suited for this application as their structure not only mimics the fibrous components of the neural ECM, but can be used to direct axon sprouting and to deliver neurotrophic factors to the site of injury [112].

Several properties of nanofiber scaffolds have been shown to affect cell proliferation and differentiation. The arrangement of the nanofibers has been shown to influence the growth patterns of neural stem cells, with neural stem cell elongation and neurite outgrowth in the direction of the aligned fibers [24], possibly due to the alignment of fibronectin networks [113]. Human ESCs cultured on aligned PLLA nanofibers displayed enhanced axon growth compared to those cultured on randomly oriented fibers [82]. Furthermore, the diameter of fibers has been shown to influence neural stem cell behavior, as smaller diameter fibers were shown to increase the rates of proliferation and differentiation [24, 114]. The conductivity of nanofibers can also be controlled, and conductive nanofibrous scaffolds with average fiber diameters ranging from 112 to 189 nm were electrospun from polyaniline, PCL and gelatin. Electrical stimulation was shown to lead to increased neural stem cell proliferation and neurite outgrowth for cells cultured on such constructs [85].

Other work has shown that self-assembled PAs with an epitope known to promote neurite sprouting and growth can be used to encapsulate neural progenitor cells. The cells encapsulated in the nanofiber network were found to more rapidly differentiate into
neurons, compared to controls [115]. Furthermore, growth factors have been used in combination with nanofibers. Immobilizing EGF and bFGF onto the surface of PLLA fibers was shown to significantly enhance the axon growth [82].

**Cardiovascular Tissue Engineering**

In cardiac tissue the ECM causes cardiomyocytes to form into fiber-like cell bundles. These fibrous bundles elongate and align allowing mechanical coupling of adjacent fibrils [116]. A polymeric structure that causes cardiomyocytes to align would mimic this specific feature of the natural tissue architecture. As such, electrospun fibers of P(LLA-CL) with average diameter of 550 nm have been shown to support the attachment and proliferation of human coronary artery smooth muscle cells (SMC), and endothelial cells [23, 39]. Constructs with aligned P(LLA-CL) fibers were found to have a higher rate of SMC adhesion and proliferation compared to controls. Cytoskeletal proteins were observed to arrange parallel to the direction of the fibers, and the cells migrated along the axis of the fibers and developed a contractile phenotype, which is desired [23]. Furthermore, the positioning density of aligned nanofibers has been varied and the best results were achieved with a density of 30-50 nanofibers/mm. In this case, cardiac cells took an elongated shape and formed excitable cardiac tissue [117].

To further mimic the native tissue, several approaches have focused on altering the surface properties of nanofibers. A hydrophobin coating was used to immobilize anti-CD31 to the surface of PCL nanofibers. The processing was shown to enhance the binding of human umbilical vein endothelial cells, which shows promise as a technique for the vascularization of small diameter vascular grafts [81]. In mimicking the basal
lamina, gelatin was covalently grafted onto surface-modified, electrospun PCL constructs. The nanofibrous constructs consisted of either aligned or random fiber orientations. The gelatin grafting was shown to enhance endothelial cell spreading and proliferation and cells cultured on aligned fibers were found to align in the direction of the fibers [25]. Other approaches have included collagen directly into the electrospinning process, creating collagen blended P(LLA-CL) fibers, which were shown to promote endothelial cell spreading, attachment and viability [86]. Similarly, core/shell nanofibers have been created with gelatin in the shell to promote cell adhesion and proliferation, while poly(glycerol sebacate) was used as the core to mimic the mechanical properties of heart muscle. These fibers were shown to support the cardiogenic differentiation of MSCs, indicating the potential of the nanofibers in the repair of myocardium [31].

Self-assembling peptides have also been shown to be a promising tool for cardiovascular tissue engineers. Peptides were injected into the myocardium and were found to be able to self assemble into a nanofiber network in vivo and after doing so, enhanced the recruitment of vascular cells [56].

**Conclusion**

Polymeric nanofibers show great potential as an effective tool for tissue engineering, serving as a scaffold to support tissue growth or as a platform for the delivery of biochemical or mechanical stimuli. While the applicability of nanofibers in tissue engineering is quickly advancing, much remains to be accomplished. In the area of nanofiber production, methods to electrospin nanofibrous scaffolds with pore size and structure adequate for cell infiltration are under development. Other production methods, such as phase separation and self-assembly are not ideally suited for large scale
production, and advances in fabrication technology would facilitate their implementation in a commercial setting. While methods to mechanically characterize individual nanofibers exist, the difficulty of implementing the procedures limits their application. As such, more efficient methods of working with individual nanofibers could lead to improved nanofiber characterization. Moreover, the field would benefit from more advanced mathematical models describing the behavior and mechanical properties of individual fibers. With regard to bioactive factor delivery, many techniques have been employed to reduce the burst release observed with nanofibrous scaffolds. As mechanisms of fiber degradation and interaction with bioactive factors are further understood, greater control of factor delivery should result. Additional understanding of such principles could lead to improved modeling of bioactive factor release from nanofibers, which is currently lacking. Finally, the ability to use chemical signals to modulate cell behavior is currently more developed than the ability to use mechanical signals from scaffold architecture to achieve the same result. However, the chemical and mechanical signals will often have a synergistic relationship and distinguishing between the effects of each is a challenge. Successfully expanding the current understanding of how nanofibrous structures modulate cell behavior would enable more rigorous design of nanofibrous scaffold architecture for tissue engineering applications. Improvement in the current level of technology in these numerous areas should have substantial impact on the field of tissue engineering.
Figure 2.1: Production methods.

This figure presents schematics summarizing methods used to fabricate polymeric nanofibers in tissue engineering. (A) Electrospinning requires only a few relatively inexpensive pieces of equipment. A syringe pump is used to extrude the polymer from the syringe. A power supply is used to apply an electric charge to the needle and ground the collecting plate. (B) Phase separation does not require specialized equipment but requires several steps to produce nanofibers. The steps are summarized in the figure. The polymer (a) and solvent (b) are combined in solution (c). The solution is then rapidly cooled, which induces phase separation (d) of the polymer and solvent. Finally, the solvent is then removed (e), leaving a network of nanofibers. (C) Peptide amphiphiles are a common building block for self-assembled nanofibers. This schematic shows the structure of peptide amphiphiles, which consist of a hydrophilic peptide sequence of four or more amino acids (a) attached by an amide bond to a hydrophobic aliphatic tail (b). The peptides will assemble into cylindrical structures with the hydrophobic tails clustered in the core. This structure leaves the hydrophilic regions positioned on the exterior of the fiber. The four amino acids closest to the core (*) have been shown to be responsible for the formation of beta-sheet hydrogen bonds oriented down the z-axis of the fiber, and the disruption of these bonds will lead to the formation of a spherical nanostructure.
Figure 2.2: Bioactive factor loading.

Nanofibrous constructs can be loaded with drugs, proteins, and nucleic acids via several mechanisms. Blending during electrospinning (A) can lead to the factors being distributed in aggregates, homogeneously dispersed or oriented on the surface of the fibers. Coaxial electrospinning (B) localizes the factors to the center of the fibers. Adsorption (C) or immobilization (D) can be used to load the factors onto the nanofiber surfaces.
Table 2:1: Applications of Polymeric Nanofibers in Bone Tissue Engineering Discussed in this Review

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Fiber Composition</th>
<th>Application</th>
<th>Key Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 nm-5 μm</td>
<td>Electrospun PCL</td>
<td><em>In vitro</em> culture of rat MSCs</td>
<td>Nanofiber scaffolds supported mineralized tissue formation</td>
<td>[98]</td>
</tr>
<tr>
<td>372±179 nm</td>
<td>Electrospun PCL</td>
<td><em>In vitro</em> culture of rat MSCs</td>
<td>Compared to controls, cells grown on nanofibers displayed increased: adhesion, proliferation, ALP activity and osteocalcin and osteopontin production</td>
<td>[118]</td>
</tr>
<tr>
<td>Alternating layers of 600 nm and 5 μm</td>
<td>Electrospun PCL</td>
<td><em>In vitro</em> culture of rat MSCs</td>
<td>Nanofibers enhanced cell spreading, but limited cell infiltration</td>
<td>[36]</td>
</tr>
<tr>
<td>100 nm-5 μm</td>
<td>Electrospun PCL</td>
<td><em>In vitro</em> implantation in rat omenta for 4 weeks with rat MSCs</td>
<td>Mineral deposits, cells and ECM found throughout scaffold, which had a rigid bone-like appearance</td>
<td>[119]</td>
</tr>
<tr>
<td>180±31 nm</td>
<td>Electrospun chitosan-based hydroxyapatite composite doped with collagen</td>
<td><em>In vitro</em> culture of human fetal osteoblasts</td>
<td>Compared to controls, cells grown on construct displayed increased: proliferation, ALP activity and mineral deposits</td>
<td>[120]</td>
</tr>
<tr>
<td>520±55 nm</td>
<td>Electrospun composite fibers containing silk, PEO, hydroxyapatite and BMP-2</td>
<td><em>In vitro</em> culture of human MSCs</td>
<td>BMP-2 and hydroxyapatite greatly enhanced bone formation</td>
<td>[121]</td>
</tr>
<tr>
<td>400 nm</td>
<td>Electrospun starch/PCL nanofibers on top of fiber bonded microfibers</td>
<td><em>In vitro</em> culture of SaOs-2 cells and rat MSCs</td>
<td>Compared to microfiber controls, nanofibers led to: stretched morphology in cells, higher viability and increased ALP activity</td>
<td>[122]</td>
</tr>
<tr>
<td>7.6±1 nm</td>
<td>Self-assembled PA with RGD amino acid sequence and phosphoserine</td>
<td><em>In vitro</em> mineralization (acellular)</td>
<td>Promoted mineralization and alignment of hydroxyapatite crystals, whose orientation replicated natural bone</td>
<td>[123]</td>
</tr>
</tbody>
</table>
Table 2.2: Applications of Polymeric Nanofibers in Cartilage Tissue Engineering Discussed in this Review

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Fiber Composition</th>
<th>Application</th>
<th>Key Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>700 nm</td>
<td>Electrospun PCL</td>
<td><em>In vitro</em> culture of bovine chondrocytes</td>
<td>Higher levels of chondrogenic gene expression on nanofibers compared to tissue culture polystyrene</td>
<td>[124]</td>
</tr>
<tr>
<td>500-900 nm</td>
<td>Electrospun PLLA</td>
<td><em>In vitro</em> culture of bovine chondrocytes</td>
<td>Compared to microfibers, chondrocytes on nanofibers: maintained rounded cell morphology and displayed higher proliferation rate</td>
<td>[105]</td>
</tr>
<tr>
<td>Not reported</td>
<td>Self-assembled SA PA KLD-12 hydrogel</td>
<td><em>In vitro</em> culture of bovine chondrocytes</td>
<td>Chondrocytes maintained rounded morphology. Produced cartilage-like ECM with enhanced mechanical properties</td>
<td>[125]</td>
</tr>
<tr>
<td>400-1400 nm 300 nm–20 μm</td>
<td>Electrospun PCL and starch-compound PCL</td>
<td><em>In vitro</em> culture of bovine chondrocytes</td>
<td>Nanofibers supported cartilage ECM production. Cells colonized both scaffolds with some migration into the interior</td>
<td>[126]</td>
</tr>
<tr>
<td>700 nm</td>
<td>Electrospun PCL</td>
<td><em>In vitro</em> culture of human MSCs</td>
<td>Nanofibers supported the multilineage differentiation of MSCs</td>
<td>[108]</td>
</tr>
<tr>
<td>492±120 nm 2796±845 nm</td>
<td>Electrospun PCL</td>
<td><em>In vitro</em> culture of human MSCs</td>
<td>Cells oriented with fibers. Could be used to create oriented tissue such as zonal organization of cartilage</td>
<td>[127]</td>
</tr>
<tr>
<td>500-900 nm</td>
<td>Electrospun PCL</td>
<td><em>In vitro</em> culture of human MSCs</td>
<td>Chondrogenesis of MSCs seeded on nanofiber scaffolds comparable to pellet culture.</td>
<td>[128]</td>
</tr>
<tr>
<td>500-900 nm</td>
<td>Electrospun PCL</td>
<td><em>In vivo</em> implantation of human MSCs in swine model</td>
<td>Led to the formation of hyaline-like cartilage with smooth surface, easily fixed to surrounding tissue</td>
<td>[129]</td>
</tr>
<tr>
<td>0.29±0.08 μm 1±0.04 μm 5±1.5 μm 9±2.0 μm</td>
<td>Electrospun PLLA</td>
<td><em>In vitro</em> culture of human MSCs</td>
<td>Chondrogenic gene expression highest in micron sized fibers</td>
<td>[130]</td>
</tr>
</tbody>
</table>
Table 2.3: Applications of Polymeric Nanofibers in Ligament and Tendon Tissue Engineering Discussed in this Review

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Fiber Composition</th>
<th>Application</th>
<th>Key Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>438±156 nm</td>
<td>Electrospun, aligned and randomly</td>
<td><em>In vitro</em> culture of meniscal fibrocartilage</td>
<td>Aligned fibers can be used to mimic the properties of musculoskeletal tissue</td>
<td>[131]</td>
</tr>
<tr>
<td></td>
<td>oriented PCL</td>
<td>cells and human MSCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>519±127 nm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>430±170 nm</td>
<td>Electrospun, aligned and randomly</td>
<td><em>In vitro</em> culture of human tendon stem cells</td>
<td>Alignment led to the upregulation of tendon specific genes</td>
<td>[132]</td>
</tr>
<tr>
<td></td>
<td>oriented PLLA</td>
<td></td>
<td>Nanofibers led to spindle shaped cells</td>
<td></td>
</tr>
<tr>
<td>450±110 nm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>657±183 nm</td>
<td>Electrospun, aligned PU</td>
<td><em>In vitro</em> culture of human ligament fibroblasts</td>
<td>Aligned fibers led to increased collagen synthesis and spindle morphology in fibroblasts</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>There was no difference in cell proliferation with fiber alignment Fibroblasts were more sensitive to strain in the longitudinal direction</td>
<td></td>
</tr>
<tr>
<td>300-900 nm</td>
<td>Electrospun PLGA nanofibers on top of</td>
<td><em>In vitro</em> culture of porcine MSCs</td>
<td>Nanofibers led to improved cell seeding and proliferation and higher levels of tendon/ligament specific gene expression</td>
<td>[133]</td>
</tr>
<tr>
<td></td>
<td>microfibers</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.4: Applications of Polymeric Nanofibers in Neural Tissue Engineering Discussed in this Review

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Fiber Composition</th>
<th>Application</th>
<th>Key Results</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| 300 nm   | Electrospun, aligned and randomly oriented PLLA        | \textit{In vitro} culture of neural stem cells | Cell differentiation was higher on nanofibers than microfibers, independent of alignment  
Cells aligned with aligned fibers, independent of fiber size                                                                                                                | [134] |
| 250 nm   |                                                        |                              |                                                                                                                                                                                                            |       |
| 800±96 nm| Electrospun, aligned poly(acrylonitrile-co-methacrylate) | \textit{In vitro} culture of Schwann cells | Fiber alignment promoted the alignment of fibronectin networks sourced from both serum and Schwann cells  
Topographically organized fibronectin networks may contribute to Schwann cell migration and neurite outgrowth                                                                 | [135] |
| Not reported | Electrospun PLLA with adsorbed or heparin immobilized bFGF or EGF | \textit{In vitro} culture of rat neural stem cells | Immobilization of bFGF or EGF promoted axon growth  
Adsorption of bFGF and EGF was not effective in increasing axon growth                                                                                                           | [136] |
| 238±45 nm | Electrospun polyethersulfone                          | \textit{In vitro} culture of human embryonic stem cell-derived neural cells | Fiber diameter influenced cell differentiation and proliferation  
Decreased diameter led to increased proliferation and cell spreading and a lower degree of cell aggregation                                                                 | [137] |
| 749±153 nm |                                                                 |                              |                                                                                                                                                                                                            |       |
| 1452±312 nm |                                                                 |                              |                                                                                                                                                                                                            |       |
| 112-140 nm | Electrospun polyaniline, PCL and gelatin               | \textit{In vitro} culture of neural stem cells | Incorporation of polyaniline allowed for electrical stimulation of cells and enhanced cell spreading and neurite outgrowth                                                                                     | [138] |
| 5-8 nm   | Self-assembled IKVAV peptide hydrogel                  | \textit{In vitro} culture of murine neural progenitor cells | Induced rapid differentiation of cells into neurons  
Discouraged the development of astrocytes                                                                                                                                                     | [139] |
Table 2.5: Applications of Polymeric Nanofibers in Cardiovascular Tissue Engineering
Discussed in this Review

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Fiber Composition</th>
<th>Application</th>
<th>Key Results</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| 400-800 nm     | Electrospun P(LLA-CL)                                                             | *In vitro* culture of human smooth muscle and endothelial cells             | Scaffold is capable of supporting cell attachment and proliferation  
Cells maintained phenotypic shape  
Mechanical properties of fibers are comparable to human coronary artery  
Smooth muscle cells attached and migrated along the axis of aligned fibers and expressed spindle-like contractile phenotype  
Adhesion and proliferation improved compared to polymer films                                                                 | [23]   |
| 550±120 nm     |                                                                                   |                                                                            |                                                                                                                                                                                                         |        |
| 400-500 nm     | Electrospun PCL, plasma treated and covalently grafted with gelatin               | *In vitro* culture of human coronary endothelial cells                      | Gelatin enhanced cell spreading and proliferation  
Cells aligned with fibers                                                                                                                                                                                   | [140]  |
| 200 nm–1 μm    | Electrospun polymethylglutamine with controlled positioning density               | *In vitro* culture of rat cardiac cells                                     | Cardiac cells elongated and grew along nanofibers to form an excitable cardiac tissue  
Best ordering and alignment was found with fibers spaced ≤ 30 μm                                                                                                                                      | [141]  |
| 200-500 nm     |                                                                                   |                                                                            |                                                                                                                                                                                                         |        |
| 500-1500 nm    | Electrospun PCL with hydrophobin coating to immobilize anti-CD31                  | *In vitro* culture of human umbilical vein endothelial cells                | Anti-CD31 enhanced the binding of human umbilical vein endothelial cells                                                                                                                                 | [142]  |
| 400-500 nm     |                                                                                   |                                                                            |                                                                                                                                                                                                         |        |
| 100-200 nm     | Electrospun, collagen blended P(LLA-CL)                                            | *In vitro* culture of human coronary artery endothelial cells              | Collagen grafting promoted cell spreading and viability and preserved the endothelial phenotype                                                                                                          | [143]  |
| 1000±125 nm    | Electrospun core/shell fibers with poly(glycerol sebacate)/gelatin                | *In vitro* culture of rabbit cardiomyocytes and MSCs                       | Gelatin promoted cell adhesion and proliferation  
Poly(glycerol sebacate) provided mechanical support  
The scaffold supported MSC differentiation into cardiomyocytes                                                                                                                                             | [144]  |
| Not reported   | Self-assembled RAD16-II peptide hydrogel                                         | Injected into mouse left ventricle                                         | Recruited endogenous endothelial and smooth muscle cells  
Exogenously injected cells survived in the nanofiber matrix                                                                                                                                              | [56]   |
Chapter 3. Design of a High Throughput Flow Perfusion Bioreactor System for Tissue Engineering

ABSTRACT

Flow perfusion culture is used in many areas of tissue engineering and offers several key advantages. However, one challenge to these cultures is the relatively low-throughput nature of perfusion bioreactors. Here, a flow perfusion bioreactor with increased throughput was designed and built for tissue engineering. This design uses an integrated medium reservoir and flow chamber in order to increase the throughput, limit the volume of medium required to operate the system, and simplify the assembly and operation.

* This chapter was published as Dahlin, RL, Meretoja, VV, Ni, M, Kasper, FK, Mikos, AG. Design of a High-Throughput Flow Perfusion Bioreactor System for Tissue Engineering. Tissue Eng Part C. 2012;18:817-820
OVERVIEW OF FLOW PERFUSION BIOREACTORS

Flow perfusion bioreactors are commonly used in many areas of tissue engineering [145-149]. Such systems are designed to perfuse culture medium through the interconnected pores of a tissue culture scaffold. This method of culture has two primary advantages. Perfusion culture can be used to increase the rates of mass transport through the interior of the scaffold compared to the rate of diffusion alone [150] and can be used to apply mechanical stimulation, in the form of shear stress, to cells in the scaffold, in order to achieve a desired cellular response [150, 151].

There are ubiquitous challenges encountered with perfusion bioreactors, and their use with specific tissues, such as cartilage, often presents several additional unique challenges. One of the primary limitations to perfusion cultures is the relatively low throughput nature of these systems. This is primarily due to space limitations, as perfusion bioreactors require relatively large amounts of space to operate in a controlled environment. However, the time intensive assembly and operation can further limit output. Chondrogenic cultures often necessitate the use of growth factors, and with the large volumes of culture medium that are often necessary to operate perfusion bioreactors, incorporating growth factors into the culture medium can be costly. Additionally, the rabbit osteochondral defect model is one of the most widely used animal models in articular cartilage engineering [152]. Here, the critical sized defect is 3 mm in diameter [152]. Thus the ideal perfusion bioreactor for cartilage engineering would be capable of culturing scaffolds of this size but be easily adaptable to other scaffold sizes.
The objective of this work was to design a flow perfusion bioreactor for tissue engineering, while addressing some of the challenges encountered with perfusion bioreactors, primarily the low throughput nature. Additionally, due to our interest in cartilage engineering, the design considered the above-mentioned challenges specific to this field. The primary design requirement of the system was the ability to provide a consistent and controllable media flow rate through the scaffolds. Additionally, the design was intended to allow for high throughput cultures, while minimizing the volume of medium required to operate the system. The bioreactor was designed to initially culture scaffolds 3 mm in diameter but to be easily adaptable to additional scaffold sizes. Additionally, the system was designed to eliminate air bubbles before reaching the scaffolds in order to not disrupt the cultures. Finally, all parts were designed to be autoclavable and easily handled and assembled under sterile conditions.

**Design Philosophy**

In many perfusion bioreactor designs, the medium is contained within reservoirs, separate from the flow chamber where the scaffolds are perfused [150]. Here, by integrating the medium reservoir and flow chamber into one unit, the amount of medium and tubing and the number of parts required to run the system is significantly reduced. In combination with perfusing the scaffolds from top to bottom, the air bubbles are prevented from accumulating below the scaffolds and are eliminated before reaching the scaffolds and disturbing the culture. Finally, culturing multiple scaffolds per flow channel provides significant flexibility when selecting a volumetric flow rate, as the culture medium is divided between multiple samples as the scaffolds are perfused.
**DESIGN**

The bioreactor design, shown in Figure 3.1, consists of four main components. The scaffold holder, medium reservoir, and peristaltic pump and tubing.

**Scaffold Holder**

Scaffolds are contained within custom-made, autoclavable, polycarbonate scaffold holders, shown in Figure 3.2. Each scaffold holder is designed to contain 10 scaffolds 3 mm in diameter, distributed axisymmetrically in order to ensure an equal division of flow through the scaffolds. This scaffold holder can be easily adapted to fit other scaffold sizes, as shown in Figure 3.3. Samples are pressfit from the bottom of the holder and supported from below by a stainless steel 316 mesh (McMaster-Carr, Elmhurst, IL). The support mesh slides into place and is held by two tabs on the bottom of the scaffold holder. The thickness of the scaffold holder was chosen so that each individual flow channel in the holder is able to contain an appropriate volume of cell solution for seeding scaffolds. This dimension (11.5 mm) is significantly more than the entrance length required for fully developed flow at the scaffold surface [153], which is necessary to minimize entrance effects.

**Medium Reservoir Lid and Base**

The medium reservoir consists of two pieces machined from stainless steel 316. The two pieces fit together to form a base and lid. The scaffold holder is placed in between the two pieces with nitrile rubber o-rings (McMaster-Carr, Elmhurst, IL) on top and bottom. The medium reservoir is compressed together and held with stainless steel bolts and wingnuts. Medium is contained both above and below the scaffolds,
approximately 25 ml contained below the scaffolds. The system can operate with as little as 10 ml in the reservoir above the scaffolds, but can store as much as 120 ml, bringing the total medium volume range in the system to 35 to 145 ml.

Medium is changed through a port of the lid that can be sealed by a silicone rubber stopper (McMaster-Carr, Elmhurst, IL). Two additional ports lead to two polypropylene, barbed, male threaded adapters (Cole Parmer, Vernon Hills, IL). One adapter leads to a syringe filter that facilitates gas exchange, and the other adapter connects to the inlet tubing. The reservoir base contains an adapter that leads to the outlet tubing.

**Peristaltic Pump and Tubing**

The inlet and outlet tubing is made of platinum-cured silicone (Masterflex tubing; Cole-Parmer, Vernon Hills, IL). Platinum-cured tubing is used to reduce the amount of leachable chemicals and is relatively low-protein binding. Furthermore, the tubing is gas permeable to oxygen and carbon dioxide. A short segment of mechanically stronger, neoprene, non-gas-permeable tubing is used within the pump (Pharmed tubing; Cole Parmer, Vernon Hills, IL). With the tubing size used in this system (Cole Parmer L/S 13) the pump is capable of accurately and consistently pumping flow rates from 0.06 to 6.0 ml/min through each flow chamber. This flow rate is divided equally between all scaffolds in one flow chamber. Each pump is capable of driving 12 flow chambers, given that the pump is a twelve-channel peristaltic pump (Cole Parmer, Vernon Hills, IL).
**Representative Results**

Scaffolds were fabricated by electrospinning poly(ε-caprolactone) meshes with an average fiber diameter of 10 μm using previously established methods [154]. Scaffolds 3 mm in diameter and 1.0-1.1 mm thick were die-punched using a 3 mm dermal biopsy punch. Bovine chondrocytes were isolated and pooled from the femoral condyle of three 7-10 day old calves (Research 87, Boylston, MA) using established methods [155, 156]. Primary chondrocytes were seeded at a density of 50,000 cells per scaffold and incubated overnight. Cultures were then continued either in the flow perfusion bioreactor or under static conditions in a 24 well culture plate. Perfusion cultures were performed with a flow rate of 0.03 ml/min through each scaffold. All constructs were cultured in chondrocyte proliferation medium [155], which consisted of DMEM, 10% FBS (Gemini Bio-Products, West Sacramento, CA), 1% non-essential amino acids, 0.4 mM proline, 10 mM HEPES buffer, and 50 mg/L ascorbic acid, and penicillin, streptomycin, and fungizone. After 14 days, samples were removed from culture, rinsed in phosphate buffered saline and fixed with 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA). Samples were then soaked in 70% ethanol, embedded in Histoprep freezing medium (Fisher Scientific, Pittsburgh, PA), frozen at -20 °C, and sectioned using a cryotome (Leica Biosystems, Richmond, IL). Sections from each scaffold (n=2 scaffolds per culture condition) were cut to a thickness of 5 μm, mounted onto Superfrost Excell glass slides (Fisher Scientific, Pittsburgh, PA), and stained using Safranin O histological stain to visualize the distribution of extracellular matrix. Images were obtained using a light microscope (Zeiss Axio Imager 2; Carl Zeiss, Oberkochen, Germany) with a video camera attachment (Zeiss Axio Cam MRc5, Carl Zeiss, Oberkochen, Germany).
Representative images are shown in Figure 3.4. In all scaffolds evaluated there was a significant increase in chondrocyte extracellular matrix in perfused scaffolds, compared to static. These results demonstrate the significant enhancement that can be seen with flow perfusion culture, consistent with previous studies.9

**Design Limitations**

As with any bioreactor design, there are several limitations to this system. To begin with, while culturing multiple scaffolds per flow channel allows for high throughput cultures, all of the scaffolds within one flow chamber share a common medium reservoir and thus should be within the same experimental group. Similarly, all of the samples in a single scaffold holder must be harvested at the same timepoint. However, multiple bioreactor units can be used to incorporate additional groups and time points in one experiment. Finally, the medium flow is divided between all scaffolds in a flow chamber, so in order for the flow rate to be evenly distributed between all scaffolds, the resistance of each scaffold to fluid flow must be equal. Thus, each scaffold must have relatively uniform interior geometries and approximately equal scaffold thicknesses. Furthermore, if one or more scaffolds were damaged or shifted during culture, the flow rate through all scaffolds in the flow chamber would be affected. However, scaffolds are both pressfit into the scaffold holder and supported by the stainless steel mesh to prevent such problems. Finally, with the integrated medium reservoir and flow channel, portions of the culture medium are contained both above and below the scaffolds. It is intended that medium changes be performed from the port on the top of the reservoir, however, approximately 25 ml of medium are contained below the scaffold holder and cannot be
changed using this method, which could allow for the undesired accumulation of waste products. However, in some cases, partial medium replacements could be advantageous as they allow endogenous cytokines to remain in low levels [157]. Furthermore, this limitation can be overcome by increasing the frequency of medium changes or increasing the volume of medium contained in the top region of the medium reservoir (capable of holding 10 to 120 ml) in order to reduce the significance of the unchangable 25 ml.

CONCLUSION

In conclusion, a flow perfusion bioreactor system has been designed and built for high throughput cultures. This system contains an integrated medium reservoir and flow chamber in order to reduce the number of parts and volume of medium required. Additionally, multiple samples per flow chamber allow for increased production capacity. Furthermore, this system is able to operate with a relatively low volume of medium and has simplified assembly and operation.
Figure 3.1: A. Schematic of assembled bioreactor unit with the integrated medium reservoir and flow chamber. B. Image of assembled bioreactor unit.
Figure 3.2: Scaffold holder and stainless steel 316 support mesh.

Ten scaffolds, 3 mm in diameter are distributed axisymmetrically and pressfit into position. A support mesh slides into place to prevent the movement of scaffolds during culture.
Figure 3.3: The scaffold holder is easily adaptable to other scaffold sizes and numbers.

Shown here from left to right are scaffold holders designed to support twenty 2 mm scaffolds, ten 3 mm scaffolds, ten 5 mm scaffolds, and four 8 mm scaffolds.
Figure 3.4: Representative histological sections of electrospun poly(ε-caprolactone) scaffolds with bovine chondrocytes cultured for 14 days under (A) static and (B) flow perfusion conditions.

Images show histological sections stained with Safranin O to visualize cartilaginous matrix. Extracellular matrix proteoglycans are stained red, and representative examples are indicated with yellow arrows. Representative examples of poly(ε- caprolactone) fibers are marked with blue arrows. Bar represents 100 μm in both images. These images demonstrate the significant increase in cartilage-like matrix as a result of perfusion culture.
Chapter 4. Hypoxia and Flow Perfusion Modulate Proliferation and Gene Expression of Articular Chondrocytes on Porous Scaffolds*

ABSTRACT

The combination of reduced oxygen tension and flow perfusion bioreactor culture is investigated for its effect on the proliferation, glycosaminoglycan production, and chondrogenic gene expression of bovine articular chondrocytes on porous polymer scaffolds. It was hypothesized that the combination of such factors would more closely replicate the *in situ* environment of these cells, leading to improvements in the cell phenotype. Chondrocytes were seeded onto electrospun poly(ε-caprolactone) scaffolds and cultured in static or perfusion culture in either normoxic or hypoxic conditions for 6 days. Results demonstrated that the combination of hypoxic and perfusion culture led to an increase in chondrocyte proliferation and glycosaminoglycan production, as well as an improvement in the ratio of collagen II/I gene expression over perfusion culture alone. The results demonstrate the need to combine multiple signals *in vitro*, in order to improve tissue growth by more closely replicating the native environment of cells.

* This chapter was published as Dahlin, RL, Meretoja, VV, Ni, M, Kasper, FK, Mikos, AG. Hypoxia and Flow Perfusion Modulate Proliferation and Gene Expression of Articular Chondrocytes on Porous Scaffolds. AIChE. 2012;59:3158-66
INTRODUCTION

Articular cartilage lesions, the result of trauma or disease, have a limited healing ability and often progress to osteoarthritis [158]. Osteoarthritis was the main contributor to the 450,000 cases of total knee arthroplasty in the United States in 2004, totaling $14.6 billion in healthcare costs, and this number is expected to rise to an astounding 3,500,000 cases by 2030 [159]. There are currently several treatments that attempt to repair articular cartilage defects [158, 160]; however, these approaches suffer from inconsistency [160] and usually result in the formation of fibrocartilage, which often fails due to inferior mechanical properties [161]. Thus, improved options to treat articular cartilage defects are needed, and tissue engineering provides a potential solution to this problem. This work focuses on investigating methods for the production of tissue-engineered cartilage with emphasis on the application of flow perfusion bioreactor culture and controlled oxygen tension on chondrocyte-seeded scaffolds in vitro.

Chondrocytes are a common cell source for cartilage tissue engineering and are currently used in the clinic during autologous chondrocyte transplantation; however there are several limitations to their use [162]. Chondrocytes can be obtained from articular cartilage biopsies and expanded in vitro [161]. However, clinically the isolation of chondrocytes causes donor site morbidity [163] and thus isolation of sufficient numbers of cells, which are relatively sparse in articular cartilage [164], presents a problem. Furthermore, upon in vitro expansion, chondrocytes dedifferentiate and express a fibroblastic phenotype [165], which leads to the production of an inferior tissue engineered construct with significantly less collagen type II than native tissue [166]. For
this reason, technologies that allow the expansion of articular chondrocytes without the loss of phenotype could be instrumental to tissue engineers.

In articular cartilage engineering, a significant volume of work has focused on the application of mechanical stimulation to cells in order to enhance the cell phenotype and matrix production [167]. Several forms of mechanical stimulation have been employed, such as hydrostatic pressure, direct compression, and shear [157, 168]. Flow perfusion bioreactors are one method of applying mechanical stimulation to cells in a three-dimensional scaffold and are commonly used in many areas of tissue engineering [169, 170]. These culture systems are used to apply shear stress to cells and to enhance mass transport through the scaffold [150]. In cartilage engineering, perfusion bioreactors have primarily been shown to enhance articular chondrocyte proliferation, matrix production, and infiltration into the scaffold porosity [146, 171, 172].

In vivo, chondrocytes are continuously exposed to a gradient of hypoxic conditions [173], [174], and low oxygen conditions are thought to play a vital role in cartilage differentiation and endochondral bone development [175, 176]. For these reasons, the role of oxygen tension has been investigated in the regulation of chondroprogenitor cells. In chondrocytes, low oxygen tension has been shown to promote both the production of cartilage-specific extracellular matrix (ECM) components and restoration of the chondrocyte phenotype[177, 178], as well as the production of mechanically superior constructs compared to chondrocytes cultured in normoxic conditions [176]. Enhancements in chondrocyte matrix production on both gene and protein levels can be seen with as little as 1 day of hypoxic exposure [179].
The beneficial effects of perfusion and hypoxia in isolation have been demonstrated by others; however the combined effects of these two factors on articular chondrocytes have not previously been evaluated. In this study, it was hypothesized that hypoxia in combination with flow perfusion culture would more closely mimic the \textit{in situ} environment of chondrocytes, and thus enhance the chondrogenic phenotype of these cells as detected by the production of cartilage-like extracellular matrix and expression of chondrogenic genes.

**MATERIALS AND METHODS**

**Experimental Design**

Bovine articular chondrocytes were seeded onto electrospun poly(\(\varepsilon\)-caprolactone) (PCL) scaffolds and cultured in static or flow perfusion culture in both normoxic and hypoxic conditions in order to investigate the combined effects of perfusion culture and hypoxia. A flow perfusion bioreactor was used, the design of which has previously been described in detail [180]. Time points were taken after 2, 4, and 6 days of culture, as well as day 0 (after the overnight adhesion period, and just prior to the start of perfusion culture). At each time point, 3 bioreactor units and an equivalent number of static samples were harvested and samples were stored for biochemical analysis (n=9), real time reverse transcriptase polymerase chain reaction (RT-PCR) (n=6), and histology (n=6).

**Scaffold Fabrication and Characterization**

Nonwoven PCL microfiber mats were fabricated with an average fiber diameter of approximately 10 \(\mu\)m (9.30 \(\mu\)m \(\pm\) 1.7 \(\mu\)m, \(n=90\)) as previously described [155, 181].
Mats were electrospun with a horizontal electrospinning set up using PCL (Inherent viscosity range 1.0-1.3 dL/g in CHCl₃; Durect Corporation, Cupertino, CA) with a number-average molecular weight ($M_n$) of 71,000 ± 2,300 Da and a polydispersity index ($M_w/M_n$) of 2.2 ± 0.07, as determined by gel permeation chromatography (Phenogel Linear Column with 5-μm particles, Phenomenex, Torrance, CA; Differential Refractometer 410, Waters, Milford, MA, n=3) and a calibration curve generated from polystyrene standards (Fluka, Switzerland). An 18 wt% polymer solution in a 5:1 volume ratio of chloroform to methanol was pumped at a flow rate of 40 mL/hour through a blunt 16 G needle. The needle was charged with a voltage of 30 kV and directed towards a grounded collecting plate 33 cm from the tip of the needle. Mats were electrospun to the desired thickness, and fiber diameter and morphology were inspected using a scanning electron microscope (FEI Quanta 400 Environmental; FEI, Hillsboro, OR). Scaffolds were determined to have an average pore size of 45 μm and a porosity of 87% as measured by mercury porosimetry (Quantachrome PoreMaster; Quantachrome Instruments, Boynton Beach, FL) and gravimetric analysis, respectively, which is consistent with previous characterization of electrospun PCL scaffolds with approximately the same fiber diameter [36, 155].

Scaffolds were prepared by die-punching 3 mm diameter discs from the electrospun mats using dermal biopsy punches. Scaffolds 1.2 to 1.3 mm thick were press-fit into custom-made polycarbonate scaffold holders designed to confine the cell seeding solution and support the scaffolds during perfusion culture. The scaffolds and scaffold holders were then sterilized by exposure to ethylene oxide (Anderson Sterilizers, Haw
River, NC) for 14 hours. Scaffolds were then prewetted by soaking in a graded series of ethanol (100% to 25%) followed by three rinses in phosphate buffered saline (PBS) and incubation in general culture medium (high-glucose DMEM, 10% fetal bovine serum (FBS) (BenchMark; Gemini Bio-Products, West Sacramento, CA), penicillin/streptomycin/fungizone (PSF)) for three days [181].

Cell Isolation and Culture

Bovine articular chondrocytes were isolated from 7-10 day old calves (Research 87, Bolyston, MA) less than 24 hours after slaughter using previously established methods [155, 181]. Briefly, articular cartilage was collected from the femoral condyle, minced to 1x1x1 mm pieces, washed with PBS and digested in chondrocyte growth medium (DMEM, 10% FBS, 1% non-essential amino acids, 50 μg/mL ascorbic acid, 46 μg/mL L-proline, 20 mM HEPES, 1% PSF) containing 2 mg/mL collagenase type II (Worthington biochemical corporation, Lakewood, NJ). Digestions were incubated on a shaker table at 37°C for 16 hours. Cells were isolated from 4 legs, pooled, aliquoted and cryopreserved in freezing medium (DMEM containing 20% FBS and 10% dimethyl sulfoxide.)

Prior to use cells were thawed, removed from freezing medium, plated at 2 million cells per T-225 flask in chondrocyte growth medium, and expanded for one passage. Cells were then trypsinized using 0.05% trypsin-EDTA and counted using a hemocytometer. Chondrocytes were seeded onto each scaffold at a density of 40,000 cells/scaffold in a 30 μL cell solution, and incubated overnight for cell attachment [155].
Static cultures were removed from the scaffold cassettes and placed in ultralow attachment 24 well plates with 1 mL of chondrocyte growth medium. Dynamic cultures were performed in a flow perfusion bioreactor as previously described [180] with 50 mL of medium, 10 scaffolds per bioreactor unit, and a flow rate of 10 μL/minute through each 3 mm scaffold. Oxygen tension was controlled and monitored using a heat jacketed CO₂ incubator with oxygen sensing and control (HeraCell 150i; ThermoScientific) [182]. Hypoxic cultures were maintained at 5% O₂ throughout the duration of the culture. Normoxic cultures were maintained at 20% O₂. At the conclusion of each culture, samples were rinsed with PBS before being stored until analysis.

**Biochemical Analysis**

Samples were stored at -20°C until analysis. Thawed samples were digested in 500 μL of proteinase K digestion buffer (50 mM Tris-HCl (pH 7.6), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM iodoacetamide, 10 μg/ml pepstatin A, and 1 mg/ml proteinase K) in a water bath at 56°C for 16 hours. Following digestion, samples underwent three rapid freeze/thaw cycles (10 minutes in liquid nitrogen/ten minutes in 36 °C water bath) followed by 20 minutes of sonication to ensure complete extraction of DNA and matrix components from the scaffolds into the supernatant.

The concentration of double stranded DNA in the supernatant was quantified using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Eugene, OR) according to the manufacturer’s instructions [22, 155]. Cell lysate, assay buffer and dye solution were pipetted into an opaque 96 well plate in duplicates and fluorescence was measured using excitation and emission wavelengths of 485 nm and 528 nm (FL x800 Fluorescence
A lambda DNA standard curve was used to calculate DNA concentrations.

Sulfated glycosaminoglycans were quantified using the colorimetric dimethylmethylene blue dye (DMMB) assay and a chondroitin sulfate standard curve [155, 183]. Cell lysate and DMMB reagent were combined in a transparent 96-well plate in duplicates. Absorbance at 520 nm was measured (Powerwave x340 Microplate Reader; BioTek Instruments). These results were converted to total sulfated GAGs per scaffold and normalized to the number of cells present in each scaffold.

**RT-PCR**

After rinsing with PBS, two scaffolds were pooled together for each RNA isolate sample (n=6 samples). Scaffolds were placed in 600 μL of lysis buffer and vortexed before storing at -80 °C until further processing. RNA isolation was performed using an RNeasy mini kit (Qiagen, Valencia, CA) [155, 181]. Cell lysate was passed through a QIAshredder homogenization column and combined with an equal volume of 70% ethanol. RNA isolation was then performed following the manufacturer’s instructions for the isolation of RNA from animal cells. Reverse transcription was performed using Oligo(dT) primers (Promega, San Luis Obispo, CA) and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR (Applied Biosystems 7300 Real-Time PCR System, Foster City, CA) was performed on cDNA samples using SYBR Green detection (PerfeCTa SYBR Green FastMix, ROX; Quanta Biosciences, Gaithersburg, MD) with previously established primer sequences (Integrated DNA
Technologies, Coralville, IA) [181]. β₂-microglobulin (B2M) was used as a housekeeping gene, as it has previously been shown to be stable under hypoxic conditions [184].

Target gene expression was first normalized to the expression of the housekeeping gene B2M in the same sample (ΔCt), then to the average baseline expression of that target gene measured in the cell stock used to seed the scaffolds (ΔΔCt). The $2^{ΔΔCt}$ method was used to convert normalized gene expression levels to fold differences [185], and statistical analysis was performed on these values. Similarly, $2^{ΔCt}$ was used to calculate the ratios of collagen II / collagen I. The sequences of primers used in this analysis were [181]: Collagen type I: 5’-CGGTTCTTGCTGGTCATCAT-3’, 5’-TGCACCAGGCTGTCCAATG-3’; Collagen type II: 5’-AGTGGAGAGCGGAGACTACTG-3’; 5’-GGTGGAGCCAGGTTGTCAT-3’; Aggrecan: 5’-AGAGAGCCAAACAGCCGACA-3’; 5’-TAGTCCCTGGGCATTGTTGTTGA-3’; B2M: 5’-CCGATAGTTAAGTGGGATCG-3’; 5’-CATGGACATGTAGCACCACAGA-3’.

**Histological Analysis**

Harvested samples were fixed using 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA). Samples were dehydrated in 70% ethanol before embedding in HistoPrep freezing medium (Fisher Scientific). Scaffolds were cut into 5 µm thick sections using a cryostat (Leica CM 1850 UV; Leica Biosystems Nussloch GmbH, Germany). Sections were then stained using Safranin O and Fast Green to visualize the presence and distribution of cartilaginous matrix and cells, respectively. Images were
obtained using a light microscope with a digital camera attachment (Axio Imager.Z2 equipped with AxioCam MRc5; Carl Zeiss MicroImaging GmbH, Germany).

Statistical Analysis

Results are reported as means + standard deviation. Statistical analysis was performed using JMP 10 software package (SAS Institute, Cary, NC). One-way ANOVA and Tukey-Kramer multiple comparison tests were used to determine significant differences (p<0.05).

RESULTS AND DISCUSSION

In previous studies, flow perfusion culture and controlled oxygen tension have been employed individually in order to enhance the production of cartilage-like ECM and maintain cell phenotype [178, 186]. Here, it was hypothesized that the combination of flow-induced shear stress and hypoxia, external stimuli that are normally encountered by chondrocytes in the physiological environment, would further enhance cartilage-like ECM production and promote the chondrocyte phenotype of cells cultured in vitro. In order to test this hypothesis, the effects of flow perfusion and hypoxia were investigated individually and in combination on the ECM deposition and gene expression profiles of bovine chondrocytes in vitro.

The first advantage that was obtained by the combination of hypoxia and perfusion cultures was observed in the DNA content of the scaffolds. As seen in Figure 3.1, the amount of DNA per scaffold had increased from the initial value after two days in culture for each experimental group, with perfusion having an additional positive effect over static samples at all time points. The amount of DNA in each group followed
an increasing trend throughout the timeframe of the study, with the level of DNA at day 6 in samples exposed to perfusion far exceeding the amount of DNA in those samples cultured under static conditions. The combination of hypoxia and perfusion demonstrated its beneficial effects on cellular proliferation as the highest level of DNA at day 6 occurred in Hypoxia Perfusion. As observed by others, the increased proliferation in perfusion culture could be the result of improved mass transport into the interior of the scaffold, the application of shear stress to the cells, or a combination of both factors [187-189].

Here, in static cultures hypoxia had no effect on the cellularity of the constructs, which is consistent with previous studies [173, 190]; however in perfusion culture, hypoxia led to a significant increase in cellularity over all other groups at day 6, demonstrating a potential benefit of combined hypoxic and perfusion cultures. Although the mechanism for this effect is not understood, it has previously been demonstrated with other cell types, as hypoxia and mechanical stimulation can have a synergistic effect on proliferation of bladder smooth muscle cells [191].

Furthermore, the quantity of GAGs in each scaffold increased from day 2 to day 6 although only slight changes were observed by day 4. As seen in Figure 4.2a the amount of GAGs at day 6 in samples exposed to perfusion exceeded the amount of GAGs in samples cultured under static conditions, and the highest level of GAGs at day 6 occurred in Hypoxia Perfusion. Normalizing the total GAG content to DNA content in the scaffolds can provide additional information about the GAG synthesis. The ratio of the amount of GAG to DNA is regarded as a measure of the ECM production of a cell population on a cell equivalent basis; however, particularly in perfusion culture, this
measure can be confounded by the loss of GAGs from the construct [192]. As shown in Figure 4.2b, in this study, GAG/DNA at day 2 was higher for samples cultured under static conditions compared to perfusion conditions. This difference could be the result of a high percentage of the GAGs being washed out of scaffolds in perfusion, particularly at the early stages of culture, when only low levels of ECM are present to increase the GAG retention [192] or a result of the increased proliferation in perfusion compared to static by day 2. A decrease in GAG/DNA was observed in all groups, from day 2 to day 4, followed by an increase from day 4 to day 6. This trend, along with the DNA content of the scaffolds, seems to indicate a period of proliferation, followed by GAG synthesis over the course of the study. Normoxia Static saw the highest level of GAG/DNA at day 6 compared to all other groups, including Hypoxia Static. Although the GAG/DNA was lower in perfusion culture at day 6 compared to Normoxia Static, this could be a result of the high levels of proliferation in those cultures, and the overall result for perfusion in general was a construct with much higher levels of cartilage ECM. However, this may indicate that the increased levels of GAGs in the perfusion scaffolds is not a result of increased GAG synthesis per cell, rather an increase in cell number.

Analysis of the histological sections, shown in Figure 4.3, corroborated the results of the biochemical assays. In Figures 4.3a and 4.3b sections are stained with Fast Green and Safranin O, respectively. The images reveal the same trend as the biochemical assays, as it is evident that perfusion cultures led to higher amounts of cells and cartilaginous matrix. Perfusion cultures typically lead to improved cellular infiltration compared to static cultures, due to the improved mass transport into the interior of the
construct [150]. In this study, the quantity of cells was significantly improved in perfusion, yet the cellular infiltration did not appear to be significantly affected. However, if this culture was conducted over a longer period of time, a more noticeable improvement in cellular infiltration may have been observed in perfusion cultures, as more ECM would be produced, potentially limiting the transport of nutrients into the interior of the static scaffolds.

The gene expression was quantified relative to a baseline value, which was established as the gene expression level of the cell population at the beginning of the study [155, 181]. As shown in Figure 4.4a, the expression of collagen I in static cultures did not change from day 2 to day 6. In perfusion, the collagen I expression showed a statistically significant increase from day 2 to day 4, but did not change from day 4 to 6. Comparing perfusion to static cultures, static cultures had higher levels of collagen I expression at day 2; however by day 4 there was no statistical difference between the groups. These results demonstrate a delay in the upregulation of collagen I gene expression in perfusion cultures, which is beneficial as collagen I expression is indicative of the dedifferentiation of chondrocytes into a fibroblastic phenotype, a transition that normally occurs very early in the cell expansion process. The subsequent fibrocartilage ECM produced by these cells is inferior to the normal articular cartilage and therefore not the desired type in cartilage tissue engineering [193]. By day 6, Hypoxia Static demonstrated an increased level of collagen I expression relative to Hypoxia Perfusion and Normoxia Static.
In Figure 4.4b, comparing the expression of collagen II at day 2 and day 6 showed a decrease in expression for all groups, except Hypoxia Perfusion. Collagen II expression was higher in static samples at day 2 and 4 compared to perfusion samples. At day 6, Hypoxia Static showed the highest level of expression. Normoxia Static showed a higher level than Normoxia Perfusion, however it was not statistically higher than Hypoxia Perfusion. These results indicate that by itself, perfusion culture led to a decrease in collagen II expression below the levels of static cultures; however hypoxia led to an increase in collagen II expression above the levels of normoxic cultures. When combined, while perfusion still leads to a decrease in collagen II expression compared to Hypoxia Static, it did not decrease the expression below the levels of Normoxia Static. The predominant type of collagen found in cartilage tissue is type II, thus high levels of collagen II expression by chondrocytes is indicative of the chondrogenic phenotype.[194] Here, perfusion led to an undesirable reduction in collagen II expression compared to static cultures; however, when combined with hypoxia, the collagen II expression is equivalent to the levels seen in static cultures, without the hypoxic treatment.

As shown in Figure 4.4c, aggrecan gene expression decreased comparing day 2 to day 6 for static cultures. Hypoxia Perfusion exhibited aggrecan expression that peaked at day 4 before returning to its day 2 value, and Normoxia Perfusion followed the same trend, although it was not statistically significant. Aggrecan expression was higher for static groups at day 2 compared to perfusion. Higher levels of aggrecan expression were observed for Hypoxia Perfusion at day 4 compared to Normoxia Static. Aggrecan expression was higher for Hypoxia Static at day 6 compared to all other conditions. It is
interesting to note that aggrecan expression peaked at day 4 for both perfusion groups; however, only the Hypoxia Perfusion was statically significant. This result is consistent with the levels of GAGs detected, as a large increase in GAG content is seen in the perfusion groups between days 4 and 6. Transient peaks in aggrecan gene expression as a result of compressive loading have previously been observed by others; however these peaks occurred over a much shorter time period [195]. The decline in gene expression from day 4 to 6 could also be attributed to the change in the shear stress experienced by the cells as a result of the change in pore structure resulting from ECM production [196].

In Figure 4.5, the collagen II/I gene expression ratio decreased in all groups from the day 0 value of 2.65, as well as from day 2 to day 6. Exposure to hypoxic conditions delayed the decrease in collagen II/I in samples exposed to perfusion at day 4, as Normoxia Perfusion exhibited a lower collagen II/I ratio compared to Normoxia Static, but at the same time point, Hypoxia Perfusion was equal to Hypoxia Static. By day 6, the collagen II/collagen I ratio in the static samples exceeded that of the perfusion samples. However, in the perfusion groups, Hypoxia Perfusion was found to have a higher collagen II/I expression than Normoxia Perfusion.

The decrease in the ratio of collagen II/I expression over the course of this study likely demonstrates a decrease in chondrogenic phenotype of the cells in these samples [197]. Multiple factors have been shown to lead to chondrocyte dedifferentiation, many of which are associated with the in vitro expansion of these cells [193]. Alternatively suboptimal levels of shear stress could lead to a reduction in chondrocyte specific gene expression compared to static cultures [157, 198]. However, using a cylindrical pore
model for the approximation of the scaffold geometry [169, 199, 200] the initial levels of shear stress used in this study were estimated to be approximately 5 mPa, which was previously found to lead to higher GAG/DNA ratios than other shear levels investigated [201], and is on the same level of shear stresses used by other investigators [202, 203]. Furthermore, the reduction in collagen I expression that was also observed in the perfusion cultures, compared to static, may be further evidence that the levels of shear stress were not too high, as chondrocytes have been shown to increase production of collagen type I in response to high shear [157]. High levels of chondrocyte proliferation have been correlated with chondrocyte dedifferentiation [193, 204, 205], as high levels of proliferation are not characteristic of differentiated chondrocytes [102], and the high levels of proliferation seen in perfusion culture may be tied to the further reduction in collagen II/I expression in the perfusion groups compared to static. Interestingly, although Hypoxia Perfusion samples saw the greatest increase in cellularity, the reduction in collagen II/I ratio was delayed, compared to the Normoxia Perfusion and although the proliferation was much higher in Hypoxia Perfusion at Day 6, the collagen II/I ratio was not lower than that of Normoxia Perfusion, demonstrating the benefit of hypoxia in perfusion culture.

Many approaches have been taken in order to prevent dedifferentiation of chondrocytes, such as the use of growth factors [206], or optimizing scaffold structure [105]. In this study, chondrocytes were cultured in FBS-containing, growth medium without any added growth factors. These conditions are likely to lead to high levels of chondrocyte proliferation, and a loss in chondrocyte phenotype, as was observed in this
study. While hypoxia in combination with flow perfusion was effective in enhancing the cell phenotype compared to perfusion alone (as observed by the ratio of collagen II/I expression), it was not sufficient to maintain the cell phenotype throughout the study, which was demonstrated by the decrease in the collagen II/I ratio over time. Future work could investigate the use of additional factors to further enhance the chondrogenic phenotype over prolonged culture times.

Hypoxia in combination with mechanical stimulation (mostly hydrostatic compression [207, 208]) has been investigated in several studies. One such study evaluated the effects of intermittent hydrostatic pressure in combination with hypoxia. Similar to the results of this study, mechanical stimulation was found to reduce type II collagen production; however in combination with hypoxia, the production was increased compared to compression alone, although not to the level of hypoxia without mechanical stimulation [208]. Similarly, it has been shown that hydrostatic pressure in combination with hypoxia can lead to increases in collagen production and enhance the phenotypic stability of chondrocytes cultured in monolayer; however, the response varied based on duration and magnitude of the loading regime [207]. Furthermore, the combined effects of such factors for chondrogenesis have been investigated with other cell types. Using human neonatal fibroblasts cultured on alginate beads, it was found that mechanical stimulation in combination with hypoxic conditions can lead to enhanced chondrogenic gene expression when combined with the addition of BMP-2 [209].

While the use of reduced oxygen tension in combination with other forms of mechanical stimulation have been evaluated, the use of flow perfusion bioreactors in
combination with controlled oxygen tension presents a unique advantage. Flow perfusion culture can be used to increase the mass transport through the pores of scaffolds [169], which includes the transport of oxygen and nutrients into the scaffold, as well as the transport of waste products out of the scaffold. Improved transport into the scaffold, in some cases may lead to higher levels of oxygen compared to static cultures, which in the case of chondrogenic cultures may not be beneficial and could contribute to the reduction in chondrogenic gene expression that was observed in perfusion culture. Thus, controlled oxygen tension in combination with flow perfusion can optimize the level of oxygen delivered to the cells, while still benefiting from the improved transport of other nutrients into and waste products out of the scaffold. Additionally, since static cultures rely on diffusion alone for the transport of oxygen, flow perfusion bioreactors are potentially a unique tool to study the effects of controlled oxygen tension in three-dimensional scaffolds, as they allow one to homogeneously control the level of oxygen in the scaffold.

In this study, flow perfusion culture in normoxic conditions led to similar effects reported by many in the literature, namely increased cellular proliferation, and extracellular matrix production [186, 188, 201], as well as a delay in the increase in collagen I expression compared to static cultures; however a reduction in the collagen II/I expression ratio was also observed. The addition of hypoxia to the perfusion culture led to significant increases in cellularity and GAG content of the scaffolds above the levels of perfusion alone. Additionally, the ratio of collagen II/I expression was increased in cells exposed to both hypoxia and perfusion compared to only perfusion. While others have investigated the effects of hypoxia combined with other factors, such as growth factors
these are the first results to demonstrate the benefits of hypoxia when combined with flow perfusion culture. As this study was the first to evaluate the combination of these factors on articular chondrocytes, and to demonstrate the beneficial effects of hypoxia and flow perfusion culture, there are several areas that could be further investigated. While this study quantified the expression of genes associated with the chondrogenic phenotype, its corresponding protein production was not evaluated. Future studies could evaluate the production of proteins, such as collagen type II. Additionally, this study employed cells from young calves. Adult human chondrocytes may exhibit a different response from the animals observed here, so future studies could evaluate the effect of these factors in human chondrocytes. Furthermore, the mechanism behind the combined effects of hypoxia and flow perfusion was not investigated in this study. Low oxygen tension can lead to a variety of responses on systemic and cellular levels, and the mechanism of these effects is often attributed to the activation of the HIF-1α transcription factor. Future studies could investigate interactions between cell signaling pathways activated by mechanoreceptors and the HIF-1α transcription factor. Finally, this study was conducted over a relatively short period of culture, as chondrocytes are thought to be more receptive to mechanical stimulation at early times, prior to the development of a pericellular matrix. Future studies could be conducted to evaluate the effects over a much longer time frame.

**CONCLUSION**

In the present work, the effect of hypoxia in combination with flow perfusion culture was investigated for its effect on the proliferation, GAG production, and
chondrogenic gene expression in bovine articular chondrocytes. Here, it was concluded that flow perfusion culture led to an enhancement in chondrocyte proliferation and GAG production, and while compared to static cultures, it delayed the increase in collagen I gene expression, it also led to a reduction in collagen II and the ratio of collagen II/I expression. However, when combined with hypoxia, flow perfusion cultures had even higher levels of chondrocyte proliferation and GAG production along with higher collagen II/I ratios compared to perfusion in normoxic conditions after 6 days. This work demonstrates the benefits of both hypoxic and perfusion cultures, particularly in combination, and the need to combine multiple signals in vitro in order to more closely replicate the in situ environment of these cells. These effects could be leveraged by tissue engineers in order to achieve more robust in vitro methods of cartilage tissue fabrication.
**Figures**

Figure 4.1: DNA content of scaffolds cultured in each condition after 2, 4, and 6 days of culture.

DNA content after the 24 h adhesion period (day 0) is represented by a dashed line. Data are presented as mean ± standard deviation. * represents a statistically significant difference from all other groups at the same time point that are not marked (p < 0.05). # represents a statistically significant difference from the previous time point in the same group (p < 0.05), and ~ represents a statistically significant difference from the day 2 time point in the same group (p < 0.05). + represents a statistically significant difference between the groups indicated (p < 0.05).
Figure 4.2: (a) GAG content per scaffold, and (b) µg of GAG normalized to µg DNA in each scaffold in each culture condition after 2, 4, and 6 days of culture.

Data are presented as mean ± standard deviation. * represents a statistically significant difference from all other groups at the same time point that are not marked (p < 0.05). # represents a statistically significant difference from the previous time point in the same group (p < 0.05). ∼ represents a statically significant difference from the day 2 time point in the same group (p < 0.05). + represents a statically significant difference between the groups indicated (p < 0.05).
Figure 4.3: Representative histological sections of day 6 samples cultured in a flow perfusion bioreactor under normoxic conditions (NP), in a flow perfusion bioreactor under hypoxic conditions (HP), in static culture in normoxic conditions (NS), and in static under hypoxic conditions (HS).

Regions pictured in the magnified images (ii) are indicated with black rectangles in the corresponding image (i). Electrospun PCL scaffolds (representative examples of PCL fibers are indicated with black arrows) are stained with (a) fast green to visualize the distribution of cells (representative examples indicated with red arrows), and (b) Safranin O to visualize the distribution of cartilaginous matrix (representative examples indicated with blue arrows). Scale bars represent 200 μm and 50 μm in (i) and (ii) images, respectively.
Figure 4.4: Quantitative gene expression of (a) collagen type I, (b) collagen type II, and (c) aggrecan for samples (n = 6) cultured in each condition after 2, 4, and 6 days of culture.

Data are presented as mean ± standard deviation. * represents a statistically significant difference from all other groups at the same time point that are not marked (p < 0.05). # represents a statistically significant difference from the previous time point in the same group (p < 0.05). ^ represents a statically significant difference from the day 2 time point in the same group (p < 0.05). + represents a statically significant difference between the groups indicated (p < 0.05).
Figure 4.5: The ratio of collagen II to collagen I expression for samples cultured in each condition after 2, 4, and 6 days of culture.

Data are presented as mean ± standard deviation. * represents a statistically significant difference from all other groups at the same time point that are not marked (p < 0.05). # represents a statistically significant difference from the previous time point in the same group (p < 0.05). ^ represents a statically significant difference from the day 2 time point in the same group (p < 0.05). + represents a statically significant difference between the groups indicated (p < 0.05).
Chapter 5. Chondrogenic Phenotype of Articular Chondrocytes in Monoculture and Co-Culture with Mesenchymal Stem Cells in Flow Perfusion

Abstract

This work investigated the effect of flow perfusion bioreactor culture with and without TGF-β3 supplementation on the proliferation, extracellular matrix (ECM) production, and chondrogenic gene expression of chondrocytes both in monoculture and in co-culture with bone marrow-derived mesenchymal stem cells. Both cell populations were cultured on electrospun poly(ε-caprolactone) scaffolds for two weeks in static or flow perfusion culture with and without TGF-β3. Overall, it was observed that without growth factors, flow perfusion culture led to increased cell proliferation and ECM with a more cartilage-like composition. While with TGF-β3 induction, flow perfusion constructs generally had lower chondrogenic gene expression than the corresponding static cultures, the growth factor still had an inductive effect on the cells with enhanced gene expression compared to the corresponding non-induced cultures. Additionally, while flow perfusion cultures generally had reduced overall ECM content, the ECM distribution was more homogenous compared to the corresponding static cultures. These results are significant in that they indicate that while flow perfusion culture has some beneficial effects on the chondrogenic phenotype of articular chondrocytes, flow perfusion alone is not sufficient to maintain the chondrogenic phenotype of chondrocytes in either monoculture or co-culture, thus demonstrating the advantages of using exogenously added growth factors in

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flow perfusion culture. Furthermore, the results demonstrate the advantages of flow perfusion culture for the creation of large tissue engineered constructs and the potential of co-cultures of articular chondrocytes and MSCs to be used in flow perfusion culture.
INTRODUCTION

Articular cartilage lines the surfaces of bones in synovial joints to allow for smooth gliding of the joints and protection of the underlying bones. Articular cartilage has a unique structure that imparts it with distinctive properties crucial to its function [194]. This unique structure leads to a very low propensity for tissue healing, and current treatment options are not able to completely heal damage. Thus, injury to articular cartilage can cause long-term pain and disability, and there is a significant need for new treatment options [102]. For this reason, tissue engineering seeks to develop new techniques and knowledge that can be used to enhance articular cartilage repair.

The culture of chondrocytes is not without its challenges. Specifically, articular chondrocytes can be difficult to isolate in sufficient numbers to be used in an effective treatment, and while they are easily expandable they dedifferentiate upon expansion and lose their phenotype to become more fibroblast-like cells [165]. For these reasons, researchers have been investigating methods that would reduce the demand for chondrocytes and/or enhance the phenotype of the cells. One area of investigation that has shown great promise has been the use of co-cultures of chondrocytes with mesenchymal stem cells (MSCs). Such co-cultures have recently been investigated, and it has been observed that co-cultures can be used to reduce the total number of chondrocytes needed for the culture as the MSCs have been observed to enhance the phenotype of the chondrocytes when in co-culture [181]. Furthermore, such co-cultures have been investigated in both normoxic and hypoxic conditions [212], with a range of chondrocyte passage numbers [213], and have been found to be more sensitive to the chondrogenic stimulus TGF-β3 than chondrocyte cultures alone [214].
Flow perfusion culture is one tissue engineering technique that has shown to be beneficial for the culture of a variety of cell types [180]. Flow perfusion culture aims to perfuse the pores of three-dimensional scaffolds with culture medium in order to improve the mass transfer into the interior of the scaffolds and to apply shear stress to the cells in the scaffolds [215]. This culture method has been shown to create tissue constructs with a more uniform distribution of cells and extracellular matrix (ECM) compared to static cultures, and the application of shear stress to the cells is known to have a variety of effects depending on the cell type. With chondrocyte cultures, the use of flow perfusion has been shown to increase the cell proliferation and cartilage-like ECM production [186, 188, 189]. While transforming growth factor-β3 (TGF-β3) is known to improve the chondrogenic phenotype of both chondrocytes and co-cultures of chondrocytes and MSCs, its effect on these cell populations in flow perfusion culture has not been evaluated.

The present study aimed to investigate the effects of flow perfusion culture, growth factor stimulation, and MSC co-culture on the phenotype of bovine articular chondrocytes over a two week culture on electrospun poly(ε-caprolactone) (PCL) scaffolds. We hypothesized that the combined effects of flow perfusion culture and TGF-β3 induction would enhance the chondrogenic phenotype of both cell populations, and we aimed to mechanistically investigate the effects of each factor.
METHODS

Experimental Design

Bovine articular chondrocytes were seeded in monoculture or in co-culture with rabbit bone marrow-derived MSCs (1:1 ratio of chondrocytes:MSCs) on electrospun PCL scaffolds and cultured for two weeks in static or in flow perfusion culture with and without 10 ng/ml of TGF-β3. After two weeks, constructs were analyzed via DNA, glycosaminoglycan (GAG), and hydroxyproline (HYP) quantification, chondrogenic gene expression analysis, and histology.

Cell Isolation

Bovine articular chondrocytes were isolated from the femoral condyles of 7-10 day old calves (Research 87, Boylston, MA) using previously described methods [216], rinsed with PBS, pooled, and frozen for storage. Bone marrow-derived MSCs were harvested and isolated from the tibiae of 5-week-old New Zealand White rabbits (Charles River Laboratories, Wilmington, MA) as previously described [212]. Briefly, isolated bone marrow was plated on tissue culture flasks and rinsed after 48 h to remove non-adherent cells. Adherent cells were then cultured in general medium (DMEM, 10% FBS, PSF) until confluent, pooled, and frozen. Similar to previous studies [181], two species of cells were used in co-culture in order to investigate the relative portion of chondrocyte gene expression.

Scaffold Preparation

Electrospun PCL fiber mats were fabricated as previously described [36], using an 18 wt% solution of PCL (Inherent viscosity range 1.0-1.3 dl/g; Durect corporation,
Cupertino, CA with a number-average molecular weight (Mn) of 71,000 ± 2,300 Da and a polydispersity index (Mw/Mn) of 2.2 ± 0.07, as determined by gel permeation chromatography (Phenogel Linear Column with 5-μm particles, Phenomenex, Torrance, CA; Differential Refractometer 410, Waters, Milford, MA, n=3) and a calibration curve generated from polystyrene standards (Fluka, Switzerland), created by dissolving the polymer in a 5:1 v/v ratio of chloroform to methanol. The polymer solution was extruded at a flow rate of 40 ml/h through a 16 G needle, charged with a voltage of 30 kV, and directed towards a grounded collecting plate, 33 cm from the needle tip. Fiber morphology was inspected using scanning electron microscopy.

Scaffolds with an average fiber diameter of 10.2±2.3 μm (n=30) were punched from electrospun mats using a 3 mm dermal biopsy punch. Scaffolds, approximately 1.5 mm in thickness, were press-fit into polycarbonate scaffold holders, designed to support the scaffolds during perfusion culture [180], sterilized by exposure to ethylene oxide, prewetted in a graded ethanol series, rinsed with PBS, and incubated in general medium for 3 days.

**Cell Seeding and Culture**

Chondrocytes and MSCs were thawed and expanded for one and two passages, respectively, lifted from culture using 0.05% trypsin-EDTA, and suspended in chondrocyte growth medium (DMEM, 10% FBS, 1% non-essential amino acids, 50 μg/mL ascorbic acid, 46 μg/ml L-proline, 20 mM HEPES, 1% PSF). All scaffolds were seeded with a total of 50,000 cells in 30 μl of culture medium. Scaffolds were seeded with chondrocytes or a 1:1 ratio of chondrocytes to MSCs. The cultures were incubated
overnight in chondrocyte growth medium to allow for cell attachment [155]. After incubation, static cultures were removed from loading cassettes and placed in ultralow attachment 24 well plates for culture. Dynamic cultures were performed in a flow perfusion bioreactor as previously described [180, 216] with 10 scaffolds per bioreactor unit, and a flow rate of 10 μL/minute through each 3 mm scaffold. Each bioreactor unit and static sample, were given an equal volume of serum-free chondrogenic medium (high-glucose DMEM, 1% ITS+ premix (BD Biosciences, San Jose, CA), 50 mg/ml ascorbic acid, 100 nM dexamethasone, PSF). Additionally half of the cultures were supplemented with TGF-β3 (PeproTech, Rocky Hill, NJ) at a concentration of 10 ng/ml of culture medium. Half of the medium was replenished three times a week with serum-free medium with or without TGF-β3 and cultured for two weeks.

**Biochemical Assays**

After culture, samples from each group (n=12-16) were rinsed in PBS and stored at -20°C for two weeks until the biochemical assays were started. Samples were thawed and digested in proteinase K solution (1 mg/ml proteinase K, 0.01 mg/ml pepstatin A and 0.185 mg/ml iodoacetamide in a 50 mM tris(hydroxymethyl aminomethane), 1 mM ethylenediaminetetraacetic acid buffer, pH 7.6) at 56°C for 16 h [217]. Following proteinase K digestion, samples underwent three freeze/thaw cycles followed by 20 min of sonication. Double-stranded DNA content of the constructs was quantified using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Eugene, OR) following previously described methods [181]. Cell lysate, assay buffer and dye solution were combined in an opaque 96-well plate in duplicates, incubated for 10 min at room temperature, and
fluorescence was measured using excitation and emission wavelengths of 485 nm and 528 nm, respectively (FL x800 Fluorescence Microplate Reader; BioTek Instruments, Winooski, VT). DNA concentrations were determined relative to a lambda DNA standard curve.

Sulfated GAG content was determined using the colorimetric dimethylmethylene blue assay, previously described [217]. Cell lysate and color reagent were combined in a transparent 96-well plate in duplicates. After being incubated at room temperature for 7 min, absorbance at 520 nm was measured (PowerWave x340 Microplate Reader; BioTek Instruments). GAG concentrations were determined relative to a chondroitin sulfate standard curve.

HYP content, an indicator of total collagen content, was measured using a colorimetric assay [217]. Cell lysate was combined with an equal volume of 12 M HCl and heated at 115°C for 4 h. Samples were then evaporated under nitrogen flow and reconstituted in ddH₂O. Samples were divided into duplicate reactions, with chloramine-T and p-dimethylaminobenzaldehyde solutions. Absorbance was read at 570 nm. HYP concentrations were determined relative to a trans-4-hydroxy-L-proline standard curve.

**RT PCR**

Pelleted cell seeding stocks and cultured constructs (n=6-8 samples) were rinsed in PBS, placed in 600 μl lysis buffer (Qiagen, Valencia, CA), and vortexed before storing at -80 °C. After thawing, cell lysate was centrifuged through a QIAshredder homogenization column and combined with an equal volume of 70% ethanol. Total RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA), following the
manufacturer’s instructions for the isolation of RNA from animal cells. Reverse transcription was performed using Oligo(dT) primers (Promega, San Luis Obispo, CA) and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR (Applied Biosystems 7300 Real-Time PCR System, Foster City, CA) was performed on cDNA samples using SYBR Green detection (PerfeCTa SYBR Green FastMix, ROX; Quanta Biosciences, Gaithersburg, MD) with previously established primer sequences (Integrated DNA Technologies, Coralville, IA) [181].

All samples were first analyzed using primer sequences designed to amplify bovine targets with a high level of specificity compared to rabbit targets. Target gene expression was first normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample (ΔCt), then to the average baseline expression of that target gene measured in the chondrocyte cell stock used to seed the scaffolds (ΔΔCt). The $2^{-ΔΔCt}$ method was used to convert normalized gene expression levels to fold differences [185], and statistical analysis was performed on these values. $2^{ΔCt}$ was used to calculate the ratios of collagen II / collagen I. The primer sequences used in this analysis were [181]: Collagen type I: 5’-CGGGTCTTGCTGGTCATCAT-3’, 5’-TGCACCAGGCTGTCCAATG-3’; Collagen type II: 5’-AGTGGAAGAGCGGAGACTACTG-3’, 5’-GTTGGGAGCCAGGTTGTCAT-3’; Aggrecan: 5’-AGAGAGCCAACACAGCCGACA-3’, 5’-TAGTCCTGGGCGATTGTTGTTGA-3’; GAPDH: 5’-GAGTCCACTGGGGTCTTCACT-3’, 5’-GCGTGGACAGTGGTCATAAGTC-3’.
Additionally, co-cultured samples were also analyzed using previously established primer sequences, designed to amplify both rabbit and bovine targets with equal efficiencies. $2^{-\Delta Ct}$ was used to calculate the ratios of bovine-specific/cross-species expression within individual samples [181]. The primer sequences used in this analysis were [181]: Collagen type I: 5’-CCCAGAATGGGAGCAGTGGTACT-3’, 5’-AGCAGACGCATGAAGGCAAG-3’; Collagen type II: 5’-GGCCTCCACTTCAGCTATGGAG-3’, 5’-GTGTGTTTCTGCGAGCAGCCATC-3’; Aggrecan: 5’-GAGCAGAGTTTGTCAACAACAA-3’, 5’-CCTCCCCAGTGGAAGAAG-3’; GAPDH: 5’-CCATCTTTCCAGGAGCGAGAT-3’, 5’-GGTTCACGCCCATACAAAC-3’. The bovine specific GAPDH signal of the co-cultures increased from 0.1 to approximately 1.5 irrespective of the culture conditions (data not shown) indicating enrichment of the bovine chondrocytes [181].

**Histological Analysis**

Samples were fixed using 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA), dehydrated in 70% ethanol, embedded in HistoPrep freezing medium (Fisher Scientific), and cut into 5 μm thick sections using a cryostat (Leica CM 1850 UV; Leica Biosystems Nussloch GmbH, Germany). Sections were mounted onto glass slides, and stained using Alcian Blue and Picrosirius Red counter stained with Fast Green to visualize the presence and distribution of GAGs and collagen and cells, respectively. Images were obtained using a light microscope with a digital camera attachment (Axio Imager.Z2 equipped with AxioCam MRc5; Carl Zeiss MicroImaging GmbH, Germany).
Statistical Analysis

Results are reported as mean + standard deviation. Results of biochemical assays were evaluated using one-way ANOVA and Tukey-Kramer multiple comparison tests to determine significant differences (p<0.05). Results of RT-PCR were evaluated using the Kruskal-Wallis test followed by the Mann-Whitney U test (p<0.05).

RESULTS

Biochemical Assays

As shown in Figure 5.1, after 14 days of culture, all groups exhibited an increase in cell content from the day 0 levels except the non-induced, static chondrocyte constructs. However, no difference in DNA content was observed between the two cell populations in any of the culture conditions. Without TGF-β3 present, there was a noticeable effect of perfusion culture, leading to an increase in DNA content in both cell populations. However, in inductive cultures perfusion and static groups had similar cellularity. In perfusion TGF-β3 increased DNA content in only the chondrocyte population, but in static culture there was a significant effect of TGF-β3 in both cell populations.

Total GAG content and GAG/DNA ratio followed similar trends (Figure 5.2). All TGF-β3 cultures demonstrated greater GAG contents and GAG/DNA ratios than non-inductive cultures. The difference in GAG production as a result of perfusion culture was most drastic in the cultures with TGF-β3, as there was no effect of perfusion culture on GAG production in the non-inductive cultures. Conversely, the inductive perfusion
cultures had significantly less GAG and lower GAG/DNA ratios than the corresponding static cultures.

Total HYP contents and GAG/HYP ratios are shown in Figure 5.3. Total HYP content of the constructs was lower in all perfused constructs compared to static constructs. Exposure to TGF-β3 increased HYP content in static culture but not in perfusion. Evaluating the type of ECM present in the constructs, perfusion increased the GAG/HYP ratio only in non-induced cultures, whereas TGF-β3 exposure always increased the ratio.

**RT PCR**

Aggrecan expression of non-induced static cultures decreased over the two week culture period with the chondrocytes in co-culture exhibiting lower aggrecan expression than the chondrocytes in monoculture. Conversely, non-induced perfusion culture led to maintenance of the initial level of aggrecan expression and higher expression compared to the static culture. TGF-β3 induction generally led to an increase in aggrecan expression compared to the initial levels and the levels in the non-induced cultures, with the exception of the chondrocyte perfusion group, whose aggrecan expression was no greater than the corresponding non-induced cultures or the initial aggrecan expression and was less than the induced static culture.

Collagen I expression increased over the duration of the culture in all groups with perfusion culture adding an additional increased effect in chondrocytes in both co-culture conditions. Interestingly, chondrocytes in co-culture demonstrated increased collagen I expression when exposed to perfusion then the corresponding co-culture in static.
Collagen II expression decreased from the initial levels in all non-induced cultures; however, perfusion culture increased the expression in chondrocyte cultures compared to the corresponding static and co-culture groups. Induced cultures generally all had greater collagen II expression than non-induced cultures, with the exception of the chondrocyte perfusion group. Induced cultures in static exhibited an increase in collagen II expression over the duration of the culture, and while the induced perfusion cultures maintained the initial level of collagen II expression, it was significantly lower than the corresponding static cultures. Furthermore, in static culture the chondrocytes in induced co-culture exhibited higher collagen II expression than the corresponding chondrocytes in monoculture.

The collagen II/collagen I ratio decreased over time in all non-induced cultures with the monoculture chondrocytes in perfusion having a higher collagen II/collagen I ratio than the co-culture chondrocytes in perfusion. TGF-β3 induction had a positive effect on the collagen II/collagen I ratio in all cultures with the effect being most noticeable in the static cultures. In static culture, induction medium led to an increase in the collagen II/collagen I ratio over time; however, in perfusion culture the ratio was not only lower than the static culture, but decreased over time. Additionally, in static conditions, co-culturing chondrocytes led to an additional increase in collagen II/collagen I ratio in comparison to monocultures.

**Histology**

Histological evaluation corroborated the results of the biochemical assays. Representative images of sections stained for GAGs and collagen are shown in Figure 5.5
and Figure 5.6, respectively. Denser Alcian Blue and Picrosirius Red staining was observed in constructs exposed to TGF-β3. Furthermore, no difference in staining was observed between chondrocyte and co-cultured constructs. Static cultured constructs had a dense peripheral region of ECM deposition, compared to flow perfusion cultured constructs, which had a more homogeneous distribution of ECM.

**DISCUSSION**

Previous studies investigating flow perfusion culture of articular chondrocytes have demonstrated that flow perfusion is capable of enhancing chondrocyte proliferation and matrix production, which could aid in the production of large tissue engineered constructs [186, 188, 189]. However, studies investigating the gene expression of chondrocytes in perfusion have generally observed a decrease in chondrogenic gene expression compared to static cultures [198, 216]. In our previous work we investigated the use of flow perfusion culture in combination with hypoxia and found that while there were beneficial effects of both flow perfusion and hypoxic culture, neither condition was capable of maintaining the chondrogenic phenotype of the cells in the absence of exogenously added growth factors [216]. Instead we observed high levels of cellular proliferation, and with that rapid dedifferentiation of the cells, as the ratio of collagen II to collagen I gene expression decreased dramatically over even a very short period of culture (6 days) [216]. Thus, while flow perfusion enhanced the chondrocyte proliferation, ECM production, and spatial distribution of cells and ECM, flow perfusion conditions were not sufficient to maintain the initial levels of chondrogenic gene expression. When cells were exposed to hypoxia under flow perfusion, the level of
chondrogenic gene expression was maintained at a higher level, but while hypoxia has been shown to support chondrogenesis [210, 212], it was not able to fully mitigate the dedifferentiation that occurred in both perfusion and static culture. Therefore, there was a need for a more potent chondrogenic signal. Previous studies in flow perfusion have traditionally used medium with 10% serum content and no additional exogenous growth factors [186, 188, 189, 198, 201-203]. While these conditions are beneficial for cell proliferation, they may not be ideal for the maintenance of the chondrogenic phenotype. Accordingly, the present study aimed to mechanistically evaluate the effects of flow perfusion culture, with minimal serum, with and without the chondrogenic stimulus TGF-β3, on the chondrogenic phenotype of chondrocytes in monoculture and co-culture. It was hypothesized that combined exposure to the chondrogenic growth factor and perfusion culture could support the chondrogenic phenotype of the cells both in monoculture and co-culture. To evaluate this hypothesis, bovine articular chondrocytes were cultured for 14 days on PCL fiber mesh scaffolds in static and flow perfusion conditions with and without TGF-β3 and in monoculture or a 1:1 co-culture ratio with rabbit MSCs.

Cell proliferation was evaluated by the total DNA content of the constructs after 14 days of culture. Similar to previous studies, flow perfusion culture increased proliferation in non-induced cultures of chondrocytes [216]. However, when exposed to TGF-β3, no difference in proliferation was observed between static and perfusion cultures, as the growth factor led to high levels of cell proliferation. These results correspond well with previous findings that when exposed to shear stress, chondrocytes
proliferate as a result of TGF-β1 upregulation and the subsequent autocrine signaling [218]. In the present study with TGF-β3 supplementation, enhanced proliferation due to perfusion was not observed, which may occur according to the described mechanism as the effect of intrinsic growth factor upregulation would be masked by supplemented TGF-β3.

Chondrogenic ECM deposition was evaluated by total GAG content, GAG/DNA, and GAG/HYP of the constructs after 14 days. The utility of co-cultures rests in the assurance that co-cultures of MSCs and chondrocytes can achieve levels of normalized GAG and total GAG on par with chondrocytes alone [181, 214]. This is a striking fact considering that in as little as 14 days of culture in the present study, one cell population starting with half the chondrogenic potential of another can deposit an equivalent level of chondrogenic ECM. An interesting question is raised as to whether this striking effect of co-cultures can persist in the presence of other inductive environments, namely perfusion with or without TGF-β3 supplementation, which was shown in the present study to be the case. In each case the co-cultures achieved chondrogenic ECM deposition on par with chondrocytes alone.

The present experiment expanded prior analysis to isolate the effects of perfusion and TGF-β3 supplementation. With added TGF-β3, perfusion cultures consistently produced lower multiples in total GAG and normalized GAG but equivalent GAG/HYP compared to static. It appears that perfusion culture decreased the levels of both GAG and HYP in the constructs so that greater overall ECM production in static did not translate to an enhanced chondrogenic make-up, which may be a result of ECM
production or poor retention of ECM in the perfusion constructs [192]. Comparing TGF-β3 supplemented to non-supplemented cultures in perfusion for the first time, TGF-β3 induced the chondrogenesis of articular chondrocytes and co-cultures creating an additive effect to perfusion alone. This proved true in terms of GAG/DNA, total GAG, and GAG/HYP.

Our gene expression analysis indicated that perfusion alone had a positive chondrogenic effect on the chondrocytes in culture. This effect was demonstrated by the level of aggrecan expression of the chondrocytes both in monoculture and co-culture without TGF-β3 supplementation. Here, the aggrecan expression was maintained in perfusion culture from the day 0 levels, as compared to the static cultures which exhibited a decreased level of aggrecan expression over the 14 day culture. This result corroborates the increase in GAG/HYP described above. With TGF-β3 supplementation, there was a further increase in aggrecan expression. However, similar to previous studies, the addition of growth factor to co-cultures led to a more dramatic chondrogenic effect compared to the chondrocytes in monoculture [214]. This effect was noticable as the growth factor supplementation led to no increase in aggrecan expression in the chondrocyte perfusion cultures. Interestingly, with TGF-β3 there was an increase in co-culture aggrecan expression to levels where no difference between static and perfusion culture was observed. This result may further indicate that the difference in GAG content of the constructs in static and perfusion may be the result of the washing out of GAGs from the scaffolds, rather than a difference in ECM production [192].
While perfusion alone was able to maintain the aggrecan expression, the ratio of collagen II/I expression was not maintained from the initial level in perfusion culture. In fact, without growth factor supplementation, there was no effect of flow perfusion culture on the expression ratio. Without TGF-β3 monocultured chondrocytes in perfusion exhibited a higher collagen II/I ratio than chondrocytes co-cultured in perfusion, but again with growth factor induction there was a more drastic increase in collagen II/I expression in chondrocytes in co-culture than in monoculture. Interestingly, collagen I expression was higher for all co-cultured chondrocytes in perfusion compared to static culture. Perhaps the flow perfusion culture lowered the density of cell-to-cell contacts causing an increase in the more fibrocartilage associated collagen [219]. The highest level of collagen II/I expression was observed in the static chondrocytes in co-culture with TGF-β3 supplementation. However, with TGF-β3 present, perfusion culture exhibited a lower level of expression than static and day 0. While the collagen II/I ratio still decreased over time in perfusion, the growth factor was still effective in enhancing the chondrogenic phenotype of the cells in perfusion culture compared to chondrocytes not supplemented with growth factors.

In static culture with TGF-β3 there were significant ECM deposits outside the scaffold, compared to the perfusion cultures, which had minimal ECM deposits outside the scaffold. This could be the result of poor diffusion of nutrients into the interior of the scaffold, a known effect of static cultures in constructs of this size [220]. Similarly, the TGF-β3 diffusion would be greatly diminished to the interior of the construct as diffusion of large proteins through dense ECM may be inhibited [221], leading to the creation of a
local growth factor gradient. Such an effect would potentially create a positive feedback to the creation of an even thicker exterior. On the other hand, in perfusion, a more even distribution of ECM can be seen in the perfusion cultures, possibly due to the more even distribution of TGF-β3 when it is supplemented to the scaffolds through convection. These results demonstrate the need for the use of flow perfusion or convection in cultures supplemented with growth factors to form large tissue engineered constructs.

**CONCLUSIONS**

The effect of TGF-β3 and perfusion, which were hypothesized to increase chondrogenesis in these cultures was confirmed in this work. Perfusion culture alone, although exhibiting advantages to the distribution of ECM production in our PCL constructs, was not capable of maintaining chondrogenesis. While perfusion alone promoted higher aggrecan expression than the corresponding static cultures, perfusion alone was not capable of maintaining the collagen II/I expression. However, when combined with TGF-β3 supplementation, the perfusion groups achieved some of the chondrogenic benefit while retaining the previously established advantages of perfusion cultures, namely a more homogenous distribution of ECM compared to static cultures. Overall the results provide rational for creating tissue engineering chondrogenic constructs on a larger scale by using perfusion culture.
Figure 5.1: DNA content of samples after 14 days of culture.

The dashed line indicates the day 0 DNA levels. x indicates a statistical difference between the day 14 and day 0 samples. + indicates a statistical difference between the corresponding static and flow perfusion samples. * indicates a statistical difference between cultures exposed to TGF-β3 and the corresponding cultures not exposed to TGF-β3. p<0.05 for all statistical comparisons. Error bars designate standard deviation for n=12-16.
Figure 5.2: (A) GAG content and (B) GAG/DNA ratio of samples after 14 days of culture.

+ indicates a statistical difference between the corresponding static and flow perfusion samples. * indicates a statistical difference between cultures exposed to TGF-β3 and the corresponding cultures not exposed to TGF-β3. $p<0.05$ for all statistical comparisons. Error bars designate standard deviation for n=12-16.
Figure 5.3: (A) HYP content and (B) GAG/HYP ratio of samples after 14 days of culture.

+ indicates a statistical difference between the corresponding static and flow perfusion samples. * indicates a statistical difference between cultures exposed to TGF-β3 and the corresponding cultures not exposed to TGF-β3. $p<0.05$ for all statistical comparisons. Error bars designate standard deviation for n=12-16.
Figure 5.4: Gene expression of samples after 14 days of culture.

Aggrecan, collagen I and collagen II expression are all normalized to the expression levels of chondrocyte seeding stock. The dashed line indicates the day 0 Collagen II/Collagen I ratio of chondrocytes in the seeding stocks. x indicates a statistical difference between the day 14 and day 0 samples. + indicates a statistical difference between the corresponding static and flow perfusion samples. * indicates a statistical difference between cultures exposed to TGF-β3 and the corresponding cultures not exposed to TGF-β3. # indicates a statistical difference between the gene expression of the chondrocytes in monoculture and the corresponding chondrocytes in co-culture. p<0.05 for all statistical comparisons. Error bars designate standard deviation for n=6-8.
Figure 5.5: Alcian blue staining for GAGs in 5 μm thick sections of constructs cultured for 14 days.

Scale bar represents 500 μm in all images.
Figure 5.6: Picrosirius red staining for collagen and fast green staining for cells in 5 μm thick sections of constructs cultured for 14 days.

Scale bar represents 500 μm in all images.
Chapter 6. TGF-β3-Induced Chondrogenesis in Co-Cultures of Chondrocytes and Mesenchymal Stem Cells on Biodegradable Scaffolds

ABSTRACT

In this work, it was hypothesized that co-cultures of articular chondrocytes (ACs) and mesenchymal stem cells (MSCs) would exhibit enhanced sensitivity to chondrogenic stimuli, such as TGF-β3, and would require a reduced concentration of TGF-β3 to achieve an equivalent level of chondrogenesis compared to monocultures of each cell type. Furthermore, it was hypothesized that compared to monocultures, the chondrogenic phenotype of AC/MSC co-cultures would be more stable upon the removal of TGF-β3 from the culture medium. These hypotheses were investigated by culturing ACs and MSCs alone and in a 1:3 ratio on electrospun poly(ε-caprolactone) scaffolds. All cell populations were cultured for two weeks with 0, 1, 3, or 10 ng/ml of TGF-β3. After two weeks growth factor supplementation was removed, and the constructs were cultured for two additional weeks. Cell proliferation, extracellular matrix production, and chondrogenic gene expression were evaluated after two and four weeks. The results demonstrated that co-cultures of ACs and MSCs require a reduced concentration and duration of TGF-β3 exposure to achieve an equivalent level of chondrogenesis compared to AC or MSC monocultures. Thus, the present work implicates that the promise of co-cultures for cartilage engineering is enhanced by their robust phenotype and heightened sensitivity to TGF-β3.

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INTRODUCTION

Articular chondrocytes (ACs) and mesenchymal stem cells (MSCs) are common cell sources for articular cartilage engineering; however there are complications associated with the use of each cell type. While ACs are isolated, expanded, and clinically implanted in autologous chondrocyte transplantation [162, 222], obtaining sufficient numbers of ACs presents a challenge, as their isolation can cause significant donor site morbidity [162]. Furthermore, in vitro expansion of ACs can lead to rapid dedifferentiation and a fibroblastic phenotype [162], resulting in an inferior tissue-engineered construct. Conversely, bone marrow-derived MSCs are readily available, easily expandable, and capable of chondrogenic differentiation [223]. While achieving MSC chondrogenesis has been demonstrated, it is not without challenges. After chondrogenic induction, MSCs often exhibit a hypertrophic phenotype [224, 225] followed by calcification of the extracellular matrix (ECM) [225, 226]. Additionally, compared to ACs the amount of cartilage-like ECM produced by differentiating MSCs is minimal [217, 227, 228] and leads to constructs with inferior mechanical properties [229]. More recently, co-cultures of ACs and MSCs are being investigated as a potential cell population for articular cartilage engineering [230, 231]. Such a cell population has several advantages, including the ability to reduce the required number of ACs, while achieving equal or greater levels of chondrogenesis compared to ACs alone [181]. Furthermore, the co-culture scheme has been shown to reduce the mineralization and hypertrophy that is often seen in chondrogenic MSCs [212, 224].

Studies investigating the mechanism of enhanced chondrogenesis in AC/MSC cultures have primarily concluded that the trophic effects of MSCs enhance AC
chondrogenesis [181, 227, 232]. Most commonly, co-cultures have been investigated using standard serum-free culture medium with 10 ng/ml of TGF-β3 [181, 212, 224, 232, 233] or TGF-β1 [227, 230, 234] as a chondrogenic inducer in vitro; however, reduced concentrations of TGF-β3 in AC/MSC co-cultures have not been investigated. Due to the ability of TGF-β3 to enhance chondrogenesis of both ACs and MSCs and thus possibly enhancing the co-culture effects, the present study hypothesized that co-cultures may have enhanced sensitivity to chondrogenic stimuli, and thus may be capable of chondrogenic induction with a reduced concentration of TGF-β3, compared to ACs or MSC cultures alone. Furthermore, due to the continued presence of MSCs and thus continued chondrogenic stimulation, it was hypothesized that the co-cultured cell populations would have a more stable phenotype upon the removal of TGF-β3.

**Materials and Methods**

**Experimental Design**

Bovine ACs and rabbit bone marrow-derived MSCs were seeded in monoculture or in co-culture (1:3 ratio of ACs:MSCs) on electrospun poly(ε-caprolactone) (PCL) scaffolds and cultured for two weeks in a chemically defined, serum-free culture medium, supplemented with one of four concentrations of TGF-β3: 0 ng/ml, 1 ng/ml, 3 ng/ml or 10 ng/ml. After the initial chondrogenic stimulation period, all groups were cultured for an additional two weeks to evaluate the stability of the construct phenotypes upon the removal of the TGF-β3. Cultures were sampled at two and four weeks of total culture time for DNA, glycosaminoglycan (GAG), and hydroxyproline (HYP) quantification, chondrogenic gene expression, and histological analysis.
Cell Isolation

Bovine ACs were isolated from the femoral condyles of 7-10 day old calves (Research 87, Boylston, MA) within 36 hours of slaughter [216]. Articular cartilage was isolated, rinsed in PBS, minced to 1 mm x 1 mm x 1 mm pieces, and digested at 37°C in chondrocyte growth medium (DMEM, 10% fetal bovine serum (FBS) (BenchMark; Gemini Bio-Products, West Sacramento, CA), 1% non-essential amino acids, 50 mg/ml ascorbic acid, 46 mg/ml L-proline, 20 mM HEPES, penicillin/streptomycin/fungizone (PSF)) supplemented with 2 mg/ml collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ). Isolated ACs were rinsed with PBS, pooled, and frozen for storage.

Bone marrow-derived MSCs were harvested and isolated from 5-week-old New Zealand White rabbits (Charles River Laboratories, Wilmington, MA) as previously described [212]. Under general anesthesia, bone marrow from the tibiae was aspirated into 10 ml syringes containing 1000 U of heparin to prevent coagulation. Bone marrow was plated on tissue culture flasks and rinsed after 48 h to remove non-adherent cells. Adherent cells were maintained in general medium (DMEM, 10% FBS, PSF) until confluent, pooled, and frozen.

Scaffold Preparation

Non-woven PCL fiber mats were fabricated using a horizontal electrospinning apparatus, as previously described [36]. An 18 wt% solution of PCL (Inherent viscosity range 1.0-1.3 dl/g; Durect corporation, Cupertino, CA) was created by dissolving the polymer in a 5:1 v/v ratio of chloroform to methanol. The polymer solution was extruded at a flow rate of 40 ml/h through a 16 G needle, charged with a voltage of 30 kV, and
directed towards a grounded collecting plate, 33 cm from the needle tip. Fiber morphology was inspected using scanning electron microscopy.

Scaffolds with an average fiber diameter of $9.8 \pm 2.5 \, \mu m$ were punched from electrospun mats using an 8 mm dermal biopsy punch. Scaffolds, $1.0 \pm 0.1 \, mm$ in thickness, were sterilized by exposure to ethylene oxide, prewetted in a graded ethanol series, rinsed with PBS, and incubated in general medium for 3 days. Scaffolds were then press-fitted into cylindrical, custom-made, loading cassettes designed to confine the cell seeding suspension above the scaffolds.

**Cell Seeding and Culture**

ACs and MSCs were thawed and expanded for one and two passages, respectively, lifted from culture using 0.05% trypsin-EDTA, and suspended in chondrocyte growth medium. All scaffolds were seeded with a total of 220,000 cells in 180 μl of culture medium. Scaffolds were seeded with ACs, MSCs, or a 1:3 ratio of ACs to MSCs. After 4 h, additional chondrocyte medium was added to completely cover the scaffolds and loading cassettes, and the cultures were incubated overnight to allow for cell attachment. The seeded constructs were then removed from cassettes and placed in individual wells of 12-well culture plates with 4 ml of serum-free chondrogenic medium (high-glucose DMEM, 1% ITS+ premix (BD Biosciences, San Jose, CA), 50 mg/mL ascorbic acid, 100 nM dexamethasone, PSF), supplemented with 0, 1, 3, or 10 ng/ml TGF-β3 (PeproTech, Rocky Hill, NJ). Half of the medium was replenished three times a week. After two weeks, medium in all experimental groups was replaced with serum-free culture medium without TGF-β3 supplementation. Five replicate samples were harvested.
after 0, two, and four weeks of culture and washed with PBS. A 3 mm biopsy punch was then used to obtain individual samples for each method of analysis.

**Biochemical Assays**

From each construct, two 3 mm biopsy samples were pooled together and stored at -20°C. Samples were thawed and digested in proteinase K solution (1 mg/ml proteinase K, 0.01 mg/ml pepstatin A and 0.185 mg/ml iodoacetamide in a 50 mM tris(hydroxymethyl aminomethane), 1 mM ethylenediaminetetraacetic acid buffer, pH 7.6) at 56°C for 16 h [217]. Samples then underwent three freeze/thaw cycles followed by 20 min sonication. Double-stranded DNA content of the constructs was quantified using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Eugene, OR) [181]. Cell lysate, assay buffer and dye solution were combined in an opaque 96-well plate in duplicates, incubated for 10 min at room temperature, and fluorescence was measured using excitation and emission wavelengths of 485 nm and 528 nm, respectively (FL x800 Fluorescence Microplate Reader; BioTek Instruments, Winooski, VT). DNA concentrations were determined relative to a lambda DNA standard curve.

Sulfated GAG content was determined using the colorimetric dimethylmethylene blue assay [217]. Cell lysate and color reagent were combined in a transparent 96-well plate in duplicates, incubated for 7 min at room temperature, and absorbance at 520 nm was measured (PowerWave x340 Microplate Reader; BioTek Instruments). GAG concentrations were determined relative to a chondroitin sulfate standard curve.

HYP content, an indicator of total collagen content, was measured using a colorimetric assay [212]. For hydrolysis, cell lysate was combined with an equal volume
of 4 N NaOH and heated at 121°C for 15 min. The samples were then neutralized with HCl to pH 6.5–7.0. Samples were divided into duplicate reactions, with chloramine-T and \( p \)-dimethylaminobenzaldehyde solutions. Absorbance was read at 570 nm. HYP concentrations were determined relative to a trans-4-hydroxy-L-proline standard curve.

**Real Time Reverse Transcription Polymerase Chain Reaction**

Pelleted cell seeding stocks and minced constructs (n=5 samples) were rinsed in PBS, placed in 600 μl lysis buffer (Qiagen, Valencia, CA), and vortexed before storing at -80 °C until further processing. Cell lysate was centrifuged through a QIAshredder homogenization column and combined with an equal volume of 70% ethanol. Total RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA), following the manufacturer’s instructions for the isolation of RNA from animal cells [181, 217]. Reverse transcription was performed using Oligo(dT) primers (Promega, San Luis Obispo, CA) and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR (Applied Biosystems 7300 Real-Time PCR System, Foster City, CA) was performed on cDNA samples using SYBR Green detection (PerfeCTa SYBR Green FastMix, ROX; Quanta Biosciences, Gaithersburg, MD) with previously established primer sequences, designed to amplify both rabbit and bovine targets with equal efficiencies (Integrated DNA Technologies, Coralville, IA).

Target gene expression was first normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample (ΔCt), then to the average baseline expression of that target gene measured in the AC cell stock used to seed the scaffolds (ΔΔCt). The \( 2^{-\Delta\Delta C_t} \) method was used to convert normalized
gene expression levels to fold differences [185], and statistical analysis was performed on these values. Similarly, \(2^{\Delta Ct}\) was used to calculate the ratios of collagen II / collagen I. The primer sequences used in this analysis were [181]: Collagen type I: 5’-CCCAGAATGGAGCGATGGTTACT-3’, 5’-AGCAGACGCATGAAGGCAAG-3’; Collagen type II: 5’-GGGTTCCTCCACTTCAGCTATGGAG-3’, 5’-GTGTGTTCGTGCGAGCCATC-3’; Aggrecan: 5’-GAGCAGGAGTGGTCAACAAAC-3’, 5’-CCTCCCAGTGCAAGAAG-3’; GAPDH: 5’-CCATTTCCAGGACGGAGAT-3’, 5’-GGTTACGCCCATCACAAC-3’.

**Histological Analysis**

Samples were fixed using 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA), dehydrated in 70% ethanol, and embedded in HistoPrep freezing medium (Fisher Scientific). Frozen constructs were cut into 5 μm thick sections using a cryostat (Leica CM 1850 UV; Leica Biosystems Nussloch GmbH, Germany) and mounted onto glass slides. Sections were then stained using Alcian Blue and Picrosirius Red counter stained with Fast Green to visualize the presence and distribution of GAGs and collagen and cells, respectively. Images were obtained using a light microscope with a digital camera attachment (Axio Imager.Z2 equipped with AxioCam MRc5; Carl Zeiss MicroImaging GmbH, Germany).

**Statistical Analysis**

Results are reported as means ± standard deviation. Statistical analysis was performed using JMP 10 software package (SAS Institute, Cary, NC). One-way ANOVA
and Tukey-Kramer multiple comparison tests were used to determine significant differences (p<0.05).

RESULTS

Two Week Biochemical Assays

The quantity of DNA was measured in all cell populations at the start of the culture to determine the baseline value and measured again after two weeks of culture (Fig. 6.1A.). At that time, ACs and co-cultures had proliferated, resulting in an increase in DNA content from the initial levels; however, the MSCs did not exhibit any change in DNA content from day 0. In general, the levels of DNA were independent of TGF-β3 concentration with the exception of non-induced co-cultures having lower DNA content than induced co-cultures. Furthermore, the baseline DNA contents of all cell populations were equal.

Even when cultured without TGF-β3, AC constructs produced more total GAG and had higher GAG/DNA ratios, than the corresponding MSC cultures, whereas there was no significant difference between co-cultures and MSCs without TGF-β3 (Fig. 6.1B. and Fig. 6.1C.). Comparing the effect of TGF-β3 concentration on each cell population at two weeks, MSC constructs did not show an increase in total GAG contents or GAG/DNA ratios, even when cultured with the highest concentration of TGF-β3. AC constructs cultured with 3 ng/ml or higher of TGF-β3 resulted in increased GAG contents and GAG/DNA ratios in comparison to the 0 ng/ml AC group. In contrast, increased GAG contents and GAG/DNA ratios were observed with as little as 1 ng/ml TGF-β3 in the co-cultures. Comparing AC to co-culture constructs directly, co-cultures with 1 ng/ml
TGF-β3 resulted in an equivalent GAG/DNA as AC constructs with 10 ng/ml TGF-β3. Compared to cultures containing ACs, MSC constructs produced low levels of GAGs at two weeks.

No clear effect of TGF-β3 was observed on the HYP content or GAG/HYP ratios in the AC monocultures (Fig. 6.2). In contrast, co-cultures showed higher HYP contents in the 3 and 10 ng/ml cultures and higher GAG/HYP in the 10 ng/ml constructs compared to the 0 ng/ml control. MSC cultures generally contained less HYP than the AC and co-culture constructs, with no effect of the TGF-β3; however GAG/HYP ratios were higher in the 3 and 10 ng/ml MSC cultures than the 0 and 1 ng/ml cultures and were similar to the AC and co-culture constructs.

**Two Week Real Time Reverse Transcription Polymerase Chain Reaction**

Gene expression levels of select chondrogenic markers after two weeks of culture can be seen in Figure 6.3, where the values were first normalized internally to GAPDH, then normalized to the day 0 AC samples, and reported as fold change relative to the day 0 AC group. The dashed line represents the day 0 gene expression of each cell population. AC aggregan expression increased from the day 0 level not exposed to TGF-β3, decreased in the 1 ng/ml culture, and remained unchanged in the other constructs. Thus, AC cultures with TGF-β3 showed lower aggregan expression than the 0 ng/ml AC control. Collagen I expression followed a similar pattern, as only the 0 ng/ml AC control increased from the day 0 level. Collagen II expression decreased in the 0 and 1 ng/ml AC cultures but remained unchanged in the 3 and 10 ng/ml cultures. Similarly, highest
collagen II/I ratio was seen in the 3 and 10 ng/ml AC cultures, with no change from the day 0 level.

In the co-cultures aggrecan and collagen II expression increased from the initial levels in all groups exposed to TGF-β3, resulting in higher expression than the 0 ng/ml control. Collagen I expression in the 0 and 1 ng/ml co-culture groups increased from the day 0 expression, but all cultures exposed to TGF-β3 had lower collagen I expression than the 0 ng/ml control. This change in gene expression resulted in a collagen II/I ratio that increased from day 0 in all co-cultures and was highest in the 3 and 10 ng/ml co-cultures.

In the MSC cultures, aggrecan and collagen II expression increased from the initial levels in the 3 and 10 ng/ml cultures, which were both higher than the 0 ng/ml control. Collagen I expression increased from the initial levels in all MSC cultures but was lowest in TGF-β3 cultures. This led to a collagen II/I ratio that was highest in the 3 and 10 ng/ml cultures.

**Two Week Histology**

Histological evaluation, presented in Figure 6.4, corroborated the results of the biochemical assays. More intense GAG staining was observed in AC and co-culture constructs exposed to TGF-β3 compared to the 0 ng/ml control, whereas, no clear effect of TGF-β3 was observed with collagen and cell staining. Additionally, only very low quantities of ECM and cells were observed in MSC sections (data not shown).
Four Week Biochemical Assays

After four weeks, ACs and co-cultures had continued to proliferate, but the MSC cultures still exhibited no increase in DNA content from the two week levels (Fig. 6.5A.). ACs and co-cultures exhibited dose-dependent increases in DNA content with the 10 ng/ml constructs containing the highest quantity of DNA. Additionally, no difference in DNA was found between the AC and co-culture populations at each concentration of growth factor.

Similarly, GAG contents and GAG/DNA ratios of the AC and co-culture constructs followed a dose-dependent trend (Fig. 6.5B.). ACs with 10 ng/ml of TGF-β3 and co-cultures with all concentrations of TGF-β3 showed an increase in GAG content from two weeks. However when normalized to DNA, this effect translated to an decrease in the GAG/DNA ratios in the 3 and 10 ng/ml AC cultures and the 3 ng/ml co-culture constructs, but no change in the 10 ng/ml co-culture group. All MSC constructs had increased GAG/DNA ratios, and MSC constructs exposed to TGF-β3 had increased GAG content from two weeks. In the AC constructs, the total GAG content in the 3 and 10 ng/ml samples was higher than in the cultures without TGF-β3, but the GAG/DNA ratio was higher only in the 10 ng/ml constructs. However, in the co-culture constructs, all groups exposed to TGF-β3 had higher GAG content and GAG/DNA ratios than the 0 ng/ml controls. In the MSC cultures, there was no difference in GAG content among any of the groups, but the GAG/DNA ratios in the 10 ng/ml cultures were higher than those cultured with 1 ng/ml or less.

HYP content in the constructs increased in all AC and co-cultured groups from two weeks, but was unchanged in the MSC cultures (Fig. 6.6A.). GAG/HYP ratios also
remained steady in all AC and co-culture groups, with the exception of the 1 ng/ml co-cultures, which decreased from the two week level (Fig. 6.6B.). MSC 3 and 10 ng/ml cultures had increased GAG/HYP ratios from the two week levels. HYP content was higher in all co-culture constructs than in the AC groups exposed to the corresponding TGF-β3 concentration. Compared to the AC and co-culture constructs all MSC groups produced minimal HYP. The GAG/HYP ratios in the constructs followed a dose-dependent pattern with the co-cultures exposed to TGF-β3 having a higher GAG/HYP than the 0 ng/ml control. In the AC groups, the 3 ng/ml and 10 ng/ml cultures had higher GAG/HYP ratios than the 0 ng/ml AC cultures. MSC cultures only resulted in an increased GAG/HYP ratio in the 10 ng/ml culture. Comparing between cell populations, the co-cultures displayed the highest GAG/HYP ratios with the 1 ng/ml co-cultures being equal to the 10 ng/ml AC cultures.

Four Week Real Time Reverse Transcription Polymerase Chain Reaction

Gene expression in all samples at four weeks was first normalized internally, to GAPDH and then normalized to the day 0 AC samples and is shown in Figure 6.7. All groups were compared to the corresponding gene expression at two weeks and to the 0 ng/ml cultures of the same cell population. AC cultures generally had decreased aggregcan expression from two weeks, such that no effect of previous TGF-β3 exposure was observed. Collagen I expression in AC cultures increased from two weeks in the 10 ng/ml cultures, but decreased in the 0 ng/ml cultures. Both the 1 and 10 ng/ml AC cultures had higher collagen I expression than the 0 ng/ml cultures. Collagen II expression and the
collagen II/I ratio decreased from the two week levels in the 3 and 10 ng/ml AC cultures and was no different among any of the growth factor concentrations at four weeks.

In the co-cultures, aggrecan expression decreased from two weeks in all TGF-β3 cultures, with the exception of the 10 ng/ml group, where the aggrecan expression remained greater than all other co-culture groups. Collagen I expression decreased in the 0 and 1 ng/ml co-cultures. Interestingly, collagen II expression increased from two weeks in the 3 and 10 ng/ml co-cultures, and both were significantly higher than the 0 ng/ml control. However, the collagen II/I ratio decreased in the 3 and 10 ng/ml co-cultures, but the 10 ng/ml culture still remained higher than the 0 ng/ml control.

MSC cultures had decreased aggrecan expression from two weeks in the 3 and 10 ng/ml cultures, and only in the 10 ng/ml culture group was there any difference in aggrecan expression compared to the 0 ng/ml control. Collagen I expression increased from two weeks in all MSC cultures, and the collagen II expression and the collagen II/I ratio decreased in the 3 and 10 ng/ml cultures. No effect of TGF-β3 was seen in the collagen I, collagen II, or collagen II/I expression of the MSCs at four weeks.

**Four Week Histology**

Histological staining at four weeks again corroborated the results of the biochemical assays. More intense GAG staining was observed at four weeks compared to two weeks, and a dose dependent increase in intensity was also observed in the AC and co-cultured constructs. Similarly, a dose dependent increase in cell staining was also observed among the sections, and more intense collagen staining was seen in the four
week, compared to the two week sections. Similar to the two week histology, very low levels of ECM and cells were observed in the MSC constructs (data not shown).

**DISCUSSION**

Co-cultures of ACs and MSCs are a promising cell source for cartilage engineering with the potential to overcome several challenges associated with the individual use of corresponding monocultures. The enhanced proliferation, matrix production, and chondrogenic gene expression of co-cultures has generally been attributed to the ACs, whose chondrogenic activity is upregulated by the presence of MSCs [181, 227, 232]. In the present study, we hypothesized that co-cultures of ACs and MSCs would exhibit enhanced sensitivity to chondrogenic stimuli and thus would require a reduced dosage of TGF-β3 in order to observe enhanced chondrogenesis. Furthermore, it was hypothesized that the presence of the MSCs in long-term co-cultures would continue to enhance AC phenotype even after the removal of TGF-β3, and would therefore lead to robust chondrogenesis with minimized need for exogenously added growth factor.

The sensitivity of the co-cultures was evaluated through investigation of the ECM production and gene expression at each level of TGF-β3 exposure. At two weeks AC cultures had proliferated independent of the TGF-β3 dose. While GAG production was TGF-β3 dose-dependent, a significant enhancement in total GAG and GAG/DNA from the 0 ng/ml cultures was only observed with the higher concentrations (3 or 10 ng/ml) of TGF-β3. At the same time, co-cultured constructs proliferated to the same extent as AC cultures, with a greater increase seen in all cultures exposed to TGF-β3 compared to the 0
ng/ml cultures. As hypothesized in this work, it was seen that co-cultures of ACs and MSCs exhibit a greater sensitivity to TGF-β3 in that enhanced matrix production compared to no TGF-β3 cultures can be observed even with the addition of as little as 1 ng/ml of TGF-β3. Furthermore, while the enhanced sensitivity was seen to improve matrix production, the same effect was also observed when evaluating gene expression. While collagen I expression was reduced in all AC cultures exposed to TGF-β3, aggrecan expression was inhibited in these same constructs. Similar to the matrix production, AC collagen II expression and the collagen II/I ratio was enhanced only in the 3 and 10 ng/ml cultures. Co-cultures on the other hand, demonstrated increased aggrecan and collagen II expression and reduced collagen I expression in all cultures exposed to TGF-β3. These results indicate that TGF-β3 could potentially be effective in co-cultures with a lower dose than in AC cultures alone.

It is known that the *in vivo* microenvironment can alter cellular response to growth factors compared to *in vitro* conditions [221], and the results of this study may be further demonstrating the impact of cell microenvironment, as the addition of MSCs to cultures of ACs altered the growth factor sensitivity. Studies evaluating the effect of TGF-β on AC proliferation with ACs of various expansion stages, and thus differentiation states, indicate that the magnitude of the growth factor’s effect decreases with AC dedifferentiation [235, 236]. Thus, at least proliferation of ACs as a result of TGF-β exposure is dependent on AC phenotype, and it is plausible that the regulatory effect is extended to matrix production and gene expression, which at two weeks were the primary responses to TGF-β3 in the present study. Potentially, the enhanced sensitivity of
co-cultures to TGF-β3 may be a result of MSCs in co-culture enhancing the AC phenotype, and in turn the AC response to TGF-β3. Indeed, the chondrocyte differentiation state and TGF-β sensitivity has been further investigated with rabbit auricular chondrocytes, and results suggested that the correlation between phenotype and TGF-β sensitivity was due to the state of the chondrocyte pericellular matrix [237]. Theoretically, more chondrogenic ACs have more mature pericellular matrices, which are known to sequester growth factors [238] and to affect the presentation of biochemical signals to the cells [239]. Thus, the alterations in the pericellular matrix of ACs either as a result of TGF-β3 exposure or MSC trophic effects could enhance the presentation of biochemical signals to ACs. Additionally, the presence of TGF-β3 may be altering the magnitude or nature of the trophic effects directly, as exogenous growth factors have been shown to regulate the endogenous expression of growth factors [240].

Directly comparing the co-cultured constructs to the AC constructs clearly demonstrates advantages of the AC/MSC co-cultures for cartilage engineering. At two weeks, co-cultures with 3 ng/ml TGF-β3 achieved equivalent chondrogenesis as 10 ng/ml of TGF-β3 in AC constructs. Thus, co-cultures allow for a reduction in both the total number of ACs and amount of growth factor necessary to reach the same level of chondrogenesis as ACs alone. These results were obtained through in vitro culture of the constructs and demonstrate the advantages of this culture model for the generation of tissue-engineered constructs in vitro. While such effects do not necessarily translate to in vivo conditions, the results may suggest the potential of delivering MSCs and TGF-β3 in order to enhance the sensitivity of endogenous or exogenous ACs to the growth factor.
Delivering effective concentrations of growth factors \textit{in vivo} can be challenging as the \textit{in vivo} environment can lead to rapid removal of the proteins from the wound site, loss of bioactivity, or low availability as a result of the slow tissue diffusion of large proteins [221]. Thus, the use of co-cultures could potentially allow for better success with the delivery of TGF-\(\beta\)3. While such an idea cannot be proven from the current \textit{in vitro} data, the results of the present study indicate the need to further investigate this concept.

By way of addressing the second hypothesis, cultures were exposed to TGF-\(\beta\)3 for two weeks, after which the growth factor was removed, and the constructs were cultured in serum-free culture medium with dexamethasone. This culture regime is well established, as several studies have demonstrated the advantages of transient exposure to TGF-\(\beta\)3 in AC cultures and have shown that a two week exposure leads to enhanced matrix production and improved construct mechanical properties compared to continuous exposure to TGF-\(\beta\)3 [241-243]. Similar to the results here, a two week initial exposure of ACs to various concentrations of TGF-\(\beta\)3 led to more drastic dose-dependent effects later at four weeks, compared to continuous exposure [243]. However, as demonstrated in the present study, while a significant amount of matrix is produced after four weeks with transient exposure, the chondrogenic gene expression of ACs sharply declines after removal of growth factor. By four weeks, the gene expression of ACs did not demonstrate any positive effect from previous exposure to any of the TGF-\(\beta\)3 concentrations evaluated. Conversely, in co-culture constructs the chondrogenic gene expression generally still decreased from the two week levels, but a significant enhancement in aggrecan, collagen II, and the ratio of collagen II/ collagen I expression
was seen in the 10 ng/ml cultures compared to the 0 ng/ml controls. Thus, transient exposure of co-cultures to TGF-β3 resulted in increased proliferation and matrix production while maintaining enhanced gene expression, compared to constructs not exposed to TGF-β3.

Consistent with previous studies, the MSC cultures in the present work exhibited very low levels of proliferation and produced inferior levels of ECM compared to the cultures containing ACs [181, 227]. While no difference in early GAG production was observed among any of the TGF-β3 concentrations in the MSC cultures, the two week culture period may not have been an adequate length of time to observe sufficient MSC differentiation. However, at the same time the 3 and 10 ng/ml MSC constructs, demonstrated improved chondrogenic gene expression and GAG/HYP ratios similar to the AC and co-culture constructs at the same TGF-β3 concentrations, indicating chondrogenesis in these cultures. At four weeks, even in the 10 ng/ml MSC constructs, a decrease in chondrogenic gene expression, and GAG/DNA and GAG/HYP ratios occurred, indicating that the chondrogenic phenotype was not strong enough to remain after the removal of the chondrogenic stimuli. Previously, brief exposure (1 week) of MSC cultures to high levels (100 ng/ml) of TGF-β3 was shown to be capable of inducing and maintaining MSC chondrogenesis [244]. Interestingly, a transient, 3 week exposure of MSCs to 10 ng/ml TGF-β3 led to improved equilibrium modulus and GAG accumulation, similar to the phenomenon seen in transient AC exposure; however the effect was only seen with MSCs encapsulated at a high cell density [245]. Thus, the MSC
cultures in the present study may have demonstrated greater stability if cultured at a higher cell density or higher TGF-β3 concentration.

The mechanism of the enhanced stability of co-cultures was not examined in the present study. As such, further investigation is needed to elucidate the mechanism and develop potential ways to further leverage its effects. Previously, using the same co-cultures as the present study with continuous exposure to 10 ng/ml TGF-β3, an increase in the proportion of AC gene expression was observed by four weeks, indicating a decrease in the proportion of MSCs relative to ACs. This result was corroborated in the present study by the disparate proliferation of ACs and MSCs in monocultures. By four weeks, the gene expression appeared to be almost completely from ACs [181]. However, at two weeks, a significant portion of MSCs was still detected. If the enhanced stability of the co-cultures is directly due to the presence of active MSCs, rather than a sustained effect of their presence at a previous point in culture, the magnitude of this effect may diminish over the duration of the culture, as the proportion of MSCs decreases. Furthermore, the co-culture ratio may be a significant factor in this effect and possibly should be investigated in future studies.

**CONCLUSIONS**

The current work evaluated the sensitivity of co-cultures of ACs and MSCs to TGF-β3 by quantifying the synthetic capacity and chondrogenic gene expression of the cultures after exposure to a range of TGF-β3 concentrations over two weeks of culture. The results demonstrated that while TGF-β3 was able to enhance the chondrogenic phenotype of ACs and MSCs, AC/MSC co-cultures required a reduced concentration of
TGF-β3 in order to achieve enhanced chondrogenesis compared to either cell type alone. Furthermore, the present study evaluated the stability of the cell phenotypes after the removal of TGF-β3 from the culture medium. Here, it was demonstrated that the phenotype of the co-cultures was more stable upon the removal of chondrogenic stimuli compared to either cell type alone. Thus, these results demonstrate that co-cultures can be used to effectively reduce the magnitude or duration of TGF-β3 exposure while achieving an equal level of chondrogenesis compared to AC or MSC monocultures.
Figure 6.1: (A) DNA, (B) GAG, and (C) GAG/DNA in constructs after two weeks of culture.

Dashed line represents the initial quantity of DNA detected after cell seeding. * indicates a statistically significant difference from the day 0 levels ($p < 0.05$). Groups not connected by same letter are statistically different ($p < 0.05$).
Figure 6.2: (A) Hydroxyproline (HYP) and (B) GAG/HYP ratio in constructs after two weeks of culture.

Groups not connected by same letter are statistically different ($p < 0.05$).
Figure 6.3: Aggrecan, collagen I and collagen II expression all normalized to chondrocyte monoculture expression upon cell seeding and the collagen II/I expression ratio.

Dashed line indicates the starting level of gene expression in each cell type. * indicates a statistically significant difference from the day 0 gene expression levels ($p < 0.05$). ‡ Indicates a statistically significant difference from the 0 ng/ml cultures of the same cell population levels ($p < 0.05$).
Figure 6.4: Histological evaluation of AC (A and B) and co-cultured (C and D) constructs after two weeks of culture.

Staining with Alcian Blue for GAGs (A and C) and Picrosirius Red for collagen with Fast Green for cells (B and D). Scale bar represents 200 μm in all images.
Figure 6.5: (A) DNA, (B) GAG, and (C) GAG/DNA in constructs after four weeks of culture.

* indicates a statistically significant difference from the two week levels \((p < 0.05)\). Groups not connected by same letter are statistically different \((p < 0.05)\).
Figure 6.6: (A) Hydroxyproline (HYP) and (B) GAG/HYP in constructs after four weeks of culture.

* indicates a statistically significant difference from the two week levels ($p < 0.05$). Groups not connected by same letter are statistically different ($p < 0.05$).
Figure 6.7: Aggrecan, collagen I and collagen II expression all normalized to chondrocyte monoculture expression upon cell seeding and the collagen II/I expression ratio.

* indicates a statistically significant difference from the two week gene expression ($p < 0.05$). ‡ Indicates a statistically significant difference from the 0 ng/ml cultures of the same cell population levels ($p < 0.05$).
Figure 6.8: Histological evaluation of AC (A and B) and co-cultured (C and D) constructs after four weeks of culture.

Staining with Alcian Blue for GAGs (A and C) and Picrosirius Red for collagen with Fast Green for cells (B and D). Scale bar represents 200 μm in all images.
Chapter 7. Co-Cultures of Articular Chondrocytes and Mesenchymal Stem Cells Seeded on Biodegradable Polymer Scaffolds for the Repair of Cartilage Defects in a Rat Osteochondral Defect Model*

**ABSTRACT**

This work investigated the ability of co-cultures of articular chondrocytes and mesenchymal stem cells (MSCs) to repair articular cartilage in osteochondral defects. Bovine articular chondrocytes and rat MSCs were seeded in isolation or in co-culture onto electrospun poly(ε-caprolactone) (PCL) scaffolds and implanted into an osteochondral defect in the trochlear groove of 12-week old Lewis rats. Additionally, a blank PCL scaffold and untreated defect were investigated. After 12 weeks, the extent of cartilage repair was analyzed through histological analysis, and the extent of bone healing was assessed by quantifying the total volume of mineralized bone in the defect through microcomputed tomography. Histological analysis revealed that the articular chondrocytes and co-cultures led to repair tissue that consisted of more hyaline-like cartilage tissue that was thicker and possessed more intense Safranin O staining. The MSC, blank PCL scaffolds, and empty treatment groups generally led to the formation of fibrocartilage repair tissue. Microcomputed tomography revealed that while there was an equivalent amount of mineralized bone formation in the MSC, blank PCL, and empty treatment groups, the defects treated with chondrocytes or co-cultures had negligible mineralized bone formation. Overall, even with a reduced number of chondrocytes, co-cultures led to an equal level of cartilage repair compared to the chondrocyte samples,

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thus demonstrating the potential for the use of co-cultures of articular chondrocytes and MSCs for the in vivo repair of cartilage defects.
INTRODUCTION

While a number of treatment options currently exist for the repair of articular cartilage defects, these options primarily lead to short-term functional repair, but are not capable of achieving stable, long-term repair of the tissue [246, 247]. Autologous chondrocyte implantation (ACI) is generally one of the most often-used procedures for the treatment of cartilage defects, and has been shown to have some success in repairing the damaged tissue [246, 248]. However, the isolation of appropriate numbers of autologous chondrocytes is not without challenges. Chondrocytes are present in relatively low densities in native articular cartilage [194], and the isolation of sufficient numbers would lead to large donor site morbidity [249]. Furthermore, the in vitro expansion of chondrocytes is associated with a rapid dedifferentiation of the cells into a more fibroblastic phenotype, which ultimately leads to the production inferior tissue [250]. Thus, numerous approaches have been investigated in order to enhance the chondrogenic phenotype of expanded cells or to reduce the demand for chondrocytes in the treatment of articular cartilage defects [251].

Co-cultures of articular chondrocytes and mesenchymal stem cells (MSCs) are one approach that has been proposed to reduce the demand for articular chondrocytes and thus improve articular cartilage treatments [181, 212-214]. When co-cultured with MSCs, articular chondrocytes have been observed to undergo enhanced proliferation and matrix production [181, 230, 233, 252]. This effect, which has been shown to be independent of MSC source or culture condition [253], would allow for the use of reduced numbers of chondrocytes to achieve an equal chondrogenic outcome [213]. Furthermore, the co-cultured cell population has been demonstrated to be more sensitive to chondrogenic
stimuli, such as transforming growth factor-β3 (TGF-β3), and to produce a phenotype that is more stable after the removal of the stimuli, compared to monocultures of either cell type [214]. While the beneficial effects of MSCs on chondrocytes are crucial to the performance of these co-cultures, chondrocytes have similarly been demonstrated to have beneficial effects on MSCs, which mitigates some disadvantages associated with MSC chondrogenesis. The chondrogenesis of MSCs is challenged by the eventual hypertrophy and mineralization of these cells after extended culture in chondrogenic conditions [254]. However, co-culture with articular chondrocytes has been demonstrated to reduce the hypertrophy of MSCs in culture [212, 224, 255]. Thus, the advantages of co-cultures of articular chondrocytes and MSCs for the in vitro generation of articular cartilage is well-documented; however the use of this cell population for in vivo repair of articular cartilage defects has not been investigated.

The objective of the present study was to investigate the use of co-cultures of articular chondrocytes and bone marrow-derived MSCs for the in vivo repair of articular cartilage in a rat osteochondral defect. We hypothesized that the use of co-cultures of chondrocytes and MSCs would lead to equal or greater cartilage repair compared to chondrocytes alone, thus allowing for the use of reduced numbers of chondrocytes. Therefore, we implanted electrospun poly(ε-caprolactone) (PCL) scaffolds, seeded with MSCs, chondrocytes, or co-cultures of chondrocytes and MSCs into the trochlear groove of rats and evaluated the tissue repair via histology and microcomputed tomography.
METHODS

Study Design
The groups investigated in this study are outlined in Table 7.1. Briefly, bovine articular chondrocytes and rat bone marrow-derived MSCs were seeded onto electrospun PCL scaffolds to create three separate experimental groups. The AC group consisted of articular chondrocytes seeded in monoculture at a density of 40,000 cells per scaffold; the MSC group consisted of MSCs seeded in monoculture at a density of 40,000 cells per scaffold. The CC group consisted of articular chondrocytes and MSCs seeded in a 1:3 ratio at a density of 40,000 cells per scaffold (i.e. 10,000 chondrocytes and 30,000 MSCs). Additionally, an empty control (empty) and a material control (PCL) were also investigated. All samples (n=8 per group) were implanted into defects created in the trochlear groove of Lewis rats for 12 weeks. Samples were analyzed for cartilage tissue formation through histological scoring and for the formation of mineralized bone through microcomputed tomography.

Scaffold Fabrication
Non-woven mats were electrospun using PCL (Sigma-Aldrich, St. Louis, MO) with a number-average molecular weight (Mn) of 114,000 ± 4,000 Da and a polydispersity index (Mw/Mn) of 2.02 ± 0.04, as determined by gel permeation chromatography (Phenogel Linear Column with 5-µm particles, Phenomenex, Torrance, CA; Differential Refractometer 410, Waters, Milford, MA, n=3) and a calibration curve generated from polystyrene standards (Fluka, Switzerland). Briefly, a 14 wt% solution of PCL was prepared by dissolving the polymer in a 5:1 volume ratio of chloroform to methanol. The polymer solution was extruded at 25 ml/h through a 16 G needle, charged
to 30 kV, towards a grounded collecting plate 40 cm away. Fiber morphology was inspected using scanning electron microscopy and determined to be 9.51±0.75 µm (n=32 fibers). Scaffolds were punched from mats using a 1.5 mm dermal biopsy punch. Scaffolds approximately 1.6 mm in thickness were used for this study.

After preparation, scaffolds were loaded into custom-designed polycarbonate blocks designed to confine the cell suspension during seeding and sterilized by exposure to ethylene oxide (Anderson Sterilizers, Haw River, NC) for 14 h. Scaffolds were then prewet by soaking in a graded ethanol series, rinsed in phosphate buffered saline (PBS) three times, and soaked in general medium (DMEM, 10% FBS, 1% PSF) for 72 h.

**Cell Isolation and Culture**

Bovine articular chondrocytes were isolated from the femoral chondyles of 7 to 10-day old calves (Research 87, Boylston, MA) within 24 h of slaughter using previously described methods [216]. Briefly, cartilage was isolated, minced to 1x1x1 mm pieces, washed with PBS, and incubated in chondrocyte growth medium (DMEM, 10% FBS, 1% non-essential amino acids, 50 µg/ml ascorbic acid, 46 µg/ml L-proline, 20 mM HEPES, 1% PSF) supplemented with 2 mg/ml collagenase type II (Worthington biochemical corporation, Lakewood, NJ) on a shaker table at 37°C for 16 h. Cells were isolated from 4 legs, pooled, aliquoted and cryopreserved in freezing medium (DMEM containing 20% FBS and 10% dimethyl sulfoxide).

MSCs were isolated from the femora and tibiae of five 6-week old, male Lewis rats (150-174 g; Harlan Laboratories, Indianapolis, IN) [256]. Care of the animals was provided in accordance with the Rice University Institutional Animal Care and Use
Committee. Isolation was performed using previously described methods [256]. Briefly, after euthanasia, the tibiae and femora were aseptically removed and the marrow was flushed from each bone using 5 ml of general media. Marrow pellets were collected, broken up, and plated in 75-cm² tissue culture flasks. Medium was replaced after one day in order to remove the non-adherent cell population. Cells were cultured for 5 days, after which they were lifted using 0.05% trypsin-EDTA, pooled, and cryopreserved in freezing medium for storage.

MSCs and chondrocytes were then thawed, plated, and expanded in chondrocyte growth medium for 5 days. Cells were then lifted using 0.05% trypsin-EDTA, suspended in chondrocyte growth medium. 30 µl of cell suspension, containing 40,000 cells, was pipetted on top of each scaffold. Scaffolds were seeded with MSCs, chondrocytes, or a 1:3 mixture chondrocytes and MSCs. Scaffolds were then incubated overnight to allow for cell attachment. Empty scaffolds were also incubated for an additional night after prewetting in chondrocyte growth medium. Prior to implantation scaffolds were removed from loading blocks and rinsed in sterile PBS.

**Animal Surgeries**

Animal surgeries were performed according to protocols approved by the Rice University Institutional Animal Care and Use Committee, and NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were observed. Forty healthy male Lewis rats (12-weeks old and weighing 300-350 g) were purchased from Harlan Labs (Indianapolis, IN). Animals were anesthetized in an induction chamber with a 4% isofluorane/oxygen gas mixture. Prior to surgery, each animal was given an
intraperitoneal injection of buprenorphine, an intraperitoneal injection of normal saline to account for fluid losses during surgery, a subcutaneous injection of enrofloxacin as a prophylaxis against infection during surgery, and a subcutaneous injection of bupivacaine along the intended line of incision. Additionally, post-operatively each animal was given periodic intraperitoneal injections of buprenorphine for post-operative analgesia.

During surgery, a lateral parapatellar longitudinal incision was made to expose the knee joint. The synovial capsule was incised, and the trochlear groove was exposed after medial luxation of the patella. With the knee maximally flexed, a defect (1.5 mm in diameter, and 1.5 mm in depth) was created in the center of the groove, using a dental drill. A 0.9 mm diameter drill bit was first used to establish a 0.9 mm diameter defect. The defect was irrigated and enlarged to 1.5 mm using a 1.5 mm drill bit, fashioned with a 1.5 mm stop to ensure a defect of precisely 1.5 mm in depth is created. All debris was removed from the defect with a curette and irrigation. Depending on the experimental group, the defect was left untreated or a scaffold was press-fit into the defect with the appropriate cell population. The patella was physically relocated, and the joint capsule and subcutaneous tissue was closed with Vicryl 5-0 sutures. The skin was closed with Vicryl 4-0 sutures, which were removed after 1 week. After 12 weeks animals were euthanized and tissue surrounding the trochlear groove was removed en bloc. Samples were fixed in 10% neutral buffered formalin for 72 h at room temperature. Following fixation samples were stored in 70% ethanol.
**μCT Imaging and Analysis**

After tissue fixation the volume of mineralized bone in the defect was analyzed by microcomputed tomography (Skyscan 1172 high-resolution micro-CT; Sky-scan) using previously established methods [257]. To determine the volume of bone in the defect, a region of interest 1.5 mm in diameter and 1.5 mm in thickness was selected by defining the bottom region of the defect and measuring 1.5 mm toward the joint surface. The total volume of mineralized bone was then calculated using thresholds 45 and 255.

**Tissue Processing**

Samples were demineralized with EDTA and formic acid (Formical 2000; Decal Corporation, Congers, NY) for 3 weeks on a shaker table at room temperature. Samples were then prepared for cryosectioning by soaking in a solution of PBS with 15 wt% sucrose, followed by PBS with 30% sucrose. Samples were then frozen in Histoprep freezing medium at a controlled rate using chilled 2-methylbutane, and sectioned using a cryotome (Leica CM 1850 UV; Leica Biosystems Nussloch GmbH, Germany). Longitudinal sections 6 µm thick were mounted on glass slides. Sections were stained with Safranin O/Fast Green and hematoxylin and eosin (H&E). Images were obtained using a light microscope with a digital camera attachment (Axio Imager.Z2 equipped with AxioCam MRc5; Carl Zeiss MicroImaging GmbH, Germany).

**Histological Scoring**

Histological sections from the lateral and medial regions of each defect (total of 16 images per group) were blindly scored by three independent evaluators (J.L., S.L., L.A.K.) based on a modified version of a previously established scoring system for
osteochondral tissue repair in rabbits [258]. Sections were scored for the extent of cartilage repair based on 8 criteria, as shown in Table 7.2.

**Statistical Analysis**

A significance level of 0.05 was used for all statistical analysis. \( \mu \)CT data was analyzed by one-way analysis of variance and Tukey’s multiple-comparison test. Data are reported as means plus the standard deviation. Histological scores were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney-U test. Data are reported as the distribution of scores for each parameter.

**RESULTS**

**\( \mu \)CT Imaging and Analysis**

\( \mu \)CT analysis (Figure 7.1) found mineralized bone regeneration in the empty, blank, and MSC samples that resulted in 28-35% of the defect site filled with new bone. No effect of the PCL scaffold or MSCs was observed on the total mineralized bone volume in the defect. In the AC and CC samples, negligible mineralized bone growth was observed with a total bone volume of only 0.75-1.0% of the defect site. Thus, significantly lower mineralized bone volume was detected in the AC and CC samples compared to the empty, blank or MSC samples.

**Histological Observation and Scoring**

Representative images of the histological sections from each group are shown in Figures 7.2-7.6, while Figures 7.7 and 7.8 show the score distributions for each of the 8 parameters listed in Table 7.2.
In general, the surface tissue of the empty samples contained large amounts of fibrous tissue or fibrocartilage with only one sample containing any hyaline cartilage on the surface. The vast majority of PCL and MSC samples only had a thin layer of fibrous tissue on the surface of the defect. CC and AC samples often had hyaline-like cartilage on the edges or interior of the defect with a portion of fibrous tissue in the center of the defect surface. In scoring the morphology of new surface tissue, it was observed that the CC samples had higher quality surface tissue compared to PCL and MSC samples.

When evaluating the morphology of the new cartilage tissue that formed in the defect site (Fig. 7.7C) it was again seen that nearly all of the empty, PCL, and MSC samples resulted in either no cartilage growth in the defect site or cartilage that consisted almost exclusively of fibrocartilage. However, a large portion of the CC and AC samples contained hyaline cartilage, rather than fibrocartilage, and while some had either no cartilage tissue or primarily fibrocartilage, histological scoring still resulted in significantly higher quality cartilage in the CC and AC samples than in the empty, PCL, and MSC samples.

Consistent with the evaluation of cartilage morphology, the evaluation of Safranin O staining in the defects revealed more intense staining in the CC and AC samples compared to the empty, PCL, and MSC groups. Little to no Safranin O staining was observed in the PCL and MSC samples, and similarly 15 of 16 images analyzed from the empty samples contained little to no Safranin O staining.

When evaluating the thickness of new cartilage tissue, the fibrocartilage in the empty, PCL, and MSC groups was generally found to be thinner than the neighboring
cartilage thickness. In the CC and AC samples, the new cartilage tissue was generally found to be thicker than the neighboring tissue. Histological scoring found that CC and AC samples had significantly thicker cartilage tissue than the empty PCL, and MSC samples.

When evaluating the density of chondrocytes in the new cartilage tissue, it was observed that the AC samples scored significantly higher than the empty, PCL, and MSC samples, and the CC samples had higher scores than the PCL samples. While a higher score would indicate a more desirable outcome, and ideally a chondrocyte density most similar to the neighboring cartilage, it should be noted that both the CC and AC groups had a large portion of samples with a higher density of chondrocytes compared to the surrounding tissue.

Evaluating the distribution of chondrocytes in the tissue, it was observed that the empty, PCL, and MSC samples mostly consisted of individual or disorganized cells, whereas the CC and AC samples primarily consisted of clustered chondrocytes. Thus, the CC and AC groups scored significantly higher in chondrocyte distribution compared to the empty, PCL, and MSC samples.

When evaluating the regularity of the joint surface as well as the chondrocyte and GAG content of the adjacent cartilage, it was observed that the AC samples had significantly worse joint regularity than the empty, PCL, or MSC samples and the CC samples had worse regularity than the PCL samples. However, the CC samples were seen to have higher quality adjacent cartilage than the empty, PCL, and MSC samples.
Consistent with the results of µCT analysis, significant bone ingrowth was observed throughout the empty, PCL, and MSC samples (Fig. 7.2-7.4). Conversely, negligible bone growth was observed in the CC and AC samples. Instead, the scaffold was primarily surrounded by fibrous tissue (Fig. 7.5E and 7.6E) with transitional tissue (Fig. 7.5D and 7.6D)[259] observed in the interior of the scaffold. The ingrown tissue had no Safranin O staining, and contained cells that were either oval or spheroid in shape with some present in lacunae (Fig. 7.6D).

**DISCUSSION**

Clinically, autologous chondrocytes have long been recognized for their ability to repair chondral defects when transplanted *in vivo* [248]. Currently, the ACI procedure involves initial biopsy of autologous cartilage for the isolation of articular chondrocytes, expansion of cells *in vitro*, and implantation of expanded cells into the defect, which is sealed by a layer of periosteum sutured over top [246]. While the technique is widely used in the clinic, the use of autologous chondrocytes presents several challenges to the long-term success of the treatment. Isolating large numbers of chondrocytes from healthy cartilage not only leads to donor site morbidity, but also is a challenge due to the relatively low density of chondrocytes in cartilage tissue [194]. Furthermore, rapid phenotypic changes are seen during *in vitro* expansion of chondrocytes, which leads to a cell population with a more fibroblastic phenotype than healthy articular chondrocytes [250]. Co-cultures of articular chondrocytes and MSCs have previously been shown, through *in vitro* analysis, to be able to provide several potential means to overcome some of the challenges posed by the use of articular chondrocytes alone [181, 213, 214].
Co-culture of articular chondrocytes and MSCs have been thoroughly investigated *in vitro*, and the utility of this approach has generally been shown to be the ability to use up to 75% fewer chondrocytes, while still achieving equal or greater levels of chondrogenesis as cultures containing 100% chondrocytes [181, 213]. While numerous studies have characterized these cultures in *vitro*, they have not been investigated for their ability to regenerate cartilage in an orthotopic site *in vivo*. The objective of the present study was to evaluate the ability of co-cultures of chondrocytes and MSCs to repair articular cartilage in a rat osteochondral defect when implanted with an electrospun polymer scaffold. We hypothesized that such co-cultures would lead to equal or greater cartilage repair, while utilizing significantly fewer chondrocytes, than chondrocytes alone. For consistency with previous *in vitro* studies [181, 212-214], we chose to use a 1:3 ratio of chondrocytes-to-MSCs seeded on electrospun PCL scaffolds. The animal model chosen for this study was a previously established osteochondral defect in the rat trochlear groove [260, 261]. Due to the impracticality of isolating rat cartilage, bovine articular chondrocytes were used for this study. Even using xenogeneic chondrocytes in this model, we observed no evidence of an immune response to the bovine cells in either the AC or CC groups. In addition we observed hyaline-like cartilage formation in these samples, demonstrating that the implanted cells were beneficial to the tissue repair. Several other studies have demonstrated similar success with xenogenic chondrocytes in chondral and osteochondral defects without signs of an immune response [262, 263].

As hypothesized, chondrocytes and co-cultures of chondrocytes and MSCs led to enhanced cartilage repair *in vivo*, as primarily characterized by repair tissue that consisted
of thicker hyaline-like cartilage with more intense Safranin O staining. Conversely, new cartilage formed in the empty, PCL, and MSC samples primarily consisted of fibrocartilage, a common form of repair tissue composed primarily of collagen type I and low amounts of type II collagen and proteoglycans [264]. Due to the inferior mechanical properties of this tissue composition compared to hyaline cartilage, the fibrocartilage is not expected to withstand long-term mechanical loading, and may deteriorate over time [247]. Consistent with this tissue characterization, the CC and AC samples were found to have more intense Safranin O staining than the other samples, indicative of higher proteoglycan content and a more hyaline-like tissue [265]. Furthermore, new cartilage formed in the CC and AC samples was not only of a superior morphology but was found to be much thicker than new cartilage formed in the empty, PCL, and MSC samples. This result is correlated to the ability of the different treatments to form cartilage but may also be due to deterioration of repair cartilage prior to twelve weeks in the empty, PCL, and MSC samples. Based on the histological scoring, the adjacent cartilage of the empty, PCL, and MSC samples showed signs of degenerative changes, relative to the CC samples, providing evidence of the likelihood of past or future deterioration of the repair tissue. Investigation of additional time points in future studies would likely shed light on the long-term stability of this tissue. Additionally, the distribution of cells in the CC and AC samples was found to be more similar to native cartilage, again indicating an improved healing response compared to empty, PCL, and MSC samples. Overall, it was observed that the CC and AC samples led to the formation of new cartilage tissue with a higher quality composition and structure compared to the empty, PCL, and MSC
samples. The quality of this tissue is more similar to native hyaline cartilage and would be expected to be a more functional and durable tissue compared to the fibrocartilage seen in the other samples.

While the newly formed cartilage of the CC and AC samples was in many ways a significantly higher quality repair tissue than the empty, PCL, and MSC samples, the surface regularity of the AC samples was worse than all groups other than CC. This may be a result of the large amount of cartilage formation that did not integrate well with the surrounding tissue: a significant challenge in cartilage repair [247]. Alternatively, the poor surface regularity could be a result of the lack of bone formation in the subchondral region, and thus lack of stability of the newly formed tissue [266, 267]. Furthermore, in many instances, the new cartilage tissue in the CC and AC samples had a slight concave structure with the center filled with fibrous tissue. This indentation may be further evidence of a lack of underlying structural support in these samples. The drastic difference in bone and cartilage healing that was observed in these samples is quite remarkable when the impact of the subchondral bone in cartilage regeneration is considered. It is well established that the healing of the subchondral bone is crucial to both the regeneration and stability of articular cartilage [268-270], so the stark improvement in cartilage repair seen in samples with no subchondral bone healing at 12 weeks was a significant outcome that demonstrates the robust ability of the CC and AC treatments to stimulate cartilage repair.

Transitional tissue has been described as a tissue that is comprised of ovoid and spherical cells, present with or without lacunae. This tissue characterization encompasses
tissues that range from fibrous tissue to hyaline cartilage and includes, but is not limited to, fibrocartilage [259, 271]. Most transitional tissue is of a higher regenerative quality than fibrous tissue, but a lower quality than fibrocartilage [272]. Studies evaluating the quality of new tissue seen in repair procedures have often observed the formation of transitional tissue after the deterioration of hyaline tissue or the presence of transitional tissue without earlier evidence of hyaline cartilage [273-275]. In the present study, transitional tissue was observed to be the primary tissue type that grew into the subchondral region of the CC and AC samples. Without earlier time points, we are unable to determine the initial morphology of the ingrown tissue; however it is possible that this tissue was of a higher quality cartilage repair tissue that deteriorated to its present state. Likewise, it is possible that this tissue would eventually remodel and bone ingrowth would occur into the subchondral regions of the scaffold. However, future studies should investigate the use of a bilayered treatment method that could accelerate the bone healing in the subchondral region of the CC and AC samples. The presence of subchondral support may further enhance the cartilage repair [268, 269].

Interestingly, when seeded on PCL scaffolds, MSCs had no measurable effect on either cartilage healing or mineralized bone volume compared to PCL scaffolds alone. While numerous studies have demonstrated enhanced cartilage [276, 277] repair with the delivery of MSCs, other work has observed no effect of delivered MSCs on cartilage repair [267]. Potentially, the method of cell delivery in this study may have not allowed for condensation of the MSCs. Other studies delivering MSCs in osteochondral defects have been capable of achieving MSC condensation, which is known to enhance
chondrogenesis [276]. Furthermore, the delivery of undifferentiated MSCs may not have provided the cells with enough chondrogenic stimuli to lead to cartilage formation. Possibly the use of chondrogenically pre-differentiated MSCs may have seen improved chondrogenesis [136].

The results of the present study indicate the potential for the use of co-cultures of articular chondrocytes and MSCs to be used for in vivo repair of articular cartilage defects. The number of cells seeded onto the PCL scaffolds in this study was a density of cells that is approximately three times lower than the density reported for use in clinical ACI procedures [248]. However, the use of a polymer scaffold makes the current approach more similar to matrix-associated ACI procedures, which have been demonstrated to be capable of success with approximately half the number of cells needed for first generation ACI procedures [278]. Furthermore, these results may indicate the mechanism for success of experimental procedures investigating the combination of ACI with microfracture [259]. Therein it was observed that the combination of ACI with microfracture led to improved cartilage repair compared to ACI alone. Based on the results of the present study, one may postulate that the combination of bone marrow progenitor cells with ACI led to enhanced repair in a similar manner as demonstrated for the effect of co-cultures of MSCs and chondrocytes. Regardless, the results here demonstrate that co-implantation of MSCs with chondrocytes can reduce the total number of chondrocytes needed for implantation when implanted with a total density of cells in the same range as ACI or matrix-associated ACI procedures. The outcome of matrix associated ACI procedures was shown to be dependent on in vitro culture
conditions, such as the cell passage number [230], and thus the addition of MSCs to chondrocytes may allow for the use of lower passaged chondrocytes and thus improved outcomes.

CONCLUSIONS

This study demonstrated the ability of co-cultures of articular chondrocytes and MSCs to repair cartilage defects in the rat trochlear groove defect. These results have important implications for cartilage tissue engineering, as they demonstrate that such co-cultures could be used to reduce the total number of chondrocytes needed for cartilage repair, while still achieving an equivalent level of cartilage repair.
Figure 7.1: Percent of defect volume filled with mineralized bone, as determined by microcomputed tomography.

Data are reported as means ± standard deviation for n=8 animals. Groups not connected by the same letter are significantly different (p<0.05).
Figure 7.2: Representative histological sections of tissue formation after 12 weeks in untreated defects in the rat trochlear groove (empty).

Sections were stained with H&E (A, C, D, and E) and Safranin O/Fast Green (B). Scale bars in A and B each represent 250 µm, and scale bar in C represents 50 µm in C, D, and E. A and B show mostly fibrous tissue with limited fibrocartilage in the chondral region and bone growth in the subchondral space. C, D, and E show high magnification images of the chondral and subchondral regions.
Figure 7.3: Representative histological sections of tissue formation after 12 weeks of implantation of electrospun PCL scaffolds (PCL).

Sections were stained with H&E (A, C, D, and E) and Safranin O/Fast Green (B). Scale bars in A and B each represent 250 μm, and scale bar in C represents 50 μm in C, D, and E. A and B show mostly fibrous tissue with limited fibrocartilage in the chondral region and new bone growth into the nondegraded PCL scaffold. Round, unstained regions of approximately 10 μm show fibers comprising the PCL scaffold. C, D, and E show high magnification images of the chondral and subchondral regions.
Figure 7.4: Representative histological sections of tissue formation after 12 weeks of implantation of electrospun PCL scaffolds seeded with rat MSCs (MSC).

Sections were stained with H&E (A, C, D, and E) and Safranin O/Fast Green (B). Scale bars in A and B each represent 250 µm, and scale bar in C represents 50 µm in C, D, and E. A and B show mostly fibrous tissue with limited fibrocartilage in the chondral region and new bone growth into the nondegraded PCL scaffold. Round, unstained regions of approximately 10 µm show fibers comprising the PCL scaffold. C, D, and E show high magnification images of the chondral and subchondral regions.
Figure 7.5: Representative histological sections of tissue formation after 12 weeks of electrospun PCL scaffolds seeded with a co-culture of rat MSCs and bovine articular chondrocytes (CC).

Sections were stained with H&E (A, C, D, and E) and Safranin O/Fast Green (B). Scale bars in A and B each represent 250 μm, and scale bar in C represents 50 μm in C, D, and E. A and B show thick regions of hyaline-like cartilage with some fibrocartilage and fibrous tissue in the chondral region. The subchondral region is composed mainly of transitional and fibrous tissue. Round, unstained regions of approximately 10 μm show fibers comprising the PCL scaffold. C, D, and E show high magnification images of the chondral and subchondral regions.
Sections were stained with H&E (A, C, D, and E) and Safranin O/Fast Green (B). Scale bars in A and B each represent 250 µm, and scale bar in C represents 50 µm in C, D, and E. A and B show thick regions of hyaline-like cartilage with some fibrocartilage and fibrous tissue in the chondral region. The subchondral region is composed mainly of transitional and fibrous tissue. Round, unstained regions of approximately 10 µm show fibers comprising the PCL scaffold. C, D, and E show high magnification images of the chondral and subchondral regions.
Figure 7.7: Histological score distribution for the (A) morphology of new surface tissue, 
(B) Safranin O staining, (C) morphology of new cartilage, and (D) thickness 
of new cartilage.

Specific scoring criteria for each category is listed in Table 2. Groups not connected by 
the same letter shown on top of the bars are significantly different ($p<0.05$).
Figure 7.8: Histological score distribution for the (A) chondrocyte cellularity, (B) chondrocyte distribution, (C) joint surface regularity, and (D) chondrocyte and GAG content of adjacent cartilage.

Specific scoring criteria for each category is listed in Table 2. Groups not connected by the same letter shown on top of the bars are significantly different ($p<0.05$).
Table 7.1: Outline of Experimental Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Abbreviation</th>
<th>Scaffold</th>
<th>Rat MSCs (cells)</th>
<th>Bovine ACs (cells)</th>
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<tr>
<td>Empty control</td>
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<td>No scaffold</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Material control</td>
<td>PCL</td>
<td>Electrospun PCL</td>
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<td>0</td>
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<tr>
<td>MSCs</td>
<td>MSC</td>
<td>Electrospun PCL</td>
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<td>0</td>
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<tr>
<td>Co-cultures</td>
<td>CC</td>
<td>Electrospun PCL</td>
<td>30,000</td>
<td>10,000</td>
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<tr>
<td>Articular chondrocytes</td>
<td>AC</td>
<td>Electrospun PCL</td>
<td>0</td>
<td>40,000</td>
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Table 7.2: Histological Scoring System for the Evaluation of Cartilage Repair in rat osteochondral defects based off of a modified version of a previously established scoring system for rabbit osteochondral tissue repair [258]

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<th>Morphology of New Surface Tissue</th>
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<tr>
<td>Exclusively AC</td>
<td>4</td>
</tr>
<tr>
<td>Mainly hyaline cartilage</td>
<td>3</td>
</tr>
<tr>
<td>Fibrocartilage (spherical morphology in &gt;75% of cells)</td>
<td>2</td>
</tr>
<tr>
<td>Mostly fibrous tissue (spherical morphology in &lt;75% cells)</td>
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<tr>
<td>No tissue</td>
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<table>
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</tr>
<tr>
<td>Mainly hyaline cartilage</td>
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</tr>
<tr>
<td>Fibrocartilage</td>
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<tr>
<td>Only fibrous tissue/ No tissue</td>
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<th>Thickness of New Cartilage</th>
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<td>Similar to surrounding cartilage</td>
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</tr>
<tr>
<td>Greater than surrounding cartilage</td>
<td>2</td>
</tr>
<tr>
<td>Less than surrounding cartilage</td>
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</tr>
<tr>
<td>No cartilage</td>
<td>0</td>
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<table>
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<th>Joint Surface Regularity</th>
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<tr>
<td>Smooth, intact surface</td>
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</tr>
<tr>
<td>Surface fissures (&lt;25% new surface thickness)</td>
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</tr>
<tr>
<td>Deep fissures (25-99% new surface thickness)</td>
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<td>Complete disruption of the new surface</td>
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<table>
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<th>Chondrocyte Distribution</th>
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Chapter 8: References


[52] Ramakrishna S, Fujihara K, Teo WE, Lim TC, Ma Z. An Introduction to Electrospinning and Nanofibers. Singapore: World Scientific Publishing Co.; 2005.


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APPENDIX A

List of manuscripts co-authored by the doctoral candidate during the course of this thesis


Pre-differentiated Mesenchymal Stem Cells in a Rabbit Model. Osteoarthritis and Cartilage. 2014; Submitted.
APPENDIX B

Enhanced Chondrogenesis in Co-Cultures with Articular Chondrocytes and Mesenchymal Stem Cells*

ABSTRACT
In this work, articular chondrocytes (ACs) and mesenchymal stem cells (MSCs) with 1:1 and 1:3 cell ratios were co-cultured in order to evaluate if a majority of primary ACs can be replaced with MSCs without detrimental effects on in vitro chondrogenesis. We further used a xenogeneic culture model to study if such co-cultures can result in redifferentiation of passaged ACs. Cells were cultured in porous scaffolds for four weeks and their cellularity, cartilage-like matrix formation and chondrogenic gene expression levels (collagen I and II, aggrecan) were measured. Constructs with primary bovine ACs had ~1.6 and 5.5 times higher final DNA and glycosaminoglycan contents, respectively, in comparison to those with culture expanded chondrocytes or MSCs harvested from the same animals. Equally robust chondrogenesis was also observed in co-cultures, even when up to 75% of primary ACs were initially replaced with MSCs. Furthermore, species-specific RT-PCR analysis indicated a gradual loss of MSCs in bovine-rabbit co-cultures. Finally, co-cultures using primary and culture expanded ACs resulted in similar outcomes. We conclude that the most promising cell source for cartilage engineering was the co-cultures, as the trophic effect of MSCs may highly increase the chondrogenic potential of ACs thus diminishing the problems with primary chondrocyte harvest and expansion.

* This appendix was published as Meretoja, VV, Dahlin, RL, Kasper, FK, Mikos, AG. Enhanced chondrogenesis in co-cultures with articular chondrocytes and mesenchymal stem cells. Biomaterials. 2012;33:6362-6369
INTRODUCTION

Articular cartilage lesions, commonly caused by trauma, erroneous biomechanical conditions or age-related degeneration, have very limited self-regeneration capacity. Due to the low biosynthetic activity in and avascular nature of mature cartilage tissue, even small cartilage lesions fail to heal on their own [1, 2]. In cases of deep defects down to the level of subchondral bone, the formation of a blood clot and the recruitment of stem cells can contribute to the healing process. Clinically, this phenomenon is commonly exploited in subchondral drilling and microfracture techniques, where bleeding is artificially induced to help in the treatment of cartilage defects [3, 4]. Furthermore, autologous chondrocyte transplantation, often in combination with a culture substrate, has recently been used to provide enough cartilaginous matrix producing cells to fill relatively large defects. Unfortunately, none of the current treatment methods are able to reliably regenerate native hyaline cartilage structure but usually result in more fibrous and mechanically inferior repair tissue.

The interdependent problems of donor site morbidity, lack of availability and rapid dedifferentiation in culture are major drawbacks associated with autologous chondrocyte transplantation. Depending on the size and quality of the original cartilage biopsy, approximately twenty to fifty fold culture expansion of the harvested cells is required to obtain clinically relevant numbers of cells [5]. Alternative cell sources and tissue engineering methods are therefore actively being researched [6, 7]. One promising cell source for cartilage engineering is mesenchymal stem cells (MSCs). MSCs can be harvested and expanded from various tissue sources including bone marrow, synovium and adipose tissue, with certain phenotypic differences depending on the source and
culture conditions [8, 9]. The most widely utilized tissue source for MSCs is bone marrow, due to the ease of harvest and high chondrogenic potential of marrow-derived MSCs. A major difficulty with MSCs is that they produce cartilage only after extensive chondrogenic induction, which however, can further lead to hypertrophy and calcification, reminiscent of endochondral ossification [10, 11].

Lately, co-cultures of articular chondrocytes (ACs) and MSCs have been proposed to mitigate the above mentioned problems. Interactions between the two cell types can maintain their chondrogenic phenotype, resulting in robust chondrogenesis while still minimizing the need for mature chondrocytes. The functionality of AC-MSC co-cultures was originally demonstrated in pellet cultures [12] and only few studies have further evaluated such co-cultures using either hydrogels [13-15] or porous scaffolds [16] as three-dimensional tissue engineering matrices. Furthermore, although both primary and culture expanded chondrocytes have been used in the co-cultures, there seem to be no previous studies comparing the two in a single culture model.

The aim of the current study was to evaluate if a majority of primary ACs can be replaced with MSCs without detrimental effects on in vitro chondrogenesis when cultured in porous scaffolds. We further examined whether such co-cultures can rescue the chondrogenic potential of culture expanded ACs.

**Materials and Methods**

**Scaffold Preparation**

Nonwoven poly(ε-caprolactone) (PCL) microfiber mats were fabricated using a horizontal electrospinning setup with a copper ring to stabilize the electric field as
previously described [17]. Mats were electrospun to an average fiber diameter of approximately 10 μm (9.6 ± 2.0 μm) using a solution of 18 wt % PCL (Inherent viscosity range 1.0 – 1.3; Durect corporation, Cupertino, CA) in a 5:1 volume ratio of chloroform to methanol. The polymer solution was pumped at a flow rate of 40 mL/h through a blunt 16 G needle, while charged with an applied voltage of 30 kV to draw microfibers toward the collector plate 33 cm from the needle tip. The resulting PCL mats of approximately 1.3 mm thickness were aerated, inspected for consistent microfiber morphology using SEM, and stored in a desiccator.

PCL scaffolds were prepared by die-punching 8-mm-diameter discs from the electrospun mats using a dermal biopsy punch. The scaffolds were then sterilized by exposure to ethylene oxide (AN74i, Andersen Sterilizers, Haw River, NC) at ambient temperature for 14 h and aerated overnight to remove residual fumes. In order to eliminate air bubbles and to improve cell adhesion, scaffolds were prewetted by centrifugation through a graded series of ethanol (100 % – 20 %), followed by two rinses in phosphate buffered saline (PBS), and incubation in general culture medium (high-glucose DMEM, 10 % fetal bovine serum (FBS), penicillin/streptomycin/fungizone (PSF)) for three days.

**Cell Harvest and Expansion**

Bovine MSCs and ACs were harvested from 7-10 day old calves (Research 87, Boylston, MA), less than 36 h after slaughter using previously established protocols [18]. Briefly, bone marrow was harvested from tibiae and femora, diluted with culture medium and separated from fat by centrifugation. Marrow isolations were plated onto culture
flasks and allowed to adhere for 48 h before washing with PBS to remove non-adherent cells and blood clots. Cultures were maintained in general medium until confluent and passaged for sub-cultures. After two passages, MSCs from a minimum of three animals were pooled, aliquoted, and frozen in freezing medium (DMEM with 20% FBS and 10% dimethyl sulfoxide). Articular cartilage (from the same animals) was collected from the femoral condyle area, minced to approximately 1x1x1 mm, washed with PBS and digested in chondrocyte culture medium (DMEM, 10% FBS, 1% non-essential amino acids, 50 μg / mL ascorbic acid, 46 μg / mL L-proline, 20 mM HEPES, PSF) containing 2 mg / mL collagenase type II (Worthington biochemical corporation, Lakewood, NJ) while incubating at 37 °C overnight. Primary ACs were pooled, aliquoted, and frozen in freezing medium.

Rabbit MSCs were harvested and isolated as previously described [19]. Briefly, bone marrow was aspirated from the tibiae of six 6 month old male New Zealand white rabbits (Myrtle's Rabbitry Inc., Thompsons Sta, TN). Under general anesthesia, bone marrow from each leg was aspirated into a 10 mL syringe containing 5000 U / mL heparin to prevent coagulation. The bone marrow was plated in tissue culture flasks and non-adherent cells were washed away after 72 h. Cultures were maintained in general medium until confluent, cells from all animals were pooled together, aliquoted, and frozen in freezing medium.

**Cell Seeding and Chondrogenic Culture**

Two independent cell cultures were performed: one with primary (p₀) bovine ACs and (p³) bovine MSCs, one with culture expanded (p²) bovine ACs and (p²) rabbit MSCs.
The purpose of the xenogeneic model was to facilitate the cell type specific gene expression analysis of co-cultured chondrocytes and MSCs. In both cases, scaffolds were seeded with pure AC and MSC populations as well as their combinations, with 1:1 and/or 1:3 AC-to-MSC ratios.

The frozen AC and MSC aliquots were thawed and sub-cultured as indicated to establish seeding suspensions of $1.25 \times 10^6$ cells / mL. In preparation for cell seeding, prewetted scaffolds were press-fitted into cylindrical poly(methyl methacrylate) cassettes designed to confine the seeding suspension. Cells (in ~200 μL of general medium) were then pipetted onto each scaffold to achieve a final seeding density of $4 \times 10^6$ cells / mL scaffold volume. Cells were allowed to adhere for 4 h before culture medium was gently added to completely cover the cassettes. Scaffolds were removed from the cassettes after 24 h and placed in 12 well culture plates with 4 mL of serum free chondrogenic medium (high-glucose DMEM, 1 % ITS+ premix [BD Biosciences, San Jose, CA], 50 μg / mL ascorbic acid, 100 nM dexamethasone, 10 ng / mL TGF-beta 3 [PeproTech, Rocky Hill, NJ], PSF). Half of the medium was replenished two to three times a week. Four replicate scaffolds were harvested after 0, 14, and 28 days of chondrogenic culture and washed with PBS. A 3 mm biopsy punch was then used to obtain individual samples for each analysis.

**Biochemical Assays**

Two 3 mm biopsy samples were pooled together from each scaffold and frozen in -20 °C until used for biochemical assays. Thawed samples were digested in 500 μL of a proteinase K solution (1 mg / mL proteinase K, 0.01 mg / mL pepstatin A and 0.185 mg /
mL iodoacetamide in a 50 mM tris(hydroxymethyl aminomethane) – 1 mM ethylenediaminetetraacetic acid buffer, pH 7.6) in a 56 °C water bath for 16 h. DNA and matrix components were extracted via two additional freeze-thaw cycles followed by 10 min sonication.

DNA content of the scaffolds was determined using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Eugene, OR). Briefly, cell lysate, assay buffer and dye solution were combined in an opaque 96-well plate in duplicates, and allowed to incubate for 10 min at room temperature. Fluorescence was measured using excitation and emission wavelengths of 485 nm and 528 nm (FL x800 Fluorescence Microplate Reader; BioTek Instruments, Winooski, VT), respectively, and DNA concentrations were determined relative to a lambda DNA standard curve.

Glycosaminoglycan (GAG) content was determined using the colorimetric dimethylmethylene blue assay [20]. Briefly, cell lysate and color reagent were combined in a transparent 96-well plate in duplicates, and allowed to incubate for 7 min at room temperature. Absorbance at 520 nm was measured (PowerWave x340 Microplate Reader; BioTek Instruments), and GAG concentrations were determined relative to a chondroitin sulfate standard curve. For glycosaminoglycan synthetic activity, the resulting GAG amounts were normalized to the amount of DNA for each sample.

**Histology**

One 3 mm biopsy sample from each scaffold was fixed for histology in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA), then immersed in 70% ethanol prior to embedding in HistoPrep freezing medium (Fisher Scientific). Frozen
sections 5 μm thick were cut using a cryostat (Leica CM 1850 UV; Leica Biosystems Nussloch GmbH, Germany), mounted onto glass slides, and placed on a 42 °C slide warmer to facilitate adhesion. Sections were stained with Safranin O to visualize the distribution of cartilaginous extracellular matrix. Images were obtained using a light microscope with a digital camera attachment (Axio Imager.Z2 equipped with AxioCam MRc5; Carl Zeiss MicroImaging GmbH, Germany).

**Real-Time Reverse Transcription Polymerase Chain Reaction**

After 3 mm samples of the scaffolds were removed for biochemical analysis and histology, the remainder of the scaffolds were minced to approximately 1×1×1 mm pieces and used for RNA isolation using an RNeasy mini kit (Qiagen, Valencia, CA). Briefly, minced scaffolds were placed into 600 μL of lysis buffer and incubated at room temperature for 30 min with periodical vortexing. Cell lysate was then passed through a QIAshredder homogenization column and stored at -80 °C until further processing. An equal volume of 70 % ethanol was added to thawed lysates and RNA isolation was continued following the animal cell protocol provided by the manufacturer. Reverse transcription was then carried out to synthesize cDNA from purified RNA samples using Oligo(dT) primers (Promega, San Luis Obispo, CA) and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Finally, cDNA was subjected to real-time PCR (Applied Biosystems 7300 Real-Time PCR System, Foster City, CA) using SYBR Green detection (PerfeCTa SYBR Green FastMix, ROX; Quanta Biosciences, Gaithersburg, MD) with custom designed primers (Integrated DNA Technologies, Coralville, IA).
Primer sequences are given in Table B.1. Species specificity (>99%) and PCR efficiency (>95%) of each primer pair was verified before use. Target gene expression was first normalized to the expression of the housekeeping gene GAPDH in the same sample (ΔCt), then to the average baseline expression of that target gene measured in the bovine AC group at the start of chondrogenic cultures (ΔΔCt). Finally, the 2^-ΔΔCt method was used to convert normalized gene expression levels to fold differences and statistics were calculated on these values [21]. Similarly, 2^-ΔCt was used to calculate the ratios of collagen II / collagen I and bovine-specific / cross-species expression within individual samples.

Statistics

Results are presented as means ± standard deviations. Statistical analysis was performed with an SPSS 16.0 software package (SPSS, Chicago, IL). Biochemical assay data were analyzed using one-way ANOVA followed by Tukey's post-hoc test, whereas RT-PCR data were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney U test. Differences were considered significant at 95 % confidence level.

RESULTS

Scaffold Cellularity

A continuous increase in scaffold cellularity was observed in all culture groups with primary bovine ACs and bovine MSCs, as seen in Figure B.1A. The presence of primary chondrocytes enhanced cell proliferation as the DNA content in the beginning of the cultures was the highest but at later times the lowest with pure MSCs. Similarly, increasing cellularity was observed also with passaged bovine chondrocytes whereas


DNA content in the pure rabbit MSC group decreased in culture (Figure B.1B). Furthermore, there were no differences between primary AC and co-culture groups at any time, whereas the passaged AC group had significantly lower DNA contents in comparison to the corresponding co-culture group.

**GAG Synthesis**

All cultures with primary bovine ACs and bovine MSCs showed continuously increasing GAG contents and synthetic activity, as seen in Figures B.2A and B.2B. In the beginning of the chondrogenic cultures, GAG synthesis was strongest with the primary AC group, whereas at later times co-cultures reached similar or even higher levels of GAG content and synthesis than pure ACs. With passaged bovine ACs and their co-cultures, GAG contents and synthesis increased during the first two weeks, but then remained at similar levels for the remainder of the cultures (Figures B.2C and B.2D). The increase was larger with co-cultures than with pure ACs. Rabbit MSCs showed relatively high synthetic activity in the early cultures, whereas GAG contents actually decreased during culture (due to low cellularity).

**Histology**

Histological analysis showed that cells and extracellular matrix (ECM) accumulated mostly on the upper part of the scaffolds, with increasing cellular infiltration during the culture. Histology corroborated the results of GAG measurements. Primary ACs and their co-cultures had similar histological appearances, whereas clearly thinner cell/ECM crust was observed in pure bovine MSC cultures (Figure B.3A). In contrast to primary cells, co-cultures with passaged ACs produced much higher tissue contents than
ACs alone (Figure B.3B). Furthermore, only very limited amounts of cells/ECM were observed with pure rabbit MSCs.

**RT-PCR**

RT-PCR analysis was performed only with passaged ACs and rabbit MSCs. As expected, the initial gene expression levels for aggrecan and collagen types I and II were lowest with undifferentiated MSCs and highest with pure chondrocytes, and the same was true also for the collagen type II-to-type I expression ratio (Figure B.4). MSC and co-culture groups showed strong chondrogenic induction already after two weeks of culture, and after four weeks they had similar or even higher gene expression levels than pure chondrocytes. Chondrogenic markers were always higher in co-culture than in the MSC group, except that the highest aggrecan expression after four weeks was seen in MSCs.

Species-specific analysis of gene expression showed a strong continuous decrease in the proportion of rabbit MSCs in the co-cultures. In the beginning, the majority of GAPDH and collagen I signal came from rabbit MSCs, whereas collagen II and aggrecan expression were predominantly of bovine origin (Figure B.5). This was consistent with the relative expression levels in pure AC and MSC populations combined with the 1:3 AC-to-MSC seeding ratio. The situation had changed already after two weeks and all studied markers indicated a mostly bovine cell population, with further increase in bovine specific signal at the end of the culture period.
DISCUSSION

Cell-based cartilage engineering strategies involve the harvesting and *in vitro* expansion of chondrogenic cells for subsequent implantation usually within a porous scaffold or embedded in a carrier matrix to repair the original cartilage defect. The most commonly used cell types are articular chondrocytes and bone marrow derived mesenchymal stem cells. Challenges with chondrocytes include lack of availability and rapid dedifferentiation upon culturing, whereas MSCs require extensive induction by exogenously added growth factors to obtain the chondrogenic phenotype, which can also lead to hypertrophy and calcification. The current study aimed to investigate different cell sources for chondrogenic cultures, and to explore the potential of AC-MSC co-cultures for cartilage engineering.

In this study, serum-free differentiation medium supplemented with chondrogenic factors was used to create a controlled culture environment suitable for multiple cell types. This kind of defined medium is commonly applied for MSC differentiation, and may decrease experimental variation related to unknown serum composition [22]. While serum-free conditions can limit chondrocyte proliferation, the medium still allows for efficient cartilaginous ECM production [23, 24]. Furthermore, although the electrospun PCL scaffolds used in the current study may not be optimal for mechanically demanding applications, they provide an excellent 3D culture model for cartilage engineering.

Much higher DNA and matrix contents were observed with primary chondrocytes than with passage two cells, consistent with the notion of rapid dedifferentiation of culture expanded chondrocytes [25-27]. In addition, passaged ACs showed decreasing collagen expression and unchanged GAG contents from two to four weeks, further
highlighting the problem of dedifferentiation during prolonged cultures. Primary chondrocytes have been previously shown to produce superior engineered cartilage in comparison to MSCs harvested from the same animals [18, 28], and this was also the case in the current study. In contrast, the outcome with passaged ACs seemed to be similar to that with MSCs. Interestingly, two recent studies with large animal models demonstrated equal or even better cartilage repair using MSCs than culture expanded chondrocytes [29, 30]. On the other hand, it has been shown that chondrocytes can retain their original phenotype or even redifferentiate in co-cultures with primary cells, potentially increasing their applicability in cartilage engineering [31-33].

Bovine and rabbit MSCs showed distinctly different behavior in the current culture model. Bovine MSCs showed clear proliferation and matrix formation, whereas rabbit cultures indicated decreasing cellularities and GAG contents, similar to our previous study [19]. Although the viability of rabbit MSCs decreased in culture, the cells obtained a highly chondrogenic phenotype. Most importantly, both MSC types induced chondrogenesis in the co-cultures to levels similar to or even higher than with pure chondrocytes. Gene expression analysis indicated progressive loss of rabbit MSCs also in co-cultures, suggesting that MSCs functioned primarily as inducers of AC proliferation and redifferentiation. This was consistent with other studies where matrix related genes were predominantly expressed by chondrocytes even when MSCs were retained in the co-cultures [12, 14, 34]. In addition, it was recently proposed that predifferentiating MSCs toward the osteogenic lineage might further enhance chondrogenesis in AC-MSC co-cultures [15]. Cultured MSCs produce a multitude of cytokines and growth factors
[35], including known chondrogenic inducers like TGF-betas [36], BMP-2 [37] and IGF-1 [38]. It seems that this trophic effect is the main driving force in AC-MSC co-cultures, although direct cell-cell contacts also contribute to the improved chondrogenesis [34]. Furthermore, the trophic effect is not limited to the culture model used in the current study, but can be observed with several different cell sources, species and culture conditions [39]. In contrast, other studies have shown that chondrocytes can induce chondrogenic differentiation of co-cultured MSCs and that those MSCs might also directly contribute to cartilage formation [16, 40, 41]. It should be noted that the few clinical trials done with MSCs have all used undifferentiated cells within a carrier matrix, therefore relying mostly on the host response to the implanted constructs [42, 43].

We tested two different AC-to-MSC cell ratios in the bovine co-cultures, namely 1:1 and 1:3. This range was selected based on current literature [34, 44] and proved to result in comparable outcomes, without any significant difference between the two. This suggests that the majority of primary ACs, the golden standard for cartilage engineering, can be replaced by MSCs without affecting the cartilage-like matrix formation. Clinically relevant numbers of cells with chondrogenic potential are more easily obtained from bone marrow than from cartilage biopsy, and decreasing the demand for primary ACs by lowering the proportion of chondrocytes in the co-culture should therefore be beneficial. In addition, MSCs are known to be immunosuppressive and could potentially provide an allogeneic cell source in the future [45, 46]. Importantly, although primary cells were superior in monocultures, the co-culture results were similar with primary and culture expanded ACs, demonstrating further potential to lessen the requirements for AC harvest.
One additional benefit of AC-MSC co-cultures, which however was not evaluated in the current study, is the capacity to decrease hypertrophy and mineralization often seen in chondrogenically induced MSC constructs [13, 41, 47]. This effect, however, may be lost in constructs with a high proportion of MSCs [14, 48], and the AC-to-MSC cell ratio should therefore be kept within the optimal range.

CONCLUSIONS

In this work, we investigated chondrogenesis in porous scaffolds using primary and culture expanded ACs, MSCs and combinations thereof as potential cell sources for cartilage tissue engineering. Engineered constructs with primary ACs had higher cellularities and cartilage-like matrix formation in comparison to those with culture expanded chondrocytes or MSCs harvested from the same animals. Equally robust chondrogenesis was also observed in co-cultures, even when up to 75% of ACs were replaced with MSCs. Most importantly, co-cultures using primary and culture expanded ACs resulted in similarly positive outcomes. The most promising cell source for cartilage engineering was the co-cultures, as the trophic effect of MSCs may highly increase the chondrogenic potential of ACs thus diminishing the problems with primary chondrocyte harvest and expansion.
Figure B.1: DNA content of cultured constructs with primary bovine ACs and bovine MSCs (A) and with expanded bovine ACs and rabbit MSCs (B).

Results are presented as mean ± SD with n = 4. #, &, and * denote statistically significant difference to the corresponding MSC and AC culture and to the previous time point, respectively (p < 0.05).
Results are presented as mean ± SD with $n = 4$. #, ◇ and * denote statistically significant difference to the corresponding MSC and AC culture and to the previous time point, respectively ($p < 0.05$).
Figure B.3: Cartilage-like matrix production within cultured constructs with primary bovine ACs and bovine MSCs (A) and with expanded bovine ACs and rabbit MSCs (B).

Images taken after 14 days of culture unless otherwise stated. Scale bar represents 200 μm. Safranin-O staining.
Figure B.4: Quantitative gene expression results for Col I (A), Col II (B), Acan (C) and Col II / Col I ratio (D) using expanded bovine ACs and rabbit MSCs.

Results are presented as mean ± SD with \( n = 4 \). #, \( \Box \) and * denote statistically significant difference to the corresponding MSC and AC culture and to the previous time point, respectively \( (p < 0.05) \).
Figure B.5: Ratio of bovine specific-to-non specific quantitative PCR signal.

* denotes statistically significant difference to the previous time point ($p < 0.05$).
Table B.1: Forward (F) and Reverse (R) Primers Used for Quantitative RT-PCR.

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REFERENCES


APPENDIX C

The Effect of Hypoxia on the Chondrogenic Differentiation of Co-Cultured Articular Chondrocytes and Mesenchymal Stem Cells in Scaffolds*

ABSTRACT

In this work, we investigated the effects of lowered oxygen tension (20% and 5% O₂) on the chondrogenesis and hypertrophy of articular chondrocytes (ACs), mesenchymal stem cells (MSCs) and their co-cultures with a 30:70 AC:MSC ratio. Cells were cultured for six weeks within porous scaffolds, and their cellularity, cartilaginous matrix production (collagen II/I expression ratio, hydroxyproline and GAG content) and hypertrophy markers (collagen X expression, ALP activity, calcium accumulation) were analyzed. After two weeks, hypoxic culture conditions had expedited chondrogenesis with all cell types by increasing collagen II/I expression ratio and matrix synthesis by ⋅2.5–11 and ⋅1.5–3.0 fold, respectively. At later times, hypoxia decreased cellularity but had little effect on matrix synthesis. ACs and co-cultures showed similarly high collagen II/I expression ratio and GAG rich matrix formation, whereas MSCs produced the least hyaline cartilage-like matrix and obtained a hypertrophic phenotype with eventual calcification. MSC hypertrophy was further emphasized in hypoxic conditions. We conclude that the most promising cell source for cartilage engineering was co-cultures, as they have a potential to decrease the need for primary chondrocyte harvest.

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and expansion while obtaining a stable highly chondrogenic phenotype independent of the oxygen tension in the cultures.
**INTRODUCTION**

Articular cartilage lesions and progressive cartilage loss caused by degenerative disease are major contributors to disability in the United States. Furthermore, cartilage related problems are expected to increase dramatically in the future due to the aging population and increasing incidence of obesity [1]. Cartilage is an avascular tissue with low metabolic activity and cell density, consisting mainly of collagen- and proteoglycan-rich extracellular matrix [2]. Poor innate access to reparative cell sources results in low regeneration capacity of damaged cartilage, and advanced treatment options relying on auto- and allografts have therefore been widely studied [3]. Current clinical therapies are unfortunately inadequate to regenerate the native hyaline cartilage structure in articulating joints, but instead produce mechanically inferior fibrocartilage highly increasing the risk of treatment failure in the long term. Therefore, novel cell sources and culture methods are needed before cell based therapies can reach their full potential [4].

The most common cell sources for cartilage engineering include articular chondrocytes (ACs) and mesenchymal stem cells (MSCs). Both of these cell types have inherent advantages and disadvantages. Autologous ACs have now been used for two decades in clinic with promising results [5]. However, the harvest of primary cells by cartilage biopsy is related with donor site morbidity [6] and the cultured cells lose their chondrocyte phenotype rapidly upon monolayer expansion [7]. MSC harvest, usually from bone marrow or adipose tissue, and subsequent expansion poses fewer complications, but effective chondrogenesis requires extensive differentiation in the presence of growth factors [8]. More problematically, the MSC differentiation does not arrest at the chondrocyte level, but can lead to hypertrophy and formation of calcified
tissue replicating the process of endochondral ossification [9]. At present, there have been only few published investigations on the use of MSCs to treat cartilage defects in humans [10]. Co-cultures of ACs and MSCs have recently been proposed to mitigate problems associated with the corresponding monocultures. Based on the current \textit{in vitro} evidence, such co-cultures are highly chondrogenic, demonstrate decreased hypertrophy and have a potential to minimize the need for chondrocyte harvest [11].

Due to the lack of vasculature, articular cartilage obtains nutrients and oxygen mainly by diffusion from synovial fluid. Oxygen tension within the tissue is estimated to range from approximately 7\% on the joint surface to as low as 1\% close to subchondral bone [12]. This range of hypoxic conditions is known to play crucial role in cartilage physiology and endochondral bone development \textit{in vivo}, largely through the actions of HIF transcription factors [13]. Consequently, the effects of low oxygen tension have been investigated also on the maintenance and gaining of chondrogenic phenotype \textit{in vitro}. Hypoxic cultures can promote the restoration of chondrogenic phenotype in passaged ACs [14] and increase the differentiation of MSCs toward chondrogenic lineage [15]. Changes on both the gene expression and protein levels can be seen already after one day of hypoxia [16] and [17]. Still, hypoxic cultures have not been widely adopted in cartilage engineering applications so far.

Hypoxia and AC-MSC co-cultures have previously been studied separately as potential approaches to produce highly chondrogenic tissue engineering constructs \textit{in vitro}, but to our knowledge there are no published records evaluating their combined effects on (re)differentiation and hypertrophy of chondrogenic 3D cultures.
The aim of the current study was to investigate the effects of hypoxia on the chondrogenesis of AC-MSC co-cultures within porous polymer scaffolds. Furthermore, the roles of different cell sources and hypoxia in the stable maintenance of chondrogenic phenotype during long term 3D cultures were evaluated.

**MATERIALS AND METHODS**

**Scaffold Preparation**

Electrospun nonwoven poly(ε-caprolactone) (PCL) microfiber mats were fabricated with an average fiber diameter of approximately 10 μm as previously described [18]. The mats were inspected for consistent fiber morphology and diameter (9.5 ± 1.7 μm) using scanning electron microscopy and stored in a desiccator until use. Scaffolds were prepared by die-punching 8-mm-diameter discs from the electrospun mats using a dermal biopsy punch. The scaffolds (1.1 ± 0.1 mm thickness) were then sterilized by exposure to ethylene oxide (Andersen Sterilizers, Haw River, NC) for 14 h and aerated overnight to remove residual fumes. In order to eliminate air bubbles and to improve cell adhesion, scaffolds were prewetted by centrifugation through a graded series of ethanol (70–20%), followed by two rinses in phosphate buffered saline (PBS), and incubation in general culture medium (high-glucose DMEM, 10% fetal bovine serum (BenchMark FBS; Gemini Bio-Products, West Sacramento, CA), penicillin/streptomycin/fungizone (PSF)) for four days.

**Cell Harvest and Expansion**

Bovine MSCs and ACs were harvested from 7–10 day old calves (Research 87, Boylston, MA), less than 24 h after slaughter using previously established protocols [19].
Briefly, marrow isolations from tibiae and femora were plated onto culture flasks and allowed to adhere for 48 h before washing with PBS to remove non-adherent cells and blood clots. Cultures were maintained in general medium until confluent and passaged for sub-cultures. After two passages, MSCs from a minimum of three animals were pooled, aliquoted, and cryopreserved in freezing medium (DMEM with 20% FBS and 10% dimethyl sulfoxide). Articular cartilage was collected from the femoral condyle area, minced to approximately $1 \times 1 \times 1$ mm, washed with PBS and digested in chondrocyte culture medium (DMEM, 10% FBS, 1% non-essential amino acids, 50 $\mu$g/mL ascorbic acid, 46 $\mu$g/mL L-proline, 20 mM HEPES, PSF) containing 2 mg/mL collagenase type II (Worthington biochemical corporation, Lakewood, NJ). Digestions were incubated on a shaker table at 37 °C for 16 h and passed through cell strainers. Primary ACs from a minimum of three animals were pooled, aliquoted, and cryopreserved in freezing medium.

**Cell Seeding and Chondrogenic Culture**

The frozen AC and MSC aliquots were thawed and sub-cultured for one or two passages to establish seeding suspensions of $1.25 \times 10^6$ cells/mL. In preparation for cell seeding, prewetted scaffolds were press-fitted into custom made cylindrical polycarbonate cassettes (with the height, inner and outer diameters of 13 mm, 7.9 mm and 19 mm, respectively) designed to confine the seeding suspension. Cells (in ~200 $\mu$L of general medium) were then pipetted onto each scaffold to achieve a final seeding density of $4.5 \times 10^6$ cells/mL scaffold volume. Scaffolds were seeded with pure AC and MSC populations as well as their combination with a 30:70 AC:MSC ratio. The co-
culture ratio was chosen based on our previous studies [19]. Cells were allowed to adhere for 4 h before culture medium was gently added to completely cover the cassettes. Scaffolds were removed from the cassettes after 24 h and placed in 12 well culture plates with 4 mL of serum free chondrogenic medium (high-glucose DMEM, 1% ITS+ premix [BD Biosciences, San Jose, CA], 50 μg/mL ascorbic acid, 40 μg/mL L-proline, 10 mM Na-beta-glycerophosphate, 100 nM dexamethasone, 10 ng/mL TGF-β3 [PeproTech, Rocky Hill, NJ], PSF). Cultures were divided into normoxic (20% O₂) and hypoxic (5% O₂) conditions, and half of the medium was replenished two to three times a week. Four replicate scaffolds were harvested after 0, 14, 28 and 42 days of chondrogenic culture and washed with PBS.

**Real-Time Reverse Transcription Polymerase Chain Reaction**

Total RNA was isolated from pelleted cell seeding stocks and minced (approximately 1 x 1 x 1 mm pieces) 3D constructs cultured for 14 days, using RNeasy mini kit (Qiagen, Valencia, CA). Briefly, samples were immersed in lysis buffer and incubated at room temperature for 30 min with periodic vortexing. Cell lysate was then passed through a QIAshredder homogenization column and stored at −80 °C until further processing. An equal volume of 70% ethanol was added to thawed lysates and RNA isolation was continued following the animal cell protocol provided by the manufacturer. Reverse transcription was then carried out to synthesize cDNA from purified RNA samples using Oligo(dT) primers (Promega, San Luis Obispo, CA) and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Finally, cDNA was subjected to real-time PCR (Applied Biosystems 7300 Real-Time PCR System, Foster City, CA) using
SYBR Green detection (PerfeCTa SYBR Green FastMix, ROX; Quanta Biosciences, Gaithersburg, MD) with custom designed primers (Integrated DNA Technologies, Coralville, IA).

Primer sequences are given in Table C.1. Target gene expression was first normalized to the expression of the housekeeping gene GAPDH in the same sample (ΔCt), then to the average expression of that target gene measured in the AC group under normoxic conditions (ΔΔCt). Finally, the $2^{-\Delta\Delta Ct}$ method was used to convert normalized gene expression levels to fold differences and statistics were calculated on these values [20]. Similarly, $2^{-\Delta Ct}$ was used to calculate the ratios of collagen II/collagen I expression within individual samples.

**Histology**

A 3 mm biopsy punch was used to obtain individual samples from randomized scaffold location for each analysis. One biopsy sample from each scaffold was fixed for histology in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA), then immersed in 70% ethanol prior to embedding in HistoPrep freezing medium (Fisher Scientific). Frozen sections 5 μm thick were cut using a cryostat (Leica CM 1850 UV; Leica Biosystems Nussloch GmbH, Germany), mounted onto glass slides, and placed on a 42 °C slide warmer to facilitate adhesion. Sections were stained with Alcian Blue, Picrosirius Red and Fast Green to visualize the distribution of glycosaminoglycan (GAG), collagen and cells, respectively, in the 3D constructs. Images were obtained using a light microscope with a digital camera attachment (Axio Imager.Z2 equipped with AxioCam MRc5; Carl Zeiss MicroImaging GmbH, Germany).
Biochemical Assays

Two biopsy samples were pooled together from each scaffold to be used for DNA, GAG and hydroxyproline (HYP) assays. The rest of the scaffold was minced (approximately 1 x1 x 1 mm pieces) and used for alkaline phosphatase (ALP) and calcium assays. All samples were stored at −20 °C until further processing. Thawed biopsy samples were digested in proteinase K solution (1 mg/mL proteinase K, 0.01 mg/mL pepstatin A and 0.185 mg/mL iodoacetamide in a 50 mM tris(hydroxymethyl aminomethane) – 1 mM methylenediaminetetraacetic acid buffer, pH 7.6) in a 56 °C water bath for 16 h, whereas minced samples were immersed in distilled water. Cell and extracellular matrix components were extracted via two additional freeze-thaw cycles followed by 10 min sonication in a water bath.

DNA content of the scaffolds was determined using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Eugene, OR). Briefly, cell lysate, assay buffer and dye solution were combined in duplicates, and allowed to incubate for 10 min at room temperature. Fluorescence was measured using excitation and emission wavelengths of 485 nm and 528 nm (FL x800 Fluorescence Microplate Reader; BioTek Instruments, Winooski, VT), respectively, and DNA concentrations were determined relative to a lambda DNA standard curve.

GAG content was determined using the colorimetric dimethylmethylene blue assay [21]. Briefly, cell lysate and color reagent were combined in duplicates, and allowed to incubate for 7 min at room temperature. Absorbance at 520 nm was measured (PowerWave x340 Microplate Reader; BioTek Instruments), and GAG concentrations were determined relative to a chondroitin sulfate standard curve. For glycosaminoglycan
synthetic activity, the resulting GAG amounts were normalized to the amount of DNA for each sample.

HYP content, an indicator for total collagen, was determined in a colorimetric assay [22]. Briefly, an aliquot of cell lysate was combined with an equal volume of 4 N NaOH and hydrolyzed by autoclaving for 15 min, 121 °C (approximately 50 min total processing time). The solution was neutralized with HCl and acetic acid to pH 6.5 – 7.0 and divided into duplicate reactions. Chloramine-T and p-dimethylaminobenzaldehyde solutions were added sequentially, the absorbance at 570 nm was measured using a plate reader and HYP concentrations were determined relative to a trans-4-hydroxy-L-proline standard curve. For collagen synthetic activity, the resulting HYP amounts were normalized to the amount of DNA for each sample.

ALP enzymatic activity in the cell lysates was measured using alkaline buffer solution and phosphatase substrate tablets (Sigma-Aldrich, St. Louis, MO). Briefly, cell lysate and the reagents were combined in duplicates and incubated at 37 °C for 1 h. The reaction was stopped by addition of NaOH, the absorbance at 405 nm was measured using a plate reader and ALP activity was determined relative to a p-nitrophenol standard curve. Enzymatic activities were normalized to the amount of DNA for each sample.

After determining the ALP activity, acetic acid was added into the cell lysate in a final concentration of 0.5 M and the samples were incubated at room temperature for 16 h to dissolve calcium present in the minced scaffold. Calcium content was then determined in a colorimetric assay. Briefly, sample was combined with calcium arsenazo III reagent (Genzyme, Cambridge, MA) in duplicates, the absorbance at 650 nm was measured using
a plate reader and Ca\textsuperscript{2+} concentrations were determined relative to a CaCl\textsubscript{2} standard curve.

**Statistics**

Results are presented as means ± standard deviations. Statistical analysis was performed with an SPSS 16.0 software package (SPSS, Chicago, IL). Biochemical assay data were analyzed using one-way ANOVA followed by Tukey's post-hoc test, whereas RT-PCR data were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney U test. Differences were considered significant at 95% confidence level.

**RESULTS**

**Real-Time Reverse Transcription Polymerase Chain Reaction**

A two week culture experiment was performed to analyze early changes in chondrogenic gene expression in 3D cultures under different oxygen tensions. With all cell types expression of collagen type II was significantly higher and type I lower in hypoxia compared to normoxia, resulting in higher collagen type II-to-type I expression ratio in these conditions (Fig. C.1). In addition, comparison to the original monolayer expanded cell stocks used to seed the scaffolds indicated clear chondrogenic differentiation with TGF-β3 induction irrespective of the cell population used, although the AC and co-culture groups showed significantly stronger chondrogenesis than MSCs, as indicated by high collagen II expression. Furthermore, similar levels of collagen type X expression were observed with both cell stocks and all 3D constructs in normoxic conditions, whereas hypoxia increased this hypertrophy marker in the MSC and co-culture groups.
Scaffold Cellularity

An independent six week culture was conducted to study long term effects of hypoxia in chondrogenic 3D cultures. Scaffold cellularity increased for 4 weeks, and at that point all cell types showed significantly decreased DNA contents in hypoxic conditions compared to normoxic conditions (Fig. C.2). Furthermore, the cellularities in the AC and co-culture groups were similar, and significantly higher than that in the MSC groups, irrespective of the oxygen tension.

Collagen Synthesis

Hypoxia increased the total collagen, measured as HYP content and synthetic activity in early cultures (Fig. C.3). After 4 weeks, however, the situation was reversed and there was higher HYP content in normoxic than hypoxic conditions. In both conditions, MSCs showed higher levels of HYP synthesis than either ACs or co-cultures. These results were confirmed qualitatively in histological analysis using collagen-specific staining (Fig. C.4 and Fig. S1). Tissue formation occurred mostly within the topmost ∼300 μm of and even over the porous scaffold.

GAG Synthesis

Oxygen tension had only minor effects on GAG deposition, and only in early cultures. GAG synthetic activity was significantly increased with all cell types after two weeks in hypoxia, but only the AC group showed corresponding increase also in GAG content (Fig C.5). At later times, normoxic and hypoxic cultures demonstrated similar GAG contents and synthetic activities, and this was confirmed in histological evaluation (Fig. C.6 and Fig. S2). While GAG deposition increased gradually throughout the culture
period with all cell types, MSCs always showed lower GAG contents than the corresponding AC and co-culture groups.

**Hypertrophy Markers**

No ALP activity was seen in any AC culture, and while co-cultures exhibited some minor activity, that was not statistically different from the corresponding AC groups (Fig. C.7A). Similarly, none of the AC or the co-culture groups showed any signs of calcification. In contrast, MSC cultures showed strong induction of ALP activity already after 2 weeks of culture in both normoxic and hypoxic conditions. A decreasing trend was subsequently observed in hypoxia, whereas the activity continued to increase in normoxia and remained high even after 6 weeks. Furthermore, MSC cultures obtained a mineralizing phenotype, and this was expedited by hypoxic conditions. Calcium accumulation started by 4 and 6 weeks in hypoxia and normoxia, respectively (Fig. C.7B).

**DISCUSSION**

Current articular cartilage repair strategies yield inconsistent treatment outcomes commonly resulting in the formation of fibrocartilaginous repair tissue and compromised long term functionality. Previous studies have demonstrated that hypoxic *in vitro* culture conditions and the use of heterotypic co-cultures can each increase the chondrogenic potential of tissue engineered 3D constructs, but there seem to be no studies investigating combined effects of these two approaches. Here, we hypothesized that AC-MSC co-cultures would show similarly increased chondrogenesis than corresponding monotypic cultures in hypoxia compared to normoxia. Furthermore, we hypothesized that the co-
cultures would obtain stable chondrogenic phenotype without hypertrophy. To test these hypotheses, we conducted parallel chondrogenic 3D cultures of ACs, MSCs and their 30:70 mixture (AC:MSC) in normoxia and hypoxia. Cells were seeded on highly porous electrospun polymer scaffolds and their proliferation, cartilage-like matrix production and hypertrophy were followed for up to six weeks.

Low oxygen tension, mimicking the physiological conditions within cartilage tissue, has been proposed to increase chondrogenic potential of MSCs and chondrocytes. Hypoxia results in posttranslational stabilization of HIF transcription factors [23], and subsequent increase in chondrogenic gene expression [24] and [25]. However, there is no established consensus for the optimal level or timing of the hypoxia for the purposes of cartilage engineering [26]. In recent literature, commonly used gas phase oxygen levels in hypoxic chondrogenic cultures seem to range from approximately 1% to 5% [27] and [28]. It is noteworthy that there are always oxygen gradients in static cultures, and the oxygen tension within a metabolically active 3D construct is much lower than the equilibrium level near the air-liquid interface [29]. In addition, it is not clear whether hypoxic conditions have greater chondroinductive effect when applied in monolayer expansion phase or subsequent differentiating 3D cultures. With ACs, Egli et al. [27] showed enhanced cartilage formation with hypoxia expanded cells, but decreased chondrogenesis within hypoxic pellet cultures. In contrast, Ströbel et al. [30] and Schrobback et al. [14] did not see any benefits of hypoxic expansion, but reported positive effects of hypoxia in pellets. With MSCs, Adesida et al. [31] observed enhanced chondrogenesis in pellet cultures only when hypoxia was first applied in cell expansion,
whereas Sheehy et al. [32] saw benefits of hypoxia only in 3D, and yet Müller et al. [33] reported best results with continuous exposure to hypoxic conditions. Furthermore, not only hypoxia but the change from a 2D to 3D culture itself is a strong chondrogenic inducer [34].

We did not compare different hypoxic regimens but chose only one set of conditions, i.e. 5% oxygen level applied in 3D cultures, and observed clear modulation of chondrogenic response in comparison to normoxic cultures. Our 3D constructs showed increased collagen type II-to-type I ratio and decreased cellularity in hypoxia with all tested cell populations. In addition, hypoxia increased matrix synthesis at early cultures but not at later times. Chondrogenic (re)differentiation upon prolonged exposure to exogenously added TGF-β3 might explain this, masking the effects of hypoxia in long term cultures [35]. Furthermore, diffusional constraints in static culture conditions commonly limit the cell growth onto the periphery of porous scaffolds and result in spontaneous development of hypoxia within the 3D constructs [36], irrespective of the surrounding atmosphere. Although medium changes were performed by replacing only half of the liquid volume at each time to retain some of the cell-produced growth factors and cytokines and to avoid major fluctuations in the pH and oxygen levels, better control of the culture environment would be desirable. Cartilage engineering might therefore benefit from bioreactor cultures to improve mass transfer while allowing precise regulation of culture pH and low oxygen conditions [37].

MSCs have garnered great attention as a potential cell source for cartilage engineering as they are relatively easy to harvest and expand, and their
immunosuppressive properties might even allow the use of allogeneic cell sources [38]. Although MSCs can be readily differentiated toward chondrogenic lineage in vitro, their main mode of action in vivo might be as synthesizers of trophic factors rather than as direct producers of cartilaginous matrix and tissue [39]. Potential problems with in vitro differentiated MSCs include their unstable phenotype, often leading to the formation of fibrocartilage and even calcified tissue [40]. The propensity for terminal differentiation seems to be dependent on the MSC cell source [41], and using bone marrow derived cells this endochondral route has even been exploited in bone engineering [42]. These aspects were highlighted also in the current study, where MSC constructs had the lowest cellularity and GAG content, obtained high ALP activity, and produced collagen (type I) rich matrix with eventual mineralization. Various HIFs (1α and 2α) can have opposing effects on MSC chondrogenesis and hypertrophy [43], and their transcription and stability are differentially regulated in hypoxic conditions [44]. Current literature generally indicates that hypoxia should decrease the hypertrophic tendency of MSCs [24, 32, 45], although increased Col X and Runx2 expression has been occasionally reported [46]. In this study, MSC hypertrophy was increased in hypoxia diminishing the benefits of low oxygen conditions. This finding could be related to the evolving oxygen gradients within porous scaffolds and use of bovine cells, as MSC hypertrophy has not been previously studied in such a culture model.

Chondrogenic (re)differentiation of cultured cells was achieved in this study with a commonly used method of continuous application of dexamethasone and TGF-β in serum free culture medium. However, recent reports with both ACs [47] and MSCs [48]
indicate that transient, two to three weeks, exposure might yield higher quality tissue engineered constructs with enhanced biochemical and biomechanical properties. The effects of such transient induction are not known in hypoxic conditions or in AC-MSC co-cultures and should warrant further investigations.

Co-cultures of MSCs and chondrocytes have recently emerged as a promising way to simultaneously increase the chondrogenic potential of tissue engineered constructs and to inhibit MSC hypertrophy [49, 50]. The major role of MSCs in such cultures seems to be the induction of chondrocytes to proliferate and produce cartilaginous matrix [51] while the chondrocytes can protect MSCs from hypertrophy [52]. In the current study, early induction of collagen X expression and minor ALP activity was detected with hypoxic co-cultures, but hypertrophy was soon arrested and the co-cultures never developed a calcifying phenotype. High proliferation rate of chondrocytes results in a gradual increase in the AC:MSC ratio [19, 53], likely increasing the hypertrophy inhibiting effect in long term cultures. The increased growth and redifferentiation of chondrocytes is important also because the strong correlation between monolayer expansion and loss of phenotype currently limits the applicability of autologous chondrocyte transplants. In addition, the microenvironment in injured cartilage tissue is often not only hypoxic but also inflammatory, and co-cultures have been shown to retain their chondrogenic capacity in such conditions [54]. Co-cultured 3D constructs need less chondrocytes at the seeding phase to produce similar end results than pure AC constructs, diminishing the requirements for the amount of harvested cartilage tissue and/or to the extent of monolayer expansion. Interestingly, the beneficial effects of co-cultures were
not dependent on the oxygen tension, making hypoxic co-cultures an attractive culture modality for future studies.

CONCLUSIONS

In this work, we investigated the effects of hypoxic culture conditions on the chondrogenesis and hypertrophy of ACs, MSCs and their combinations as potential cell sources for cartilage engineering. All tested cell populations showed early enhancement of cartilaginous matrix production within porous scaffolds in hypoxia compared to normoxia, but this effect was diminished in prolonged cultures. The co-cultures were able to inhibit MSC hypertrophy in both conditions, whereas pure MSC cultures showed increased hypertrophy in hypoxia. The most promising cell source for cartilage engineering was co-cultures, as they have a potential to decrease the need for primary chondrocyte harvest and expansion while obtaining a stable highly chondrogenic phenotype independent of the oxygen tension in the cultures.
TABLES AND FIGURES

Figure C.1: RT-PCR analysis of chondrogenic and hypertrophic gene expression after 2 weeks of culture with various cell types and oxygen tensions. Collagen type II (A), type I (B), type II/I ratio (C) and collagen type X (D). Results are presented as mean ± SD with $n = 4$. The dashed line represents the AC stock used to seed the scaffolds. # and * denote statistically significant difference to the corresponding normoxic culture condition (same cell type) and AC group (same oxygen tension), respectively ($p < 0.05$).
Figure C.2: DNA content of cultured constructs with various cell types and oxygen tensions.

Results are presented as mean ± SD with $n = 4$. The dashed line represents the cell stocks used to seed the scaffolds. # and * denote statistically significant difference to the corresponding normoxic culture condition (same cell type) and AC group (same oxygen tension), respectively ($p < 0.05$).
Figure C.3: HYP content (A) and synthetic activity (B) with various cell types and oxygen tensions.

Results are presented as mean ± SD with $n = 4$. The dashed line represents the cell stocks used to seed the scaffolds. # and * denote statistically significant difference to the corresponding normoxic culture condition (same cell type) and AC group (same oxygen tension), respectively ($p < 0.05$).
Figure C.4: Histological analysis of collagenous matrix production within porous scaffolds after 6 weeks of culture with various cell types and oxygen tensions.

Scale bar represents 500 μm. Picrosirius Red and Fast Green staining.
Figure C.5: GAG content (A) and synthetic activity (B) with various cell types and oxygen tensions.

Results are presented as mean ± SD with \( n = 4 \). The dashed line represents the cell stocks used to seed the scaffolds. # and * denote statistically significant difference to the corresponding normoxic culture condition (same cell type) and AC group (same oxygen tension), respectively \((p < 0.05)\).
Figure C.6: Histological analysis of GAG production within porous scaffolds after 6 weeks of culture with various cell types and oxygen tensions.

Scale bar represents 500 μm. Alcian Blue staining.
Figure C.7: ALP activity (A) and calcium content (B) with various cell types and oxygen tensions.

Results are presented as mean ± SD with n = 4. # and * denote statistically significant difference to the corresponding normoxic culture condition (same cell type) and AC group (same oxygen tension), respectively (p < 0.05).
Table C.1: Forward (F) and Reverse (R) Primers Used for Quantitative RT-PCR.

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APPENDIX D

Articular Chondrocyte Redifferentiation in 3D Co-cultures with Mesenchymal Stem Cells*

ABSTRACT

In this work, we evaluated the ability of 3D co-cultures with mesenchymal stem cells (MSCs) to redifferentiate monolayer expanded articular chondrocytes (ACs) and produce cartilaginous extracellular matrix at varying stages of the dedifferentiation process and further examined the dependency of this effect on the culture medium composition. Primary bovine ACs were expanded in monolayers for up to nine population doublings to obtain seven cell stocks with gradually increasing levels of dedifferentiation. Culture expanded ACs were then seeded as monocultures and co-cultures with rabbit bone marrow-derived MSCs (30:70 ratio of ACs-to-MSCs) on porous scaffolds. Parallel cultures were established for each cell population in serum-containing growth medium and serum-free induction medium supplemented with dexamethasone and TGF-β3. After 3 weeks, all groups were analyzed for DNA content, glycosaminoglycan (GAG) and hydroxyproline (HYP) production, and chondrogenic gene expression. Significant enhancements in cellularity, GAG content and GAG/HYP ratio, and chondrogenic phenotype were observed in the induction medium compared to growth medium at all levels of AC expansion. Furthermore, primary co-cultures showed similarly enhanced chondrogenesis compared to monocultures in both culture media, whereas passaged ACs benefitted from co-culturing only in the induction medium. We

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conclude that co-cultures of ACs and MSCs can produce superior *in vitro* engineered cartilage in comparison to pure AC cultures, due to both heterotypic cellular interactions and decreased need for monolayer expansion of biopsied chondrocytes. While the initial level of AC dedifferentiation affected the quality of the engineered constructs, co-culture benefits were realized at all stages of AC expansion when suitable chondroinductive culture medium was used.
INTRODUCTION

Articular cartilage is responsible for the low friction and even distribution of loads in all synovial joints, making the normal pain-free movement possible [1]. Mature cartilage is avascular and has a low cell density and metabolic activity. Therefore, damage to this tissue is unable to elicit an adequate healing response to produce functional repair of even small defects, and surgical interventions are often required. To improve articular cartilage repair, chondrogenic cell populations are traditionally brought to the defect site in the form of marrow stimulation, auto- and allograft implantation, and ex vivo expanded autologous chondrocyte transplantation [2]. However, these strategies tend to result in the formation of mechanically and functionally inferior repair tissue, resulting in further complications in the long term [3]. Therefore, improved tissue engineering strategies are needed to reach the full potential of cell-based therapies [4].

Articular chondrocytes (ACs) and mesenchymal stem cells (MSCs) are the most common cell sources in cartilage engineering [5]. Although easily harvested and expanded, multipotent MSCs require extensive in vitro manipulation to achieve stable chondrogenic differentiation [6], and the end results often bear signs of fibrocartilage and hypertrophy rather than mature articular cartilage [7, 8]. On the other hand, risk of donor site morbidity limits the number of harvested ACs [9], and unfortunately, these cells dedifferentiate rapidly during monolayer culture, acquiring a fibroblast-like phenotype and producing inferior cartilaginous tissue in comparison to primary cells [10, 11]. Minimally expanded human ACs seem to be more potent cartilage producers than MSCs both in vitro and in vivo [12, 13], but it is not clear if this holds true for more dedifferentiated ACs.
Typical cartilage engineering schemes consist of two steps: chondrogenic cells are first expanded in serum-containing growth medium and cartilaginous tissue formation is subsequently achieved in serum-free induction medium commonly supplemented with known chondrogenic factors, such as dexamethasone and TGF-β [14]. Prior research has shown that while AC dedifferentiation in monolayer cultures is a gradual process [15], already four population doublings can eradicate the intrinsic chondrogenicity of the resulting cell population [16]. However, highly expanded ACs can regain their chondrogenicity in the induction medium, and this redifferentiation process can be further enhanced by other suitable external stimuli like hypoxia [17] and mechanical loading [18] .

Recently, trophic factors produced by co-cultured MSCs have been implicated as a novel promising chondroinductive signal to enhance AC proliferation, phenotype, and extracellular matrix (ECM) production in 3D cultures [19, 20]. The benefits of such co-cultures have been demonstrated using chondrocytes and MSCs from various sources [21, 22], but to the best of our knowledge, there are no detailed reports on the effectiveness of this approach at varying stages of AC dedifferentiation. In the current study, we investigated the relationship of chondrocyte redifferentiation capacity in AC-MSC co-cultures with the level of AC expansion and further examined the dependency of this trophic effect on the culture medium composition.

**Materials and Methods**

**Experimental Design**

Primary bovine ACs were expanded in monolayers for up to nine population doublings to obtain seven cell stocks with gradually increasing levels of dedifferentiation.
Culture expanded ACs were then seeded as monocultures and co-cultures with rabbit bone marrow-derived MSCs (30:70 ratio of ACs-to-MSCs) on electrospun poly(ε-caprolactone) (PCL) scaffolds. Parallel cultures were established for each cell population in serum-containing growth medium, and serum-free induction medium supplemented with dexamethasone and TGF-β3. After 3 weeks, all groups were analyzed for DNA content, cartilaginous ECM production, and chondrogenic gene expression.

**Scaffold Preparation**

PCL fibers with ~10 μm diameter were electrospun into a millimeter thick nonwoven mat as previously described [23]. Discoid scaffolds with 8 mm diameter were then cut using a dermal biopsy punch. The scaffolds were sterilized by exposure to ethylene oxide (Andersen Sterilizers) for 14 h and aerated overnight to remove residual fumes. To remove air bubbles and improve cell adhesion, sterilized scaffolds were prewetted in a graded ethanol series, washed with phosphate-buffered saline (PBS), and incubated in general culture medium (high-glucose DMEM, 10% fetal bovine serum [BenchMark FBS; Gemini Bio-Products], penicillin/streptomycin/fungizone [PSF]) for 4 days before use.

**Cell Harvest and Expansion**

Bovine ACs and rabbit MSCs were harvested as previously described [19]. ACs were obtained from 7- to 10-day-old calves (Research 87), less than 24 h after slaughter. Briefly, articular cartilage from femoral condyles of three donor animals was collected, minced to ~1x1x1 mm, washed with PBS, and digested in the chondrocyte growth medium (DMEM, 10% FBS, 1% nonessential amino acids, 50 μg/mL ascorbic acid,
46 μg/mL L-proline, 20 mM HEPES, PSF) containing 2 mg/mL collagenase type II (Worthington Biochemical Corporation). Digestions were incubated on a shaker table at 37°C for 16 h and passed through cell strainers. Harvested primary ACs were then aliquoted and cryopreserved. Every 2 days, two to four frozen aliquots were plated at a density of 6600 cells/cm² in the growth medium. Medium was changed once at 4 days, and semiconfluent cultures were passaged the first time at 6 days. Subsequent passaging occurred every 2 days with a constant plating density. Fourteen days after the first plating, all cultures were terminated simultaneously to form seven AC stocks with gradually increasing expansion levels to be used for scaffold seeding.

Bone marrow-derived MSCs were obtained from 5-week-old New Zealand White rabbits (Charles River Laboratories). Under general anesthesia, bone marrow from the tibia was aspirated into 10-mL syringes containing 1000 U heparin to prevent coagulation. The marrow was suspended in general medium and plated in tissue culture flasks. Nonadherent cells were washed away after 72 h, and cultures were maintained until confluent. Cells from six donor animals were pooled together, aliquoted, and cryopreserved. Frozen MSCs were plated at a density of 3300 cells/cm² in general medium and passaged once at day 5. After 5 more days of culture, an MSC stock (passage 3) was formed for scaffold seeding.

**Cell Seeding and 3D Culture**

Seeding suspensions of ~1×10⁶ cells/mL were established for the MSCs, each of the AC passages, and corresponding co-cultures with 30:70 AC-to-MSC cell ratio. The cell ratio was chosen based on our previous studies, and the xenogeneic culture model
was used to facilitate AC gene expression analysis without interference from co-cultured MSCs [19]. Prewetted scaffolds were press-fitted into custom-made cylindrical polycarbonate cassettes designed to confine the seeding suspension, and cells in the chondrocyte growth medium were pipetted onto each scaffold to achieve a final seeding density of 4.5×10⁶ cells/mL scaffold volume. Cells were allowed to adhere for 4 h before more medium was gently added to completely cover the cassettes. After 24 h, scaffolds were removed from the cassettes and divided into the growth medium and induction medium (high-glucose DMEM, 1% ITS+ premix [BD Biosciences], 50 μg/mL ascorbic acid, 40 μg/mL L-proline, 100 nM dexamethasone, 10 ng/mL TGF-β3 [PeproTech], PSF) in 12-well culture plates. Cultures were continued for 3 weeks, with half of the medium replenished two to three times a week. MSC controls were kept only in the induction medium.

**Biochemical Assays**

Four replicate scaffolds from each culture group were harvested and washed in PBS. A 3 mm biopsy punch was used to obtain individual samples from randomized scaffold locations for biochemical and histological analysis, and the rest of the scaffold was used for RNA isolation.

Two biopsy samples were pooled together from each scaffold and stored at −20°C until further processing for DNA, glycosaminoglycan (GAG), and hydroxyproline (HYP) assays. Thawed samples were digested in proteinase K solution (1 mg/mL proteinase K, 0.01 mg/mL pepstatin A, and 0.185 mg/mL iodoacetamide in a 50 mM tris(hydroxymethyl aminomethane)–1 mM ethylenediaminetetraacetic acid buffer, pH
7.6) in a 56°C water bath for 16 h. Cell and ECM components were extracted via two additional freeze-thaw cycles followed by 10 min sonication in a water bath.

DNA content of the scaffolds was determined using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). Briefly, cell lysate, assay buffer, and dye solution were combined in duplicates and allowed to incubate for 10 min at room temperature. Fluorescence was measured using excitation and emission wavelengths of 485 and 528 nm (FL×800 Fluorescence Microplate Reader; BioTek Instruments), respectively, and DNA concentrations were determined relative to a lambda DNA standard curve.

GAG content was determined using the colorimetric dimethylmethylene blue assay [24]. Briefly, cell lysate and color reagent were combined in duplicates and allowed to incubate for 7 min at room temperature. Absorbance at 520 nm was measured (PowerWave×340 Microplate Reader; BioTek Instruments), and GAG concentrations were determined relative to a chondroitin sulfate standard curve.

HYP content, an indicator for total collagen, was determined in a colorimetric assay [25]. Briefly, an aliquot of cell lysate was combined with an equal volume of 4 N NaOH and hydrolyzed by autoclaving for 20 min, 121°C (~55 min total processing time). The solution was neutralized with HCl and acetic acid to pH 6 and divided into duplicate reactions. Chloramine-T and p-dimethylaminobenzaldehyde solutions were added sequentially, the absorbance at 570 nm was measured using a plate reader and HYP concentrations were determined relative to a trans-4-hydroxy-L-proline standard curve.
**Histology**

One biopsy sample from each scaffold was fixed for histology in 10% neutral buffered formalin (Fisher Scientific), then immersed in 70% ethanol before embedding in the HistoPrep freezing medium (Fisher Scientific). Frozen sections of 5 μm thick were cut using a cryostat (Leica CM 1850 UV; Leica Biosystems Nussloch GmbH), mounted onto glass slides, and placed on a 42°C slide warmer to facilitate adhesion. Sections were stained with Alcian Blue, Picrosirius Red, and Fast Green to visualize the distribution of GAG, collagen, and cells, respectively, in the 3D constructs. Images were obtained using a light microscope with a digital camera attachment (Axio Imager.Z2 equipped with AxioCam MRC5; Carl Zeiss MicroImaging GmbH).

**Real-Time Reverse Transcription–Polymerase Chain Reaction**

Total RNA was isolated from pelleted cell seeding stocks and minced (~1x1x1 mm pieces) 3D constructs, using RNeasy mini kit (Qiagen). Briefly, samples were immersed in lysis buffer and incubated at room temperature for 30 min with periodic vortexing. Cell lysate was then passed through a QIAshredder homogenization column and stored at −80°C until further processing. An equal volume of 70% ethanol was added to thawed lysates and RNA isolation was continued following the animal cell protocol provided by the manufacturer. Reverse transcription was then carried out to synthesize cDNA from purified RNA samples using Oligo(dT) primers (Promega) and SuperScript III reverse transcriptase (Invitrogen). Finally, cDNA was subjected to real-time PCR (Applied Biosystems 7300 Real-Time PCR System) using SYBR Green
detection (PerfeCTa SYBR Green FastMix, ROX; Quanta Biosciences) with custom-designed primers (Integrated DNA Technologies).

Primer sequences are given in Table 1. Target gene expression was first normalized to the expression of the housekeeping gene GAPDH in the same sample ($\Delta C_t$), then to the average expression of that target gene measured in the p0 1 day AC seeding stock ($\Delta \Delta C_t$). Finally, the $2^{-\Delta \Delta C_t}$ method was used to convert normalized gene expression levels to fold differences, and statistics were calculated on these values [26]. Similarly, $2^{-\Delta C_t}$ was used to calculate the ratios of collagen II/collagen I expression and bovine-specific/cross-species GAPDH signal within individual samples.

**Statistics**

Results are presented as means±standard deviations. Statistical analysis was performed with an IBM SPSS 20.0.0 software package. Biochemical assay data were analyzed using one-way ANOVA followed by Tukey's post-hoc test, whereas RT-PCR data were analyzed using the Kruskal–Wallis test with stepwise stepdown multiple comparisons. Differences were considered significant at 95% confidence level. For the sake of presentation clarity, results for 3D cultures are shown with only four levels of AC passaging. However, statistical analysis and data interpretation are based on all seven expansion levels, and quantitative measurements for all experimental groups are included in supplementary data (Supplementary Tables S1–S10; Supplementary Data are available online at www.liebertpub.com/tec).
RESULTS

Monolayer Expansion of ACs

Proliferation of ACs during monolayer expansion is depicted in Figure D.1. Some nonviable and poorly adherent cells were eliminated from the frozen cell stocks upon initial plating, resulting in ~40% cell loss in the p0 seeding stock. For all other groups, the first medium change was performed after 4 days, and at that time, the cells had obtained a more flattened morphology and started to proliferate. Furthermore, a gradual increase in the proliferation rate was observed at each passage. Gene expression analysis (Fig. D.2) showed immediate increase in dedifferentiation markers collagen type I and type III upon plating, whereas chondrogenic markers collagen type II, aggrecan, and COMP started to decline only after the first passage at day 6.

Scaffold Cellularity

DNA contents in the 3D scaffolds were measured at the end of the 21-day culture period. Clear increase in cellularity was observed for all experimental groups, except the pure MSC control, which had very low DNA content (Fig. D.3). Co-culturing and induction medium resulted in significant increases in cellularity in comparison to pure AC cultures and growth medium, respectively. In addition, highest cellularities were observed with p1 ACs and primary co-cultures, and cellularity tended to decrease with higher passages.

ECM Production

Cultured constructs were analyzed for their contents of major cartilaginous ECM components, that is, sulfated GAGs and total collagen (HYP). Induction medium
increased GAG and decreased collagen production in comparison to the growth medium (Fig. D.4). Co-culturing typically increased both GAG and HYP contents, especially with primary cultures. Highly passaged cells showed strong decline in GAG contents, whereas HYP contents remained relatively stable from p1 to p5. MSC controls produced only small amounts of ECM. The GAG/HYP ratio was calculated for each construct as an indicator for hyaline or fibrocartilage nature of the produced matrix. A steady decline was observed upon increasing levels of AC passaging, whereas the use of the induction medium highly increased the ratio (Fig. D.4c). The effect of co-culturing was strongest with highly passaged cells, increasing the ratio in the induction medium but decreasing the ratio in the growth medium. MSC controls had low GAG/HYP ratio, similar to p5 chondrocytes.

**Histology**

Histological evaluation of the 3D constructs corroborated with the quantitative ECM analysis. GAG production was highly increased in induction medium, and the overall amount of cartilaginous tissue was highest using p1 ACs (Fig. D.5). Dense GAG-rich matrix with embedded cells was largely confined to the seeding surface of the scaffolds, whereas acellular collagenous matrix was more evenly distributed throughout the porous structure (data not shown).

Gene expression in 3D scaffolds

The cellular proportions and chondrogenic phenotype of the cultured constructs were analyzed using real-time RT-PCR. The ratios of bovine-specific to cross-species PCR signal for a housekeeping gene GAPDH were equal with bovine AC monocultures
and xenogeneic co-cultures (1.26±0.08 and 1.25±0.09, respectively) across all expansion levels and medium compositions, indicating negligible proportion of rabbit MSCs present in the co-cultures at the end of a 21-day culture period.

Induction medium decreased collagen type I and increased collagen type II expression, resulting in highly increased collagen II/I expression ratio (Fig. D.6). The highest ratios were observed with primary co-cultures, with a gradual decrease upon increasing levels of AC passaging. Aggrecan and COMP expression acted similarly to collagen II. Again, induction medium and primary co-cultures increased these chondrogenic markers, with a gradual decrease on increased AC passaging. Collagen type III expression was analyzed as a dedifferentiation marker, and it was clearly decreased in the induction medium. Co-culturing or the level of AC passaging did not have a consistent effect on this marker. In addition, certain degree of redifferentiation (relative to the corresponding seeding stocks) was observed in chondroinductive cultures using p3–p5 ACs, but none of the 3D constructs were able to regain the initial chondrogenic phenotype of primary cells.

**DISCUSSION**

Despite over 20 years of tissue engineering efforts, stable regeneration of articular cartilage defects still remains a challenge [27]. One of the major difficulties encountered is the frequent formation of fibrous repair tissue instead of proper hyaline cartilage, indicating the need for optimized cell sources and in vitro culture conditions. Co-cultures of ACs and MSCs have recently shown promise for cartilage engineering, avoiding some of the common problems seen in the corresponding monocultures [5]. The current study
evaluated the ability of such 3D co-cultures to redifferentiate ACs and produce cartilaginous ECM at varying stages of culture expansion and further examined the dependency of this trophic effect on the culture medium composition.

Tissue engineering approaches using autologous chondrocytes to repair clinical cartilage defects typically require a minimum of 20-fold expansion of harvested cell numbers in monolayers before seeding into 3D scaffolds and eventual transplantation back to the patient [28, 29]. That would correspond to more than four population doublings, and approximately passage 3 in the current experimental setup. Extended cell cultures typically result in a significant loss of the chondrogenic phenotype in vitro and the ability to form hyaline cartilage in vivo [10, 30]. The problem of dedifferentiating chondrocytes is further reflected in the fact that although clinical products for autologous chondrocyte transplantation are designed with an upper limit to the level of monolayer expansion (the exact process parameters are not publicly available), the resulting constructs still exhibit signs of dedifferentiation [31].

As current gene expression analysis indicated, robust redifferentiation of culture expanded ACs remains an elusive goal, and it should therefore be beneficial to limit the degree of dedifferentiation in the first place. Recent proposals to achieve this include the use of physiological oxygen tension [32], chondrogenic growth factors [33], ECM-coated culture substrates [34], and constant maintenance of high cell densities [35] during monolayer culture. Surprisingly, however, some culture schemes have shown enhanced 3D chondrogenesis following treatments that increased proliferation rates but also favored dedifferentiation in monolayers [33, 36].
In the current study, rapidly increasing collagen type I and type III expression levels indicated that primary ACs started to dedifferentiate immediately on monolayer plating, although typical chondrogenic markers remained high until the first passaging at 6 days of culture. Whereas diffusional nutrient and oxygen limitations [37, 38] commonly confine 3D cultured ACs to the periphery of porous scaffolds, the seeded cells adhere, continue to proliferate, and slowly invade the available macropores. The observed increase in cellularity within our 3D constructs is in contrast to relatively flat or even decreasing cell numbers typical to hydrogel and pellet cultures.40,41 A possible downside to this proliferation is the progression of the dedifferentiation process even in 3D. Indeed, signs of chondrogenic redifferentiation were seen only with our most expanded AC seeding populations and only in induction medium.

Surprisingly, 3D monocultures in the growth medium using the p1 ACs resulted in more matrix production and exhibited more chondrogenic phenotype in comparison to constructs derived from primary ACs. Possible reasons for this might include lower seeding efficiency and an initial adaptation period to the in vitro culture conditions with the primary cells, corresponding to the observed decline in cell numbers in the beginning of monolayer cultures. In line with our findings, Ng et al. [39] recently showed enhanced mechanical and biochemical cartilage properties using p1 rather than primary chondrocytes in hydrogel cultures. Most importantly, however, primary ACs were highly responsive to the trophic effects of MSCs in our co-culture models, yielding engineered constructs with significantly higher GAG contents as well as GAG/HYP and collagen type II/I ratios in comparison to culture expanded cells. Previous xenogeneic studies have
shown that the proportion of ACs dramatically increases during 3D culture due to high proliferation rate and MSC apoptosis, and the final constructs therefore originate mainly from the ACs [19-22]. Furthermore, this phenomenon has been demonstrated using cells from multiple species and tissue sources and was again confirmed in the current study. Thus, it is interesting that passaged ACs benefitted from the co-culturing (in terms of ECM quality and cell phenotype) only in the induction medium, and our ongoing studies indicate that co-cultures are also more responsive to transient low-dose exposure to TGF-β3 in comparison to AC monocultures [40]. Consequently, the trophic effect seems to be at least partially dependent on the differentiation status of the MSCs. On the other hand, the use of the induction medium increased the quality of our 3D constructs similarly at all levels of AC expansion in both mono- and co-cultures.

Assuming a fixed number of harvested chondrocytes and final seeding density, replacing 70% of ACs with MSCs in the current co-cultures would correspond to roughly one less passage in monolayer. Furthermore, a recent study by Sabatino et al. [41] demonstrated enhanced in vivo cartilage formation in co-culture constructs with as little as 5% (primary) AC fraction. Co-culturing can therefore be an effective way to limit the degree of AC expansion and concomitant dedifferentiation in cartilage engineering. Taking into account previous reports on the robust chondrogenic potential of co-cultures in hypoxic [42] and inflammatory conditions [43] typical to injured cartilage, the use of co-cultures shows great promise for clinical applications.
CONCLUSIONS

Major enhancements in cellularity, ECM quantity and quality, and chondrogenic gene expression of AC-MSC co-cultures were observed in the induction medium compared to the growth medium at all levels of AC expansion. Primary co-cultures showed increased chondrogenesis compared to AC monocultures in both culture media, whereas passaged ACs benefitted from co-culturing with MSCs only in the induction medium. Co-cultures decrease the need for AC expansion, thus enabling the use of less dedifferentiated seeding stocks, resulting in improved quality of cartilage-engineered constructs.
Figure D.1: Articular chondrocyte (AC) proliferation in monolayer cultures.

Each marker represents cumulative population doublings (mean±standard deviation [SD]) measured from 2 to 10 independent platings. Arrows indicate passage numbers for subsequent three dimensional (3D) cell seeding stocks discussed in the main article (data related to the unlabeled seeding stocks are included in the supplementary files.)
Figure D.2: Progression of AC dedifferentiation in monolayer cultures.

Fold change in gene expression of selected (a) chondrogenic and (b) dedifferentiation markers relative to the p0 seeding stock. Each marker represents an individual seeding stock used in the subsequent 3D cultures. The analyzed genes were collagen type I (Col1a2), type II (Col2a1) and type III (Col3a1), aggrecan (Acan), and cartilage oligomeric matrix protein (COMP).
Results are presented as mean±SD with $n=4$. The bolded dashed line represents the mesenchymal stem cells (MSC) control group, which was cultured in the induction medium only. The used seeding protocol results in $\approx 3\mu g$ of DNA per scaffold at the beginning of the cultures.¹⁹ #, ◊, and * denote statistically significant difference to the corresponding monoculture and growth medium (same passage) and to the lower AC expansion level (same cell population and culture medium), respectively ($p<0.05$).
Figure D.4: Extracellular matrix production in 3D cultures.

(a) GAG content, (b) HYP content, and (c) GAG/HYP ratio. Results are presented as mean±SD with \( n=4 \). The bolded dashed line represents the MSC control group, which was cultured in the induction medium only. #, *, and * denote statistically significant difference to the corresponding monoculture and growth medium (same passage) and to the lower AC expansion level (same cell population and culture medium), respectively (\( p<0.05 \)). GAG, glycosaminoglycan; HYP, hydroxyproline
Figure D.5: Histological evaluation of GAG production and tissue distribution in 3D cultures. Scale bar represents 500 μm. Alcian Blue staining.
Figure D.6: Fold change in gene expression of selected chondrogenic markers in 3D cultures.

Results are normalized (except for Col II/I ratio) in relation to the p⁰ seeding stock and presented as mean±SD with n=4. #, σ, and * denote statistically significant difference to the corresponding monoculture and growth medium (same passage) and to the previous AC expansion level (same cell population and culture medium), respectively (p<0.05). Note the different scales on primary and secondary Y-axes.
Table D.1: Forward and Reverse Primers Used for Quantitative RT-PCR

F, forward; R, reverse; *Col1a2*, collagen type I; *Col2a1*, collagen type II; *Col3a1*, collagen type III; *Acan*, aggrecan; *COMP*, cartilage oligomeric matrix protein; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

<table>
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<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product length</th>
<th>GenBank No.</th>
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<tr>
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REFERENCES


APPENDIX E

Flow Perfusion Co-Culture of Human Mesenchymal Stem Cells and Endothelial Cells on Biodegradable Polymer Scaffolds*

ABSTRACT
In this study, we investigated the effect of flow perfusion culture on the mineralization of co-cultures of human umbilical vein endothelial cells (HUVECs) and human mesenchymal stem cells (hMSCs). Osteogenically precultured hMSCs were seeded onto electrospun scaffolds in monoculture or a 1:1 ratio with HUVECs, cultured for 7 or 14 days in osteogenic medium under static or flow perfusion conditions, and the resulting constructs were analyzed for cellularity, alkaline phosphatase (ALP) activity and calcium content. In flow perfusion, constructs with monocultures of hMSCs demonstrated higher cellularity and calcium content, but lower ALP activity compared to corresponding static controls. ALP activity was enhanced in co-cultures under flow perfusion conditions, compared to hMSCs alone; however unlike the static controls, the calcium content of the co-cultures in flow perfusion was not different from the corresponding hMSC monocultures. The data suggest that co-cultures of hMSCs and HUVECs did not contribute to enhanced mineralization compared to hMSCs alone under the flow perfusion conditions investigated in this study. However, flow perfusion culture resulted in an enhanced spatial distribution of cells and matrix compared to static cultures, which were limited to a thin surface layer.

* This appendix was published as Dahlin RL, ‡ Gershovich JG, ‡ Kasper FK, Mikos AG. Flow Perfusion Co-culture of Human Mesenchymal Stem Cells and Endothelial Cells on Biodegradable Polymer Scaffolds. Annals of biomedical engineering. 2013.
‡ These authors contributed equally to this work.
INTRODUCTION

Bone marrow is populated by a variety of stem and progenitor cells that endow the tissue with a high regenerative capacity [1]. One such cell population is mesenchymal stem cells (MSCs), which are capable of differentiating into bone-forming osteoblasts, and reside in the perivascular niche of the bone marrow [1-3]. The localization of MSCs around blood vessels has prompted the investigation of cross-talk between MSCs and vascular cells for a variety of tissue engineering applications. In some cases, the co-culture of MSCs and vascular cells, such as endothelial cells (ECs) has been investigated in an effort to enhance the osteogenic or angiogenic properties of tissue engineered constructs. For example, the addition of ECs to cultures of MSCs has been previously shown to enhance the osteogenic differentiation of the cultures [4-8].

While numerous studies have investigated such co-cultures in various culture conditions [5], the majority of research to-date has focused on the co-culture of these cells in static conditions [9]. In order to better recapitulate the bone marrow perivascular niche, the dynamic mechanical environment in vivo should also be considered in an in vitro co-culture system [10]. Flow perfusion bioreactor systems can be used to apply mechanical stresses to three-dimensional cell cultures [11]. Furthermore, relative to static culture, flow perfusion culture has been shown to be capable of improving the infiltration, proliferation, and osteogenic differentiation of MSCs in three-dimensional scaffolds through the enhancement of mass transfer and the application of shear stress to the cells [11-14]. While several studies have investigated the effects of shear stress on the vascularization of EC and MSC co-cultures [15, 16], the effect of flow perfusion culture on the osteogenic differentiation of MSCs in such co-cultures has not been evaluated.
Thus, the goal of this study was to investigate the effects of flow perfusion culture on the mineralization of co-cultures of human MSCs (hMSCs) and human umbilical vein endothelial cells (HUVECs) on three-dimensional porous polymer scaffolds. It was hypothesized that flow perfusion culture would enhance the osteogenenic differentiation of hMSCs and that addition of HUVECs would have a beneficial effect in flow perfusion. In order to evaluate this hypothesis, osteogenically precultured hMSCs were cultured alone or with HUVECs on electrospun microfiber scaffolds in flow perfusion and static conditions and analyzed after 7 and 14 days.

MATERIALS AND METHODS

Experimental Design

Osteogenically precultured hMSCs were seeded onto electrospun poly(ε-caprolactone) (PCL) scaffolds and cultured in osteogenic medium for up to 14 days. In order to investigate the effects of hMSC co-culture with ECs, HUVECs were cultured with hMSCs in a 1:1 ratio on the PCL scaffolds (MH). Additionally, to control for the effects of cell density, low-density (M1) and high-density (M2) hMSC monocultures were investigated. The low-density monocultures (M1) consisted of half the number of hMSCs as the high-density hMSC group (M2) and the same number of hMSCs as the co-culture groups (MH). In order to investigate the effects of flow perfusion on these cell populations, cultures were conducted in static (St) and flow perfusion (Fl) conditions [11]. For each time point, two bioreactor units (with 10 constructs in each unit) were employed, and constructs were analyzed for DNA, alkaline phosphatase (ALP) activity, and calcium content (n=10, flow perfusion; n=6, static). Additionally, histological
analysis (n=6, flow perfusion; n=2, static) and scanning electron microscopy (SEM) (n=4, flow perfusion; n=2, static) were performed to visualize the presence and distribution of cells and extracellular matrix.

**Scaffold Fabrication and Characterization**

Nonwoven PCL microfiber mats with an average fiber diameter of approximately 10 µm (9.4±1.0 µm) were fabricated as previously described,[12] using a horizontal electrospinning setup. An 18 wt% PCL solution was prepared by dissolving PCL (inherent viscosity 1.0-1.3 dl/g; Lactel, Pelham, AL) in a solution of 5:1 v/v chloroform:methanol. The PCL solution was pumped at a flow rate of 25 ml/h through a blunt 16 G needle, which continuously received an applied voltage of 25 kV. The needle tip was directed at a copper collecting plate 33 cm away. Fiber diameter and morphology were inspected via SEM. Scaffolds (1.2 to 1.3 mm in thickness) were punched from electrospun mats using a 3 mm biopsy punch and loaded into custom-made polycarbonate holders designed to confine the cell solution during seeding and support the scaffolds during perfusion culture [11]. Scaffolds were then sterilized by exposure to ethylene oxide (Anderson Sterilizers, Haw River, NC) for 14 hours and aerated overnight to remove residual gas. Following sterilization, scaffolds were pre-wetted by soaking in a decreasing ethanol gradient (100% to 25%), rinsed three times in phosphate buffered saline (PBS), and soaked in culture medium (α-MEM, 13% fetal bovine serum (FBS) (Atlanta biologicals, Norcross, GA), 1% penicillin/streptomycin/fungizone (Gibco, Grand Island, NY)) for three nights at 37°C prior to cell seeding.
Cell Culture and Seeding

Frozen bone marrow-derived hMSCs were provided by Dr. Darwin Prockop from the Texas A&M University Health Science Center in Temple, Texas, USA. Per the supplier, cells were confirmed to possess widely accepted CD-markers, including CD90, CD105 and CD73, and to be capable of osteogenic and adipogenic differentiation up to passage 4 [3]. For this experiment, the hMSCs were thawed and cultured for 2 passages in expansion medium (α-MEM without nucleosides and ribonucleosides (Gibco) with 13% v/v FBS, 100 units/ml penicillin, 100 µg/ml streptomycin (Gibco) and 2-4 mM L-glutamine (Sigma, St. Louis, USA)) and osteogenically precultured for 7 days in osteogenic medium (expansion medium supplemented with 10 nM dexamethasone, 10 mM β-glycerol-phosphate and 0.2 mM ascorbic acid (all from Sigma)).

Frozen primary HUVECs pooled from several donors were purchased from American Tissue Culture Collection, (ATCC, Manassas, VA). According to the supplier’s certification, the cells were von Willebrand factor positive and smooth muscle α-actin negative. HUVECs were thawed and cultured until passage 4 in vascular cell basal medium containing 0.2% bovine brain extract (BBE), 5 ng/ml rhEGF, 10 mM L-glutamine, 0.75 units/ml heparin sulfate, 1 µg/ml hydrocortisone, 50 µg/ml ascorbic acid, 2% v/v FBS (Endothelial Cell Growth Kit-BBE) (all from ATCC) and 10 units/ml penicillin, 10 µg/ml streptomycin and 25 ng/ml amphotericin B (Gibco).

Cells were detached from T-225 Falcon flasks with 0.05% Trypsin-EDTA (for HUVECs) or 0.25% Trypsin-EDTA (for hMSCs) (Sigma), centrifuged at 1200 rpm for 10 min, and resuspended in expansion medium. Cell suspensions in 30 µl of culture medium were then pipetted onto the scaffolds at a density of 35,000 and 70,000 cells per
scaffold for monocultures of hMSCs (M1 and M2) and 70,000 total cells at a 1:1 ratio for co-cultures (MH: 35,000 hMSCs and 35,000 HUVECs). After an overnight attachment period, perfusion samples were cultured in a flow perfusion bioreactor with 45 ml of osteogenic medium at a flow rate of 0.03 ml/min through each 3 mm scaffold, as previously described [11, 17]. At the same time, static cultures were removed from loading cassettes and transferred to 12 well plates with 3 ml of osteogenic medium in each well. Half of the medium in flow perfusion and static cultures was changed twice a week for up to 14 days. Six constructs from each group were harvested the day after seeding to assess the seeding efficiency. At each time point twenty constructs from two bioreactor units and ten static constructs were harvested, rinsed with PBS, and frozen at -20°C for storage.

**Biochemical Assays**

Prior to biochemical analysis, samples underwent two freeze/thaw cycles in 250 µl of ddH₂O, followed by 10 min of sonication [18]. The concentration of double stranded DNA (dsDNA) in the supernatant was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Eugene, OR) according to the manufacturer’s instructions and as previously described [18, 19]. Briefly, cell lysate, buffer, and dye were pipetted into an opaque 96 well plate in duplicates, and fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 528 nm (FL x800 Fluorescence Microplate Reader; BioTek Instruments, Winooski, VT). DNA concentrations were determined relative to a lambda DNA standard curve.
ALP activity of different constructs was measured using a previously established colorimetric assay [18]. Briefly, samples were combined with buffer and p-nitrophenyl phosphate disodium salt hexahydrate (ALP-substrate) (Sigma, USA) in a clear 96-well plate in duplicates. Plates were incubated for 1 hour at 37°C, and the absorbance of each well was measured at 405 nm (PowerWave 340 Microplate Reader; BioTek Instruments). The ALP activity of samples was determined relative to a p-nitrophenol standard curve and normalized to the total DNA content of each individual sample.

Calcium content of constructs was determined using an established colorimetric assay, as previously described [18]. Constructs were placed into 0.2 ml 1 N acetic acid, and left on a shaker table at 200 rpm overnight to dissolve calcium in the constructs. The supernatant and calcium reagent (Arsenazo III, Diagnostic Chemicals, Oxford, CT) were pipetted into a clear 96-well plate, and the absorbance at 650 nm was measured. Samples were run in duplicate and diluted to fit within the range of a standard curve generated using CaCl₂.

**Histology**

Constructs from each group were rinsed with PBS, fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA) for 30 min at room temperature, and then dehydrated for 30 min in 70% ethanol. Samples were then embedded into HistoPrep freezing medium (Fisher Scientific) overnight and frozen. Sections, 7 µm thick, were made using a cryostat (Leica CM 1850 UV; Leica Biosystems Nussloch GmbH, Germany) and mounted onto glass slides. Slides were placed on a 42°C slide warmer for 2-3 days to enhance scaffold adhesion. Sections were stained with Fast Green and von
Kossa stains to visualize the distribution of cells and matrix and mineralized matrix, respectively. Images were taken using a ZeissAxio Imager.Z2 microscope equipped with AxioCam MRe5 (Carl Zeiss MicroImaging GmbH, Germany).

**Scanning Electron Microscopy**

Constructs from each group were rinsed with PBS, fixed in 2.5% v/v glutaraldehyde in PBS for 30 min at room temperature. They were then dehydrated with a gradient alcohol series and dried under laminar air flow in a culture hood for 1 day. Dried samples were mounted onto aluminum stubs and sputter-coated with 15 nm of gold. Extracellular matrix morphology of constructs was evaluated via SEM (FEI Quanta 400 Environmental, Hillsboro, OR) under high vacuum at a 30 kV voltage.

**Statistical Analysis**

Results were analyzed using one-way ANOVA with Tukey’s post-hoc test using JMP 10 software. Differences were considered significant at p<0.05, and results are presented as mean + standard deviation for n=10 (flow perfusion) and n=6 (static).

**RESULTS**

**Construct Cellularity**

Over the time course of the experiment, all groups showed an increase in DNA content from the day 0 samples, and all cell populations showed an increase in cellularity as a result of flow perfusion culture (Figure E.1). As expected, the DNA contents of the high-density monocultures (M2) were greater than the low-density monocultures (M1) immediately after the initial attachment period. However, after 7 days no difference was observed in cellularity between the two seeding densities in either static or flow
perfusion. While the DNA content of the high-density static monocultures (M2 St) was greater than the lower density (M1 St) at 14 days, the opposite effect was seen in the flow perfusion cultures (M2 Fl and M1 Fl). The high-density flow perfusion monoculture (M2 Fl) showed an increase in DNA content at day 7 but did not show any significant change in cellularity from day 7 to 14. In contrast, the low-density flow perfusion monoculture (M1 Fl) showed a continuous increase over the course of the study, resulting in a higher cellularity than the high-density monoculture by day 14. The initial cell density in the co-cultures (MH Fl and MH St) was equal to that of the high-density monocultures (M2 Fl and M2 St). However, after 14 days in static culture, the co-cultures (MH St) resulted in lower DNA content than the high-density monocultures (M2 St) and an equivalent amount of DNA as the low-density monocultures (M1 St). Similarly, in flow perfusion, the co-cultures followed the same trend as the low-density monocultures, where after 7 days the co-cultures had lower cellularity than the high-density monocultures but after 14 days exceeded the cellularity of the high-density monocultures.

**ALP Activity**

ALP activity was higher in static samples compared to the corresponding perfusion samples at all time points (Figure E.2). No difference was observed in ALP activity between the high- and low-densities of hMSCs in either static or flow perfusion. However, a higher level of ALP activity was found after 7 days in the co-cultures in both culture conditions compared to the hMSCs alone. A decrease in ALP activity from 7 to 14 days was observed in the static high-density monoculture (M2 St) and both co-culture
(MH St and MH Fl) groups, and by day 14 there was no statistical difference in ALP activity between any group.

**Calcium Content**

In the monoculture groups, calcium content was higher in flow perfusion than in static at both time points and at both seeding densities (Figure E.3). In static, no difference in calcium deposits was observed between the high- and low-densities at either time point, but after 14 days in flow perfusion, the high-density monoculture (M2 Fl) led to greater levels of mineralization compared to the low-density (M1 Fl). Calcium content in the static co-culture group (MH St) was greater than both static monocultures and the flow perfusion co-culture at both time points. In flow perfusion, the co-cultures had no significant effect on the mineralization compared to the low-density monocultures and had lower calcium contents compared to the high-density monocultures.

**Histology**

Staining of histological sections was consistent with the results of the biochemical assays (Figure E.4). Higher intensity Fast Green staining was observed in flow perfusion samples compared to static samples, indicating higher cellularity and matrix production. Similarly, greater von Kossa staining was observed in the static co-cultures and high-density flow perfusion samples (MH St and M2 Fl). A more homogeneous spatial distribution can be observed in the sections of flow perfusion samples, as staining in static samples was primarily localized to the top region of the construct.
**Scanning Electron Microscopy**

SEM imaging (Figure E.5) demonstrated cells and extracellular matrix completely covering the scaffold surfaces as well as cells infiltrating the pores between PCL fibers. SEM imaging confirmed that static co-cultures exhibited the greatest mineralization and that both monocultures in flow perfusion exceeded the mineralization of those in static. Furthermore, high-density flow perfusion cultures displayed the most abundant mineral deposition among all flow perfusion groups.

**Discussion**

Previous research has shown that when co-cultured with osteoblasts or MSCs, ECs enhance osteogenic potential as demonstrated by greater ALP activity and mineralization.[4-6, 20] Furthermore, previous studies from our laboratories have demonstrated that co-culturing osteogenically precultured hMSCs with HUVECs in a 1:1 ratio under static conditions led to enhanced osteogenic differentiation compared to cultures of hMSCs alone [9]. Both cell populations have previously been shown to be highly responsive to mechanical forces [13, 21, 22], and the effect of mechanical stimulation on the behavior of endothelial cells and vascularization in co-cultures has been investigated [15, 16]. However, the effect of mechanical forces on the osteogenic differentiation of hMSC/HUVEC co-cultures has not been evaluated. Thus, the objective of this study was to investigate the effects of flow perfusion culture on co-cultures of hMSCs and HUVECs. Specifically, it was hypothesized that flow perfusion would enhance the osteogenic differentiation of hMSCs in such co-cultures.

In the present study, flow perfusion culture was shown to significantly enhance cell proliferation and cell and matrix distribution through the constructs in all groups.
These results are consistent with previous studies [13, 18, 23, 24], which demonstrated enhanced proliferation and distribution of MSCs as a result of enhanced mass transfer,[13] as well as through the application of shear stress to the cells [14]. Here, it was observed that flow perfusion culture stimulated the proliferation of both high- and low-density hMSC cultures, although the timing of proliferation varied with seeding density. Similarly, previous work investigating the effect of flow perfusion on co-cultures of rat MSCs and aortic ECs found increased proliferation compared to either cell type alone [15]. In contrast to the previous study, co-cultures of the present study did not stimulate cell proliferation in flow perfusion conditions, which could be a result of the difference in the culture conditions or cell species used in the two studies [15]. Furthermore, while quantifying the total DNA content indicates the proliferation within the construct as a whole, it cannot distinguish between the proliferation rates of the hMSCs or HUVECs in co-culture.

The co-culture ALP activity was enhanced in both static and perfusion culture; however when comparing static to flow perfusion conditions, perfusion culture led to a reduced level of ALP activity at all time points evaluated. The enhanced ALP activity in the co-cultures is consistent with previous studies of co-cultures in static conditions [4-6, 9] and with the enhanced mineralization that was also observed in these cultures. While ALP activity is a transient marker and thus no definitive conclusions can be drawn from this result, the enhanced ALP activity of the perfusion co-cultures at the time points evaluated here, suggests that the addition of HUVECs to hMSC cultures may enhance early stages of osteogenesis in both static and flow perfusion. While reduced ALP
activity was observed in flow perfusion compared to static conditions, the cultures were only assayed at 7 and 14 days, and thus different results may have been observed at earlier time points.

Previous research has shown enhanced osteogenic differentiation of rat MSCs in high-density compared to low-density cultures as a result of increased cell-cell communication [25, 26]. In the present study, no effect of the seeding density on either ALP activity or calcium content in monocultures was observed in static conditions. While no effect was observed in static cultures for the specific densities evaluated, increased mineralization (characteristic of late-stage osteogenic differentiation) was observed in the high-density compared to the low-density flow perfusion monocultures after 14 days. The increased levels of calcium could be a result of increased cell number. However, after noting the equivalent levels of ALP activity between these two groups, the enhanced late stage osteogenesis may also be attributed to a higher applied shear stress resulting from a reduced scaffold pore size due to greater quantities of ECM being produced by the high cell density. Previous studies have demonstrated that the quantity of ECM produced by osteogenically differentiating MSCs is sufficient to reduce the average pore size in fibrous scaffolds [27] and that a lower scaffold mesh size in flow perfusion can lead to greater mineralization [26]. Furthermore, increased shear stress has demonstrated a capacity to enhance mineralization, without significantly affecting ALP activity, which led to the conclusion that variation in shear stress primarily affects the later stages of osteogenic differentiation [13, 14]. Additionally, this result is consistent with studies evaluating hMSC seeding density and flow rate in perfusion, in which it was observed
that while seeding density affected construct mineralization, seeding density was a less significant factor than flow rate (i.e. shear stress) [28].

While the mineralization of hMSC monocultures was enhanced in flow perfusion compared to static culture, the opposite outcome was observed in the co-cultures where the calcium content was reduced under flow perfusion conditions. The most likely explanation for this outcome includes the application of suboptimal flow perfusion parameters as well as reduced cell-cell contacts. A flow rate of 0.03 ml/min through each 3 mm scaffold was selected for this study in order to provide an average shear stress approximating the level, which was previously shown to be beneficial for osteogenic differentiation of rat MSCs [13, 14]. The initial shear stress level was estimated based on a cylindrical pore model previously used to calculate the level of shear stress in scaffolds of this geometry [12, 13, 29]. Based on this approximation, the initial shear stress was estimated to be 0.15 dynes/cm². While this flow rate proved to be beneficial to the osteogenic differentiation of rat MSCs alone, an optimal level of shear stress for the co-culture of human cell populations has not been established. The ideal range of shear stress for the osteogenic co-cultures of these cell types may be a balance between the level that alone is beneficial for osteogenic differentiation and that which promotes the proliferation or specific gene expression of the endothelial cells. For example, studies have demonstrated improved EC proliferation and microvasculature formation in co-cultures with MSCs when exposed to approximately 5 dynes/cm² in collagen gels [15]. ECs are highly sensitive to shear stress, and can exhibit vastly different responses and gene expression patterns depending on the magnitude of shear stress experienced [22, 30,
Thus, a different effect may have been observed with different flow rates, a potentially interesting phenomenon for future study.

Reduced cell-cell contacts resulting from increased cellular infiltration into the scaffolds, as seen in Figure E.4, is another possible explanation for perfusion-dependent reductions in calcium content in co-cultures. The relationship between seeding density, scaffold pore size, and flow rate for perfusion cultures was emphasized previously [28, 32, 33] generating outcomes in agreement with the data presented herein. While soluble signaling is certainly significant in the osteogenesis of co-cultures, bone marrow-derived MSCs are thought to be especially sensitive to cell-cell contacts [6, 20]. An enhancement of osteogenic differentiation may result from increased cell density in perfusion co-cultures, which also increased the number of cell-cell contacts. The fact that mineralization was greatest in the high density monoculture group and was virtually equal between the low density monoculture and co-culture groups further supports the conclusion that optimization of the seeding density may enable better osteogenic outcomes in co-cultures under flow perfusion conditions.

Here, a 1:1 ratio of hMSCs to HUVECs was implemented, as previous studies have shown that a 1:1 ratio of hMSCs to HUVECs led to the highest levels of mineralization and ALP activity compared to the other ratios evaluated (80:20 to 98:2), and that the cell ratio had a significant effect on the osteogenic outcome [5]. In the present perfusion cultures, the change in cell ratio in the co-culture over the duration of the experiment is unknown. However, the high-density monoculture exhibited the greatest calcium mineralization, indicating that enhanced osteogenesis may be achieved
by increasing the proportion of hMSCs relative to HUVECs in the perfusion co-cultures above a 1:1 ratio.

**CONCLUSIONS**

Flow perfusion culture was found to enhance the mineralization of hMSC cultures at both high- and low- densities. While hMSC density had no effect on mineralization in static culture, increased hMSC density led to enhanced mineralization in perfusion cultures, possibly due to the higher effective shear stress experienced by cells in these constructs. While co-cultures of hMSCs and HUVECs had enhanced mineralization compared to monocultures in static conditions, the same effect was not observed in perfusion cultures. Co-cultures of hMSCs and HUVECs did not contribute to enhanced mineralization compared to hMSCs alone under the flow perfusion conditions investigated in this study. However, flow perfusion culture did result in an enhanced spatial distribution of cells and matrix compared to static cultures.
Figure E.1: DNA content of constructs cultured for 14 days in osteogenic medium in static (St) or flow perfusion (Fl) conditions.

Scaffolds were seeded with either 35,000 hMSCs (M1), 70,000 hMSCs (M2) or 35,000 hMSCs and 35,000 HUVECs (MH). Samples were taken after 0, 7, and 14 days of culture. Data are presented as means ± standard deviation. At a given time point, statistical differences (p < 0.05) between co-cultures (MH) and low-density monocultures (M1) are indicated by #, and differences (p < 0.05) between co-cultures (MH) and high-density monocultures (M2) are indicated by *. At a given time point, statistical differences (p < 0.05) between high-density monocultures (M2) and low-density monocultures (M1) are indicated by x, and statistical differences (p < 0.05) between a flow perfusion group (Fl) and the corresponding static group (St) are indicated by +. Within a group, statistical differences (p < 0.05) compared to previous time points are indicated by ‡.
Figure E.2: ALP activity normalized to DNA in constructs cultured for 14 days in osteogenic medium in static (St) or flow perfusion (Fl) conditions.

Scaffolds were seeded with either 35,000 hMSCs (M1), 70,000 hMSCs (M2) or 35,000 hMSCs and 35,000 HUVECs (MH). Samples were taken after 7 and 14 days of culture. Data are presented as means ± standard deviation. At a given time point, statistical differences (p < 0.05) between co-cultures (MH) and low-density monocultures (M1) are indicated by #, and differences (p < 0.05) between co-cultures (MH) and high-density monocultures (M2) are indicated by *. At a given time point, statistical differences (p < 0.05) between high-density monocultures (M2) and low-density monocultures (M1) are indicated by x, and statistical differences (p < 0.05) between a flow perfusion group (Fl) and the corresponding static group (St) are indicated by +. Within a group, statistical differences (p < 0.05) compared to previous time points are indicated by ‡.
Figure E.3: Calcium content of constructs cultured for 14 days in osteogenic medium in static (St) or flow perfusion (Fl) conditions.

Scaffolds were seeded with either 35,000 hMSCs (M1), 70,000 hMSCs (M2) or 35,000 hMSCs and 35,000 HUVECs (MH). Samples were taken after 7 and 14 days of culture. Data are presented as means ± standard deviation. At a given time point, statistical differences (p < 0.05) between co-cultures (MH) and low-density monocultures (M1) are indicated by #, and differences (p < 0.05) between co-cultures (MH) and high-density monocultures (M2) are indicated by *. At a given time point, statistical differences (p < 0.05) between high-density monocultures (M2) and low-density monocultures (M1) are indicated by x, and statistical differences (p < 0.05) between a flow perfusion group (Fl) and the corresponding static group (St) are indicated by +. Within a group, statistical differences (p < 0.05) compared to previous time points are indicated by ‡.
Figure E.4: Representative histological sections of constructs cultured for 7 or 14 days in static (St) or flow perfusion (Fl) conditions.

Scaffolds were seeded with either low (M1) or high (M2) densities of hMSCs, or cocultures of hMSCs and HUVECs (MH). Samples were taken after 7 and 14 days of culture and were stained with Fast Green (green) to visualize the distribution of cells and matrix (first and third row) and von Kossa (brown) to visualize the presence of minerals (second and fourth row). Scale bar represents 100 μm in all images.
Figure E.5: Scanning electron micrographs of the top surfaces of constructs cultured for 7 or 14 days in static (St) or flow perfusion (Fl) conditions.

Scaffolds were seeded with either low (M1) or high (M2) densities of hMSCs, or cocultures of hMSCs and HUVECs (MH). Samples were taken after 7 and 14 days of culture. Panels A and C are magnified at 300×, and the scale bar is 200 μm and applies for all images in these panels. Panels B and D are magnified at 1600×, the scale bar is 30 μm and applies for all images in these panel.
REFERENCES


