EXPANSION AND DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS IN A PERFUSION AND SURFACE SHEAR STRESS BIOREACTOR FOR ENGINEERED TISSUE CARTILAGE

by

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EXPANSION AND DIFFERENTIATION OF HUMAN MESENCHYMAL STEM
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BIOMEDICAL ENGINEERING

ABSTRACT
The demand for tissue engineered articular cartilage for implantation in patients with
osteoarthritis requires the development of stable and robust process production systems.
We have developed a shear stress and perfusion bioreactor (SSPB) for tissue engineering
cartilage to enhance nutrient diffusion and mechanical stimuli. Using static and bioreactor
culture in 3D scaffolds we have identified tissue culture conditions using transforming
growth factor beta-1 (TGFβ-1), bone morphogenic protein-2 (BMP-2) and micromass
cell seeding of hMSCs in polycaprolactone (PCL) scaffolds. hMSCs were seeded on
porous PCL scaffolds using the following conditions: no grow factors, TGFβ-1 or BMP-2
alone, TGFβ-1+BMP-2 simultaneously and transient application of BMP-2 in the
presence of TGFβ-1. We found that the transient use of BMP-2 in the presence of TGFβ-
1 greatly enhances differentiation of hMSCs as shown by GAG deposition and collagen
II expression. We tested this condition in the rotating disc bioreactor as a proof of
concept. We found an increase in cell proliferation and extra cellular matrix production in
the bioreactor compared to static culture.

Keywords: bioreactor, tissue engineering, cartilage
DEDICATION

To my parents, Concepcion Moran-Rodriguez and Carlos Carmona-Zepeda and my girlfriend Ana Rosa Tenorio-Sandoval.
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CHAPTER 1

BACKGROUND AND RELEVANCE

1.0 Introduction

The average age of the world population is increasing as a consequence of the improvements in medicine and health care [1]. This increase in life expectancy in most industrialized nations contributes to a general increase in degenerative illness [1]. It is believed that by the year 2050 the median life span around the world will extend another 10 years [2]. This steady increase in population age will generate an impact on the medical and social services [2]. Therefore it is critical to assess the cost effectiveness of treatments that improve the quality of life for persons with degenerative illness. This opens the opportunity for therapies based on the treatment of degenerative diseases such as bone and joint disease.

Among the new treatments being developed, tissue engineering (TE) is a promising and relatively new interdisciplinary field that combines clinical medicine, biological science and engineering [3]. Tissue engineering aims to duplicate the body’s regenerative capacity by creating a suitable environment in which tissue regeneration can be performed and controlled in vivo or in vitro [4]. Tissue engineering has grown faster in the last 10 years and has become a feasible solution for repair or replacement of damaged tissue and organs such as cartilage, vascular tissue and bone [4]. Tissue
engineering incorporates the use of cells, novel scaffold materials, signaling factors, and bioreactors for the creation of biological implants that can restore tissue damage [3].

One of the degenerative illnesses that can be treated using tissue engineering is joint degeneration. Joint degeneration is typically caused by the disruption of the joint’s cartilage due to osteoarthritis or trauma and its common symptom is joint pain [5,6]. In 2003 the number of adults reported with rheumatic conditions in the U.S was about 46.1 million [7]. The total expenditures in 2003 generated by all the rheumatic conditions were $321.8 billion and caused a total of $47 billion in labor losses [7].

Joint cartilage or articular cartilage is a connective tissue that covers the end of the bones and its main function is to provide cushioning between the bones that form the joint. Though the origins are still not clear, osteoarthritis is a disease that produces inflammation in the bone joints and causes a gradual degeneration of the joint cartilage [8]. Available treatments for osteoarthritis fall in two categories: non-surgical options and surgical options [9]. Non-surgical options primarily treat the symptoms whereas the surgical alternatives produce different outcomes in patients. Non-surgical options include nonpharmacologic treatments such as heat and cold modalities, physical therapy and lifestyle activity modification such as weight loss and physical therapy; whereas pharmacologic treatments include anti-inflammatory medication, mild analgesics, nonsteroidal anti-inflammatory drugs, and local cortico-steroid injections [9].

Surgical treatments include a wide variety of methods to either stimulate the intrinsic repair processes of the joint or by replacing damaged tissue from another source. Drilling the subchondral bone (Arthroscopy drilling method) is used to stimulate the
body’s own repair mechanisms [10]. This procedure creates a micro perforation that reaches the bone marrow so the cells in the bone marrow can now migrate into the injured area and begin the healing process. However the outcome for this technique is variable and in many cases the new tissue acquires fibro-cartilage properties [9]. Another procedure involves the use of cartilage transplants either from autologous source or from a donor. This procedure requires the creation of a secondary injury in another area of the joint to extract the cartilage [10]. A promising approach is the direct transplantation of cells or tissue into the cartilage defect with a biologic or synthetic polymer. Among these available treatments using direct transplantation, tissue grafts are one of the most successful approaches [11]. However, none of these procedures have been proven to produce the best results. The variability associated with them and the risk of rejection by about 25% of the patients makes these procedures the last alternative when treating osteoarthritis [11]. There is a need for new treatments that address this variability and rejection risk while providing a functional replacement material for the injured or damaged area. One of these new promising treatments is tissue engineering.

Tissue engineering uses new therapies that produce biological substitutes for tissue and organ repair or replacement in which other treatments have found it difficult or impossible to address successfully using the existing tools of medicine [12]. Tissue engineering is an interdisciplinary field that combines several disciplines such as chemical engineering, material science and cell biology for the development of tissue suitable for implantation. With this in mind, it is possible to expect that one of the first applications of tissue engineering might be the fabrication or growth of cartilage using a biomaterial in a device that provides a controlled environment for tissue growth such as a
bioreactor. A bioreactor is an engineered device capable of producing biological products of interest and whose design allows control and monitoring of the operating conditions.

Another important element in tissue engineering is the cell type to be used to create new tissue. An ideal cell should be easily expandable, nonimmunogenic and receptive to differentiation cues for the tissue to be replaced [13]. The cell source can be primary cells or stem cells [14]. Primary cells are differentiated cells that are derived from mature tissues and can be autologous cells (same individual) or allogenic cells (different individual). Stem cells are undifferentiated cells that have the ability to differentiate to one or more specific cells. They can be subdivided in embryonic stem cells and adult stem cells.

The other 2 components in tissue engineering are the porous scaffolds and growth factors. The scaffold is the basic architecture that provides support and promotes the free growth and communication of the cells. The scaffold properties depend on the type of material used for its construction. This material must enhance the desired final characteristics of the product while being bio-absorbable by the body and at the same time it has to be porous to allow nutrient diffusion [15]. Growth factors are essential for the development and transition of the cells into the desired tissue; especially in instances where the regeneration potential or growing of tissue is very low [4].

1.1 Articular Cartilage Formation and Tissue Architecture

Articular cartilage is a dense connective tissue in which the chondrocyte is the cell in charge of the production and maintenance of the extracellular matrix [16].
Cartilage originates from mesenchymal condensation during embryogenesis [17]. In this embryonic period, joint formation passes through two principal stages: the formation of the cartilaginous anlagen that creates the mold for the subsequent skeletal elements and joint formation [17]. Mesenchymal condensation is called skeletal blastema and is a highly dense avascular aggregation. The first cartilaginous nodule that leads the anlagen formation appears in the middle of the blastema. This cartilage nodule is characterized by the appearance of flattened and elongated cells at the periphery that create the perichondrium. Once created the cartilaginous model the chondrocytes on it can start the process of hypertrophic maturation and become long bones.

In a human body the main function of articular cartilage it is provide a protective layer between joints. The tissue architecture is designed to minimize friction between joints while providing cushioning against compressive forces [18]. Articular cartilage composition is mostly water (75% - 80%), cells (10%-15%) and proteins (10%-15%). The main function of the protein mesh within the cartilage is to attract water molecules within the mesh and use it as a cushion. When pressure is applied water molecules are squeezed out of the protein frame thus diminishing the impact force on the joint.

The tissue architecture of articular cartilage in the adult human body can be defined by 4 zones of morphological demarcation: 1) superficial, 2) middle 3) deep 4) and calcified [9]. Each of these zones present a different extracellular matrix protein composition and they can be subdivided into three different regions according to proximity to the chondrocyte: pericellular, territorial, and interterritorial (Figure 1) [19,20]. It is believed that this change of cell density and protein arrangement in different regions is closely related to the mechanical forces presented in cartilage tissue.
Cartilage tissue on the surface is exposed to shear stress whereas the cells in deeper layers perceive pressure [21]. The cell exposure to shear stress and mechanical loading causes a reaction of the cells that optimizes the protein production and remolds tissue architecture according to the forces received on each layer of cartilage tissue [22].

Figure 1. Zonal organization in cartilage and collagen II fiber alignment.
The superficial zone is the outer most layer that consists mainly of tangentially arranged collagen type II fibers and is covered by a thin film called lamina splenda. The lamina splenda consist of proteoglycan monomers, collagen II and link protein that provide a smooth surface against shear stress [23]. This protein configuration allows for the movement of the joint while minimizing friction. Collagen II is a structural protein arranged in a triple helix of three identical polypeptide chains [24]. These collagen fibers self arrange into a fibrillar mesh joined together by aldimine-derived cross linker molecules. The main function of these collagen fibers along with the other proteins present in cartilage, is to provide a structural mesh capable of withstanding mechanical forces. Proteoglycans in cartilage are aggrecan aggregates bond to link molecules of hylauronic acid. Aggrecan has a core protein that is stabilized by action of disulfide bonds [24]. The negative charge of these sulfated molecules allows the entrapment of water molecules within cartilage tissue. The morphology of the chondrocytes in this superficial zone tends to be more like a disc shaped and they are aligned along the surface. The cell number in the superficial zone is the highest of all the zones presents in hyaline cartilage. These chondrocytes produce a special lubricating proteoglycan called lubricin [25].

The transitional or middle zone has thicker collagen II fibers and a higher content of proteoglycans. The arrangement of the fibers tends to be obliquely and randomly distributed. That is because in this region the shear forces that act in the superficial layer are transformed into compressive forces. In this zone the chondrocyte morphology changes from disc-shape to spherical like shape [9].
The deep region has the thickest collagen fibers of all the zones and most of them are attached vertically into the tidemark. The tidemark is the demarcation line that indicates the beginning of calcified tissue. The high compressive forces expressed in this region create a perpendicular arrangement of collagen fibers and the proteoglycan content is also the highest here [9]. The cell shape adopts an ellipsoid conformation and they form columns of two to six cells orientated perpendicularly toward the radial articular surface. The calcified layer helps to decrease excessive shear stress between bone and cartilage [24]. The chondrocytes in this zone are smaller and are surrounded by calcified ECM.

1.2 Articular Cartilage Degeneration

Most cartilage degeneration is caused by osteoarthritic wear of the joint. However there is a high number of cartilage degeneration injuries due to trauma. Joint injuries can be classified in 3 types depending on the degree of penetration in the three layers of cartilage [9]. The first type of lesion just includes the superficial and middle zone. These types of lesion cause a matrix disruption that in most of cases is not permanent and can be restored by the chondrocytes in the region [16]. The second type of lesion compromises all the layers in articular cartilage but without reaching the bone marrow. This type of lesion is reversible if the chondrocytes within the tissue respond quickly to produce new ECM molecules and degrade the damaged molecules [26]. The third type of lesion penetrates all the layers reaching the bone marrow and causing a migration of stromal cells into the injury site [26].
1.2.1 Osteoarthritis

Osteoarthritis or degenerative joint disease is a severe condition caused by the mechanical and biochemical breakdown of the articular cartilage [9]. It also causes the remodeling of the subchondral bone and eventually inflammation of the synovial membrane. It is believed that during the first stages of osteoarthritis the breakdown of the macromolecular ECM releases particles into the synovial fluid creating inflammation of the joint [19]. This change in the ECM composition produces a change in the homeostatic conditions within the tissue that affects the chondrocytes and lead to the failure of maintain the homeostatic conditions between the extra cellular matrix production and degradation [5]. This creates a change in the protein content in the ECM causing a softening of the tissue that leads to the eventual breakdown of collagen II fibers [26]. The softening of the cartilage tissue causes an increase in the force transmitted to the subchondral bone, therefore increasing the overall stress in the damaged zone. This cascade of events produces inflammation and the production of inflammatory cytokines that switch chondrocytes from anabolic metabolism to catabolic metabolism. This catabolic metabolism enhances the breakdown of ECM molecules and exacerbates the damage of the joint.
1.3 The Tissue Engineering Approach for Cartilage Regeneration

Tissue cartilage engineering aims to produce suitable implants for injured tissue with feasible industrial production and medical application. To produce quality implants with mechanical properties similar to those found in native tissue it is necessary to enhance the production of cartilage specific proteins while trying to mimic in vivo tissue architecture [27]. This main goal has to be achieved with a feasible clinical treatment that improves medical costs over current treatments. Tissue engineering uses cells, biomaterials and biochemical factors to develop artificial solutions to tissue degeneration problems. Tissue engineering combines these 3 factors to produce quality implants in a large scale process that will satisfy the market demand.

1.3.1 Cell Source

The principal element in cartilage tissue engineering is the biological component that under the right environment conditions produces the matrix proteins necessary for cartilage growth. Large quantities of cells are required to build the complex series of proteins and biochemical signals needed to create a tissue [14]. The first cell types used in tissue engineering cartilage were chondrocytes. These primary cells are found in mature cartilage tissue within the body and are fully differentiated cells. The harvest technique used to collect the cells requires invasive surgery that might cause damage in the harvested region. Once the cells are collected is necessary to expand the chondrocytes in large numbers [28]. However some problems are presented when these cells are grown outside the body. A monolayer expansion causes dedifferentiation of chondrocytes into fibroblast and the lack of mechanical stimulus causes a decrease in proteoglycan
synthesis and collagen type II [29]. There is also an increase in collagen type I production which is expected in fibroblastic cultures [30]. This low ratio of secreted collagen II and GAG proteins affects the final mechanical properties needed for functional cartilage for implants. The low proliferation rate and difficulty of obtaining chondrocytes from patients started a new search for cells that can be used in tissue engineering.

In 1981 embryonic stem cells were isolated from mice [31] and eventually stem cells started to be isolated from human embryos and mature tissues. Stem cells are characterized by being able to differentiate into different lineages (pluripotency) and they have the ability to self-renew their population. Stem cells can be classified into pluripotent human embryonic stem cells or human adult somatic stem cells derived from adult tissues [32]. Embryonic stem cells can be extracted soon after the fertilization of the ovum during the blastocyst stage. These cells have the ability to differentiate into any type of cell in the organism. However still there are some ethical and biological problems associated with the use of embryonic stem cells [33]. Additionally, due to the high proliferative capacity of these cells teratoma formations is another concern [34].

The most prominent adult stem cells are those originating in bone marrow called human mesenchymal stem cells. These cells are able to differentiate into several types of cells such as chondrocytes, myocytes and neurons by just changing the culture conditions [35]. They also can be easily expanded outside the body which gives them a great potential for tissue engineering regeneration. Bone marrow containing mesenchymal stem cells is extracted; usually from the iliac crest in humans. After bone marrow extraction, nucleated cells are isolated using Percoll tubes and finally the cells are cultured in tissue culture flasks and non-adherent cells are extracted. The adherent cells are tested for
differentiation into chondrogenic, osteogenic and adipogenic lineages [36]. Currently there have not been developed specific markers for mesenchymal stem cells. Extracted cells need to show negative surface markers for hematopoietic cells such as: CD34, CD45, CD14, CD31, CD133, and they have to show positive for CD105, CD166, CD54, CD55, CD13 and CD44 [37].

1.3.2 Scaffolds for Tissue Engineering

In vivo growth of tissue follows a 3 dimensional (3D) pattern. This pattern is regulated by the complex interaction of cells, extracellular matrix and chemicals signals that direct the 3D growth of developmental tissue within the body [15]. In order to achieve good tissue development in vitro and to promote well developed tissue architecture, a 3D growth of cells is required. Biomaterials with a 3D structure started to be used as scaffolds to slow the dedifferentiation process associated with 2D cultures. The scaffold is the structure that provides the basic architecture and promotes growth of the cells. The scaffold must enhance the desired properties of the final product, in this case human tissue [15]. The surface properties of the scaffold material are crucial for cell adhesion, development and differentiation [38].

Biomaterials used for tissue engineering are classified as a) natural materials and b) synthetics materials [39]. Natural materials offer greater biocompatibility than synthetic materials and usually are extracted from animal origin [39]. Most of these materials consist of extra cellular matrix proteins that are similar to human extra cellular matrix proteins. Also some natural materials like collagen [40], fibrinogen [41] and
chitosan [42] have bioactive properties and in some cases the protein structure is similar to those found in vivo. Since these materials are extracted from animal tissue the purification and cleaning process affects the overall biological and mechanical properties [39]. On the other hand synthetic materials have the advantage that they can be created by mass production techniques and they can be produced to meet specific material properties such as stiffness, hardness, and degradation rates. They can be classified as polymers and non polymeric materials. The mechanical properties of polymeric biomaterials can be modified by varying the molecular weight of the polymer and copolymerization ratio. These elements affect the degradation rate and surface properties to meet desired values [43].

Other important properties related with scaffold design include: biocompatibility, porosity, surface properties and chemistry (including pH, surface charge and surface energy). Interconnected geometry and large surface area to volume ratio facilitates cell growth within the scaffold [44]. This internal configuration must be balanced with the entire mechanical properties desired for tissue engineered cartilage. The surface properties of the material in the scaffold is a main characteristic since the cells are in contact with it, thus a good material allow the attachment of the cells without affecting the development.

1.3.3 Designing Cartilage Tissue Using Mesenchymal Stem Cells

The design of cartilage using tissue engineering requires the selection of the cell source, scaffold material and the right culture conditions that lead to cartilage zonal
organization and maximize protein production therefore enhancing the mechanical properties. As mentioned above mesenchymal stem cells have become a feasible alternative for tissue engineering. These cells can be easily extracted by a simple non-hospitalization procedure. Though the normal fate of mesenchymal stem cells is bone; several studies have shown that these cells differentiate into different connectives tissues under the right chemical culture conditions.

1.3.4 Material Selection for Tissue Engineering Cartilage

Tissue engineering of cartilage requires the use of scaffold constructs that support the earliest stages of tissue development [45]. Currently, a broad variety of natural and synthetic materials have been developed for use as scaffolds in tissue engineering cartilage. The important elements that made a scaffold material suitable for tissue engineering cartilage are: biodegradability, mechanical properties, a high surface/volume ratio and a good porosity [46,47].

Polymeric materials meet all these elements and at the same time they can be modified to meet specific biodegradability, mechanical and energy surface values [46]. Polymeric materials that are derived from aliphatic polyesters such as polylactic acid or polycaprolactone have been already approved by the FDA for medical applications [48]. These two types of materials can be tailored to meet specific physical and chemical properties by modifying the molecular weight or by altering their surface chemistry [49,50]. The modification of the molecular weight alters the degradation time and the
mechanical properties. The alteration of the surface chemistry has a bigger impact on cell
attachment, proliferation and differentiation.

The hydrophobic properties associated with PCL decrease its biodegradation rate
compared with other aliphatic polyesters [51]. Not only does this relatively slow
degradation rate makes PCL suitable for tissue engineering of cartilage; but the
mechanical properties of PCL are higher than PLLA or other copolymers [50]. Porous
PCL scaffold can have a compression module in the range of 54.5 to 90.9 kPa [52].
However the mechanical properties of PCL are still low compared to native cartilage (0.5
MPa to 1.0 MPa of compression modulus) [53]. One of the disadvantages of PCL scaffold
is the high hydrophobicity that affects cell attachment. However this disadvantage can be
overcome with a combination of cell seeding techniques such as micromass or
mechanical cell seeding. Scaffolds made out of PCL have been used in in vivo studies
for cartilage tissue regeneration [54]. When the interaction of PCL with mesenchymal
stem cells was compared with well known differentiation process such as pellet cultures;
the interaction with PCL did not produce alterations in the grade of differentiation as
showed by GAG production and gene expression [55].

1.3.5 Cell Culture

Differentiation of mesenchymal stem cells into cartilage is a complex process
regulated by several biochemical and mechanical pathways. To understand these
pathways, scientists started to study the early steps that occur during cartilage embryo
formation [56]. One of this crucial conditions identified from cartilage development is
cell density [57]. Mesenchymal stem cell growth and differentiation is highly sensitive to cell culture density. When hMSCs are cultured under low densities they proliferate until they reach confluency. Once hMSCs reach confluency they do not proliferate anymore and they start to differentiate. One way to increase this differentiation potential of hMSCs is to use high density cultures of hMSCs or micromass cultures. These micromass cultures are well known to induce cartilage differentiation of hMSCs [58]. This type of results can be explained from an embryo development point of view [59]. Mesenchymal stem cells are part of mesenchyme tissues and during embryogenesis cartilage development starts with mesenchymal cells recruiting, condensation (micromass), proliferation and differentiation [59]. In vitro differentiation of mesenchymal stem cells when cultured in high density follows a series of pre-cartilaginous stages before reaching a complete differentiation. These stages can be identified by morphological inspection of the cells under the microscope. In culture, mesenchymal stem cells exhibit a flattened and elongated form. When mesenchymal stem cells are cultured under micromass conditions they start to create small aggregates that eventually lead to a small cluster. Those small clusters go a pre-cartilaginous condensation and finally they become a cartilage nodule [60,61].

There are several examples of micromass cultures used in the literature [62,63,64]. Micromass refers to a technique that uses a high cell concentration suspension (In the range of 5 X 10^6 cells/ml to 40 X 10^6 cells/ml) for culture [65,66]. Small aliquots of the cell suspension (ranging from 5 to 100 μl) are placed in tissue culture plates. These small drops of cell suspension are maintained in that high density state from 2 hours up to 5 hours before more media is added to culture reservoir. That period of time allows the
condensation and creation cell-cell junctions that start the differentiation process. After that period of time even the addition of more media to the reservoir does not destroy the micromass zone.

1.3.6 Growth Factors

Another important aspect of mesenchymal cell differentiation is the use of the appropriate chemical signals that enhance cell proliferation, trigger cell commitment and eventually differentiation into chondrocytes. Growth factors are cytokines produced by the cells during various stages of development. These different signals interact with specific cell membrane receptors and initiate a signaling cascade mechanism that activates or silences protein production, gene expression, proliferation, cell death and cell movement. These cytokines also guide the development of long bones and cartilage during embryo development. This is a sequential process that starts with the production of hyalurionan and collagen type I and type II [67].

Transforming growth factor beta (TGFβ) is a family of signaling proteins capable of stimulating cell growth and differentiation [68]. The TGFβ family includes a broad variety of polypeptide proteins. There are at least five known of these proteins with a 70% to 80% of amino acid sequence identity that constitute the TGFB family (from TGFβ-1 through TGFβ-5) [69]. Besides of their multiple roles in cell growth in other types of cells, members of the TGFβ family have been shown to play a role in cartilage and bone formation. Specifically several studies have shown that exposure of mesenchymal stem cells to TGFβ induces cartilage differentiation [70]. TGFβ signaling
process occurs via the serine/theronine kinase receptors (named type I and type II). When these receptors are activated by the presence of TGFβ a phosphorylation process takes place and activates several Smad mechanisms [71]. These smad mechanisms acts in the cell nucleus to regulate or suppress the expression of genes involved in cell regulation, apoptosis and differentiation [71]. TGFβ is found during the earliest stages of mesenchyme condensation during embryogenesis. It is believed that the role of TGFβ is to set the boundaries of the mesenchymal condensation. When TGFβ2 receptors are deleted from mice chondrogenic differentiation occurs normally but leads to malformation of long bones suggesting a crucial role in skeletal development and a partial role in cell differentiation [72].

Bone morphogenic protein (BMP) is found in the same family of signaling proteins as TGF-β. BMP proteins bind type IA, IB and type II receptors. It is believed that the main role of BMP in cartilage is to induce endochondral ossification and stimulate the maturation of full differentiated chondrocytes to undergo hypertrophic chondrocytes. It also has been demonstrated that BMP is involve in the formation stages of bone and cartilage. The principal role during this process is in setting the frontiers for the condensate mesenchyme tissue [73]. When used in vitro studies BMP-2 along with high density cultures enhances chondrogenesis in mesenchymal cells [63].

The use of TGFβ and BMP in tissue engineering cartilage has been broadly addressed [74]. Studies with hMSCs treated with TGFβ-1 showed an increase and maintenance of cartilage differentiation phenotype when TGFβ-1 was used in the range of 1 ng/ml to 5 ng/ml [75]. Though the use of lower concentrations of TGFβ-1 (0.3 ng/ml to 1 ng/ml) also enhances cartilage formation it does not prevent the formation of bone
during differentiation of mesenchymal tissue [76]. On the other hand the use of BMP produces a faster and more robust cartilage differentiation that any of the other TGFβ family members [77]. However there are certain concerns about the potential of bone formation associated with the use of BMP proteins. When BMP-2 was used at a concentration of 50 ng/ml in murine mesenchymal stem cells; it was found necessary the modulation of cadherin molecules expressed during micromass cultures to maintain chondrogenesis [63]. Other studies have addressed the proliferative effect of TGFβ-1 when combined with 3D scaffolds materials [78]. In addition, the exposure of chondrocytes to TGFβ-1 represses their terminal differentiation into hypertrophic chondrocytes [79]. This hypertrophic differentiation is associated with osteoarthritis. When TGFβ-1 and BMP-2 were used in combination an enhancement of the chondrogenic potential of hMSC was observed [80]. The combination of TGFβ-3 and BMP-6 caused an increase in the expression of collagen II genes. In this study it also was noted that the application of TGFβ-3 or BMP-6 alone caused a partial differentiation of hMSCs identified by the production of collagen I expression [81]. Similar studies have shown the same results when using TGFβ at concentrations of 10 ng/ml in combination with BMP-2 at 50 ng/ml [82].
1.3.7 Bioreactor Development

Bioreactors generate the mechanical stimulus necessary to promote nutrient diffusion for cell growth [27]. A bioreactor needs to be designed in a form that not only allow the growth and monitoring of the tissue but also it has to be capable of enhance tissue growth by generating a physical stimuli. The use of mesenchymal stem cells for cartilage regeneration requires the use of a novel bioreactor capable of generating different mechanical stimuli like shear stress and perfusion.

Bioreactor technologies for tissue engineering started with the use of stirred tank bioreactors [83]. These types of bioreactors hold scaffolds inside of a reservoir and mix a cell suspension. The cells eventually attach to the scaffolds due to the convective movement generated. Scientist started to use this type of bioreactors because they are not complex and they are easy to handle and operate [84]. They have a good performance in nutrient diffusion through the constructs because of the mechanical agitation. However, stirred tank bioreactors have certain disadvantages, like low seeding efficiencies, excessive shear stress and non-uniform distribution of the cells [85,86]. These results leaded the development of new systems more suitable for tissue engineering and mammalian cell cultures. It soon became evident that new designs were needed to fulfill the different nutrient and mechanical forces required for the broad variety of specialized cells. Soon an ample variety of bioreactors were developed like bioreactors that applied shear stress in a more controlled fashion or that employed direct perfusion for nutrient transfer.

The rotating wall bioreactor is one of the most used bioreactors in cell cultures. This bioreactor was developed by the NASA for testing cellular growth under
microgravity environments [87]. It consists of a horizontal cylindrical chamber filled with media. The outer wall of the chamber rotates around 15 to 20 rpm. The constructs inside of the bioreactor are kept in a free fall state during all the time due to the rotation of the chamber. Even though large constructs have been grown in this bioreactor, some necrosis has been observed in tissue constructs larger than 3 mm [88].

Another bioreactor that uses rotation to produce shear stress is the concentric cylinder bioreactor [89]. This bioreactor uses an inner polypropylene bob that holds the scaffolds inside of a cylindrical reservoir. There is a small gap between the inner bob and the outer spinning cylinder. The rotation of the cup generates a laminar shear stress within the gap. This bioreactor allows an efficient seeding of the cells into the scaffolds while providing a well defined shear stress stimulus to the cells. Other bioreactors use perfusion to enhance nutrient diffusion and to minimize the necrosis zones in constructs [84].

Tubular bioreactors are also a good alternative for tissue growth. These types of bioreactors allow the perfusion of media in the constructs increasing the nutrients mass transfer rate. These bioreactors also have good seeding efficiencies [90]. The cells can be directly transported within the scaffold structure due to the mechanical drag force generated by perfusion.

The basic requirements for a bioreactor are good nutrient mass transfer properties, a controllable and well modeled shear stress and fluid development [91]. In vivo, a tissue is a complex system that is nourished through a complex network of capillaries. Cells in vivo tissues usually are no more than 100 µm to 200µm away from one of these
capillaries [92]. Oxygen is one of the most important nutrients for developing of cells. A good control of the oxygen levels is necessary for good tissue development. This control can be either to improve oxygen diffusion or to suppress oxygen levels. The choice depends on the type of tissue used in the bioreactor. For cartilage tissue a low oxygen tension of 40 mmHg is necessary to maintain cartilage anabolic metabolism [93].

The other important factor that needs to be addressed during bioreactor design is shear stress. Shear stress is a mechanical stimulus generated by effect of the viscous properties of the fluid with the exposed surface of any component inside the bioreactor. Many types of cells respond when they are stimulated with shear stress [94]. For example valve leaflets must sustain shear stresses in the range of 22 dyne/cm$^2$ while shear stress levels in cartilage tissue in an adult human can reach levels from 2.0 MPa to 3.5 MPa [93,94]. A less specialized type of cell like mesenchymal stem cells or epithelial cells respond to even lower levels in the range of 0.01 dyne/cm$^2$ to 0.1 dyne/cm$^2$ [95].

Shear stress affects the overall performance of tissue cartilage growth. When cultured in a concentric cylinder bioreactor, chondrocytes expressed more collagen deposition within the scaffold when a shear stress with a range between 2.6 dynes/cm$^2$ and 5.1 dynes/cm$^2$ was applied compared to a lower values of shear stress [89]. Other studies using chondrocytes have found the same trend with shear stress in the range of 0.01 dyne/cm$^2$ to 0.1 dyne/cm$^2$ [96]. The higher the shear stress used, the higher the content of collagen II. However in these studies a decrease of GAG molecules is associated with the higher shear stress. Glycosaminoglycan content decreased mostly because of the higher hydrodynamic force creates within the bioreactor that detached the GAG molecules deposited in the scaffold [89,96]. On the other hand studies performed
using mesenchymal stem cells demonstrated the high sensitivity of these cells to shear stress [95]. When shear stress in the range of 0.001 dyne/cm\(^2\) was applied to hMSCs, an increase in proliferation was observed compared with higher shear stress [95].

Another important mechanical force in bioreactor design is media perfusion. Perfusion allows the flow of media through all the thickness of the scaffold. This increase in fluid exchange enhances the nutrient diffusion within the scaffold-cell system [97]. Perfusion also generates shear stress within the scaffold in the same direction that the fluid is perfused. When tissue perfusion was used in scaffold seeded with chondrocytes, an alignment of the cells along the direction of the fluid was observed [98]. Another important advantage of perfusion bioreactors is the increase in nutrients transport inside the 3D scaffold. Seeding efficiency increases drastically when perfusion is applied especially if the fluid direction is changed alternatively [99].

The combination of shear stress and perfusion must enhance tissue growth within the scaffold and influence tissue morphology to create a more natural appearance and function. Perfusion systems also enhance cartilage tissue growth in bioreactors [97]. However, high perfusion rates might lead to a loss of cells and extra cellular matrix components in the scaffold [99]. When perfusion at a flow rates of 0.5 ml/min and 0.8 ml/minute were used in chondrocyte seeded scaffolds; a high content of cells and GAG deposition was associated with the flow rate of 0.5 ml/min compared to 0.8 ml/min [97]. For hMSCs perfusion values of 0.1 ml/min increased cell proliferation without disturbing differentiation potential [99].
The mechanisms by which shear stress and perfusion affect cell behavior are still not well understood at the molecular level. However, their effects in cell regulation, extracellular matrix production and cell proliferation have been registered in several experiments [89,97]. These mechanical forces translated in the form of stress, tension or shear stress can regulate the expression of important numbers of genes [100]. The transmission of the forces is carried by the cell surface receptors toward the cytoskeleton network within the cell. After that is passed to the microfilament and microtubule network and ultimately transmitted to nucleus to affect gene transcription [101]. The entire basic internal support network of the cell becomes affected and cell morphology becomes influenced. It has been demonstrated that the cell shape has a remarkable effect on cell differentiation [102].

1.4 Motivation for Current Research and Hypothesis

One of the key points for tissue engineering cartilage using hMSCs is to identify the proper conditions that enhance cell proliferation, cell differentiation and extracellular matrix deposition. Tissue engineered cartilage made using HMSCs has 1/10 of the mechanical properties when compared with tissue engineered cartilage created using chondrocytes [103]. Therefore it is important to elucidate and optimize the known hMSCs conditions that produce cell differentiation toward cartilage. We hypothesized that differentiation and phenotypic commitment of human mesenchymal stem cells can be enhanced by optimizing a combination of micromass culture, specific growth factors use such as TGFβ-1 and BMP-2 and physical stimulus generated by a bioreactor.
Specific aim 1: hMSCs differentiation induced by micromass culture can be optimized based on cell concentration used to create the micromass condition.

One of the models used for study cartilage formation and development is micromass culture [65]. During culture time micromass bodies follows a series of morphological changes such as increase in size of the micromass zone. The increase of size of the micromass zone is associated with two main processes: ECM production and cell migration [62,63]. We therefore test 2 different cell concentrations to create micromass bodies and measured the increase in the micromass spreading area in a 2D surface as an indicative of micromass ability to colonize a seeding area.

Specific aim 2: The sequential use of bone morphogenic protein and transforming growth factor will promote a more specific hyaline cartilage formation when combined with micromass seeding of hMSCs in PCL scaffolds.

Another key point for cartilage formation it is the use of growth factors to enhance and accelerate cell differentiation and matrix deposition. In the present work we explore the use of two different growth factors to induce hMSC’s differentiation and cartilage formation; TGFβ-1 and BMP-2 in different conditions. We explore the effect of the growth factors alone, in combination or the sequential use of them.

Specific aim 3: To test the effect of the conditions proposed in aim 1 and 2 in a novel shear stress and perfusion bioreactor.

Based in previous experiments, mechanical stimulus in tissue engineered cartilage promotes collagen formation and enhances nutrient diffusion. A new bioreactor design was developed to incorporate shears stress and perfusion. The combination of these two
mechanical stimuli will enhance ECM production and nutrient diffusion. Short experiments of 14 days have been carried out in the shear stress and perfusion bioreactor as proof of concept.

The future impact of tissue engineering will depend on the practical applications of the new technology developed. New procedures must be developed as a multidisciplinary approach that considers all the important elements such as clinical and industrial feasibility. Therefore this new bioreactor design will provide industrial feasibility allowing the production of tissue cartilage in situ in the hospital under good manufacturing practices.
CHAPTER 2

MATERIALS AND METHODS.

2.0 Bioreactor Design

A shear stress and perfusion bioreactor was developed to enhance tissue cartilage native architecture. This bioreactor uses media perfusion to enhance nutrient diffusion through the scaffolds and provides mechanical stimulation (shear stress) to promote cell alignment within the growing tissue. A rotating disc mechanism exposes constructs to shear stress on the scaffolds surface to stimulate chondrogenesis. The design is based on a tubular chemical reactor. Tubular reactors have been used in industry during many years. They offer a great advantage over stirred tanks due to the plug type flow developed inside of the chamber. This plug flow can be easily modeled and characterized compared to a stirring tank reactor whose fluid dynamics become complex as the turbulent fluid starts to develop [104]. The basic design used in here is a conical chamber made out of clear casted acrylic with a rotating construct support disc also made out of acrylic held by a disc support seal that prevents media leakage around the edges of the disc (Figure 2). The support disc was designed to hold eight 1.1 cm in diameter scaffolds that are 4 mm thick. The construct holders are designed to allow the insertion of gasket seals that prevent fluid from going around the edges of the scaffolds (Figure 4). This configuration avoids any fluid perfusion loss through the edges of the scaffold. A thin stainless steel mask with holes just slightly smaller than the disc holes prevents the scaffolds from
falling out from the disc (Figure 4). The system is connected to a **peristaltic pump** that allows the control of the media flow rate. The design incorporates a basic manometer that monitors the drop in pressure across the constructs during the culture time.

The rotating disc inside the conical chamber produces a shear stress on the surface of the disc [105]. This shears stress is dependent on the rotational velocity (ω) and the distance from the center of the disc (r) (Figure 2). The solution for the system of equations generated can be approximated numerically by the equation 1 [106].

\[
\tau_w = 0.8\rho\sqrt{\nu}\omega^{3/2}r
\]

Equation 1

Where \(\tau_w\) is the shear stress in the surface of the disc in Pa, \(\rho\) is the density of the media in kg/m\(^3\), \(\nu\) is the kinematic viscosity expressed m\(^2\)/s, and \(r\) is the distance from the center of the disc to the radius value where the shear stress is calculated. Previous studies have shown that shear stress levels around 0.01 dyne/cm\(^2\) to 0.1 dyne/cm\(^2\) stimulate chondrocytes and increase protein production [96]. However matrix deposition within the scaffolds decreases at higher shear stress levels [89]. On the other hand some studies have demonstrated that hMSCs are highly sensitive to shear stress in the range of 0.001 dyne/cm\(^2\) to 0.01 dyne/cm\(^2\) [95,99]. We decided to use an average value of shear stress of 0.01 dyne/cm\(^2\) for our bioreactor experiments with hMSCs. We solve equation 1 for \(\omega\) using a shear stress value of 0.01 dyne/cm\(^2\) and the radius used was \(R_2-R_1/2= 0.16\) cm (Figure 3). We found that at 10 rpm the shear stress in the middle of the scaffolds is 0.012 dyne/cm\(^2\). The shear stress at \(R_1\) is 0.0087 dyne/cm\(^2\) and the shear stress at \(R_2\) is 0.017 dyne/cm\(^2\). Although the shear stress on the scaffold surface is not constant across the radius, the variability of the shear stress falls within a relatively narrow range of shear stress (0.0087 to 0.017 dyne/cm\(^2\)).
Figure 2. Shear stress and perfusion bioreactor design and flow profile schematic. A) The bioreactor consists of a rotating disc enclosed in a tubular chamber. The disc is tightly fitted in a disc support seal that allows the free rotating of the disc while preventing fluid from passing through the edges of the disc. B) Mathematical components that describe the fluid dynamics generated by a rotating disc immerse in a fluid. C) Fluid dynamic profile generated by a rotating disc and shear stress generated on the surface of the disc due to rotation.
This bioreactor also uses perfusion through the constructs to enhance nutrient diffusion through the scaffold and improve ECM architecture. Perfusion rates used for chondrocytes ranged from 0.1 to 1.0 ml/minute per construct. However, it is believed that mesenchymal stem cells are more sensitive to mechanical forces [95]. Based on previous work with hMSCs in perfusion bioreactors [95,99], we decided to use a perfusion rate of 0.1 ml/min. The perfusion rate (Q) through each scaffold was calculated by assembling the bioreactor with empty scaffolds and taking measurements of the flow rate for different pump capacity. The flow rate was divided by the number of scaffold inside of the bioreactor, in this case 8. The average linear velocity was calculated by dividing the flow rate by the surface area of each scaffold.
Figure 4. Bioreactor disc design. The disc design allows fitting 8 constructs with a 1.3 cm diameter and 4 mm in thickness. The scaffolds are placed in top of silicon O-rings that tightly seal the edges of the scaffold. The disc is joined to the shaft by the same screw that holds the disk mask.
2.1 Cell Culture and Expansion

All reagents used for cell culture were obtained from Fisher Scientific (Fairlawn, NJ) unless otherwise specified. Human mesenchymal stem cells (hMSCs) obtained from Lonza (Walkersville, Maryland) were expanded in 75 cm$^2$ tissue culture flasks using proliferative media consisting of Dulbecco’s Modification of Eagle’s medium with high glucose, L-glutamine and pyridoxine hydrochloride, 1% amphotericin B, 1% streptomycin and 10% fetal bovine serum. Cells were detached using 0.25% trypsin in EDTA and frozen in 8% dimethyl sulfoxide (DMSO). Cells used in all the experiments were thawed and plated in 75 cm$^2$ tissue culture flask at a concentration of $5 \times 10^3$ cell/cm$^2$. Cells were passaged usually after 75%-85% confluence (4 to 5 days after plated in the 75 cm$^2$ tissue culture flask) and expanded until passage 5.
2.2 Cell Density Experiments in Micromass Cultures

Cells at passage 5 were collected using 3 ml of 0.25 trypsin in EDTA for 5 minutes and then 3 ml of proliferative media was added to inhibit trypsin. The cell content of the flasks was suspended in 50 ml tubes and cell concentration was adjusted to 8 X 10^6 cells/ml or 16 X 10^6 cell/ml using hematocytometer. The tubes were centrifuged at 1000 rpm for 5 minutes and the supernatant with the trypsin was carefully extracted. Immediately after trypsin removal fresh media was added and the cells in the bottom were carefully resuspended using a 1 ml micropipette. The cell concentration was again obtained by making a dilution with 10 µl of the high concentrated cell suspension and 990 µl of PBS.

Aliquots of 10 µl containing either 80,000 or 160,000 hMSCs were seeded in the center of the well of 24 well non tissue culture plates (BD Falcon, Japan) and the cells were allowed to settle for 2 hours. After this, 1 ml of either proliferative media or differentiation media consisting of DMEM-high glucose with L-glutamine and pyridoxine hydrochloride, 50 µg/ml L-ascorbic acid -2-phosphate (Sigma-Aldrich, St. Louis, MO), 0.4 mM L-proline (Sigma-Aldrich, St. Louis, MO) 10^{-7}M dexamethasone (Sigma-Aldrich, St. Louis, MO) and 1% ITS+1(Sigma-Aldrich, St. Louis, MO) was carefully added without disrupting the micromass condition. Cultures were maintained for 4 days with triplicates for each condition. Pictures of the cell micromass were taken every day using bright field microscopy (Nikon TE-240, Nikon, Japan) and a digital camera model coolsnap sf (Photometrics, Tucson, AZ).

The spreading area was calculated using NIS image analysis software (Nikon, Japan). Briefly, images were captured and stored in the image software. The software
allows the measurement of one point to another point in the image. Measurements of the
diameter of the micromass bodies were taken in 3 different directions and the average
value was used to calculate the area of a circle (the diameter was measured from the
zones where no cells were observed to the other zone where no cells were observed).

2.3 Micromass Histology

All the reagents for micromass cultures were obtained from Sigma-Aldrich (St.
Louis, MO) unless otherwise specified. At day 4, micromass cultures were fixed in 10%
formalin in a buffer solution for 24 hours. After that cells were dehydrated with 70%
reagent grade alcohol for 2 hours followed by immersion in absolute reagent alcohol for 2
hours. Cells were immediately transferred to xylene for 2 hours. After clearing with
xylene the micromass cultures were embedded in a 50/50 mixture solution of xylene and
melted Paraplast (Fisher, Fairlawn, NJ) at 60 ºC for 2 hours and then transfer to pure
melted paraplast for another 2 hours. The samples were placed in paraffin blocks and
stored for future sectioning.

Paraffin blocks were sliced using a microtome (Leica mycrosystems, Germany)
and 12 µm ribbons were obtained. The ribbons were placed in a water bath at 40ºC and
transfered to a glass slide (Fisher, Fairlawn, NJ). The slides were dried and baked at 60ºC
and stained the next day with alcian blue for GAG distribution (Table 1).
2.4 PCL Scaffold Formation

PCL beads with an average molecular weight of 40,000 were purchased from Sigma-Aldrich (St. Louis, MO). Salt leaching was used to create 90% porous scaffolds [107]. Briefly, 1 gram of PCL beads were poured in 6 ml of methylene chloride in a glass bottle, covered and left overnight on a plate shaker to dissolve the PCL. Nine grams of RNA and DNA free NaCl (Fisher, Fairlawn, NJ) with a diameter of 150μm-250μm were mixed with the PCL-methylene chloride solution in a glass bottle. Rectangular molds (11 cm long, 5 cm wide, 4 mm thickness) made out of stainless steel were used to cast the PCL solution and slowly evaporate the methylene chloride (Figure 6). After 1 day, the NaCL-PCL scaffolds were taken out of the molds and transferred into a vacuum container for 1 day to extract the remaining methylene chloride. Then the scaffolds were punched in circular discs (1.1 cm diameter, 4 mm thick) and transferred into a beaker with deionized water. The deionized water was changed twice a day for two days to remove NaCl. After salt leaching the scaffolds were sterilized using a series of 2 washes with 70% ethanol for 4 hours each and then washed extensively with sterile deionized water.

Figure 6. Stainless steel molds used to cast PCL scaffolds with a thickness of 0.4 cm.
Table 1

Alcian blue protocol for paraffin embedding slides. Sections were made of the micromass cultures and stained with alcian blue for GAG content.

<table>
<thead>
<tr>
<th>Step</th>
<th>Objective</th>
<th>Procedure</th>
<th># washes</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Paraffin removal</td>
<td>Xylene</td>
<td>2</td>
<td>1 min each</td>
</tr>
<tr>
<td>2</td>
<td>Xylene removal</td>
<td>100% Ethanol</td>
<td>1</td>
<td>30 seconds</td>
</tr>
<tr>
<td>3</td>
<td>Rehydrate slides</td>
<td>90% Ethanol</td>
<td>1</td>
<td>30 seconds</td>
</tr>
<tr>
<td>4</td>
<td>Rehydrate slide</td>
<td>70% Ethanol</td>
<td>1</td>
<td>30 seconds</td>
</tr>
<tr>
<td>5</td>
<td>Ethanol removal</td>
<td>DI water</td>
<td>1</td>
<td>30 seconds</td>
</tr>
<tr>
<td>6</td>
<td>Sample Preparation</td>
<td>Acetic acid 3%</td>
<td>1</td>
<td>2-3 minutes</td>
</tr>
<tr>
<td>7</td>
<td>Proteoglycan stain</td>
<td>Alcian blue</td>
<td>1</td>
<td>30 minutes</td>
</tr>
<tr>
<td>8</td>
<td>Wash slide</td>
<td>Tap water</td>
<td>1</td>
<td>5 minutes</td>
</tr>
<tr>
<td>9</td>
<td>Nucleus staining</td>
<td>Nuclear fast red</td>
<td>1</td>
<td>5 minutes</td>
</tr>
<tr>
<td>10</td>
<td>Wash slide</td>
<td>Tap water</td>
<td>1</td>
<td>5 minutes</td>
</tr>
<tr>
<td>11</td>
<td>Remove water</td>
<td>70% ethanol</td>
<td>1</td>
<td>30 seconds</td>
</tr>
<tr>
<td>12</td>
<td>Remove water</td>
<td>95% ethanol</td>
<td>2</td>
<td>2-3 dips</td>
</tr>
<tr>
<td>13</td>
<td>Remove water</td>
<td>100% ethanol</td>
<td>2</td>
<td>2-3 dips</td>
</tr>
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<td>14</td>
<td>Clearing slide</td>
<td>Xylene</td>
<td>2</td>
<td>2-3 dips</td>
</tr>
<tr>
<td>15</td>
<td>Mounting slide</td>
<td>Permount</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2.5 Micromass Seeding of PCL Scaffolds with hMSCs

Sterile PCL scaffold discs were placed in 24 well non tissue culture plates. The scaffolds were gently aspirated to extract all the remnant water from the sterilization process. A suspension of hMSC at a concentration of 8X10^6 cells/ml were prepared as described previously and 25 µl of cell suspension were dropped on top of the scaffold for a total of 200,000 cells. The cells were allowed to attach to the scaffold for 2 hours and
after that 1 ml of proliferative media was added to the wells gently to not disturb micromass conditions.

2.6 Growth Factor Exposure of hMSCs Seeded on PCL Scaffolds

After seeding hMSCs, PCL scaffolds were left in 2 ml of proliferative media for 4 days; the media was changed every other day. At day 4, media was replaced with 2 ml of differentiation media (DM). Differentiation media was supplemented with TGFβ-1 (5 ng/ml) (R&D Systems,), BMP-2 (50 ng/ml) (R&D Systems), combined TGFβ-1 (5 ng/ml) and BMP-2 (50 ng/ml) or sequential induction with TGFβ-1 (5 ng/ml) for 4 days followed by combined TGFβ-1 (5 ng/ml) and BMP-2 (50 ng/ml) for another 4 days before switching back to TGFβ-1 alone (5 ng/ml) the last 4 days of culture (Table 2). One ml of fresh media supplemented with growth factor(s) was changed every other day to avoid growth factor depletion. The control group was differentiation media with no growth factors. Constructs were harvested at day 0, 4, 8, 12, 16. Three experiments were carried out (N=3) with triplicates for each of the groups for biochemical analysis (n=3) and duplicates for histology analysis (n=2).

All the growth factors were reconstituted following manufacturer’s procedures. Briefly a 4 mM HCl solution containing 1mg/ml bovine serum albumin was used to create 1 ml solution of each growth factor with a concentration of 1 µg/ml. Aliquots of the suspended growth factor solutions were frozen and stored at -70ºC until ready to use.
Table 2

Growth factor experimental design. hMSCs were seeded on PCL scaffolds in micromass conditions and after four days in proliferative media (PM) were switched to differentiation media (DM) with different combination of TGFβ-1 and BMP-2.

<table>
<thead>
<tr>
<th>Experiment Condition</th>
<th>Timeline</th>
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<tbody>
<tr>
<td></td>
<td>0-2 hrs (cell seeding)</td>
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<tr>
<td></td>
<td>Day 1-4</td>
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<td>Day 4-8</td>
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<td>Day 8-12</td>
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<td></td>
<td>Day 12-18</td>
</tr>
<tr>
<td>1</td>
<td>Micromass PM</td>
</tr>
<tr>
<td></td>
<td>DM+TGFβ-1</td>
</tr>
<tr>
<td></td>
<td>DM+TGFβ-1</td>
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<td>DM+TGFβ-1</td>
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<td></td>
<td>1</td>
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<tr>
<td>2</td>
<td>Micromass PM</td>
</tr>
<tr>
<td></td>
<td>DM+BMP-2</td>
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<td></td>
<td>DM+BMP-2</td>
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<tr>
<td>3</td>
<td>Micromass PM</td>
</tr>
<tr>
<td></td>
<td>DM+TGFβ-1+1+BMP-2</td>
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<tr>
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<td></td>
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</tbody>
</table>

2.7 Digestion of hMSCs Seeded PCL Constructs for Biochemical Analysis

For biochemical analysis, constructs at each data point were collected (4 hours, days 4,8,12,16, N=3, n=3) washed with PBE, freeze dried, ground and digested with 1ml of papain solution in phosphate buffered solution at an activity of 35 U/mg (Worthington Biochemical Corporation, Lakewood, NJ). The papain solution was prepared by
dissolving 0.1 ml of papain at an activity of 35 U/mg in 100 ml of PBE solution. Samples were placed in a water bath at 54ºC for 4 hours and stored at -20 ºC until ready to test.

2.8 DNA Quantification

The DNA content of the samples was measured using a fluorescent dye that binds double strand DNA (dsDNA). Picogreen dsDNA quantification kit was purchased from Invitrogen (Carlsbad, CA) and used as manufacturer’s procedure. The kit includes a picogreen fluorescent dye, calf thymus DNA standard at a concentration of 100 µg/ml and a 20X TE (tris(hydroxymethyl) aminomethane ethylenediaminetetraacetic acid buffer) (10 mM Tris-HCl, 1mM EDTA, pH 7.5). According to the manufacturer’s protocol, DNA serial dilutions were made according to Table 3 and TE buffer was diluted to 1X with distilled water. Digested constructs were freeze-thawed and sonicated for 20 minutes (3 cycles). Samples were vortexed and briefly centrifuged to settle down all PCL debris. One hundred microliters of the solution were extracted out of the samples and diluted in 900 µl of PBS. Picogreen dye was diluted 1:200 in 1X TE buffer. Fifty microliters of the diluted samples were added to 96 well plates in triplicates (Fisher, Fairlawn, NJ) followed by 100 µl of the diluted picogreen dye previously prepared in a dark room. The plates were covered with aluminum foil and placed on an orbital shaker for 15 minutes. All the plates were read in a synergy 2 multi-Mode microplate Reader (BioTek, Winooski, VT) at excitation of 480 nm and emission of 520 nm. Linear interpolation with the graph made with the standard solutions of dsDNA was used to determine the dsDNA concentration in the samples. A value of 7.7 pg of dsDNA per cell was used to calculate cell number [108].
<table>
<thead>
<tr>
<th>DNA Stocks</th>
<th>Volume TE 1X buffer (µl)</th>
<th>Final DNA concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µl from 100 µg/ml stock of DNA solution</td>
<td>475</td>
<td>5</td>
</tr>
<tr>
<td>250µl from 5.0 µg/ml stock of DNA solution</td>
<td>250</td>
<td>2.5</td>
</tr>
<tr>
<td>250µl from 2.5 µg/ml stock of DNA solution</td>
<td>250</td>
<td>1.25</td>
</tr>
<tr>
<td>250µl from 1.25 µg/ml stock of DNA solution</td>
<td>250</td>
<td>.625</td>
</tr>
<tr>
<td>250µl from .625 µg/ml stock of DNA solution</td>
<td>250</td>
<td>.313</td>
</tr>
<tr>
<td>250µl from .313 µg/ml stock of DNA solution</td>
<td>250</td>
<td>.157</td>
</tr>
<tr>
<td>250µl from .157 µg/ml stock of DNA solution</td>
<td>250</td>
<td>.079</td>
</tr>
<tr>
<td>250µl from .079 µg/ml stock of DNA solution</td>
<td>250</td>
<td>.040</td>
</tr>
<tr>
<td>250µl from .0395 µg/ml stock of DNA solution</td>
<td>250</td>
<td>.020</td>
</tr>
<tr>
<td>0</td>
<td>250</td>
<td>0</td>
</tr>
</tbody>
</table>
2.9 Construct GAG Content Determination

All reagents used for GAG content determination were obtained from Sigma-Aldrich (St. Louis, MO). Proteoglycan content in the constructs was determined by the reaction of the sulfate contained in the GAG molecules with dimethilmethylene blue dye (DMMB) [109]. DMMB dye was prepared by dissolving 16 mg of the dye in 5 ml of ethanol in a 1 L bottle covered with aluminum foil and mixed with a stirring bar for 12 hours. Immediately after this, 950 ml of distilled water containing 3.04 g (40.5 mM) of glycine, 2.37 g (40.5 mM) of NaCl and 8.7 ml of 1M HCl (8.7 mM). The pH of the solution was adjusted to 3.0 by adding 1 M of HCl or NaOH. Chondroitin sulfate (CS) stock solution was prepared by dissolving 0.105 g of cystein in 60 ml of PBE and then making a solution of 50 mg/ml of chondrotin sulfate from bovine trachea in a PBE/cystein solution. Standard solutions were prepared as shown in Table 4.

Samples were vortexed and centrifuged for 20 seconds and 100 µl were extracted and diluted in 900 µl of distilled water. The assay was carried out in a 96 well plates using a plate reader (BioTek, Winooski, VT) with a wavelength of 525 nm. Eight µl of the sample or the standard were added to each well and then 200 µl of the DMMB dye was added. After 2 minutes of incubation at room temperature the plates were read. PCL scaffold with no cells was used as a negative control. The standard curve was obtained and the values of the samples were obtained by linear interpolation.
Table 4
GAG assay standards

<table>
<thead>
<tr>
<th>CS 50 µg/ml solution (µl)</th>
<th>DI water (µl)</th>
<th>Final CS concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>500 µl</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>495</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>490</td>
<td>1</td>
</tr>
<tr>
<td>100</td>
<td>400</td>
<td>10</td>
</tr>
<tr>
<td>200</td>
<td>300</td>
<td>20</td>
</tr>
<tr>
<td>300</td>
<td>200</td>
<td>30</td>
</tr>
<tr>
<td>400</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
<td>50</td>
</tr>
</tbody>
</table>

2.10 Construct Histology

Constructs (N=3, n=2) were harvested at day 8, 12 and 16, washed with PBS and fixed with 10% buffered formalin for 24 hours. After fixation, constructs were placed in 20% sucrose in PBS at 4°C for 24 hours and incubated in 40% sucrose solution for another 24 hours. Then samples were transfer to plastic molds and covered with optimal embedding media containing 10% poly vinyl alcohol and snap-frozen using liquid nitrogen. All the samples were stored in Ziploc bags in the freezer. Sections were cut using a cryostat (Leica mycrosystems, Germany) and mounted in coated slides (Fisher, Fairlawn, NJ). The slides were placed in a 50°C oven overnight and stored in the refrigerator at 5 °C. Hematoxylin and eosin stain (Table 5) was used for cell identification and morphology. Alcian blue staining (Table 1 starting from step 6 for frozen sections) was used for GAG expression.
Table 5

Hematoxylin and eosin staining protocol used for frozen sections.

<table>
<thead>
<tr>
<th>Step</th>
<th>Objective</th>
<th>Procedure</th>
<th># washes</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nucleus staining</td>
<td>Hematoxylin solution</td>
<td>1</td>
<td>2 minutes</td>
</tr>
<tr>
<td>2</td>
<td>Wash excess dye</td>
<td>Tap water</td>
<td>1</td>
<td>5 minutes</td>
</tr>
<tr>
<td>3</td>
<td>Remove water</td>
<td>70% ethanol</td>
<td>1</td>
<td>2 dips</td>
</tr>
<tr>
<td>4</td>
<td>Staining cytoplasm</td>
<td>Eosin solution</td>
<td>1</td>
<td>30 seconds</td>
</tr>
<tr>
<td>5</td>
<td>Remove water</td>
<td>70% ethanol</td>
<td>1</td>
<td>3 dips</td>
</tr>
<tr>
<td>6</td>
<td>Remove water</td>
<td>95% ethanol</td>
<td>2</td>
<td>2-3 dips</td>
</tr>
<tr>
<td>7</td>
<td>Remove water</td>
<td>100% ethanol</td>
<td>2</td>
<td>2-3 dips</td>
</tr>
<tr>
<td>8</td>
<td>Clearing slide</td>
<td>Xylene</td>
<td>2</td>
<td>2-3 dips</td>
</tr>
<tr>
<td>9</td>
<td>Mounting slide</td>
<td>Permount</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2.11 Construct immunohistochemistry

Immunohistochemistry was used to identify collagen I, collagen II and collagen X expression in constructs. Sections used for immunohistochemistry were affixed to a glass slide using acetone and stored in the refrigerator at 5°C until ready for use. Slides were treated with a solution of 0.01 M Ph2-HCl containing 0.05% pepsin (Sigma-Aldrich) at 37°C for 20 minutes and then at room temperature for 10 minutes. After pepsin treatment the slides were carefully rinsed with a Tris buffered saline (TBST) solution consisting of 6.9 g of trizma (Fisher Fairlawn, N), 8.5 g of NaCl, 5 drops of TritonX (Fisher Fairlawn, NJ ) in 1 liter of distilled water. Then a solution consisting of 0.1 % sodium azide
(Sigma-Aldrich, St. Louis, MO) in 0.3% H₂O₂ was added to the slides for 10 minutes at room temperature followed by a TBST washing. Three percent goat serum (Fisher, Fairlawn, NJ) was added for 10 minutes at room temperature. Collagen I antibody (St. Cruz Biotechnology, CA) was diluted following manufacturer’s instruction 1:200 in PBE. Collagen II and Collagen X antibodies (Developmental Studies Hybridoma Bank, Iowa City, IA) were applied at a concentration of 30 µg/ml each one. Antibodies were applied for 2 hours at room temperature; negative control sections did not receive antibodies. Sections were washed in TBST and a polymer + HRP complex was applied (Dako, Germany) for 30 minutes followed by a TBST wash. DAB solution (diaminobenzidin 0.05%, 50 mM Tris/HCl pH 7.4, 0.01% H₂O₂) was applied to develop the specific sites.

2.12 RT-PCR Analysis of PCL Constructs

Total RNA of the constructs was extracted using the TRIZOL method (Invitrogen). Briefly, constructs were placed in a 1 ml eppendorff tube and dissolved by adding 1 ml of trizol for 5 minutes followed by an addition of 0.2 ml of chloroform for 5 minutes at 4ºC. The cell lysate was shaken by hand for 2 minutes and centrifuged at 13,000 rpm for 12 minutes at 4ºC. After centrifugation the clear upper layer was removed and placed in a new eppendorff tube and an equal volume of isopropanol was added. The mixture was incubated at 4Cº for 30 minutes followed by 15 minutes of centrifugation at 13,000 rpm at 4 Cº. The supernatant was discarded and the pellet was rinsed with 70%-75% ethanol and centrifuged for 5 minutes at 13,000 rpm at 4 ºC. The remaining ethanol was removed and the pellet was suspended in RNA free distilled water. Samples were stored at -75ºC until gene expression analysis.
Total RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, DE). Reverse transcription was carried out using Firsts strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). Amplification of DNA was done using Applied Biosystems 7300 real-time PCR system. Green reaction mixture and intron-spanning primers were used to mark the cDNA. TaqMan gene expression assays (Applied Biosystems, Foster City, CA) were used to amplify expression of Collagen I (Hs00164004_m1), Collagen X (Hs00166657_m1) and aggrecan (Hs00153936_m1).

2.13 Bioreactor Experiments

2.13.1 Disc Seeding

The rotating construct support disc with the gasket seals was washed with distilled water packed in a self-sealing bag and sterilized in an autoclave. Eight PCL scaffolds were sterilized as described above and fitted into the disc holders of the sterile disk inside of a laminar flow hood. The construct support disc with scaffolds was placed in a sterile petridish (Fisher, Fairlawn, NJ). All the time the disk was handled with sterile tweezers.

Before seeding, remnant water in the PCL scaffolds was extracted by carefully aspirating the scaffolds. A high density cell suspension of $8 \times 10^6$ hMSCs was prepared as described and 125 µl of this cell suspension was placed on top of each PCL scaffold ($1.0 \times 10^6$ cells per scaffold, $8.0 \times 10^6$ cells total in the bioreactor). The petridish containing the disk and the seeded scaffold was placed in an incubator at 37 °C, 20% oxygen and 5% CO$_2$ for 2 hours to generate micromass condition on top of the PCL scaffolds.
The bioreactor chamber, tubing and accessories were placed inside of autoclavable bags and sterilized. Two hours after disc seeding; the bioreactor was assembled inside of a laminar flow hood and the construct support disc with the hMSCs seeded PCL scaffolds was fitted onto the shaft of the bioreactor. The bioreactor was closed and filled 60 ml of warm differentiation media containing 5 ng/ml of TGFβ-1 using a peristaltic pump.

2.13.2 Bioreactor Set Up and Operation

The sealed and sterile bioreactor with the scaffolds was transferred to a clean bench. In that area all the components of the bioreactor loop were assembled (Figure 5 and Figure 7). Differentiation media with 5 ng/ml of TGFβ-1 was pumped through the bioreactor using a peristaltic pump and a reservoir of 200 ml of media. This media reservoir was bubbled with a gas mixture containing 20% oxygen, 5% CO₂ and 75% nitrogen. The gas was bubbled in the media to maintain oxygen levels and CO₂ levels necessary for pH control. Media in the bioreactor was perfused at a rate of 0.1 ml/min per construct for a total of 0.8 ml/min.

The media residence time in the bioreactor or the media reservoir was calculated as the total volume in the bioreactor or the media reservoir divided by the perfusion rate (60 ml/0.8 ml/min and 200 ml/0.8 ml/min respectively). This configuration yields a residence time for the media of 75 minutes in the bioreactor and 250 minutes in the media reservoir (4.2 hours). Due to the relatively long residence time of the media in the media reservoir we decided to put the media reservoir in an ice bath to minimize degradation of
growth factors and nutrients. The media that is pumped into the bioreactor is warmed by placing the tubing that transports the media from the reservoir in contact with the flow of the air curtain incubator. The low flow rate in the tubing allows the heat exchange from the air and warms up the media before it reaches the bioreactor.

The bioreactor was heated by using an air curtain incubator. Before the experiment set up; several reading were taken with the operation conditions mentioned before (media reservoir in ice bath at 4 ºC, 0.1 ml/min media perfusion). We found that the air curtain incubator temperature had to be adjusted to 39º to compensate for all the heat loss due to convection. Using this air curtain incubator temperature the bioreactor operated at 37ºC. The temperatures inside of the bioreactor were taken using a thermocouple (Fisher, Fairlawn, NJ) and resulted in a consistent 37ºC.

Figure 7. Bioreactor configuration. The bioreactor was assembled in a laminar flow hood and operated in a lab bench under sterile conditions. The bioreactor used a peristaltic pump connected to media reservoir kept in ice. The bioreactor system is kept at 37 ºC by using an air curtain incubator.
2.13.3 Glucose Consumption in the Bioreactor

Media samples were extracted from the bioreactor media reservoir on days 1, 5, 8 and 14. Glucose content of the samples was obtained using quantichrome glucose assay kit (BioAssay Systems, Hayward, California). Briefly, glucose standards were diluted with distilled water as showed in Table 6. Five microliters of the media samples and standards were transferred to a 1.5 ml eppendorff tube (Fisher, Fairlawn, NJ) and mixed with 500 µL of kit reagent. The tubes were placed in boiling water for 8 minutes and cooled down in ice for 4 minutes. Two hundred microliters of the solution were transferred to wells of a 96 well plate (Fisher, Fairlawn, NJ) and absorbance was read at 620-650 nm in a synergy 2 multi-Mode microplate Reader (BioTek, Winooski, VT). Glucose concentration obtained was multiplied by the volume of media used in the bioreactor (200 ml) to obtain the total glucose content in the bioreactor.

Table 6
Glucose standard used for the glucose analysis of the bioreactor at days 1, 5, 8 and 14.

<table>
<thead>
<tr>
<th>Standard solution (300 mg/dL) + DI water (µL)</th>
<th>Final Glucose concentration (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 µL + 0 µL</td>
<td>300</td>
</tr>
<tr>
<td>100 µL + 50 µL</td>
<td>200</td>
</tr>
<tr>
<td>50 µL + 100 µL</td>
<td>100</td>
</tr>
<tr>
<td>25 µL + 125 µL</td>
<td>50</td>
</tr>
<tr>
<td>0 µL + 150 µL</td>
<td>0</td>
</tr>
</tbody>
</table>
2.13.4 Pressure Drop Measurement in the Bioreactor and Construct Permeability

The pressure drop in the bioreactor was measured using a custom made U tube manometer coupled to the bioreactor (Figure 5). The U tube contained oil with a viscosity of 0.82 gm/cm$^3$. This value was used to calculate the pressure drop between the entrance and the exit of the bioreactor with the equation 2. Oil height readings were taken in the manometer arm using a digital caliper (Mitutoyo, Japan)

$$\Delta P = \rho gh$$  \hspace{1cm} \text{Equation 2}

Where $\rho$ is the media density (1.02 g/cm$^3$ at 37ºC), $g$ is the gravity constant and $h$ is the difference in heights measured in the U tube.

The pressure drop value was used to estimate the permeability value of the constructs. The permeability was calculated using Darcy’s law (Equation 3)

$$Q = \frac{-kA(P_b-P_a)}{\mu L}$$  \hspace{1cm} \text{Equation 3}

Where $Q$ is the total discharge (0.1 ml/s), $k$ is the permeability (m$^2$). $A$ is the sum of the superficial area of all the 8 scaffolds ($A = \pi R^2 \times 8$). $\mu$ is the dynamic viscosity of the media (0.80 N s/m$^2$ X 10$^{-3}$). $L$ is the thickness of the scaffolds (4 mm).
CHAPTER 3

RESULTS

3.0 Introduction

The production of tissue engineered cartilage from hMSCs involves the use of scaffolds, growth factors, cell seeding techniques, and mechanical loadings to enhance cell proliferation, matrix deposition and cell differentiation. The purpose of this work is to combine the use of some of the known conditions that induce cartilage differentiation such as micromass seeding of scaffolds and addition of growth factors. After identifying conditions that enhance matrix deposition and cell proliferation in static cultures, we tested the condition that we considered yield the best results in a new designed rotating disc bioreactor.

In the static experiments we used micromass culture at two different hMSC concentrations to assess whether cell seeding concentration affects hMSC proliferation, spreading and chondrogenic differentiation (as measured by GAG deposition). Micromass culture follows a well known series of stages. One of these initial stages is micromass spreading. This spreading is generated by a combination of cell proliferation and cell migration on newly deposited extra cellular matrix [60].

Our next step was to elucidate the role of TGFβ-1, BMP-2 in chondrogenesis for hMSCs seeded in micromass on a porous PCL scaffolds. The use of growth factors to promote hMSCs growth and differentiation is well documented [81,82]. We explore the use of TGFβ-1 and BMP-2 with micromass seeding and we explore the transient
application of BMP-2 in hMSCs. We then select the best conditions for cell
differentiation and proliferation in PCL scaffolds to test in a newly designed shear stress
and perfusion bioreactor.

3.1 Effect of Micromass Density on hMSCs Seeded on Culture Plates

The objective of these experiments is to compare the effect of 2 different cell
countactions; $8.0 \times 10^6$ cells/ml (LOW) and $16.0 \times 10^6$ cells/ml (HIGH) to create
micromass hMSCs bodies. Aliquots of 10 µl of the cell suspension were used to create
the micromass bodies. Cell numbers were 80,000 cells for the LOW group and 160,000
cells for the HIGH group. Micromass bodies were exposed to differentiation media (DM)
during all the culture time (4 days) and proliferative media (PM) was used as a control.
Micromass body spreading area created with either LOW and HIGH hMSC concentration
was measured using imaging software ($N=3, n=3$). Images of the micromass cultures for
the HIGH cell density condition resulted too large to be measured with one single picture.
Measurements were taken by measuring the distance in one panoramic image created
with several pictures of the HIGH condition (Data not shown). All the micromass bodies
showed similar initial sizes at day 0 (after 4 hours of micromass induction) (Figure 8B).

At day 1 of culture all the micromass cultures started to show clear signals of cell
migration at the edges of the nodule. For example micromass bodies made with LOW
cell concentration (Figures 9A-F) showed a morphological change over time. This change
also was observed in HIGH micromass bodies (Data not shown). The cell
migration/micromass spreading area increase effect was more pronounced in
LOW concentration micromass bodies grown under differentiation media (Figure 10A, 10C and 10E) compared to LOW concentration micromass bodies grown in proliferative media.

Figure 8. Effect of cell concentration and media condition on micromass bodies cultures. A) Cell number obtained during micromass cultures. B) Spreading area obtained during 4 days of culture. C) Normalized spreading area values to its initial values at day 0. LOW: $8 \times 10^6$ cells/ml suspension. HIGH: $16 \times 10^6$ cells/ml suspension. DM: Differentiation media. PM: Proliferative media.
The spreading area after 4 days increased for all the conditions but the migration/growth effect was most prominent for micromass bodies grown in differentiation media. The spreading area at day 4 for LOW DM was $3.5 \text{ mm}^2 \pm 0.4 \text{ mm}^2$. In contrast the spreading area in LOW micromass growth in proliferative media (LOW PM) at day 4 was $1.0 \text{ mm}^2 \pm 0.2 \text{ mm}^2$ (Figure 8B). For micromass bodies using the HIGH concentration there were also differences in the spreading area. For HIGH concentration micromass bodies grown in DM (HIGH DM) the spreading areas was $4.8 \text{ mm}^2 \pm 0.5 \text{ mm}^2$ at day 4. In contrast for HIGH concentration micromass bodies growth in proliferative media (HIGH PM) the spreading area was $3.3 \text{ mm}^2 \pm 0.2 \text{ mm}^2$ at day 4 (Figure 8B).

When we compare the spreading size of the micromass at day 4 with the initial size at day 0 we observe that the highest increase in size from the initial value occurs with the low cell concentration cultured under differentiation media (LOW DM) (Figure 8C). This condition increased 3.5 times its initial size. This increase was the highest and it might indicate that there is certain optimal seeding density for micromass growth.

Cell numbers in the micromass bodies were estimated using DNA quantification. DNA analysis revealed a low proliferation rate in all the micromass bodies. At day 4 cell number for LOW DM and LOW PM respectively were $103,000 \pm 8,000$ cells and $117,000 \pm 9,000$ cells. Cell numbers for HIGH DM and HIGH PM micromass bodies were $191,000 \pm 14,000$ and $198,000 \pm 18,000$ (Figure 9A). Cell proliferation in micromass bodies for all the conditions increased about 20% to 30% (Figure 8A).
Figure 9 Micromass spreading observed during culture. A), C), E) Low concentration cell micromass bodies \(8.0 \times 10^6\) cells/ml in DM at days 0, 1 and 4 respectively. B), D), F) Low concentration cells in PM at days 0, 1 and 4 respectively. Scale bar 200 µm.
Nuclear red staining revealed differences in the cell distribution within the micromass bodies. In micromass cultures growth with the LOW cell density, the number of cells in the center of the micromass appears to decrease (Figure 10A and 10C) while the number of cells in the outer layers increases regardless of the media condition. In contrast micromass bodies grown in HIGH cell density showed a higher cell distribution in the center of the micromass region (figure 10B and 10D).

Alcian blue staining of the micromass cultures showed glycosaminoglycan production in cells grown under differentiation media and proliferative media (Figure 10). However the GAG distribution and cell distribution appears to change within the groups. Greater GAG deposition is observed in the LOW cell density micromass bodies. Other studies using micromass cultures have shown the same trend, a migration of cells from the center to the outer layers of the micromass zone, and the gradual expansion of the micromass center due to ECM molecules deposition [62,110]. The GAG deposition in the HIGH cell density micromass bodies seems to be more poorly distributed than the micromass cultures grown in LOW conditions. One important point is that all the micromass bodies produced GAG molecules within 4 days. This effect shows the potential of micromass to induce cartilage differentiation.
3.2 Micromass hMSCs Seeded in Porous PCL Scaffold Growth

Alcian blue and nuclear red staining showed that LOW cell density micromass bodies obtained a better cell response in terms of cell migration and GAG deposition within the micromass region. Based on those results we decided to use the LOW cell concentration (8.0 X 10^6 cells/ml) for the creation of micromass cultures on porous PCL scaffolds. In scaffold experiments, hMSCs were cultured in proliferative media in the
PCL scaffold for the first 4 days to promote cell proliferation and then switched to differentiation media for days 4-16 to increase chondrogenesis. Scaffolds were seeded with 25 µl of this hMSCs suspension (approximately 200,000 cells). After 4 hours of seeding the number of hMSCs found in the PCL scaffolds was 188,000 ± 9,000 cells. After 4 days of growth in the PCL constructs under proliferative media; cell number did not increase significantly (Figure 11). hMSCs growth in PCL scaffolds was slower than cell growth on tissue culture plastic (Figure 8A). This lag in growth might be attributed to the fact that the cells were adapting to the new substrate; in this case the PCL scaffold. At day 4 the media was replaced with differentiation media containing different combination of growth factors (as shown in table 2).

Figure 11. Three dimensional porous PCL scaffolds seeded with 200,000 hMSCs in proliferative media. Cells at 4 hours and after 4 days.
3.2.1 Effect of Growth Factors on Mesenchymal Stem Cell Proliferation and Differentiation Seeded in Porous PCL Scaffolds

Differentiation of hMSCs into cartilage can be achieved using growth factors. We chose to use TGFβ-1 and BMP-2 to stimulate hMSC differentiation and promote chondrogenesis. Chondrogenesis is a multistep process that involves several cytokines in a temporal dependant manner [59]. Therefore one of our approaches involved the transient exposure of hMSCs to BMP-2.

The primary objective of these experiments was to determine the effect of TGFβ-1 and BMP-2 on hMSC proliferation, differentiation and ECM production in 3D porous PCL scaffolds. After 4 days of culture in proliferative media, hMSCs seeded on porous PCL scaffolds were switched to differentiation media with either TGFβ-1 or BMP-2 alone, a combination of TGFβ-1 and BMP-2 together and continuous exposure of TGFβ-1 (day 0-14) with transient exposure of BMP-2 from day 4 to 8. (Table 2, Figure 12).

Cells exposed to TGFβ-1 alone showed higher proliferation than control group (DM) or cells exposed to BMP-2 alone or TGFβ-1+BMP-2 (P< 0.05). Cell number found in TGFβ-1 exposed PCL scaffolds at day 16 was 384,000 ± 35,000 cells (Figure 12), a 2 fold increase from day 0 cell number. The addition of only BMP-2 to the differentiation media caused a small decrease in cell number from day 4 to day 8 (188,000 ± 9,000 to 150,000 ± 15,000 cells respectively). Beyond day 8, the cell population in the BMP-2 treated group proliferated and reached a value of 227,000 ± 17,000 cells at day 16. The combination of TGFβ-1 and BMP-2 together during all the culture led to a steady increase in cell number from 188,000 ± 9,000 at day 4 to 291,000 ± 27,000 at day 16. In the TGFβ-1 with transient application of BMP-2 group cell number increased from
188,000 ± 9,000 at day 4 to 361,000 ± 44,000 at day 16. This increase was the second largest increase in cell number (TGFβ-1 treated group obtained the largest increase in cell proliferation). When application of BMP-2 was removed in the transient group, cell number went up dramatically (Figure 13, DM+TGF-1+BMP-2 transient group at day 16). When compared with the initial number of cells all the groups showed a small proliferation rate during the first 4 days following application of growth factors (Figure 12).

Figure 12. Effect of growth factors on hMSC proliferation in porous PCL scaffolds. Cell numbers achieved after switching to differentiation media at day 4, control group DM. Dash line indicates cell number at day 4 (before switch into differentiation media and growth factors application). * P< 0.05.
3.2.2 GAG Production of hMSCs Seeded in Porous PCL Scaffolds Exposed to Growth Factors.

The main objective of these experiments was to quantify the GAG production of hMSCs seeded under micromass conditions on porous PCL scaffolds and exposed to growth factors. TGF\(\beta\)-1 treated samples showed an increase in GAG production over time. This value started at 7.1 \(\mu\)g \(\pm\) 2.3 \(\mu\)g at day 8 and increased to 32.3 \(\mu\)g \(\pm\) 5.7 \(\mu\)g at day 16 (Figure 13A). BMP-2 treatment of hMSC seeded PCL scaffolds caused a higher deposition of GAG compared to TGF\(\beta\)-1 treated group at day 16 however this value was not statistically significant (Figure 13A). The GAG deposition in the BMP-2 treated PCL scaffolds at day 8 was 6.1 \(\mu\)g \(\pm\) 1.9 \(\mu\)g which increased to 42.1 \(\mu\)g \(\pm\) 7.8 \(\mu\)g at day 16. The application of TGF\(\beta\)-1 and BMP-2 together during the culture time increased GAG content of scaffolds from 9.7 \(\mu\)g \(\pm\) 2.6 \(\mu\)g at day 8 to 47.2 \(\mu\)g \(\pm\) 1.3 \(\mu\)g at day 16.

The effect of transient application of BMP-2 while applying TGF\(\beta\)-1 caused an even greater effect on GAG deposition in the samples compared to all the other groups. GAG content at day 8 for the transient group was 7.9 \(\mu\)g \(\pm\) 1.9 \(\mu\)g. At day 16 the GAG deposition achieved 68.4 \(\mu\)g \(\pm\) 5.1 \(\mu\)g; the highest value within all the groups. One important result is that immediately after BMP-2 application in the transient group (Day 12) the GAG deposition value went up to 36.7 \(\mu\)g \(\pm\) 6.2 \(\mu\)g making this increase in GAG deposition the largest of all the groups (Figure 13A). Also this increase was statistically significant (Figure 13A) compared to either TGF\(\beta\)-1 group, or BMP-2 group at day 12 (Figure 13A). These result showed a positive stimulus in GAG production generated by the transient exposure of BMP-2. The control group was hMSCs seeded on PCL scaffolds in DM without addition of growth factors. The control group had a GAG deposition of
1.9 µg ± 1.8 µg at day 8 and ended with a value of 17.4 µg ± 2.2 µg at day 16. GAG deposition observed at day 16 in the TGFβ-1 and BMP-2 together group and transient exposure of BMP-2 group was statistically significantly higher than TGFβ-1 and no treatment (P<0.05) (Figure 13A).

Figure 13. Effect of growth factors on glycosaminoglycan (GAG) production in the PCL scaffolds. A) GAG deposition found in the PCL constructs at day 8, 12 and 16. B) GAG/DNA ratio within PCL scaffolds. *p<0.05.
3.2.3 GAG/DNA Ratio

The GAG/DNA ratio results are good indicator of the efficiency of the matrix deposition kinetics from an engineering point of view. The main interest is to achieve the better outcome (GAG deposition) with the lowest use of materials (in this case the cells). GAG/DNA ratios for the TGFβ-1 treatment group were the lowest of all the conditions (Figure 13B). The GAG/DNA ratio at day 8 was 2.9 and increased to 10.9 at day 16. For BMP-2 treated samples the GAG/DNA ratios were 3.5, 18.2 and 24.1 at days 8, 12, and 16 respectively (Figure 13B). For simultaneous application of TGFβ-1 and BMP-2 GAG/DNA ratios for days 8, 12, and 16 were 5.0, 20.0 and 21.1 respectively (Figure 13B). Finally the GAG/DNA ratios for transient exposure of BMP-2 were 3.2, 19.5, 24.6 for days 8, 12 and 16 respectively (Figure 13B). The BMP-2 treated group and transient exposure to BMP-2 treatment group showed the highest ratio at day 16 (24.0 and 24.6 respectively) compared with all the other groups (Figure 13B). The TGFβ-1 showed the lowest GAG/DNA ratio compared with all the other groups.

3.2.4 Hematoxylin and Eosin Staining for Cell Identification in hMSCs Seeded PCL Scaffolds Treated with Growth Factors

Cell seeded PCL constructs were snap frozen, sectioned and stained for hematoxylin and eosin to stain cell nuclei and cell cytoplasm respectively or alcian blue to stain GAG molecules. All the samples revealed the presence of cell growth on the PCL scaffolds (Figure 14). Small amounts of cells in the sections were found for all the groups at day 8 of treatment (day 4 after application of growth factors). These results agree with the low cell numbers found in the scaffolds at day 8 (Figure 12) in which in most of all
the conditions cell numbers were close to the initial cell seeding. At day 12 all the groups responded to the treatment and a higher number of cells is observed in the sections. The level of organization observed in the scaffolds also increases with time and it was possible to observe the formation of a layer of cells (Figure 14 blue arrows).

Sections of TGFβ-1 treated constructs group at day 12 revealed the formation of a layer of cells on top of the PCL scaffolds (Figure 14B). At day 16 in this group, the level of organization seems to be different from that observed on day 12 (Figure 14C).

We also observe a layer formation at day 12 in the BMP-2 group. However this layer seems to be more compacted and the cells exhibited less spreading (Figure 14E). At day 16 in this group we observe a decrease in the number of cells in the scaffold (Figure 14F).

Simultaneous application of TGFβ-1 and BMP-2 also caused the formation of a layer of cells on top of the PCL scaffolds at day 12, but this layer appears to be more ordered than either the TGFβ-1 or the BMP-2 groups (Figure 14H). Simultaneous TGFβ-1 and BMP-2 group also showed a high aggregation of cells at day 16 (Figure 14I) and the layer organization decreased.

Sequential application at of BMP-2 group at day 12 caused the formation of a compact layer on top of the PCL (Figure 14K). However, at day 16 we did not observe this level of organization; instead we observe a higher amount of cells in the sections (Figure 14L).
Figure 14. Hematoxylin and eosin stain of PCL constructs. Figures A), B) and C). TGFβ-1 treated constructs at days 8, 12 and 16 respectively. Figures D), E) and F). BMP-2 treated constructs at days 8, 12 and 16 respectively. Figures G), H) and I). Simultaneous application of TGFβ-1 and BMP-2 at days 8, 12 and 16 respectively. Figures J), K) and L). Transient application of BMP-2 while applying TGFβ-1 at days 8, 12 and 16 respectively. Dark arrows: residual PCL scaffold. Blue arrows: cell layer. Scale bar: 100 µm.
3.2.5 *Alcian Blue Staining for GAG Identification in hMSCs Seeded PCL Scaffolds Treated with Growth Factors*

Glycosaminoglycan deposition in the PCL scaffolds was observed after day 12 (Figure 15). Before that it was not possible to observe GAG deposition using alcian blue staining (Data not show). All the samples showed the presence of GAG expression at day 12 and day 16. The GAG distribution within the groups was different but increased with time of culture for each group.

TGFβ-1 treated samples showed little deposition of GAG molecules at day 12 (Figure 15A). On day 16 GAG expressions increased in the constructs, especially in the cell layer area on top of the scaffolds. BMP-2 treatment caused a GAG deposition higher than the TGFβ-1 group but rather than be more evenly distributed; it was found only in the areas with the higher cell density (Figure 15D). Samples treated with TGFβ-1 and BMP-2 together produced the earliest formation of GAG molecules (day 12) (Figure 15E). The level of expression increased at day 16 (figure 15F) and was well distributed within the PCL scaffolds. Sequential application of BMP-2 showed the highest GAG expression compared with all the other groups at day 16 (Figure 15H). Interestingly, the transient application of BMP-2 did not promote GAG deposition by day 12 (Figure 15G). However the increased GAG deposition levels were observed at day 16 (Figure 15H).
Figure 15. Alcian blue staining revealing GAG distribution within the PCL scaffolds. Figures A and B show the effect of TGF-1 on GAG distribution. Figures C and D show the GAG distribution caused by the use of BMP-2. Figures E and F show the effect of Simultaneous use of TGF-1 and BMP-2. Figures G and H shows the effect of transient exposure to BMP-2. Scale bar 100 µm.
3. 2.6 Effect of Growth Factors on Collagen I, II and X Deposition in hMSCs Seeded PCL Scaffolds

Collagen I, collagen II and collagen X deposition were observed using immunohistochemistry on frozen sections of the samples at day 16. All the samples showed expression of collagen I and collagen II. Small amounts of collagen X were observed in the BMP-2 treated and simultaneous TGFβ-1 and BMP-2 treated constructs. TGFβ-1 treated samples showed lower deposition of collagen I and collagen II and no deposition of collagen X. BMP-2 treated samples presented a higher deposition levels of collagen I and an even higher deposition of collagen II, denoted for the darker stain of the fibers (Figure 16D, 16E). However most of this expression was found within the cells and only small amounts were found surrounding the cells. Additionally the production of either collagen I or II did not follow any order. A slight stain of collagen X revealing small expression levels was found in the BMP-2 treated group (Figure 16F). In the simultaneous treatment group of TGFβ-1 and BMP-2 higher expression levels of collagen I and collagen II were observed compared to TGFβ-1 group or BMP-2 group (Figure 16G, 16H).

Collagen I fibers were found parallel to the top of the surface and within the scaffold (Figure 16G). Collagen II expression was founded to be the highest in the simultaneous treatment group (TGFβ-1 and BMP-2) (Figure 16H). The collagen fibers were found to be randomly oriented and with a lack of organization; probably due to the lack of a mechanical stimulus. A slight expression of collagen X was also found, especially on the top layer of the scaffolds (Figure 16I). Transient application of BMP-2 presented a lower expression of collagen I (Figure 16J) than simultaneous use of TGFβ-1
and BMP-2 group or BMP-2 treated samples; but this expression was higher than the
TGFβ-1 treated group. Collagen II expression was lower than simultaneous use of TGFβ-
1 and BMP-2 group and higher than all the other groups (Figure 16K). This collagen II
expression was found evenly distributed within the scaffold and also created a layer on
top of the constructs. The expression of Collagen X was slightly higher than the
simultaneous use of TGFβ-1 and BMP-2 group, but still low enough not to be detected in
the entire scaffold (Figure 16L).

3.2.7 Gene Expression of HMSC Seeded PCL Scaffolds Treated with Growth Factors

Quantitative reverse transcription PCR of the hMSC seeded PCL construct treated
with growth factors at day 16 confirmed the expression of aggrecan and collagen I
(Figure 17). Gene expression levels for the TGFβ-1 group were used to normalize the
other growth factor groups because gene expression values of the samples treated only
with differentiation media resulted in expression levels too low to be used. Gene
expression levels of aggrecan in the BMP-2 treated group resulted in a 13.0 fold increase
in expression levels compared to the TGFβ-1 treated group and resulted in the highest
expression for all the groups. Collagen X gene expression levels found in simultaneous
use of TGFβ-1 and BMP-2 group or transient BMP-2 group were relatively high
compared with TGFβ-1 treated samples. Simultaneous treatment of TGFβ-1 and BMP-2
resulted in 3.4 fold increase in collagen X gene expression and transient BMP-2 resulted
in 2.5 fold increase in gene expression of collagen X (Figure 17). Interestingly no
collagen X gene expression was observed in the BMP-2 treated group.
Figure 16. Immunohistochemistry analysis PCL scaffold treated with growth factors. Analysis on frozen sections at day 16 showing the effect of growth factors on collagen I (first column), collagen II (Second column 2) and collagen X (Third column). Levels of expression are denoted by the light brown areas.
Figure 17. Gene expression levels on hMSC seeded PCL scaffold treated with growth factors. All the samples presented high values of aggrecan expression. relatively significant levels of collagen X were found in the Simultaneous TGFβ-1 and BMP-2 group and in the transient BMP-2 group. The TGFβ-1 group was used to normalize the other groups.
3.3 Shear Stress and Perfusion Bioreactor Results

The next step of the project consisted of testing one of the growth factor conditions in our newly designed rotating disc bioreactor (Figure 2). Because of the higher GAG deposition achieved using hMSC seeded PCL scaffolds (Figure 13A) and the relatively good cell proliferation rate observed (Figure 13) the sequential application of BMP-2 while applying TGFβ-1 condition was used in bioreactor culture. TGFβ-1 was added during the first 4 days of culture in the bioreactor, and then at day 4 BMP-2 was applied simultaneously with TGFβ-1 for another 4 days (From day 4 to day 8) after day 8 media with both growth factors was removed and new media containing only TGFβ-1 was applied for the next 6 days (experiment lasted 14 days).

3.3.1 Scanning Electron Microscopy (SEM) of Constructs

Scaffold samples were used for SEM analysis to evaluate the micromass cell growth in the PCL scaffolds following bioreactor growth with transient exposure of BMP-2. Constructs at day 8 showed a region of cell growth on top of the PCL scaffolds (Figure 18A arrow and circled area). This region increased in size at day 14 (Figure 17B arrow and circle area). These results show that the growth of the cells not only occurred within the scaffolds but also on the surface of the scaffolds. A magnification of the images shows the cellular region as a smooth surface (Figure 18C, 18D) that covers in the circle area the pores of the scaffold.
Figure 18. Scanning electron microscope images taken to PCL constructs cultured in the bioreactor. Figures A and B show PCL scaffolds images at day 8 and 14 respectively. Red circles shown the cellular region. Scale bar 1 mm. C and D show a magnification of the border of the cellular region and the PCL scaffold. The surface of the cellular region is smooth and filled the pores on top of the PCL scaffold.
3.3.2 Effect of Bioreactor culture on Cell Proliferation and GAG Content

The purpose of these experiments was to evaluate the effect of shear stress and perfusion on hMSC proliferation and differentiation in PCL scaffolds using differentiation media supplemented with growth factors. PCL constructs were seeded under micromass conditions with $1 \times 10^6$ hMSCs per construct using the cell concentration previously selected ($8 \times 10^6$ cells/ml, 125 µl). We decided to operate the bioreactor using differentiation media from the beginning of the experiment because of the null effect that proliferative media had on cell growth for PCL scaffolds during static culture (Figure 11) and to avoid another possible source of bacterial contamination due to the several changes in media.

After 8 days in bioreactor culture, construct cell number increase from $1.0 \times 10^6$ to $1.5 \pm 0.2 \times 10^6$ in the first bioreactor experiment and from $1.0 \times 10^6$ to $1.3 \pm 0.2 \times 10^6$ in the second bioreactor experiment (Figure 19A). At day 14, cell proliferation reached a value of $3.1 \pm 0.2 \times 10^6$ cells in the first bioreactor experiment and a value of $3.5 \pm 0.2 \times 10^6$ cells in the second experiment (Figure 19A). During the first 8 days of culture cell number did not increase dramatically. However; after 14 days in culture, cell number increased 3 fold the initial seeding.

GAG deposition in constructs after 8 days of bioreactor culture revealed an increase of proteoglycan accumulated in the scaffolds. At day 8 of bioreactor culture GAG content achieved $60 \mu g \pm 20 \mu g$ and $90 \mu g \pm 40 \mu g$ for experiments 1 and 2 (Figure 19B). At day 14 GAG content in the PCL constructs increased to $260 \mu g \pm 90 \mu g$ and $296 \mu g \pm 97 \mu g$ for experiments 1 and 2 respectively (Figure 19B).
Figure 19. Cell number content and GAG deposition in PCL constructs cultured in the bioreactor. A) Cell number in the PCL constructs in each of the two bioreactor experiments. B) GAG content of constructs.
3.3.3 Histological Analysis of HMSC Seeded Constructs

Constructs were removed from the bioreactor at day 8 and 14 and frozen sections were made for histological analysis and immunohistochemistry. Hematoxylin and eosin stain of the constructs at day 8 revealed a strong aggregation of the cells on the surface of the scaffolds (Figure 20A). This layer of cells was parallel to the surface and it is possible to observe that the morphology of the cells changed significantly compared with the cells growing under static conditions. The morphology of the cells in the top layer in the bioreactor at day 8 is more rounded with the cell spreading following the direction of the applied shear stress profile (Figure 20A arrows, Figure 20E). This type of morphology is often associated with initial; differentiation into chondrocytes [104].

Alignment of the cells in the bioreactor experiments at day 14 differs from that observed on day 8. On the surface of the constructs the alignment of the cells is parallel to the surface and in the direction of the shear stress. The alignment of the cells within the scaffold changes and becomes more aligned to the pathway of the flow perfusion (Figure 20F arrow). Although the layer of cells at day 8 on the construct was bigger than in static culture (Figure 14J); the penetration of the cells into the scaffold only achieve a value of 150 µm. However, at day 14 the cell penetration into the scaffolds increased dramatically when compared with the 8 day bioreactor culture (Figure 20B). Cell penetration went 1000 µm deep within the scaffold. PCL constructs also showed a complete filling of the pores of the scaffold (Figure 20B). Cell morphology was found to be a more rounded shape (Figure 20F) compared to cell morphology in static culture (Figure 14J, 14K).
Figure 20. Histological analysis of constructs grown in the bioreactor. Figures A and B, hematoxylin and eosin stain of bioreactor samples at days 8 and 14 respectively showing cells on the constructs. Figures C and D, alcian blue staining showing GAG distribution within the PCL scaffolds at day 8 and 14 respectively. Figures E and F 400 X magnification of H&E staining at day 8 and 14 respectively Scale bar for figures A, B, C and D 100 µm. Scale bar for figures E, and F 50 µm. Arrows indicate direction of the shear stress and perfusion.
3.3.4 Alcian Blue Staining of Bioreactor Constructs

Alcian blue staining after 8 or 14 days in bioreactor culture demonstrated GAG deposition within the constructs. At day 8, the GAG distribution occurs mainly in the top layer of cells found in the PCL scaffolds (Figure 20C). At day 14 the GAG deposition is deep inside of the scaffold (Figure 20D). Whereas the GAG deposition at day 8 seems completely uniform within the cellular region; at day 14 some gaps of GAG content can be observed within the cellular region of the scaffolds. This might be the effect of long term exposure of perfusion of the constructs that eventually washes out some of the GAG molecules.

3.3.5 Collagen I, Collagen II and Collagen X Expression

Immunohistochemistry of the sections revealed the presence of collagen type I and type II and small deposition levels of collagen X. Collagen I deposition at day 8 was found within the cellular region of the layer of cells on top of the PCL scaffolds (Figure 21A, light brown). Deposition of collagen I was also found in the center of the cellular region and also in a lower level on the edges of this region (Figure 21A arrows). Collagen II deposition at day 8 was lower than the levels of collagen I but the total area of deposition is larger (Figure 21C red marked zone) than the collagen I area. Collagen X deposition was not found in the constructs on day 8 (Figure 21E). At day 14 collagen I and collagen II levels increased in the constructs (Figure 21B and 21D respectively). Collagen I deposition was found within the entire cellular region and also strongly concentrated in certain regions (Figure 21B arrows). Collagen II expression also was
found evenly distributed within the cellular region and in some zones of elevated concentration (Figure 21D arrows). These zones of high level of deposition of collagen I and collagen II are overlapping. Small levels of collagen X deposition at day 14 were found (Figure 21D). These levels are significantly lower compared with the level of expression found for collagen I and collagen II.
Figure 21. Immunohistochemistry analysis of PCL constructs grown in the bioreactor. Images A and B collagen I expression at day 8 and 14 respectively. Arrows indicates the region of collagen I expression. C) and D) collagen II expression at day 8 and 14 respectively. Red zoned demarked on image C denotes the area of collagen II. Arrows in figure D denotes the zones of high concentration of collagen II expression. E) and F) collagen X expression at days 8 and 14 respectively.
3.3.6 Pressure Drop across Constructs

One of the advantages of the bioreactor design is the ability to measure pressure drop across the constructs to estimate the permeability. The permeability of the samples is related to the tissue growth in the scaffolds. Pressure drop at day 0 (initial seeding) was 185 Pa for the first 14 days bioreactor and 288 Pa for the second 14 day bioreactor. Both bioreactors showed a gradual increase in the pressure drop (Figure 22A) from day 0 to day 8 and 9 respectively. After that the pressure drop in both bioreactors fall from 525 Pa to 461 Pa in the first 14 days bioreactor and from 431 pa to 398 Pa in the second bioreactor. Both bioreactors followed the same trend with a gradual increase in pressure drop for the first 8-9 days and then falling 12% and 8% respectively.
Figure 22. Bioreactor pressure drop and permeability values. A) the pressure drop increased for the 2 bioreactor experiment and stabilized at the end of the culture period. B) Permeability values were calculated using Darcy’s law. The permeability values represent the total of the system (8 scaffolds).
3.3.7 PCL Constructs Permeability

Construct permeability values were calculated and plotted using Darcy’s law. The permeability values of the constructs decreased over time (Figure 22B). These values started at $3.3 \times 10^{-14}$ m²/Pa·s and $2.1 \times 10^{-14}$ m²/Pa·s in the case of the 14 day bioreactor and ended at $1.3 \times 10^{-14}$ m²/Pa·s and $1.5 \times 10^{-14}$ m²/Pa·s respectively. For the 7 day bioreactors the values of the permeability at day 0 started at $2.6 \times 10^{-14}$ m²/Pa·s and 2.27 x $10^{-14}$ m²/Pa·s respectively and slowly decreased over time to $1.2 \times 10^{-14}$ m²/Pa·s and $1.4 \times 10^{-14}$ m²/Pa·s respectively. These permeability values are still higher compared to permeability values of human articular cartilage ($0.1-10 \times 10^{-15}$ m²/Pa·s)[111].

3.3.8 Bioreactor Glucose Consumption

During the bioreactor experiments, samples of the media were taken to calculate the glucose consumption of the cells in the bioreactor. Glucose consumption is used to measure the metabolic activity of the cells in a bioreactor and therefore can be used as a control parameter when designing a large scale process. The glucose content in the media inside the bioreactors decreased over time (Figure 23). The initial glucose content found in the bioreactor media (200 ml) was 902 mg ± 37.2 mg (the manufacturer’s data sheet states a content of 900 mg in 200 ml or a glucose concentration of 450 mg/dl). At day 1 the amount of glucose found in the experiment 1 bioreactor 14 days was 874 mg ± 45 mg and in the experiment 2 bioreactor 2 was 865 mg ± 15 mg (Figure 23). The glucose content in the media decreased with time and at day 8 the glucose concentration observed in the media was 786 mg ± 24 mg and 766 ± 39 mg for experiment 1 and experiment 2, respectively. At day 8 the media was replaced with fresh media and glucose in the
bioreactor increased to 900 mg. Finally, the glucose contents at day 14 for experiment 1 and experiment 2 were 799 mg ± 13 mg and 781 mg ± 25 mg.

The glucose consumption rates were calculated for the time periods from day 0 to day 8 and from day 8 to day 14 (table 7). We use the average value of the total number of cells found in the bioreactor at day 0 and at day 8 for the first period and the average value of the total number of cells found at day 8 and at day 14 for the second period of time. We found that the glucose consumption rate (glucose/(cell*day)) decrease during the second period of culture (from days 8 to 14).
Figure 23. Total glucose content in the bioreactor media as a function of time. The glucose content in the media decreased over time as a sign of cell consumption of glucose. *: Media was changed at day 8; the total bioreactor glucose was 900 mg after media change in both bioreactors.

Table 7

Average glucose consumption rates in the bioreactor

<table>
<thead>
<tr>
<th>Average glucose consumption rate experiment 1 bioreactor 14 days</th>
<th>Average glucose consumption rate experiment 2 bioreactor 14 days</th>
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<tbody>
<tr>
<td>Day 8</td>
<td>1425 pg/(cell*day)</td>
</tr>
<tr>
<td>Day 14</td>
<td>935 pg/(cell*day)</td>
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CHAPTER 4

DISCUSSION

4.0 Introduction

Cartilage is a complex tissue with a well defined architecture designed to withstand the compressive and shear stress forces generated in the joint. Cartilage damage due to osteoarthritis or trauma injury represents a common problem in developed countries. Tissue engineering can treat cartilage damage by combining cell biology, engineering and medicine to produce new tissue in vitro that can replace the damaged tissue. Tissue engineering aims to create tissue engineered cartilage with tissue architecture similar to native cartilage to assure a complete functionality of the in vitro made tissue cartilage. One feasible alternative is to produce tissue engineered cartilage using human mesenchymal stem cells. hMSCs are cells that can be differentiated in several tissues such as cartilage, bone and others. Differentiation of hMSCs into cartilage requires the use of several growth factors and cell culture conditions. Some of the factors known to induce hMSCs differentiation include high cell density cultures, the use of growth factors such as TGFβ-1 and BMP-2 and mechanical stimuli such as shear stress and perfusion.

In the present work we designed a new bioreactor for cartilage tissue engineering. Then we optimized micromass concentration and the use of TGFβ-1 and BMP-2 to induce hMSCs chondrogenesis in static cultures. We found that one of the most viable strategies to be tested in the bioreactor is the use of micromass bodies made with a cell
concentration of $8 \times 10^6$ cells/ml in combination with the use of transient BMP-2 (50 ng/ml) in the presence of TGFβ-1 (5 ng/ml). We found that over 14 days in culture GAG production is enhanced due to the stimulus generated by the transient exposure of BMP-2. We also found that the shear stress and perfusion in the bioreactor enhances cell proliferation compared to static conditions and the perfusion caused a deeper penetration of the cells within the scaffold.

4.1 Effect of Cell Density on hMSC Micromass Culture

Micromass cultures grown in the presence of differentiation media on tissue culture plates showed a significant increase in the spreading area compared to micromass cultures grown in the presence of proliferative media. This difference in the spreading size can be attributed to the effect of the differentiation media on micromass cultures. Other studies working either with micromass or pellets have shown a steady increase in micromass size when micromass cultures grown in differentiation media [57,58]. We also observed a migration effect of the cells at the edges of the micromass region (Figure 9). We did not see a remarkable increase in cell number in the micromass cultures; therefore we cannot say that the increase in spreading area was attributed to the increase in cell number. We attributed the increase in spreading area of the micromass bodies to a combination of ECM molecules produced and cell migration. Other studies using the same type of differentiation media and hMSCs have shown that the increase of size in the micromass or pellet cultures is due to the secretion of GAG molecules and collagen fibers generated by an early chondrogenesis process [58,112].
Another interesting result is related the effect of cell concentration on micromass spreading. Micromass cultures made with a cell concentration of $8 \times 10^6$ cells/ml cultured in differentiation media (LOW DM) showed a more significant spreading increase than micromass cultures made with a cell concentration of $16 \times 10^6$ cells/ml cultured in differentiation media (HIGH DM). The LOW DM group quadrupled its spreading area at day 4 compared with HIGH DM cell density that only increased its initial spreading area 2.5 times. These results showed that there is a relationship between micromass spreading area and cell density. The spreading area does not increase in proportion to the cell number as would be thought. Instead we observe that the HIGH DM cell density decrease the spreading rate compared with the spreading rate of LOW DM cultures. One of the limitations of the interpretation of these results is the fact that the spreading observed might also indicate a flattening effect of the micromass bodies. Therefore, the spreading area might not be entirely correlated with the increase in volume of the micromass area. Although our alcian blue staining showed differences in the deposition of GAG molecules between the HIGH and LOW groups, more studies are necessary to understand the complete role of cell density in the cartilage formation process.

Alcian blue staining of the micromass bodies counterstained with nuclear red dye confirmed the presence of GAG molecules at day 4 for all the conditions. The early expression of alcian blue regardless of the media condition revealed the strong effect of micromass seeding on cell differentiation. These results support previous findings that micromass culture enhances chondrogenesis [66,113]. We also observe differences in GAG staining between the LOW and HIGH cell density micromass cultures. The highest amount of GAG staining is observed in the LOW concentration micromass bodies. These
results indicate that some part of the expansion observed in the micromass cultures is attributed to ECM molecules deposited within the micromass bodies and that this deposition is not proportional to the cell density. These results confirm previous thoughts that cell density affects the development of the micromass [77]. Specifically the use of LOW cell density achieves a higher cell staining of GAG molecules.

Nuclear red dye confirmed differences in the cell distribution within the micromass bodies. Whereas HIGH cell concentration seeding groups showed higher cell density in the center and at the edges of the micromass bodies (Figure 11B and D), LOW cell concentration groups showed a lower cell density within the center but a higher deposition of GAG. This highest amount of cells found in the center of the HIGH cell density micromass bodies might have affected the rate of nutrient diffusion, especially oxygen. This decrease in oxygen diffusion might explain the decrease in the spreading observed and the lower amount of GAG staining observed in the HIGH cell density cultures.

The diffusion of oxygen in vivo tissues is limited to about 200 μm [91]. However, some studies have addressed the advantages of low oxygen environments for cartilage formation [114]. Low oxygen levels are found in cartilage because this tissue lacks blood vessels [114]. Low oxygen levels found in the center of the micromass might promote the differentiation of hMSC cells by switching to anaerobic metabolism and by the strong signaling generated by cell-cell interaction [115]. Comparing these results with the literature we found that the micromass condition is necessary to enhance chondrogenesis, however there is a delicate equilibrium between the cell concentration used and the efficiency of the differentiation. Although this reduction in nutrient diffusion rate is
believe to induce differentiation of hMSCs into cartilage, there must be a threshold value that once overcome, down regulate the production of ECM molecules and promotes a higher rate of cellular death.

Ichinose et al. showed in detail the different stages that appear during micromass culture [62]. They showed that during the early stages an elevated number of cells in the center of the micromass region die. The rest of the cells become a chondrocyte cell like and they begin to produce high amounts of ECM proteins that increase the size of the micromass region. Ichinose used a micromass density of 500,000 cells/ml which is lower than the cell concentration that we used. Although we did not use the same cell concentration, we observe that the micromass cultures change over time and produced higher deposition of GAG molecules with the lower cell density. When looking to other studies using micromass we found that there is no consistency about the cell concentration used to create the micromass cultures in the literature [77,116]. For example, Xu Y et al. measured the mechanical properties of micromass bodies generated with a cell concentration of $10 \times 10^6$ cells/ml. They found that the dynamic modules and the micromass size increased over time due to an increase in the extra cellular matrix content of the micromass bodies [113]. This concentration was similar to one of the concentration that we use. In another study, micromass bodies made with a concentration of 200,000 cells/ml showed a more efficient GAG production when compared with micromass made with higher cell concentrations [77]. Another important result that adds support to the theory that the growth of the micromass bodies is due to ECM production and cell reconfiguration is the relatively small cell proliferation observed. In our experiments we observe only an increase of 20% to 30% in cell population in all the
micromass conditions. These rates of proliferation are low compared to the normal rate of proliferation of hMSCs which have doubling times of 36 to 48 hours when cultured in low cell densities [117].

4.2 Micromass Seeding of hMSCs in PCL Scaffolds and Effect of Growth Factors on hMSCs Seeded PCL Scaffolds

For this series of experiments we seeded micromass bodies in top of porous PCL scaffolds. We let the cells stabilize on the PCL substrate for 4 days in proliferative media (PM) and then we switched to a differentiation media supplemented with a combination of TGFβ-1 and BMP-2. From day 0 to day 4 cells in the PCL scaffolds did not proliferate at the same rate (Figure 11) as cells cultured in micromass condition in tissue culture (Figure 8A) even though they were cultured in proliferative media. This type of outcome can be possibly attributed to the change of substrate on which the cells were growing (from tissue culture plate to PCL) [118]. PCL is a hydrophobic material and its surface properties might affect cellular development by decreasing proliferation rate [119,120]. Other studies have found that without the use of TGFβ-1 hMSCs proliferation in PCL scaffold decreases [55].

Our next step was to study the effects of TGFβ-1 and BMP-2 on the proliferation and differentiation of hMSCs into cartilage using PCL as a scaffold and micromass seeding of hMSCs. We use TGFβ-1 and BMP-2 growth factors either alone, simultaneously or transiently (BMP-2 in the presence of TGFβ-1) (Table 2).
Although we observe differences in cell proliferation among all the groups, the differences between samples on each group at day 8 and day 12 were less clear. For example in all the groups we observe that the highest increase in cell number occurred at day 16 for all of them. From day 8 to day 12 we did not see a remarkable difference in the cell proliferation in each group. Although this decrease in cell proliferation might be related to the use of PCL as mentioned before, we also think that this effect observed in the samples might be caused by an interference of the PCL scaffold with the growth factors. In studies were pellet culture was combined with the use of TGFβ-1 at the same concentration with no use of scaffolds material, a cell increase up to 2 fold of the control group (no growth factors) was observed at day 7 [121]. However, we did not observe a 2 fold increase in cell number at day 8 in our experiment. We observe this increase at day 16 of the experiments and only in the TGFβ-1 treated group and in the transient BMP-2 in the presence of TGFβ-1 group.

TGFβ-1 and BMP-2 are polypeptides proteins that have affinity for plastic surfaces [122]. It might be possible that during the first day of treatment most of the growth factors might have bound to the PCL scaffold and only a relatively small amount of growth factors molecules were available for the cell receptors. However we did not find specific bibliography that investigated the specific interaction of growth factors with PCL. In most of the studies that use PCL and one type of growth factor, the interaction of the growth factor with the material has not been addressed [55,123]. However, some studies have shown the crucial role of the interaction of the materials with the growth factors [124,125]. Although the scope of this project was not to investigate these effects, we believe that further research must address this problem.
We addressed that one of the possible causes for the slow response of the hMSCs seeded in the PCL scaffold during the first 8 days of culture was the combining effect that the material had on the cells and the possibility that some of the growth factors bound to the scaffold material instead of the cell receptors. However, it was possible to observe the effect of the growth factors on the hMSCs. We observed that the use of TGFβ-1 increased cell proliferation compared with groups that did not receive TGFβ-1 treatment (control group and BMP-2 treated group). In contrast the BMP-2 treated group had just half the number of cells found in the TGFβ-1 group. These findings agree with previous studies that show that the members of the TGFβ family are better able to induce cell growth [68].

In our experiments a small role for TGFβ-1 in chondrogenic differentiation was observed since GAG production levels found in the TGFβ-1 treated scaffolds were higher than the control group with no growth factors (Figure 13A). However the GAG production in TGFβ-1 group was lower than those obtained in either TGFβ-1 + BMP-2 simultaneous group or in the transient application of BMP-2 group. Some studies have shown the importance of TGFβ-1 to induce chondrogenesis [55,126], other studies have demonstrated that that TGFβ-1 role is more necessary to maintain the phenotypic characteristic associated with cartilage differentiation of mesenchymal stem cells [58] and to avoid further differentiation into osteoblastic cells [70].

Although the GAG content found in the BMP-2 treated group was similar to the GAG levels found in the TGFβ-1 treated group, the lower amount of cells found in the constructs in the BMP-2 treated group showed a much higher production rate of GAG molecules per cell (Figure 13B). These results are confirmed with the gene expression analysis of the samples at day 14 that showed that the BMP-2 treated group had higher
expression levels of the aggrecan gene than the TGFβ-1 treated group (Figure 18). These findings agree with studies showing that BMP-2 inhibits cell proliferation by antagonizing Wnt3a signaling [127]. Some studies have reported a greater differentiation effect of BMP-2 when compared with TGFβ on hMSCs cultures [82]. Also previous studies have reported that differentiation of hMSCs is faster when BMP-2 is used compared to TGFβ-1 [128,129].

This pro-chondrogenic effect of BMP-2 is better observed in the experiments analyzing the transient application of BMP-2 in the presence of TGFβ-1 treatment group. In this experimental condition GAG deposition at day 8 was the same as that obtained in the TGFβ-1 treated group (At this time both groups were treated with TGFβ-1). At day 12 (after 4 days of application of BMP-2 while maintaining TGFβ-1) the GAG content in the transient exposure of BMP-2 group was 50% higher (p<0.05) than the TGFβ-1 treated group. This type of response is a clear signal that the cells were stimulated by the transient application of BMP-2 to secrete GAG proteins. Other studies that have used different combinations of growth factors in hMSCs found that the strongest expression of aggrecan and collagen II was related to a transient application of TGFβ-3 and BMP-6 [81]. However, in that study the transient application followed a cyclic pattern of TGFβ-3 followed by BMP-3 and the 2 growth factors never were present at the same time in culture.

The increase in GAG deposition observed in the transient BMP-2 in the presence of TGFβ-1 group was not the only positive response observed. The cell number obtained in the transient BMP-2 group was comparable to the cell number obtained in the TGFβ-1 treated group and was higher than the simultaneous use of TGFβ-1 and BMP-2 treated
group. This type of result might be of significance from the point of view of an industrial process. Since the cells are a limited source, it is important to develop conditions that allow the expansion of cells in large amounts while they keep their ability to differentiate into cartilage. This type of behavior somehow might resemble the cartilage formation process that occurs in vivo. In this process a condensation and expansion of mesenchymal cells occurs first [59]. Then a differentiation process takes place. We tried to resemble this process by using TGFβ-1 in the beginning and then applying BMP-2 just to create a signal that enhances the differentiation process. Our results seem to show that there is a relationship between the differentiation process of hMSCs and the temporal application of growth factors. Although the simultaneous application of TGFβ-1 and BMP-2 also achieves cell numbers comparables to those achieved in the transient application of BMP-2 in the presence of TGFβ-1 group, the GAG content in the transient group was 45% higher (p< 0.05). Thus, not only did transient use of BMP-2 show a positive outcome in stimulating hMSCs into cartilage but this condition also is a more economical and viable bioprocessing condition because a lower quantity of reagents are needed. It is important to address the economical viability of the final product, especially if bioreactor cultures are involved.

4.3 Bioreactor Culture of hMSCs Seeded PCL Scaffolds

Large scale production of tissue engineered cartilage requires the use of efficient bioreactor systems to produce tissue reliably under sterile conditions. Bioreactors for tissue engineered cartilage need support monitoring of the operating conditions to control tissue development. Conditions such as pressure drop, cellular metabolic activity and pH
can be correlated with tissue growth and matrix deposition and can be used for quality control. Tissue engineered cartilage also needs the mechanical stimulus generated in the bioreactor to enhance cell growth and differentiation [29]. A bioreactor needs to address all the important conditions necessary for tissue development such as mass transfer and mechanical forces. A bioreactor also needs to combine different mechanical stimuli such as shear stress and perfusion to develop tissue cartilage in vitro with tissue architecture similar to in vivo cartilage. We therefore developed a shear stress and perfusion bioreactor that combines these two forces to mechanically stimulate the cells and enhance nutrient diffusion to promote chondrogenesis. This bioreactor allows the monitoring of pressure drop in real time and allows an easy change of the operating conditions.

Bioreactor experiments are large scale projects that consume significant time and reagents. One of the initial objectives of this project was to optimize a bioprocessing condition in static culture to generate chondrogenesis using a combination of TGFβ-1 and BMP-2 in a PCL scaffold and then test one of these conditions in the shears stress and perfusion bioreactor. Based on the results on the first two static experiments we decided to use hMSC seeded on PCL scaffolds using transient exposure of BMP-2 while applying TGFβ-1. TGFβ-1 (5 ng/ml) was applied during the first 4 days. From day 4 to 8 BMP-2 (50 ng/ml) was added in the presence of TGFβ-1. At day 8 BMP-2 was removed and TGFβ-1 was used until the end of the experiment. We choose the transient use of BMP-2 while applying TGFβ-1 because we observe a positive stimulus that increased GAG production over all the other conditions in static cultures (Figure 13A). We also observed a higher proliferation rate compared with the simultaneous use of TGFβ-1 and BMP-2 or just BMP-2 and at the same level that the TGFβ-1 treated group.
We operated the bioreactor at 10 RPM; this translates to a shear stress range on the scaffold surface area of 0.087 dyne/cm$^2$ to .017 dyne/cm$^2$ and a perfusion rate of 0.1 ml/min per construct. Bioreactor culture of hMSCs seeded PCL scaffold under these conditions showed a higher cell proliferation (Figure 19A) compared to static experiments (Figure 12). Although the initial cell number used in the bioreactor experiments was significantly higher than the cell number used during static conditions (1.0 X 10$^6$ and 2 X 10$^5$ respectively); cell proliferation increased 3.25 fold (the average of 2 experiments) in the bioreactor in 14 days compared with 1.91 fold under static conditions. This increase in cell proliferation rate observed might be related to the mechanical stimulation generated by the bioreactor and the increase in nutrient diffusion generated by perfusion. Some recent works has shown that hMSCs are highly sensitive to mechanical stimulus [95]. Perfusion rates in the order of 0.01 to 0.1 ml/construct stimulate hMSCs proliferation on porous scaffolds [99]. However, when perfusion rates greater than 1 ml/min per constructs were applied to hMSCs the proliferation rate decreased [99]. In one interesting study using perfusion, Sikavitsas et al. showed that the mechanical stimulation of marrow osteoblasts is independent of the nutrient transport rates [130]. In this experiment the viscosity of the media was increased using dextran. This increase in viscosity caused an increased in the shear stress while maintaining the same nutrient diffusion rates. Based on these studies the author concludes that the increase in matrix deposition observed is due to higher shear and not due to increase in nutrient transport. Other experiments have shown that the shear stress magnitude affects hMSCs proliferation and differentiation. When shear stress in the range of 0.04 dyne/cm$^2$ to 0.07 dyne/cm$^2$ was applied to porous scaffolds; an increase in the cellular proliferation
was observed [131]. These results agree with previous findings that shear stress in that range affects cell proliferation [95]. However, higher rates of shear stress in other studies (0.37 dyne/cm$^2$ to 2.7 dyne/cm$^2$) decrease cell proliferation and increase extra cellular matrix production [132].

These values of shear stress and perfusion rates used for hMSCs differ from the actual values used to stimulate fully differentiated chondrocytes. For example, chondrocytes are more responsive to a higher shear stress. Shear stress in the range of 1.52 dyne/cm$^2$ increased cell proliferation and GAG production in bovine chondrocytes [114]. These differences might be explained because hMSCs are not fully differentiated cells and therefore they are more sensitive to mechanical stimulus [95].

Chondrogenic markers such as collagen II and proteoglycans are detected at day 8 in bioreactor culture. The expression of these markers indicates that cells began to differentiate before day 8. From day 8 to day 14 cell growth increased and also the expression of chondrogenic markers such as GAG deposition and collagen II deposition. These results differ from the results that we obtained in static culture in PCL. During static culture we only observed a small amount of matrix GAG at day 8 (7.5 µg/construct with 252,000 cells) whereas in the bioreactor we observed a high increase in GAG content (73 µg/construct with 1.5 X 10$^6$ cells). Though the amount of cells in the bioreactor was 6 times higher than the amount of cells in the static the amount of GAG produced was 9.73 times higher than the static conditions. At day 14 the amount of GAG content in scaffolds in the bioreactor was 5 times higher than the GAG value obtained in scaffolds under static culture. However, the cell number in the bioreactor was 10 times higher than the cell number in the static conditions. Thus we observe a small decrease in
the GAG deposition per cell in the bioreactor after 14 days. However, we did not test the GAG released in the media to test whether the GAG production rate per cell decrease or if the ECM molecules deposited in the scaffold were washed out due to the constant perfusion. Other studies with chondrocytes have shown that although mechanical stimulation enhances ECM production, the rate of matrix deposition eventually decreases over the culture period [89]. This GAG decrease was attributed to the release of GAG molecules due to the perfusion and shear stress from the ECM matrix in formation in the constructs [89].

Scanning electron microscope images showed an increase in size in the overall seeding area from day 8 to day 14 (Figures 18A and 18B). The cells filled the pores and created a smooth and continuous surface on top of the scaffolds (Figures 18C and 18D). The expression of collagen I and collagen II in the histological sections (Figure 21) confirmed the smooth layer of cells and ECM that was observed in the SEM images. In other studies using bioreactors, this smooth layer formation on top of the scaffolds has been attributed to the effect of shears stress [89,96]. Shear stress modulates the matrix deposition of the cells and also rearranges the orientation of the collagen fibers [133].

In bioreactor culture the cell growth not only occurred in the surface of the scaffold but also within the scaffold pores (Figure 20B). After 14 days of bioreactor culture, the cell penetration reached 1000µm into the scaffold. This penetration was higher than the penetration observed in static cultures (500 µm). This higher penetration of the cells into the scaffold can be attributed to the effect of media perfusion in the scaffolds. Several studies have shown that cell seeding and cell penetration increase with perfusion [99,134].
Although we observe a smooth layer formation on the surface of the scaffolds and expression of ECM components within the scaffold, immunohistochemistry of the constructs revealed that this layer was a combination of collagen I and collagen II fibers. This combination of collagen is often associated with fibro cartilage and is usually found in tissue engineering cartilage made either with chondrocytes or with hMSCs [89,96,103]. This collagen I production can be also associated with an immature state of cartilage [96]. However we did not run long term experiments to evaluate whether or not a reduction in collagen I expression can be observed. Further studies need to be addressed to evaluate the effect of long term culture in the transition of collagen I production into collagen II in hMSCs cultured in bioreactor.

During the culture time, the pressure drop in the bioreactor increased from day 0 to day 4 (Figure 22A). After day 4 the pressure drop stabilized around 450 Pa. In preliminary studies with the bioreactor we observe that continuous exposure of PCL scaffolds to perfusion eventually wears the porous structure of the scaffolds and disrupts the delicate network structure of the scaffolds. In some scaffolds we observe this erosion then leads to complete channels generated by the flow perfusion in the scaffolds (Data not shown). This event might explain why even though the cells were growing on top of the scaffold and were filling the internal pores, the pressure drop kept relatively constant from days 5 to 14.

The glucose consumption rate was not constant throughout all the bioreactor culture (Figure 23B). From day 0-8 an average of $10 \times 10^6$ cells/bioreactor consumed approximately 125 mg of glucose in bioreactor 1 and an average of $9.1 \times 10^6$ cells/bioreactor consumed approximately 135 mg of glucose in bioreactor 2. By day 14 an
average cell number of $18.6 \times 10^6$ cells/bioreactor consumed only 105 mg of glucose in bioreactor 1 and an average of $19.0 \times 10^6$ cells/bioreactor consumed 119 mg. Although the cell number increased 1.86 fold from day 8 to day 14 in bioreactor 1 and 2.1 fold in bioreactor 2, the glucose consumption rate observed decrease in both bioreactors during the second time period (from day 8 to 14). This effect can be better observed with the glucose consumption rate in the bioreactor. During the first stage of culture (from day 1 to 8) the glucose consumption rate of the cells was relatively higher in both bioreactors ($1425 \text{ pg/(cell*day)}$ and $1231 \text{ pg/(cell*day)}$) than during the second stage of culture ($935 \text{ pg/(cell*day)}$ and $1041 \text{ pg/(cell*day)}$ respectively) (from day 8 to 14). This suggests a switch in the metabolic activity of hMSCs grown in the bioreactor. Some studies have shown that the metabolic rate of hMSC is dependent on the initial cell number [117]. Interestingly the same study found that glucose consumption rate of hMSCs is higher for lower cell densities than a higher cell numbers. The authors explain that one possibility for this behavior is the contact inhibition generated by either cell growth or ECM deposition. This ECM/cell growth generates isolated areas where the cells only consume the nutrients that are within that particular region therefore switching the metabolism to a low glucose consumption metabolism. However, we cannot use this explanation because clearly the bioreactor enhances nutrient diffusion through the constructs due to the perfusion. Another explanation might be related with differentiation of hMSCs into a less metabolic cell such as chondrocytes [135]. Therefore the use of glucose consumption rate might be used along with pressure drop as a control variable for tissue maturation.
4.4 Conclusion and Recommendations

The present work describes a methodology and bioreactor processing conditions for cartilage tissue engineering using a combination of micromass cell seeding, growth factors (TGFβ-1 and BMP-2), and bioreactor conditions to enhance tissue production in vitro. We designed and tested a rotating disc bioreactor for tissue cartilage engineering to stimulate hMSCs into chondrocytes. We found that this new bioreactor design is feasible and can be used for long term experiments using hMSCs.

In vitro production of cartilage is a complex process regulated by several cellular, biochemical, and mechano-biological factors. In this study we investigated a combination of several of these specific conditions believed to improve differentiation of hMSCs into cartilage tissue. The use of micromass as a cell culture technique plays an important role in cartilage differentiation. However some concerns about nutrient diffusion in high density culture testing 2 different cell concentrations for micromass formation. We found that when micromass bodies are created using 10 µl of a cell concentration of 8 X 10^6 a better response in the spreading area of the micromass is observed. Spreading or growth of the micromass has been related to better ECM production and differentiation. Although the measure of the spreading is an indicative of cell stimulation due to cell density and differentiation media, one of the limitations of these results is that we are measuring the 2D projection of a 3D event. Further studies are necessary to address the real change in volume in the micromass bodies.

When we tested micromass seeding condition in PCL scaffold and tested several combinations of growth factors; we found that one of the most efficient mechanisms to enhance chondrogenesis is using transient BMP-2 while applying TGFβ-1. This
combination of growth factors increased the GAG deposition in PCL constructs compared with all the other groups. Although we find that the PCL material that we use have an interference with the proliferation of the cells, this effect seems to be overcome after certain time of culture. One way to optimize this lag observed because of the use of PCL is by modifying the surface of the scaffold. This process can be implemented by either adding specific sequence group to the scaffold or by altering the hidrophobicity of the PCL by using plasma treatment.

When we tested the transient use of BMP-2 in the presence of TGFβ-1 in the shear stress and perfusion bioreactor we found that the use of the bioreactor and growth factors greatly enhances cell proliferation and matrix deposition as noted by specific cartilage markers such as GAG deposition and collagen II expression. However, one of the limitations of our experiment was the culture time. We only made 14 days experiments, further studies need to address the effect of long term culture in this specific bioreactor.

Cartilage production involves several bioprocessing conditions. For the bioreactor cultures we only carried out experiments using a fixed shear stress and a fixed perfusion rate that were extracted from the literature to prove the feasibility of GF in bioreactor culture. Some studies have demonstrated that tissue cartilage productions might be improved by the using of dynamic stimulation [89]. We did not test any dynamic stimulation in the bioreactor but future experiments might evaluate the appropriate conditions of shear stress and perfusion for cartilage production using hMSC. Due to the high sensitivity of hMSC to shear stress it is recommended to start with low levels of
shear stress in the range of 0.01 dyne/cm\(^2\) and perfusion in the range of 0.1 ml/min per construct and gradually increase those values [95].

We also did not carry on any long term experiment. The main goal of these experiments was to show the potential use of this new designed bioreactor and its reliability for cell culture. However tissue engineering cartilage requires long term experiments usually around 28 to 35 days to produce mature tissue suitable for implantation [29,89,96]. In addition to long term experiments, the bioreactor culture can also be implemented with low levels of oxygen. This condition seems to enhance the differentiation of hMSCs into cartilage by switching the metabolism of the cells via the hypoxia inducible factor (HIF-1\(\alpha\)) [136].

Future experiments with this bioreactor might address the use of dynamic stimulation in long term cultures using hMSCs. It also might be interesting the use of two culture stages, one stage for expansion of the cells in aseptic bioreactor conditions, and the second stage for differentiation. If this idea is implemented, is necessary to find the right mechanical conditions just for expansion and another set of mechanical stimuli conditions just for differentiation. One of the advantages of this design is that the tissue growth in the PCL scaffold can be monitored with the permeability values obtained by measuring the pressure drop in the bioreactor. An increase in pressure drop across the scaffolds likely indicates that more cells and ECM are deposited in the scaffolds. These changes in pressure drop can be correlated to different stages during tissues culture. For example, cells would be expanded until certain value of pressure drop is achieved, and then automatically the bioreactor could be switch from proliferative stage to
differentiation stage. The implementation of these control parameters will allow to closely monitoring the bioreactor conditions.

Another aspect of the bioreactor that might be addressed is the use of multiple chambers. This bioreactor might be developed as a series of stacked chambers that uses the same pump and with different media reservoirs. Therefore with this configuration, each chamber might be used for a different patient or to increase the overall production capacity of each batch. Using this type of configuration will help to reduce the operation cost of the bioreactor and facilitate the production of tissue.

In summary the present work has identified bioprocessing conditions using a combination of seeding techniques and cytokines in a shear stress and perfusion bioreactor. We demonstrate feasibility in our design and we found that it is possible to culture hMSC in this new bioreactor. Further experiments can be done to test long term cultures and modify the bioprocessing conditions to optimize the production of large scale tissues.
APPENDIX

DATE: 4/10/06

MEMORANDUM

TO:  Timothy Wick  
     Principal Investigator

FROM:  Sheila Moore, CIP  
        Director, IRB

RE:  Request for Determination—Human Subjects Research
     IRB Protocol #N080410006 – Expansion and differentiation of human mesenchymal stem cells in a perfusion and surface shear stress bioreactor for engineered tissue cartilage

An IRB Member has reviewed your application for Designation of Not Human Subjects Research for above referenced proposal.

The reviewer has determined that this proposal is not subject to FDA regulations and is not Human Subjects Research. Note that any changes to the project should be resubmitted to the Office of the IRB for determination.

SM/In
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