# NOTE TO USERS

# EVALUATION OF CHONDROGENIC DIFFERENTIATION OF HUMAN STEM CELLS DERIVED FROM ADULT BONE MARROW

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**ABSTRACT**

Tissue engineering of the intervertebral disc using mesenchymal stem cells (MSCs) induced to differentiate into a disc-cell phenotype has been considered as an alternative treatment for disc degeneration. Since it is not known how to differentiate stem cells into disc cells, our rationale is to differentiate stem cells into chondrocyte-like cells. This proposal is based on the fact that cartilage and immature nucleus possess similar macromolecules in their matrix. Our hypothesis is that these cells can produce a matrix that mimics native nucleus pulposus with properties resembling that found in healthy intervertebral disc. We used a pellet culture system to promote *in vitro* chondrogenesis of MSCs, in which the cells were cultured in defined chondrogenic medium and supplemented with TGF-β1 or TGF-β3, IGF-1, with or without dexamethasone. Markers of chondrogenesis include collagen type II and aggrecan, with collagen type X being used as a marker of late stage chondrocyte hypertrophy. The purpose of this study was to investigate the above growth factors on the chondrogenic differentiation pathway using these markers to follow cell differentiation. Our results show evidence of constitutive expression of aggrecan message and early expression of type X collagen message, surprisingly before the appearance of that for type II collagen. This raises the question whether they are good markers of chondrogenesis and chondrocyte hypertrophy, respectively, during MSC differentiation.

**RESUME**

Le génie tissulaire du disque intervertébral à l'aide de cellules souches mésenchymateuses qui sont induites à se différencier en phénotype qui ressemble à celui du disque est considéré comme une alternative au traitement courant pour la dégéneration du disque. Comme il n'est pas encore connu comment différencier les cellules souche en cellules du disque, notre but est d'abord de les différencier en cellules qui ressemblent à celles du cartilage. Cette supposition est basée sur le fait que le cartilage et le nucleus du disque immature possèdent des molécules en communs dans leur matrice. Notre hypothèse est que ces cellules différenciées peuvent produire une matrice qui ressemble à celle produite par nucleus avec des caractéristiques en commun avec un disque en santé. On a utilisé un système de culture cellulaire agrégée pour induire les cellules souches à la chondrogénèse. Dans ce système, les cellules étaient en culture dans un milieu chimiquement défini et supplémenté avec le TGF-β1 ou TGF-β3, IGF-1, avec ou sans dexaméthasone. Les marqueurs de chondrogénèse incluent le collagène de type II et l'aggrecan, avec le collagène de type X étant un marqueur de différentiation terminale. Le but de notre étude était d'évaluer les effets des différents facteurs de croissances sur la différentiation à la chondrogénèse en utilisant ces marqueurs comme guides pour suivre le processus de la différentiation. Nos résultats démontrent que le message de l'aggrecan est exprimé à travers toute la période de culture et que le collagène du type X apparaît très top en culture, avant même l'apparition du message pour le collagène du type II. Ces résultats nous amènent à questionner la validité de ces marqueurs pour la chondrogénèse et la différentiation terminale de chondrocytes, respectivement, durant la différentiation de cellules souches.

*To my parents, Jake,*
*and David*

## 1. CLINICAL RELEVANCE

Low back pain is a common medical and social problem in the adult population today, and is estimated as one of the most costly ailments in working-age adults (Frymoyer 1996). Although the etiology of low back pain is often unclear, it is believed that intervertebral disc degeneration plays a major role (Kraemer 1995). Disc degeneration is already seen in late teens, and after 60 years of age a completely normal disc is rarely found (Ishihara and Urban 1999).

Although the majority of those affected by lower back pain will not require prolonged medical care or absence from work, a considerable number will require extensive care involving hospitalization (Woolf and Pfleger 2003). Present management of disc pathology has been focused on symptoms associated with degeneration, and much less study has been devoted to disc regeneration. Conservative therapy is largely based on anti-inflammatory oral medication, bed rest, and physiotherapy to relieve pain (Wunschmann *et al.* 2003). Should conservative therapy not relieve pain, surgery is often considered. Surgical treatments to treat low back pain associated with lumbar degenerative disease include disc excision and vertebral fusion (Abild *et al.* 1990; Abramovitz and Neff 1991). Although these treatments offer good short-term clinical result in relief of pain, they alter the biomechanics of the spine, leading to further degeneration of surrounding tissue and disc at adjacent levels. Clinical and radiological evidence shows that spinal fusion leads to the narrowing of the disc space and accelerated degeneration of adjacent motion segments and early failure of this treatment (Abramovitz and Neff 1991). The failure rate for lumbar fusions is 20% to 40% after five years (Snider *et al.* 1999). Surgical removal may therefore not always relieve the patient's complaints.

There has recently been increased focus on regeneration of the disc as opposed to pain management of low back pain associated with degenerate disc disease. Biological repair is therefore being considered as an alternative.

## 2. INTRODUCTION

### 2.1. The Intervertebral Disc

The human spine is divided into several sections (Figure 1): 7 cervical vertebrae, which make up the neck, 12 thoracic vertebrae comprise the chest section and have ribs attached to them and 5 lumbar vertebrae, which lie below the last thoracic bone and the top of the sacrum. The sacral vertebrae are enclosed within the bones of the pelvis, and the coccyx represents the terminal vertebrae.

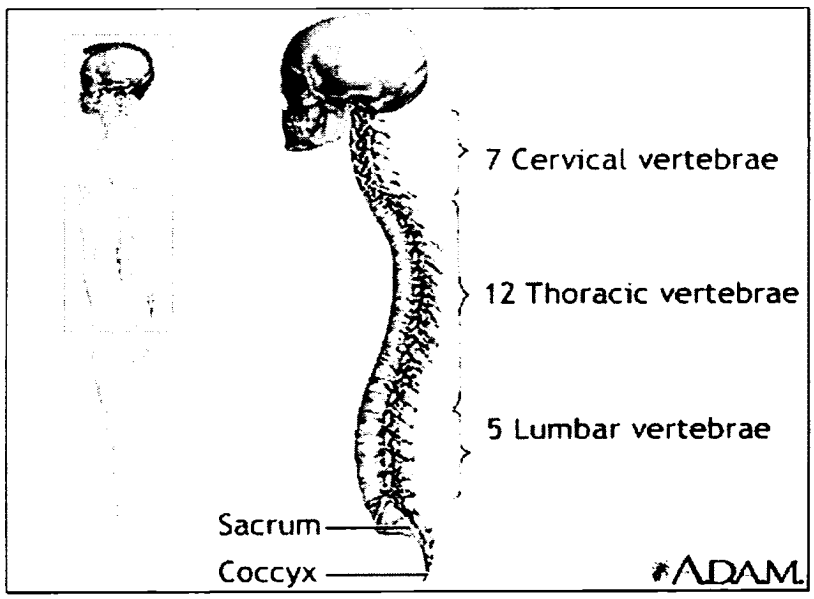


Figure 1. Structure of the human spine illustrating the major vertebral sections (National Institute of Health 2004).

Intervertebral discs extend from the neck to the sacrum and act as elastic cushions between the vertebral bodies (Figure 2). They stabilize the spine by anchoring adjacent vertebral

bodies to each other (Buckwalter 1995). End plates composed of hyaline cartilage separate the intervertebral disc from the vertebral body. Intervertebral discs provide the flexibility required for bending and twisting of the spine, while resisting compression inflicted by gravity in an upright posture by distributing loads applied to the spine (Aguiar *et al.* 1999). They are able to perform these functions because of their unique design.

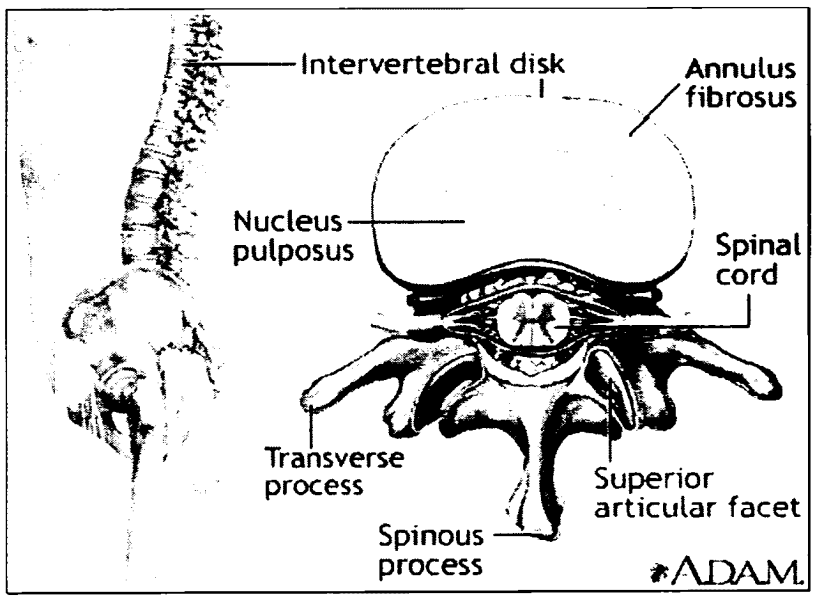


Figure 2. Location of the intervertebral disc between the vertebrae of the spine (National Institute of Health 2004).

### 2.2. Disc structure and function

The mature intervertebral disc consists of two major regions: the annulus fibrosus and the nucleus pulposus. The soft, pulpy nucleus is filled with a gelatinous material, and occupies the central part of the disc. It is surrounded by the annulus, which is made up of concentric collagenous lamellae (Figure 3). The annulus links the vertebral bodies together via their

cartilage endplates (Hayes *et al.* 2001). The lamellae in the inner region of the annulus are widely spaced. The tissue there is firmer and less hydrated than the nucleus and appears fibrocartilagenous. The outer annulus has a more fibrous appearance, with more densely packed lamellae. It is the least hydrated region of the disc (Horner *et al.* 2002). The collagen fibres of the annulus are parallel within a lamella but successive lamellae have collagen fibres at pronounced angles to one another, forming a radial ply structure (Hayes *et al.* 2001). The result is that the annular fibres form an intricate crisscross arrangement that can rotate in either direction and flex before the fibrils come into tension (Adams 1988). The structure of the annulus allows flexion and twisting of the spine. The nucleus is less organized, and exposed to compressive gravitational and muscular loads, some of which it transfers to the inner part of the annulus (Hayes *et al.* 2001). These two structures interact together to withstand the compressive forces placed on the spine (Adams 1988).

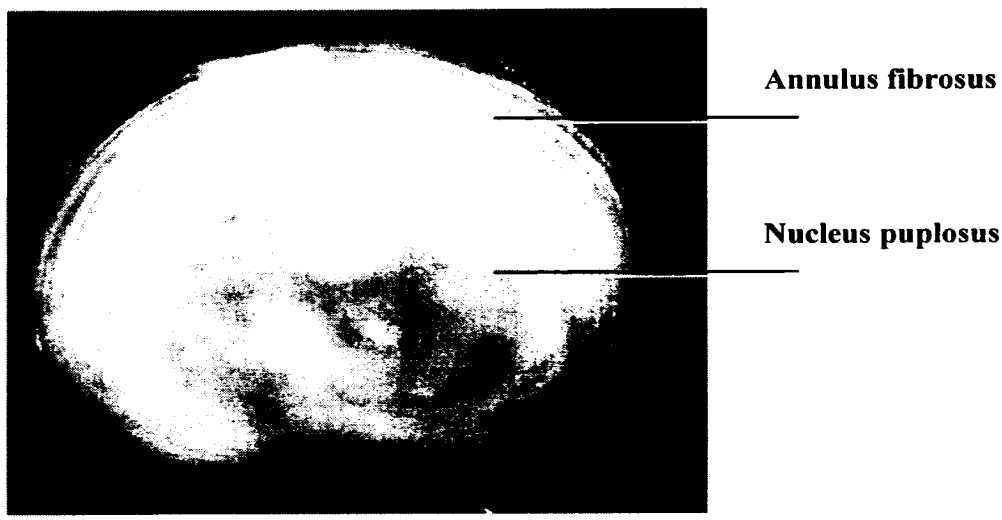


Figure 3. Section of an isolated bovine intervertebral disc showing the lamellar structure of the annulus and the gelatinous nucleus (Modified from Mauro Alini).

The disc is thought to resist compressive forces by a swelling pressure that exerts tensile forces on the fibrous annulus (Humzah and Soames 1988). During compressive loading, the stress is transferred from the vertebral end plates to the intervertebral disc. This compression increases pressure in the nucleus, and the fluid exerts hydrostatic pressure on the annulus. The central portions of the vertebral end plates are pushed away from each other, and the annular bands are pushed radially outward. The bulging annular fibres develop tensile stress in all directions.

### 2.3. Biochemical composition

The biochemical composition of the mature intervertebral disc has been extensively studied (Eyre and Muir 1976; Eyre 1979; Oegema 1993). It contains various amounts of collagens, proteoglycans, and glycoproteins. Collagens and proteoglycans constitute the two major structural components of the disc (McDevitt 1988). The collagen fibers give the tissue its tensile strength. They are a strong, durable framework, which supports the cells and confine the proteoglycan aggregates (Hukins 1988). The proteoglycans, through their interactions with water, give the tissue stiffness and ability to resist compression. The proportion and organization of these two structural molecules varies considerably across the disc (Eyre *et al.* 1989).

Collagens account for as much as 70% of the dry weight of the annulus, but less than 20% of the dry weight of the nucleus (Buckwalter 1995). At least seven distinct collagen types have been identified in the intervertebral disc: types I, II, III, V, VI, IX and XI (Eyre *et al.* 1989). Type I and II collagens are most important for the integrity of the disc, as they constitute about 80% of its collagen content (Eyre 1988). The outer part of the annulus is

rich in type I collagen, typical of fibrous, tension resisting tissues (Hayes *et al.* 2001). The concentration of type I collagen progressively falls toward the center of the disc, with no type I collagen present in the nucleus (Cassinelli *et al.* 2001). Type II collagen follows an opposite trend and increases toward the center. The nucleus is rich in type II collagen which is typical of cartilaginous, compressive resisting tissues (Hayes *et al.* 2001). The inner annulus has characteristics of both tissues since it experiences both tension and compressive loading (Eyre and Muir 1976; Eyre 1988). Collagen types III, V, VI, IX and XI are also present in small quantities in the disc, and play a role in collagen fibril organization (Cassinelli *et al.* 2001). In addition, type X collagen has been shown to be present in discs with histomorphological alterations consistent with disc degeneration (Aigner *et al.* 1998).

Proteoglycans account for only a few percent of the dry weight of the outer annulus, and about 50% of the dry weight of the nucleus (Buckwalter 1995). The ability of the disc tissue to resist compressive forces is largely due to their high content of the proteoglycan aggrecan, a molecule that is also a functionally important component of articular cartilage (McDevitt 1988). Aggrecan consists of a core protein with several distinct domains, which have either globular or extended conformations (Figure 4). Three globular domains are present on the newly synthesized aggrecan; two at the N-terminus G1 and G2, and one at the C-terminus, G3. G1 and G2 are two structurally related globular domains separated by a short extended region known as the interglobular domain (IGD), whereas the G2 and G3 domains are separated by glycosaminoglycan (GAG)-attachment domains bearing predominantly keratan sulphate (KS) or chondroitin sulphate (CS) chains. These GAG chains have a high charge density, which results in a high degree of hydration for the

proteoglycan aggregate (Sztrolovics *et al.* 1997). In the disc matrix, many aggrecan molecules can bind to a single hyaluronate chain via the G1 domain, producing large proteoglycan aggregates with each interaction being stabilized by the further interaction of a link protein (Oegema *et al.* 1979). The disc also contains versican, a large chondroitin sulphate proteoglycan belonging to the aggrecan family (Sztrolovics *et al.* 2001). In addition to aggregating proteoglycans, the disc also contains decorin, biglycan, fibromodulin and lumican (Johnstone *et al.* 1993; Sztrolovics *et al.* 1999). The compression-resisting properties of the disc proteoglycan depend on the high concentration of the aggregates, their large size and their GAG content (Roughley *et al.* 2002).

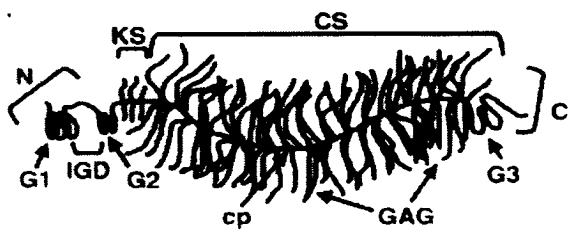


Figure 4. Structure of aggrecan: N, amine-terminal; G1, G2, G3, globular domains; IGD, interglobular domain between G1 and G2; cp, core protein; KS, keratin sulfate region; CS, chondroitin sulfate region; GAG, glycosaminoglycan chains; C, carboxyl-terminal (modified from Ng *et al.* 2003).

### 2.4. Extracellular matrix

Discs are characterized by their abundant extracellular matrix and low cell density. The cells synthesize the macromolecules of the matrix and then maintain and repair this framework (Buckwalter *et al.* 1993). Because of this, the profiles in extracellular matrix

composition across the disc presumably arise from differences in cellular activity. These differences may be due to the cells' extracellular environment in different regions of the disc (Horner *et al.* 2002). In particular, exposure to mechanical stress varies across the disc. The cells of the nucleus and the inner annulus are exposed to high hydrostatic pressure (McNally and Adams 1992). The cells of the outer annulus undergo tensile strains during flexion, extension or torsion of the disc (Stokes 1987). These signals have been found to have a powerful influence on matrix synthesis and turnover (Handa *et al.* 1997). The concentration of metabolites also varies across the disc (Horner *et al.* 2002). The disc relies on the movement of small solutes from the blood through the disc matrix by diffusion. The resulting concentration gradient depends on the balance between the rate of transport through the matrix and the rate of consumption by the cells (Urban 1990). For solutes such as $O_2$, whose rate of consumption is high relative to the rate of transport, steep concentration gradients develop. The oxygen levels in the center of the nucleus of humans have been measured to be as low as 0.7% in the adult lumbar and thoracic discs (Ishihara and Urban 1999). These steep oxygen concentration gradients in the disc lead to high lactate concentrations in the nucleus, a result of anaerobic metabolism. Consequently, this acidifies the matrix and lowers the pH values (Holm *et al.* 1981). As will be discussed in the following sections, these factors can strongly influence cellular metabolism.

### 2.5. Intervertebral disc degeneration

Discs are subject to injury, disease, and degeneration with use over time. Disc degeneration is progressive and almost universal in the human spine (Kraemer 1995). The intervertebral disc degenerates much earlier than other load bearing tissues such as cartilage (Ishihara and Urban 1999). With increasing age, discs undergo striking alterations in volume, shape,

structure and composition that decrease motion and alter spine biomechanics (Buckwalter 1995). The result is the destruction of annular structure and flattening of the disc (Osti *et al.* 1992). The frequency of spine-related pain also changes with age, being the most common cause of impairment for middle aged and older people. The relationship of these clinical problems to the age-related deterioration of the intervertebral discs remains unclear. Multiple factors contribute to disc degeneration including genetic factors, environmental factors and aging (Buckwalter 1995). Discs have a limited ability to regenerate because they are relatively hypovascular and nutritionally dependent by diffusion at the endplates (Lipson and Muir 1981). Once the degenerative process has started, it is difficult to decelerate and is ultimately considered to be an irreversible condition (Nishida *et al.* 1999).

All discs eventually develop similar age-related changes, but within the same person and among persons they vary in rate and extent. In some persons degeneration of one or more discs advances more rapidly than the changes in their other discs, or in the discs of other persons of the same age (Buckwalter 1995). These changes may decrease spinal mobility and lead to disc herniation and degeneration of the facet joints. Herniation occurs when part of the soft material of the disc pushes out through a tear or weakening in the outer annulus, and pinches on the nerve roots, resulting in pain (Figure 5). Excessive mechanical loads can also produce a herniation in the disc (Goupille *et al.* 1998)

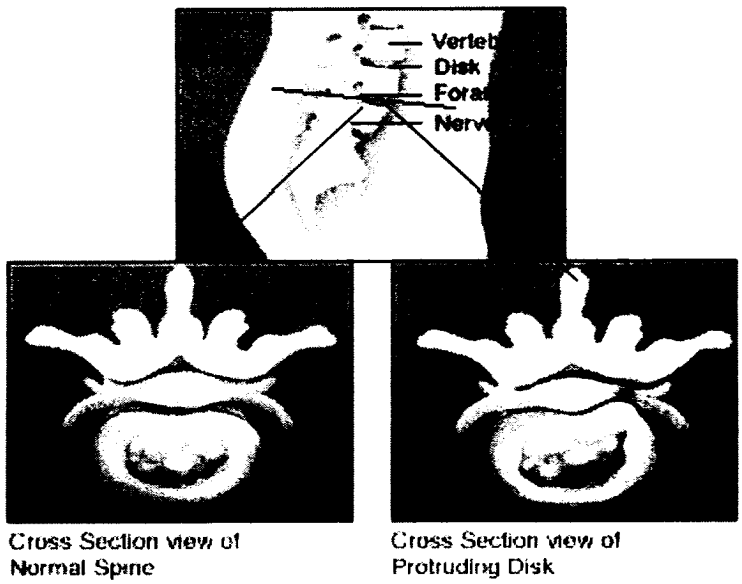


Figure 5. Disc herniation. Annular tears allow the nucleus to leak out and cause pain when it touches the nerve root (American Academy of Orthopaedic Surgeons 2004).

Thompson and group developed a five-category grading scheme for assessing the gross morphology of midsagittal sections of the human lumbar intervertebral disc (Figure 6). The classification goes as follows: Grade I is described as have a bulging gel in the nucleus and discrete fibrous lamellae in the annulus. In Grade II, there is fibrous tissue that periphally forms in the nucleus and there is appearance of mucinous material between lamellae. The hyaline cartilage in the endplate starts to have an irregular thickness. Grade III nucleus has a consolidated fibrous tissue, and in the annulus there are extensive mucinous infiltrations, as well as a loss of annular-nuclear demarcation. In the Grade IV nucleus, horizontal clefts start to and there are focal disruptions in the annulus. The endplate has fibrocartilage extending from the subchondral bone. Finally, Grade V is characterized by extensive clefts through the nucleus and annulus (Thompson *et al.* 1990). This sequence of events results in

progressive loss of normal tissue structure, composition, and biologic and mechanical function of the disc. Two characteristics of disc degeneration were considered in the evaluation of this grading scheme developed by Thompson: the frequency of disc degeneration increases with age, and the discs of the lumbar spine do not show severe degeneration until most of the proteoglycan is lost (Pearce *et al.* 1987). The proteoglycan is lost from the disc between grade I and III (Thompson *et al.* 1988; Eyre *et al.* 1989). On this basis the majority of discs in the mature adult fall in the grade III-IV category.

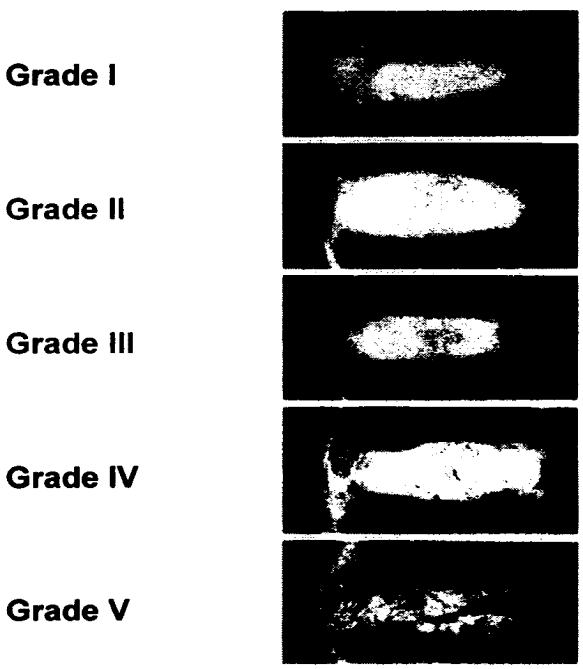


Figure 6. Thompson grading scheme for disc degeneration. Diagram illustrates grades I-V of the progressive degeneration of the human intervertebral discs. Modified from Thompson *et al.* 1990.

## 2.6. Biochemical alterations

The earliest and most extensive biochemical changes in disc occur in the nucleus, where the first known alteration includes fragmentation of proteoglycan by proteinases, followed by a decrease in water content associated with proteoglycan diminution of the nucleus pulposus and inner annulus (Pearce *et al.* 1987). Loss of these large molecules reduces the amount of hydration in the disc, changing its volume, and affects its ability to absorb and distribute load effectively. Aggrecan undergoes proteolytic cleavage at a number of different sites within the molecule. However, the most damaging site for cleavage is within the IGD domain, as this results in the loss of all the GAG-attachment regions (Figure 7). This cleavage results in the accumulation of free G1 domains in the aging disc. The proteinases that have been found to have major involvement in cleaving within the IGD domain are the matrix metalloproteinases and aggrecanases (Sztrolovics *et al.* 1997).

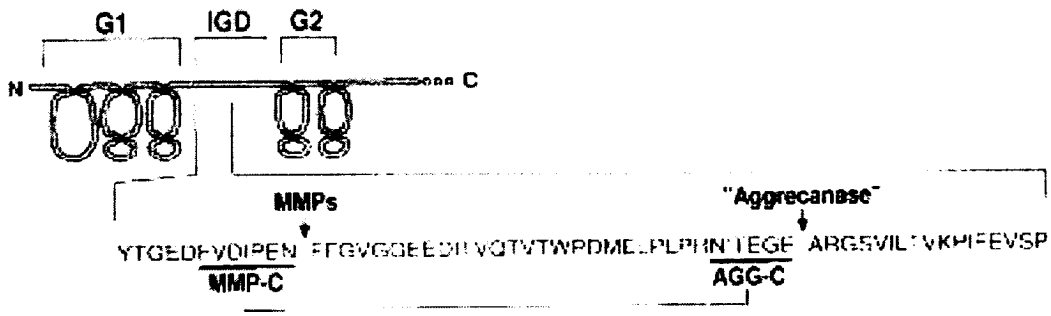


Figure 7. Proteolytic cleavage within IGD domain of the aggrecan protein. Diagram depicts the structure of the N-terminus of the aggrecan core protein. The amino acid sequence of part of the IGD is shown below the diagram. The cleavage sites within the IGD domain by matrix metalloproteinases (MMPs) and aggrecanase are indicated. Modified from Sztrolovics *et al.* 1997.

These alterations in proteoglycan structure begin early in life, years before the age-related changes in disc morphology (Buckwalter *et al.* 1989; Buckwalter *et al.* 1994). With increasing age, the proportion of nonaggregated proteoglycans progressively increases and the size of proteoglycan molecules decreases dramatically (Buckwalter 1995). Because of the important role of aggrecan in conferring the disc its mechanical properties, proteolytic degradation during aging might alter tissue function. This might be due to aggrecan depletion from the tissues, as well as accumulation of degradation products that may compete with newly synthesized molecules for space within the matrix. The functional properties of the disc may be altered, which in turn may predispose the tissue to age-related degeneration (Sztrolovics *et al.* 1997). Although these changes begin during growth and development, they may form the changes that occur subsequent to skeletal maturity (Buckwalter 1995).

Studies have shown that there are few changes in collagen content with age (Lyons *et al.* 1981). However, collagen cross-links formed by enzymatically mediated glycosylation, which are believed to play an important role in the normal functioning of collagen, decrease with age (Pokharma and Phillips 1998). There is also an increase in the amount of nonenzymatically mediated cross-links, and this may contribute to degeneration by making the tissue more susceptible to mechanical failure (Monnier *et al.* 1984).

The end result of these alterations in proteoglycan and collagen is a decrease in the proteoglycan/collagen ratio of degenerated discs, particularly in the nucleus, where much of the proteoglycan is lost (Pearce *et al.* 1987). What results with skeletal maturity, is an increasingly fibrotic nucleus that exhibits altered load-bearing capabilities.

## 2.7. Factors involved in disc degeneration

Early disc degeneration might be due to the absence of blood vessels and the decrease in cell nutrition (Kraemer 1995). These changes alter matrix biomechanics, and as a consequence lead to the later age-related changes. Other mechanisms that may accelerate or contribute to normal age-related deterioration include loss of notochordal cells, loss of viable cells, cell senescence, protein modification, accumulation of degraded matrix molecules, and fatigue failure of the matrix (Buckwalter 1995).

Decreased cell nutrition has been implicated as the critical event responsible for disc degeneration (Nachemson *et al.* 1970). Disc cells rely on the diffusion of nutrients through the matrix from blood vessels on the periphery of the annulus and within the vertebral bodies (Buckwalter *et al.* 1993). Nutrients such as glucose and oxygen required for energy metabolism or amino-acids necessary for protein synthesis diffuse through the disc matrix to the cells under the concentration gradients set up by cell metabolism (Urban *et al.* 1982). Transport of these molecules through the matrix depends on the composition and organization of the macromolecule framework, and the matrix water content, which is largely determined by the proteoglycan concentration (Buckwalter *et al.* 1993). Factors that may impair delivery of nutrients include age-related decline in the number of arteries supplying the periphery of the disc and possibly the calcification of the cartilage endplates with age (Eyre *et al.* 1989; Buckwalter *et al.* 1993). Factors such as smoking may also influence peripheral blood circulation and can also decrease nutrient transport into the central disc (Ishihara and Urban 1999). As the blood supply to the periphery declines, the accumulation of degraded matrix macromolecules and decreasing water concentration may interfere with diffusion through the matrix, further compromising cell nutrition.

The decrease in cellular nutrition has an effect on various aspects of cellular metabolism. As mentioned previously, steep oxygen concentrations develop in the disc, leading to high lactate concentrations (and low pH values) in the nucleus. The low oxygen concentrations affect cellular metabolism directly by decreasing proteoglycan synthesis, particularly in the nucleus (Ishihara and Urban 1993). The resulting high lactate levels (a result of the increased lactate production and decreased rate of lactate removal) and low pH values, which have been found in degenerated disc surgical specimens (Cassinelli *et al.* 2001), also could affect cellular metabolism (Ohshima and Urban 1992). Inadequate nutrient supply can also threaten disc cell survival (Horner and Urban 2001).

However, even if nutrient supply is sufficient to maintain viability, it might not be adequate to support matrix production. Cells may be able to survive under low levels of oxygen or relatively low pH, but proteoglycan synthesis falls steeply (Ishihara and Urban 1999). This can eventually lead to disc degeneration due to the fall in proteoglycan concentration (Thompson *et al.* 1990). Nutritional factors therefore do not have to cause cell death to lead to disc degeneration (Horner and Urban 2001).

During skeletal growth, the number of notochordal cells decreases and chondrocyte-like cells appear in the central regions of the disc (Roberts *et al.* 1991). At about this time, morphological signs of degeneration can be seen (Butler *et al.* 1990). The loss of notochordal cells may therefore signal the alteration of the nucleus matrix from its normal gelatinous structure to a degenerative fibrotic structure (Aguiar *et al.* 1999)

The cells of the disc have the role of maintaining its health because they make and maintain the extracellular matrix. Newly synthesized molecules replace the older ones, which are enzymatically degraded in the matrix. However, with aging, the accumulation of partially degraded molecules may alter properties of tissue due to proteolysis and accumulation (Buckwalter 1995). It may also prevent turnover and replacement (Roughley *et al.* 1985).

Even without an alteration to cell nutrition, cells may become senescent with increasing age. They remain viable, but lose their capacity to replicate DNA and their capacity to synthesize new matrix molecules. Experiments have shown that these changes result from changes in gene expression, and that transcription factors control these age-related changes (Buckwalter 1995).

Another important factor that may play a role in disc degeneration is the alteration of the already existing extracellular matrix of the nucleus. The loss of elasticity and strength of the intervertebral disc matrix may result from postsynthetic modification of the protein components of elastin, proteoglycan and especially collagen macromolocules in the matrix. The changes in collagen cross-links that occur through nonenzymatic glycation or lipid peroxidation may alter collagen properties such as solubility and mechanical strength, and contribute to tissue degeneration (Monnier *et al.* 1984). In addition to their potential effects on the mechanical properties of the disc, glycation products can also stimulate cells, including chondrocytes, to release cytokines and proteases that contribute to tissue degeneration (Buckwalter *et al.* 1993).

The effect of mechanical loads on the intervetebral disc matrix may also cause degeneration. Loading and deformation of discs are normal spinal movements that are followed by recovery of disc shape. In the upright and sitting positions, disc height decreases due to water being forced out of disc matrix. Prolonged recumbancy then restores the original disc volume and shape as water and nutrients return to the matrix (Kraemer 1995). These repetitive deformations may lead to fatigue failure of the matrix. Failure of the matrix may appear as fissures, cracks or more subtle changes such as fragmentation of proteoglycans and disruption of collagen fibrils and their interaction with other matrix macromolecules. As a consequence, the cells may be exposed to increased loads that compromise their function. Disc matrix synthesis rates decrease abruptly if the tissue either swells excessively or loses fluid (Bayliss *et al.* 1986). Static loads on the disc result in fluid extrusion, which depends on the size of the load and the composition of the tissue (Urban and McMullin 1988). Age-related changes may make the tissue less able to recover from deformation and more vulnerable to fatigue failure of the matrix (Buckwalter 1995).

### 2.8. Disc repair

Despite the degenerative changes of the intervertebral disc, repair of the tissue may be possible. As an alternative to surgical removal for symptomatic herniation of the intervertebral disc, the use of protease has been proposed. It has been used extensively throughout North America and Europe in the treatment of lumbar-disc disease (McCulloch 1980). This procedure, termed chemonucleolysis, consists of a chemical dissolution of the intervertebral disc by chymopapain. The chymopapain rapidly degrades the proteoglycans of the disc producing small proteoglycan fragments, increases the permeability of the annulus and endplates, and allows the rapid loss of proteoglycan. The water-binding

capacity of the disc is lost, reducing disc height and intradiscal pressure and hence pressure on the nerve root (Bradford *et al.* 1983). Over the several months after injection, there is a reconstitution of disc height (Garvin *et al.* 1977). Studies have shown that the restoration of disc height is due to the replacement of the matrix, with proteoglycan similar to that of normal nucleus pulposus (Bradford *et al.* 1983). Although results have suggested that the injection of this enzyme into the intervertebral disc may alter the course of lower back pain, there has been considerable discussion on the merits of this procedure. Alternative treatments are therefore needed.

### 2.9. Tissue engineering of the intervertebral disc

Studies have increasingly been focused on biological repair. Using an experimental animal model, Nishimura and Mochida found that reinsertion of autogenous nucleus pulposus cells into a degenerated disc results in a retardation of degeneration (Nishimura and Mochida 1998). However, this proved to be problematic because autogenous transplantation requires more cells than can be harvested from a single intervertebral disc, since the cell cultures of nucleus cells alone have poor viability. Okuma and group advanced the efficacy of this procedure by activating the biochemical properties of the reinserted nucleus pulposus cells by using a nucleus/annulus coculture method (Okuma *et al.* 2000). They found that coculture with annulus cells resulted in enhanced proliferation of nucleus cells, which was reflected by an acceleration of DNA synthesis. Preliminary evidence presented showed that reinsertion of nucleus cells activated by coculture may retard disc degeneration.

From these studies, the concept of utilizing the contained structure of the disc for cell transplantation in attempt to treat disc degeneration was explored by Sakai and group

(Sakai *et al.* 2003). They hypothesized that maintenance of the proteoglycan content in disc is achieved by avoiding the depletion of nucleus pulposus and preserving the structure of the annulus is a primary factor in decelerating disc degeneration. In this study, they embedded autologous mesenchymal stem cells embedded in Atelocollagen ® gel and transplanted them into the discs of rabbits which have undergone a procedure that induces degeneration (Lipson and Muir 1981; Okuma *et al.* 2000). Their results suggest that mesenchymal stem cell transplantation is effective in decelerating degeneration in experimental models. Atelocollagen ® permitted proliferation, matrix synthesis and differentiation of stem cells. The cells differentiated to spindle-shaped cells arranged in longitudinal layers, which resembled original disc cells. Extracellular matrix was suspected to originate from stem cells and stained for proteoglycan with Safranin-O, as well as with immunohistochemical staining. This transplantation was also effective in preserving annular structure.

### 2.10. Mesenchymal stem cells

In recent years, there has been an increased interest in the biology of adult-derived stem cells. This interest was partly sparked by the controversy surrounding the potential use of embryonic stem cells in the treatment of human diseases (Jackson *et al.* 2002). Stem cells are defined as progenitor cells capable of self-renewal and multilineage differentiation (Caplan 1991). At least two stem cell populations reside within the bone marrow: hematopoetic stem cells (HSCs) and mesenchymal stem cells (MSCs). HSCs give rise to all of the differentiated blood lineages, whereas MSCs give rise to the stromal cells of the marrow. Mesenchymal stem cells represent 2-3% of the total mononuclear cells of the

marrow (Majumdar *et al.* 2000). MSCs are thought to be multipotent cells (Figure 8), that have the capacity to differentiate into a variety of connective tissue types including osteogenic (Jaiswal *et al.* 1997), chondrogenic (Johnstone *et al.* 1998; Mackay *et al.* 1998; Barry *et al.* 2001) or adipogenic (Pittenger *et al.* 1999) lineages when placed in the appropriate culture conditions.

In culture, MSCs can be isolated from HSCs and other cells of the bone marrow by their tendency to adhere to tissue culture plastic and their prolonged proliferative ability (Haynesworth *et al.* 1992; Pittenger *et al.* 1999). In monolayer cultures, the cells display a spindle-like formation, and maintain a similar morphology with passage (Majumdar *et al.* 2000). Monoclonal antibodies to CD73 and CD105 have been shown to recognize cell surface molecules on MSCs (Barry *et al.* 1999; Barry *et al.* 2001a). CD73 is a membrane-bound 5' nucleotidase, a molecule present on many cell types. CD105 has been identified as endoglin, a soluble TGF-β receptor, commonly associated with endothelial cells. In contrast, MSCs have been found to lack expression of hemapoetic markers such as lipopolysaccharide receptor CD34, and the leukocyte common antigen CD45 (Pittenger *et al.* 1999).

MSCs can be isolated using standard techniques and expanded in culture through many generations while retaining their ability to differentiate when exposed to the appropriate conditions (Barry *et al.* 2001). These properties represent a potentially powerful tool in tissue engineering for the development of new therapeutic strategies for the repair of various tissues damaged as a result of trauma or disease (Pittenger *et al.* 1999).

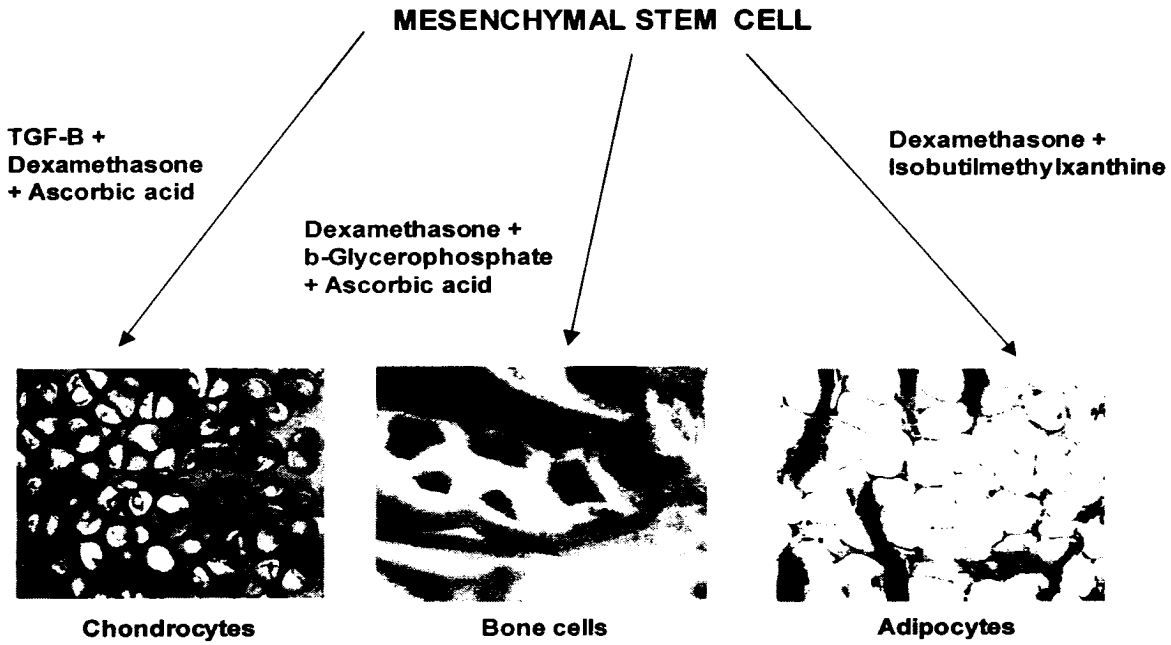


Figure 8. Schematic representation of differentiation potential of MSCs to various connective tissue lineages. Tissues were all stained with Ehrich's H&E. Cartilage stains dark purple, and lacunae are clearly visible (left), calcium deposits of bone stain light purple (middle) and adipose tissue is evidenced by its lipid globules (right). Modified from Deans *et al.* 2000.

### 2.11. Potential of MSCs for tissue repair

The properties of MSCs have opened up therapeutic opportunities for the treatment of defects in articular cartilage, among many others (Caplan *et al.* 1997). MSCs have been successfully used to engineer cartilage both *ex vivo* and *in vivo* with the goal of resurfacing damaged joints with articular cartilage or tissue reconstruction of the nose, ear, or trachea (Javazon *et al.* 2004). Articular cartilage damaged by trauma or disease has a limited ability for repair due to the lack of vascular supply and a dense extracellular matrix sparsely embedded with chondrocytes (Majumdar *et al.* 2000). Transplantation using autologous

chondrocytes to resurface joint cartilage is limited by the lack of suitable donor sites where large cartilage samples could be harvested and by their limited ability for *in vitro* expansion (Bosnakovski *et al.* 2004). Therefore, MSCs that can differentiate into chondrocytes are an attractive alternate source for repairing cartilage defects (Prockop 1997).

Despite the fact that the cells of the nucleus pulposus possess a chondrocyte-like phenotype (Horner *et al.* 2002), as previously mentioned, the association between disc cells and MSCs has not been defined in the literature. However, since it is not yet known how to differentiate stem cells into authentic disc cells, one would like to differentiate stem cells into equivalent chondrocyte-like cells, in order to test the hypothesis that these cells can produce a matrix that mimics native nucleus pulposus of a healthy disc. This is not an unreasonable supposition as cartilage and immature nucleus possess similar macromolecules in their matrix, but differ in their relative amounts.

### 2.12. Factors promoting chondrogenic differentiation

For inducing differentiation, MSCs have to be cultivated under appropriate culture conditions and stimulated with some bioactive factors. Growth factors are polypeptides that can serve as signaling molecules for cells. These small proteins can stimulate or inhibit functions inside the cell. Growth factors are the principal effectors of critical functions, such as cell division, matrix synthesis, and tissue differentiation. Many growth factors, including transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF), and insulin-like growth factor-1 (IGF-1) have been evaluated for their potential to enhance chondrogenesis (Mastrogiacomo *et al.* 2001).

The TGF-βs are a family of polypeptide growth factors that have many isoforms. Members of the TGF-β family have been found to play a major role in bone and cartilage development (Barry *et al.* 2001). TGF-β has been shown to stimulate collagen type II and proteoglycan expression in MSCs (Johnstone *et al.* 1998; Barry *et al.*2001). TGF-β isoforms have been found to differ in their effects on chondrogenic differentiation (Barry *et al.* 2001). While TGF-β has been shown to enhance chondrogenesis, IGF-1 has been shown to enhance extracellular matrix synthesis by MSCs (Worster *et al.* 2000). Similarly, bFGF has been found to have an effect on the chondrogenic potential of adult MSCs. Studies have found that bFGF maintains human MSCs in an immature state allowing their *in vitro* expansion and their maintenance as chondro-osteo-progenitor cells (Mastrogiacomo *et al.* 2001). It selects for the survival of a particular subset of cells enriched in pluripotent mesenchymal precursors and is useful in obtaining a large number of cells with preserved differentiation potential for mesenchymal tissue repair (Bianchi *et al.* 2003).

The induction of chondrogenesis requires particular culture conditions. *In vitro* chondrogenesis has been demonstrated by using a high-cell density pellet culture system (Johnstone *et al.* 1998; Mackay *et al.* 1998; Barry *et al.* 2001). Chondrogenesis is initiated as the densely packed precursor cells form appropriate cell-cell contacts (Tacchetti *et al.* 1992). The optimization of an *in vitro* culture system was formed on the basis of the understanding of *in situ* chondrogenesis. During embryonic development, MSC condensations provide physical and biochemical environmental factors conducive for cartilage formation (Johnstone *et al.* 1998). This pellet culture system is an adaptation to one that was previously described as a method to maintain the differentiated phenotype of

chondrocytes *in vitro*. Prerequisites of cell density and three-dimensional format are reflected in the physical culture formats used to induce differentiation of MSCs. Many micromass culture systems have been used to induce MSC chondrogenesis, including agarose, alginate beads, alginate layers and pellet culture systems.

Other factors have been found to have an effect on the differentiation pathway of these cells. The cells' environment plays a very important role. The chondrogenic differentiation pathway also needs a serum-free chemically defined medium (Lennon *et al.* 1995) to which are added certain bioactive factors in addition to the growth factors, such as dexamethasone (Dex). Dex is a glucocorticoid that conditions the differentiation of human bone marrow stromal cells to osteoblast-like cells in the appropriate conditions. Dex induces expression of alkaline phosphatase and mineralization in human MSCs (Cheng *et al.* 1994). However, the addition of Dex has also been reported important for the chondrogenic differentiation of MSCs (Mackay *et al.* 1998; Barry *et al.* 2001).

The sequential events leading from an undifferentiated stem cell with a fibroblastic morphology to a mature chondrocyte were investigated by the analysis of key matrix components. The rapid biosynthesis of GAG, the deposition of an integrated extracellular matrix that includes type II collagen and aggrecan expression, and the chondrocytic appearance of the cells have been used as benchmarks to signify that the chondrocyte phenotype has been achieved (Johnstone *et al.* 1998; Pittenger *et al.* 1999; Barry *et al.* 2001).

## 3. RATIONALE AND OBJECTIVES

The integrity of the gelatinous matrix of the disc is essential for the load bearing function of the disc (Aguiar *et al.* 1999). Because it would be technically difficult to regenerate a whole disc, the rationale at this point would be to regenerate the nucleus pulposus of the degenerate disc. Since it is not known how to achieve a nucleus cell phenotype, the objective at this point is to differentiate MSCs into chondrocyte-like cells, and test the hypothesis that these cells can produce a matrix that would have similar functions to the nucleus pulposus based on its production of matrix molecules (Figure 9). However, as attempts at chondrogenic differentiation produced unexpected results, demonstrated in the following sections, only objective 1 was studied.

**Specific objectives:**

(1) To investigate the effects of different factors on the chondrogenic differentiation pathway of isolated MSCs.

(2) To then modulate these cells into nucleus-like cells.

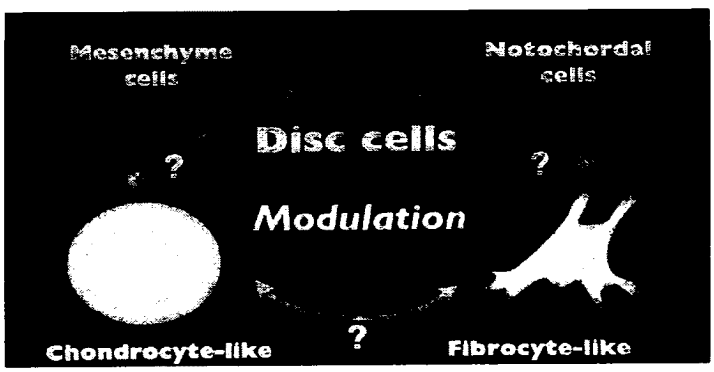


Figure 9. Schematic representation of the modulation of MSCs to the desired nucleus cell phenotype. Modified from National Institute of Health 2003.

## 4. METHODS

### 4.1. Source of stem cells

Human stem cells were obtained from 10 to 35 ml bone marrow (BM) aspirates from the femoral intramedullary canal of donors undergoing total hip replacement, using a protocol approved by the Research Ethics Committee of the Jewish General Hospital. The marrow donors included both males and females ranging in age from 38 to 89 years (Table I).j

**Table I.** Isolated stem cells from human donors (n=31).

| Age/Gender | Pass # | Age/Gender | Pass # |
|---|---|---|---|
| 63/M | 3 | 76/F | 4 |
| 53/M | 3 | 73/M | 3 |
| 59/F | 5 | 80/F | 5 |
| 80/M | 4 | 80/F | 4 |
| 53/F | 4 | 68/M | 3 |
| 71/F | 3 | 62/F | 2 |
| 53/M | 5 | 89/F | 5 |
| 65/M | 5 | 38/M | 4 |
| 74/F | 4 | 68/M | 4 |
| 82/F | 3 | 66/F | 5 |
| 61/F | 3 | 74/F | 5 |
| 57/F | 4 | 69/F | 4 |
| 79/M | 3 | 87/M | 5 |
| 76/F | 5 | 59/F | 4 |
| 67/M | 4 | 79/F | 4 |
| 67/M | 4 | | |

Pass #, is the number of subcultures; M, male; F, female.

### 4.2. Isolation of mesenchymal stem cells

Isolation of MSCs was carried out using methods previously described (Jaiswal *et al.* 1997), illustrated in Figure 10. Each aspirate was transferred to a 50 ml sterile tube and diluted 1:1 with Dulbecco's Modified Eagle Medium (DMEM; HyClone, Chateauguay,

Qc) supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin (1% PS; HyClone). The tube was centrifuged at 600 *x g* for 10 minutes. The top fatty layer was discarded and the remaining mixture was resuspended and gently layered over 1:1 Ficoll (1.073 g/ml Ficoll-Plaque; GE Healthcare, Baie d'Urfe, Qc) and centrifuged at 900 *x g* for 30 minutes. After centrifugation, the low-density MSC-enriched fraction was collected from the interface, completed to 40 ml with DMEM supplemented with PS, and centrifuged at 600 *x g* for 10 minutes.

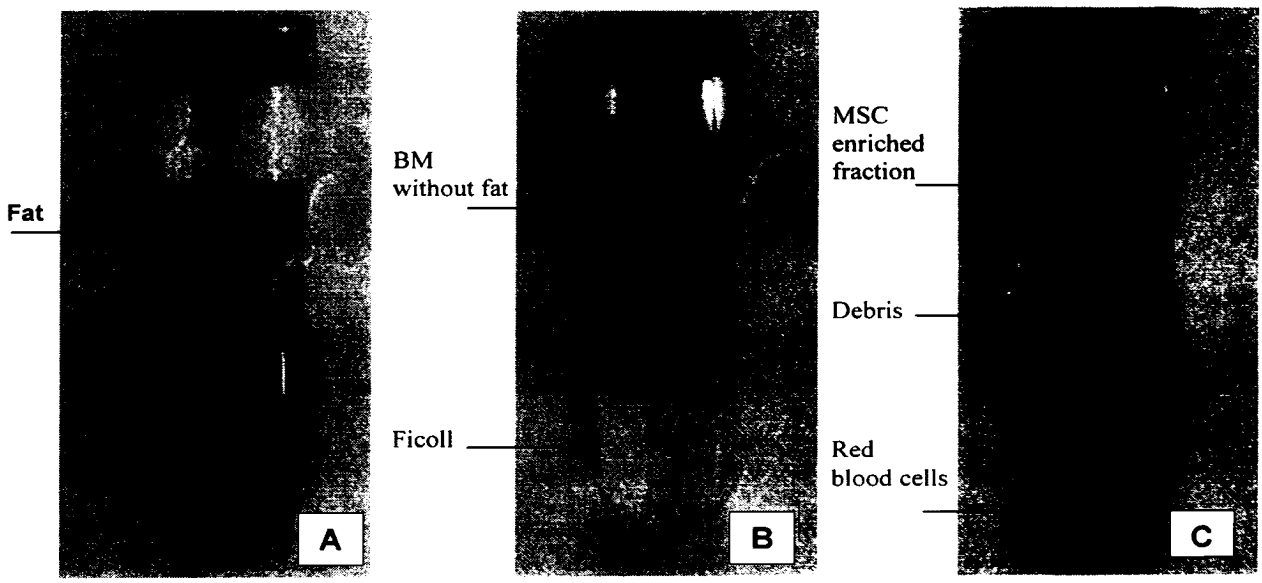


Figure 10. Preparation of BM samples for cell culture. After centrifugation, the sample separates into three layers (A). The top fatty layer is discarded and the remaining mixture loaded onto Ficoll (B). After centrifugation, the top enriched fraction was collected (C). These cells were washed and resuspended in complete culture medium and plated in dishes.

After two washes, the cells were resuspended in 20-ml MSC complete culture medium (DMEM supplemented with 10% Fetal Bovine Serum (FBS;HyClone) and 1% PS) and cultured under pre-confluent monolayer conditions in 20 cm culture dishes. The cells were

maintained at 37°C with 5% humidified $CO_2$. After 48 hours, the non-adherent cells were discarded when changing medium. Adherent cells represent approximately 1 in $10^5$ nucleated cells of this mixture. The culture medium was then changed every 3 to 4 days. For some experiments, 1 ng/ml of human recombinant bFGF (Medicorp, Montreal, Qc) was added to cells grown in monolayer at the beginning of each culture and added fresh with every medium change.

### 4.3. Passaging cells

When culture dishes became near confluent (1.5-2x$10^6$ cells per 20 cm dish), the cells were detached by treatment with 0.25% trypsin/EDTA (Invitrogen) for 7 minutes at 37°C. The action of trypsin was stopped by adding 10:1 of media supplemented with 10% FBS. The cells were centrifuged at 600 *x g* for 6 minutes to pellet the cells, and then resupended in DMEM medium. The cells were counted with a hemocytometer, and either split 1:3, and replated at 0.5-0.7 x $10^6$ cells per 20 cm dish in complete medium or cryopreserved until further use. Aliquots of 1x$10^6$ cells were cryopreserved in 50% FBS, 40% (Phosphate Buffered Saline (PBS) and 10% dimethyl sulfoxide (DMSO) freezing medium and stored at -80°C.

### 4.4. Flow cytometry

Analysis of cell surface molecules was performed on stem cell preparations to assess purity. Expanded cells were trypsinized and counted as described above. The cells were centrifuged at 600 *x g* for 10 minutes and resuspended in PBS with no calcium, no magnesium, with 1% PS, 0.05% sodium azide ($NaN_3$) and 0.5% FBS at 10 x $10^6$ cells/ml.

One million cells per 5 ml conical tube were incubated as per the manufacturer's instructions at room temperature for 10 minutes with monoclonal antibodies labeled with PE against one of CD34, CD45 (Beckman Coulter Canada Inc., Ville St-Laurent, Qc), or CD73 (BD Pharmingen, Oakville, ON). One million cells were also incubated with 2 ng of mouse anti-CD105 at 4°C for 30 minutes, and then with 1:100 goat anti-mouse IgG-FITC as per the manufacturer's instructions (Beckman Coulter). Data collection for the markers was done using separate protocols for the FITC and PE fluorochromes. Flow cytometry data for these antibodies is presented in Table II.

**Table II.** Flow cytometry data for selected antibodies

| Gender (M/F) | Age (years) | CD34 | CD45 | CD73 | CD105 |
|---|---|---|---|---|---|
| M | 80 | 0.2 | 0.1 | 100 | 100 |
| M | 38 | 1.1 | 3.6 | 99.2 | 99.7 |
| M | 68 | 0.9 | 2.1 | 99.6 | 98.3 |
| M | 87 | 0.6 | 0.5 | 97.8 | 97.6 |
| M | 79 | 0.5 | 0.7 | 98.9 | 98 |
| M | 63 | 0 | 0.1 | 78.8 | 30.9 |
| F | 62 | 13 | 31.6 | 95.3 | 93.3 |
| F | 89 | 6.5 | 8.9 | 99.2 | 98.8 |
| F | 74 | 1.9 | 2.4 | 99.8 | 92.3 |
| F | 66 | 2.7 | 4.4 | 99.5 | 92.6 |
| F | 69 | 1.3 | 0.6 | 98.7 | 98.6 |
| F | 59 | 0.4 | 0.4 | 99.3 | 94.3 |
| F | 61 | 0 | 0.1 | 52.3 | 50.8 |
| F | 61 | 0.1 | 0.1 | 88.5 | 29.5 |
| F | 58 | 0.1 | 0 | 79.2 | 60.6 |

Flow cytometry analysis of cell preparations for expression of the MSC surface markers showed that the cells were positive for CD73 and CD105, as previously described (Barry *et al.* 1999; Barry *et al.* 2001a), and depleted of CD34 and CD45, which are markers of the

hematopoietic lineage (Pittenger *et al.* 1999), as shown in Table II. Flow cytometry was used as an additional means of characterization of the MSC preparations.

**4.5. Differentiation of stem cells**

Stem cell differentiation was induced using a previously published pellet culture method (Johnstone *et al.* 1998; MacKay *et al.* 1998). When the number of cells needed was obtained, adherent cells were trypsinized and counted. A total of $1x10^6$ cells (if not otherwise specified) were placed in 15 ml conical polypropylene tubes, centrifuged at 600 *x g* for 6 minutes and resuspended in serum-free chondrogenic medium consisting of DMEM, 1% ITS (insulin (5 μg/ml), transferrin (5 ng/ml) and sodium selenite (5 ng/ml)), 1% PS and bovine serum albumin (BSA; 1 mg/ml). Sodium pyruvate (1 mM) and ascorbic acid (50 μg/ml) were also added to cultures. Cells were recentrifuged at 600 *x g* to form aggregates. The pelleted cells were cultured with or without dexamethasone ($10^{-7}$ M), human recombinant TGF-β1 (10 ng/ml; Biosource, Medicorp) or -β3 (10 ng/ml; Sigma), and human recombinant IGF-1 (100 ng/ml; Biosource, Medicorp). Standard chondrogenic medium consisted of the above serum-free medium supplemented with dexamethasone and TGF-β, as previously described (Johnstone *et al.* 1998). The pellets were cultured in a total of 1 ml medium per tube and incubated at 37°C in 5% humidified $CO_2$.

Medium was changed every 2-3 days and pellets were centrifuged with every medium change. Pellets were harvested at different time points up to 16 days of culture. Pellets that were harvested were washed with PBS to remove medium and transferred to RNase-free 1.5 ml microcentrifuge tubes. Proteinase K (1 mg/ml; Sigma) was added to tubes to help

digest pellet and incubated at 56°C for 1 hour. Proteinase K was found not to alter gene expression (Figure 11)

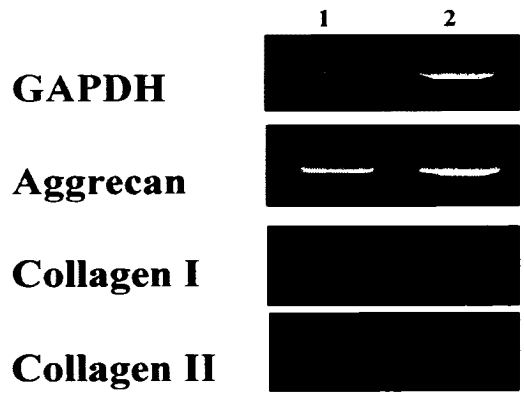


Figure. 11. Effect of proteinase K digestion on gene expression. MSCs were cultured in pellets in standard chondrogenic conditions and harvested on day 16 of culture. Some pellets were digested with proteinase K solution. Controls were maintained under the same conditions with equal amounts of control solution. RNA was extracted from pellets, and equal weight of total RNA was used for RT-PCR for GAPDH, aggrecan, collagen I and II genes. Figure illustrates analysis of pellets with (1) or without (2) digestion.

### 4.6. Total RNA isolation

Total RNA was isolated by the method of Chomczinski and Sacchi (Chomczynski and Sacchi 1987). The pellets were homogenized by adding 1 ml Trizol reagent (Invitrogen) per $1x10^6$ cells and then vortexed. For some samples (day 0), RNA was isolated from the cells after they were trypsinized and before they were used for pellet cultures. The homogenized samples were incubated for 5 minutes at room temperature. Samples were stored at –80°C and thawed for the extraction process. Samples were thawed and analyzed one experiment at a time.

The phases were separated by adding 0.2 volumes of chloroform, vortexed, incubated for 2-3 minutes, and then centrifuged for 15 minutes at 12,000 *x g* at 4°C. The mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The aqueous phase was removed for RNA isolation, and the organic phase saved for subsequent protein isolation. RNA was precipitated with 0.5 volumes of isopropyl alcohol and centrifuged at 12,000 *x g* for 10 minutes at 4°C. The RNA pellet was then briefly air-dried and resuspended in 30 µl diethyl pyrocarbonate treated (DEPC) water and incubated at 55-60°C for 10 minutes. The optical density (OD) at 260/280 nm was quantitated using a spectrophotometer, and this ratio was used to assess the purity of the preparation. The concentration of RNA was determined using the following formula: $OD_{260}$ x nucleic acid factor (40ug/ml) x dilution.

### 4.7. Reverse-transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was used to detect aggrecan and collagen I, II, and X messenger RNA. The isolated RNA samples were transcribed to cDNA using Superscript II Rnase H-Reverse Transcritase (SuperScript First-Strand Synthesis System, Invitrogen). The RT reaction was performed in a total volume of 20 µl, containing 2 µl 5x First strand PCR buffer, 2 µl 100 mM DTT, 1 µl Rnase OUT Recombinant Ribonuclease Inhibitor (40 units/µl), 1 µl of 10 M dNTP Mix (10 mM each of dATP, dGTP, dCTP and dTTP), 1 µl Random primers, 1 ng to 5 µg of total RNA isolated, and 1 µl (200 U) of Superscript (as recommended by Invitrogen). The amount of RNA added to tubes was equal for each experiment. The reverse transcription reaction was carried out for 50 min at 42°C and then inactivated by

heating at 70°C for 15 minutes before chilling on ice. The solution was then used directly for PCR amplification or stored at -20°C.

**4.8. PCR reaction**

PCR primer sets used for amplification are shown in Table III. PCR was performed in a total volume of 25 µl. PCR amplification involved the use of 1 µl of the cDNA from the reverse transcription mixture plus 24 µl of amplification mixture (2.5 µl 10x PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 1 µl 10 mM dNTP Mix, 0.5 µl specific upstream primer (10 µM) and 0.5 µl specific downstream primer (10 µM), 0.25 µl (5 units/µl) Taq DNA polymerase (5 units/µl) and 18.5 µl of sterile water). $MgCl_2$ concentrations were adjusted accordingly for each primer set (0.75 µl (1.5 mM) for GAPDH, aggrecan, and collagen X primers, and 1 µl (2 mM) for collagen I and II primers). The 30 cycles of PCR for GAPDH, aggrecan, and collagen I and II, included denaturation at 95°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 60 s (Grover and Roughley 1993; Melching, Cs-Szabo et al. 1997). The 30 cycles of Collagen type X PCR included denaturation at 95°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 5 minutes (Tchetina, Mwale et al. 2003). Amplified products were analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide. For each set of primers, a PCR reaction not containing the cDNA template was performed, and in all cases no reaction product was seen. PCR products were sequenced at the Shriners Hospital for Children (Montreal) to ensure product purity.

**Table III.** Primers used for PCR amplification. Primers are presented in a 5' to 3' orientation.

| Molecule | Primers | Reference |
|---|---|---|
| Aggrecan | 5'CTACGACGCCATCTGCTACA<br>5'ACGAGGTCCTCACTGGTGAA | Melching *et al.* 1997 |
| Collagen I | 5'CGGTTACCCTGGCAATATTG<br>5'TCCAGTGCGACCATCTTTT | Grover and Roughley 1993 |
| Collagen II | 5'CTGGCTCCCAACACTGCCAACGTC<br>5'TCCTTTGGGTTTGCAACGGATTGT | Grover and Roughley 1993 |
| Collagen X | 5'ACAGGAATGCCTGTGTCTGCTTTTACT<br>5'GCTCTCCAGAACATCATCCCTGCC | Tchetina 2003 |
| GAPDH | 5'GCTCTCCAGAACATCATCCCTGCC<br>5'CGTTGTCATACCAGGAAATGAGCTT | Melching *et al.* 1997 |

### 4.9. Histology

Pellets were harvested, washed with PBS, and embedded in liquid Histogel (Richard-Allan Scientific, Kalamazoo, MI). The pellets were fixed in 10% phosphate buffered formalin overnight at room temperature. The samples were then dehydrated by treatment with a series of graded alcohols, cleared by treatment with xylene, and infiltrated with paraffin. The 5 μm cut sections were stained with H&E to assess morphology. For proteoglycan (predominantly aggrecan) detection, sections were stained with Safranin-O.

### 4.10. Glycosaminoglycan assay

Pellets were harvested at 1 and 16 days for glycosaminoglycan (GAG) content. The pellets were washed with PBS and digested with 50 μl proteinase K (1 mg/ml) for 1 hour at 56°C. GAG content was measured by the reaction with 1,9-dimethylmethylene blue (DMMB) dye (Farndale *et al.* 1986) using shark chondroitin sulfate as a standard. Measurements were made on a spectrophotometer at a wavelength of 530 nm.

### 4.11. Collagen isolation

Proteins from pellets were isolated for Western blots. These pellets were processed by two different methods. In the first method, pellets were rinsed with PBS without calcium and magnesium and centrifuged at 1200 *x g* for 5 minutes. The medium was removed and proteins were extracted by treating pellets with 4 M guanidine hydrochloride buffered in 50 mM Tris (pH 7.5) overnight at 4°C. The solution was then dialyzed overnight at 4°C into 200 mM Tris (pH 7.5) to remove the guanidine. The solution was then transferred to a 1.5 ml microcentrifuge tube and stored at -20°C until further use.

In the second method, the proteins were extracted following RNA extraction using the Trizol method. After the colorless upper RNA-aqueous phase was completely removed, proteins were isolated from the supernatant obtained after precipitation of DNA from the phenol organic phase with ethanol. Proteins were separated with 1.5 ml isopropyl alcohol, stored at room temperature for 10 minutes, and precipitated at 12,000 *x g* for 10 minutes at 4°C. The pellet was then washed 3 times with 2 ml of 0.3 M guanidine hydrochloride solution. After the final wash, the pellet was washed in ethanol and centrifuged. The pellet was vacuum dried and then dissolved in 40 μl 0.1 % SDS. Any insoluble material was sedimented by centrifugation. Protein samples were stored at -20°C until used for Western blots.

### 4.12. Protein expression

The expression of type X collagen protein was measured by Western blot. Samples were dissolved in 2x sodium dodecyl sulfate (SDS) sample buffer and 10% mercaptoethanol,

denatured at 100°C for 3 minutes, and separated by electrophoresis in a 4 to 20% polyacrylamide gel before being transferred to a nitrocellulose membrane. After blocking with 3% Bovine Serum Albumin (BSA), blotted proteins were immunostained with rabbit anti-human type X collagen IgG (Kirsch and von der Mark 1991) using peroxidase-conjugated goat anti-rabbit IgG (Zymed, San Fransisco, California) in a 1:1000 dilution as second antibody. Proteins were detected using Western Lightning Chemiluminescence reagent (Perkin-Elmer). Protein expression was detected and analyzed using a Bio-Rad VersaDoc system equipped with a cooled CCD 12-bit camera.

## 5. RESULTS

MSCs were isolated from human bone marrow and expanded in monolayer in the presence of 10% FBS, as previously described (Jaiswal *et al.* 1997). For inducing chondrogenesis, MSCs from donors ranging from age 38 to 89 years (Table I) were cultured in serum-free medium using a pellet culture system (Lennon *et al.* 1995; Johnstone *et al.* 1998). Cells from a single donor were cultured for each experimental condition. Repeat studies of other donors have revealed essentially the same results for each condition.

RT-PCR was performed to examine the expression profiles of genes involved in chondrogenic differentiation (Johnstone *et al.* 1998). Specifically, we examined genes encoding aggrecan, type I, II and X collagens. GAPDH was used as an internal control to monitor RNA loading.

### 5.1. Cell morphology

Fibroblastic cells attached to cell culture dishes were observed after 48 hours of culture, at the first change of the medium. Cells displayed a colony formation, as previously described (Pittenger *et al.* 1999). After 10 to14 days of culture, the cells reached confluence. Cells in further passages showed more uniform monolayers and needed approximately 10 to 20 days of culture between subcultures to reach confluence depending on the donor, the number of cells needed and whether the cultures were supplemented with bFGF. Cultures supplemented with bFGF were faster to reach confluence than those cultured without bFGF.

### 5.2. Pellet cultures

1 x $10^6$ cells, if not otherwise specified, were used for each pellet (Reyes *et al.* 2001). The pellet culture system was chosen because it has been found to promote chondrogenesis (Johnstone *et al.* 1998). After centrifugation, a single pellet was formed. Within 24 hours of incubation, the cells formed essentially spherical aggregates that did not adhere to the walls of the tube (Figure 12). In order to investigate the effects of different growth factors and supplements on chondrogenic differentiation, cells in pellet culture were exposed to serum-free media supplemented with 10 ng/ml TGF-β1 or TGF-β3, with or without dexamethasone (Dex). In some experiments, pellets were also exposed to IGF-1.

Pellets supplemented with TGF-β1 and dexamethasone were 2 to 3 times bigger than those supplemented with TGF-β1 alone (Figure 12).

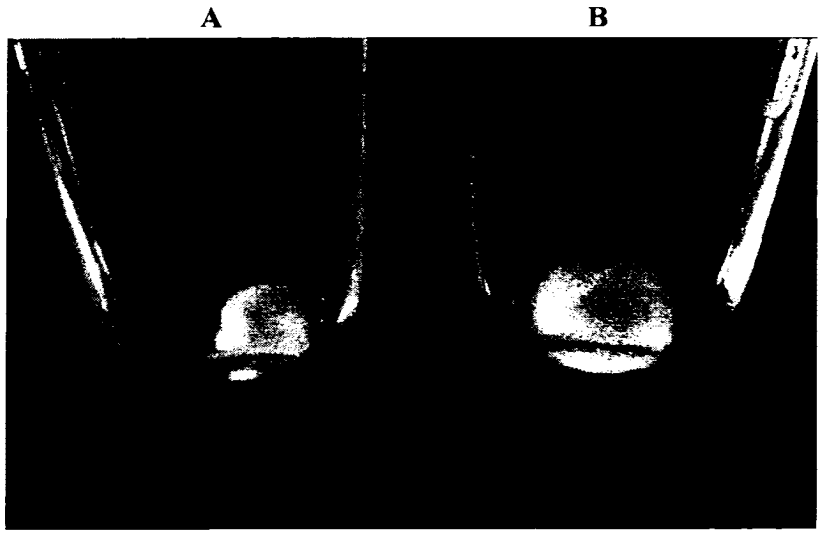


Figure 12. MSC pellet cultures. Cells were cultured (A) without dexamethasone and (B) with dexamethasone in media supplemented with TGF-β1 for 16 days.

### 5.3. Gene expression profiles of aggrecan and collagens

*Upregulation of genes during standard chondrogenic differentiation*

Our first objective was to expose MSCs to standard chondrogenic conditions (TGF-β1 and dexamethasone supplemented cultures), as found in the literature (Johnstone *et al.* 1998; Barry *et al.* 2001). Our results demonstrate that expression of type X collagen, a definitive marker of hypertrophic chondrocytes, was increased in standard chondrogenic conditions. The increase in type X collagen on day 16 of culture was accompanied by the onset of aggrecan and type II collagen expression. This upregulation of genes has been reported previously (Barry *et al.* 2001). In contrast, collagen I expression was detected throughout cultures (Figure 13).

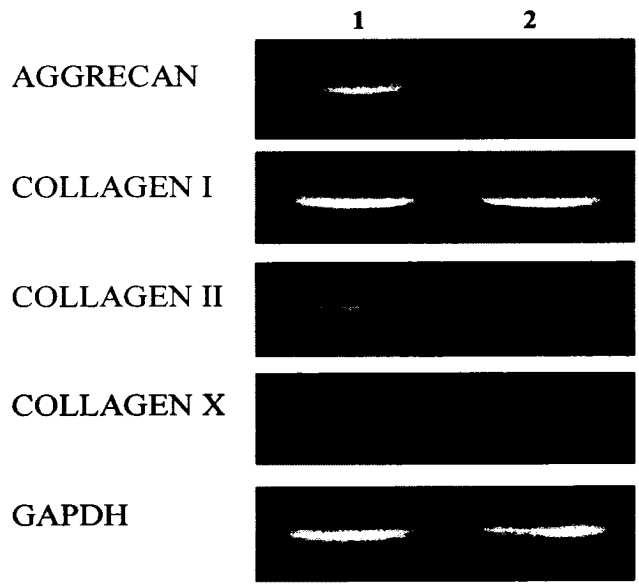


Figure 13. Upregulation of genes during standard chondrogenic differentiation. PCR products from the RT-PCR analysis of aggrecan, type I, II and X collagen mRNA expression: MSCs in pellet culture on day 16 (1) treated with TGF-β1 and dexamethasone; (2) without growth factors or supplements.

*Effect of dexamethasone on upregulation of genes*

In view of the previous results, where type X collagen appeared in standard chondrogenic conditions, and with the understanding that mineralization of the matrix synthesized by MSCs requires the presence of dexamethasone (Cheng *et al.* 1994), the rationale was to remove dexamethasone from the pellet system in order to attempt to remove type X collagen message expression. MSCs were cultured in pellets supplemented with TGF-β1 with or without dexamethasone for 16 days. Except for type II collagen, TGF-β1 increased the expression of aggrecan and type X collagen. The decrease in type II collagen message was associated with the removal of dexamethasone from the culture system (Figure 14).

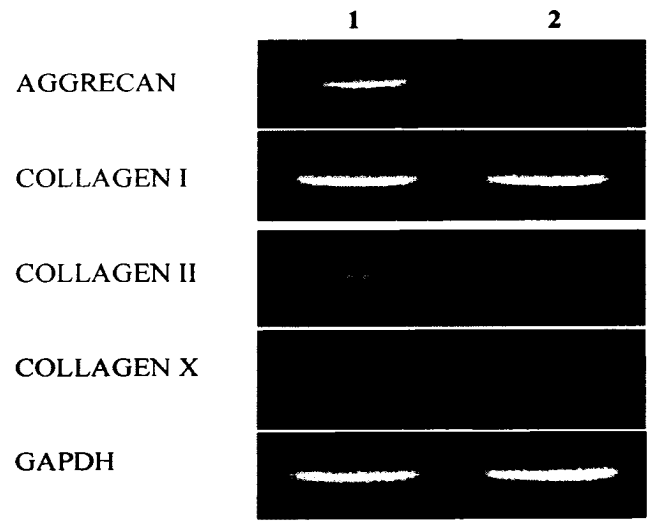


Figure 14. Effect of dexamethasone on upregulation of genes. PCR products from RT-PCR analysis of aggrecan, type I, II and X mRNA expression: MSCs in pellet culture on day 16 (1) treated with TGF-β1 and dexamethasone; (2) treated with only TGF-β1.

*Effect of bFGF on upregulation of genes*

Our next objective was to pre-treat the cells with bFGF in order to examine whether it would have an effect on their chondrogenic potential, as has been previously studied (Bianchi *et al.* 2003). Stem cells were expanded in monolayer with or without 1 ng/ml bFGF and then cultured in previously described standard chondrogenic conditions for 16 days. Aggrecan and type I collagen levels remained unchanged whether or not cells were treated with bFGF in monolayer. Collagen II and X expression was slightly up-regulated when cells were pre-treated with bFGF. However, their ratio of expression does not change (Figure 15). This observation was consistent with repeat experiments when different donors were exposed to these conditions. Expression profile of aggrecan, type I, II and X collagens in standard chondrogenic conditions was previously reported (Figure 13).

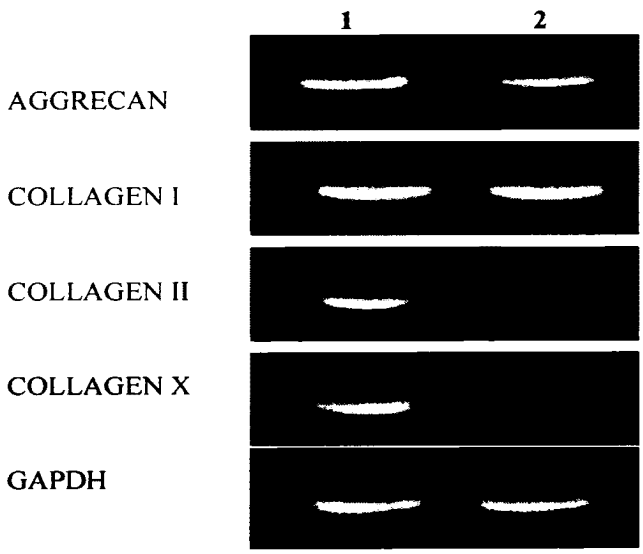


Figure 15. Effect of bFGF on upregulation of genes. PCR products of aggrecan, type I, II and X collagen mRNA expression: MSCs in pellet cultures for 16 days in standard conditions, (1) after being pre-treated with bFGF in monolayer expansion; (2) without pre-treatment.

*Effect of dexamethasone on bFGF pre-treated cultures*

In view of the appearance of collagen type II and X in bFGF pre-treated cultures from the donor shown in figure 15, one could assume that bFGF might play a role in chondrogenic differentiation. In order to see whether bFGF can be used without the addition of dexamethasone (with its inevitable appearance of type X collagen), MSCs were pre-treated in monolayer expansion with bFGF before pellet cultures, and then supplemented with TGF-β1 with or without dexamethasone for 16 days. There was downregulation of type II collagen with the removal of dexamethasone (Figure 16), which is similar to what was observed in figure 14, when pellets were not pre-treated with bFGF. The expression profile of aggrecan, type II and X collagen was consistent with that already observed (Figure 13).

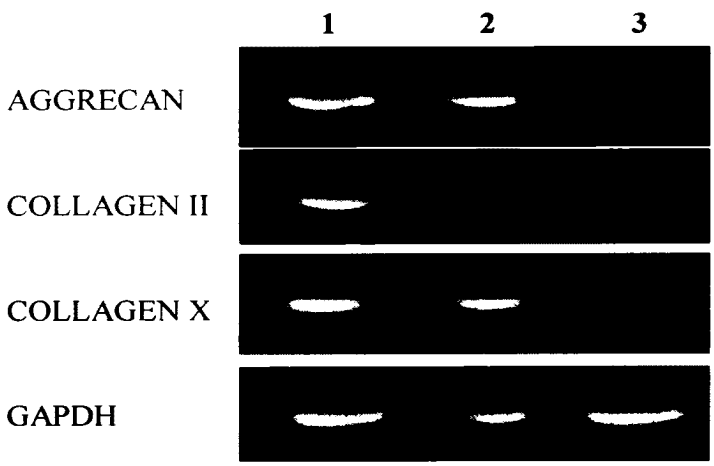


Figure 16. Effect of dexamethasone on bFGF pre-treated cultures. PCR products of aggrecan, type II and X collagen expression: MSCs in pellet cultures on day 16 (1) treated with TGF-β1 and dexamethasone; (2) with TGF-β1 only; (3) without growth factors or supplements (control).

*Time course of aggrecan and collagens*

Our next objective was to study the time course of aggrecan and collagen gene expression throughout different time points of the culture period. MSCs were pre-treated with bFGF before pellet cultures. Aggrecan and type I collagen were highly expressed in monolayer cultures, and throughout culture periods. However, aggrecan expression was down-regulated in control pellets, whereas type I collagen remained constitutively expressed. Interestingly type X collagen message was weakly expressed in monolayers, and gradually increased throughout culture. Surprisingly type X message appeared before that for type II collagen, which was upregulated on day 9 of culture. In contrast, the control pellets show a downregulation of type X collagen message level, where it is weakly expressed (Figure 17).

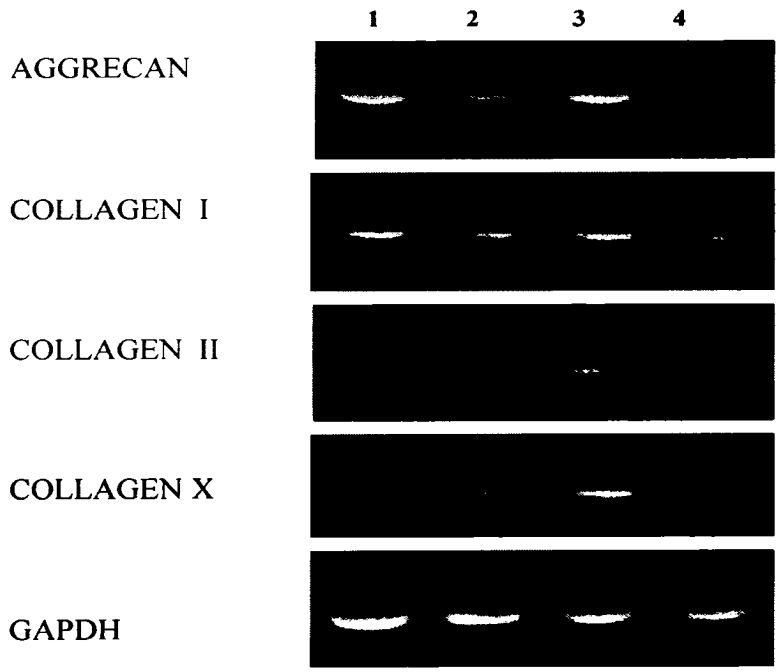


Figure 17. Time course of aggrecan and collagen gene expression. PCR products of chondrogenic markers: MSCs (1) from monolayer cultures (day 0); (2) in pellet cultures on day 9 treated with TGF-β1 and dexamethasone; (3) on day 16 treated with TGF-β1 and dexamethasone; (4) on day 16 without growth factors or supplements (control).

*Effect of TGF-β isoforms: comparison between TGF-β1 and TGF-β3.*

In order to compare the effects of TGF-β1 and TGF-β3 on gene expression of chondrogenic markers, MSCs were pre-treated with bFGF and then cultured in pellets cultures supplemented with TGF-β1 or TGF-β3 with or without dexamethasone for 16 days. Our results demonstrate that these two isomers seemed to have the same effect on the pattern of gene expression in all cultures. The removal of dexamethasone from the system is associated with the down-regulation of collagen type II, and surprisingly aggrecan (Figure 18), which was not consistent with observations from a previous donor (Figure 16). Only collagen type I expression was observed in control pellets.

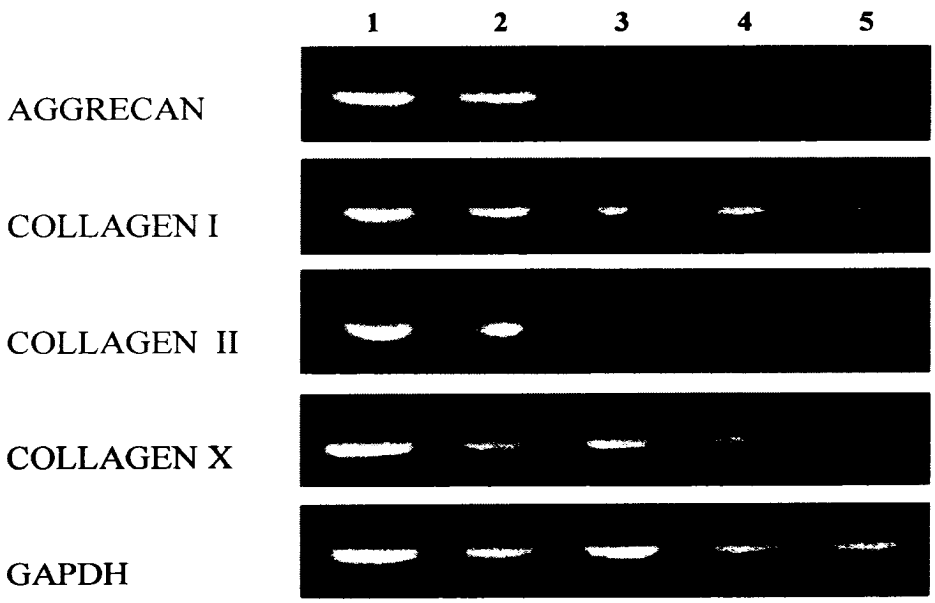


Figure 18. Effects of TGF-β1 and TGF-β3 on gene expression. PCR products of chondrogenic marker expression: MSCs in pellet cultures on day 16 treated with (1) TGF-β1 and dexamethasone; (2) TGF-β3 and dexamethasone; (3) only TGF-β1; (4) only TGF-β3; (5) without growth factors or supplements (control).

*Effect of IGF-1 on upregulation of genes.*

In order to compare the effects IGF-1 and TGF-β1 on gene expression, MSCs were cultured in IGF-1 or TGF-β1 supplemented pellet cultures for 16 days. Expression of aggrecan and type I collagen in monolayers was consistent with expression for the donor shown on figures 17. The upregulation of type II collagen was evident in both TGF-β1 and IGF-1 supplemented cultures. Surprisingly, type X collagen was not present on day 16 of IGF-1 supplemented cultures (Figure 19).

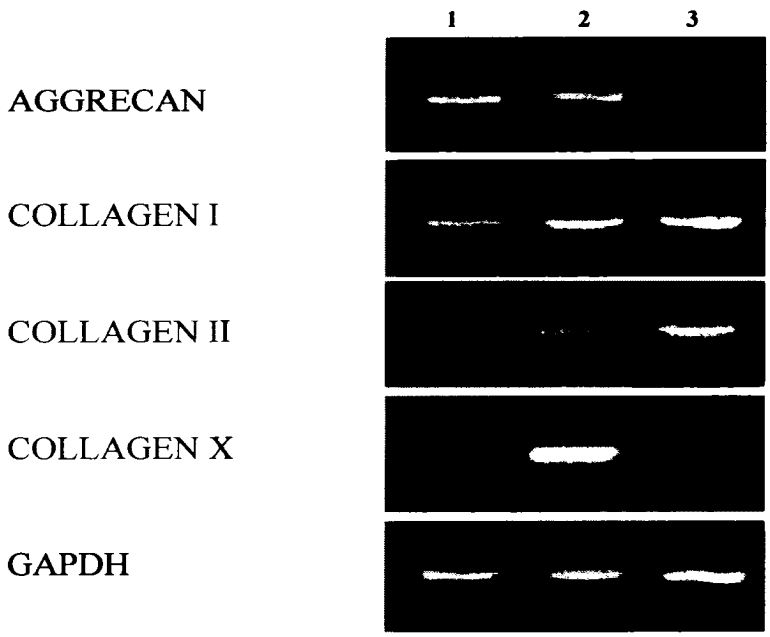


Figure 19. Effect of IGF-1 on upregulation of gene expression. PCR products of chondrogenic marker expression: MSCs (1) from expanded monolayer cultures (day 0); in pellet cultures on day 16 treated with (2) TGF-β1; (3) treated with IGF-1.

*Effect of cell density on gene expression*

In order to examine whether the cell density of the pellet cultures has an effect on the ability for the cells to undergo chondrogenesis, a total of 0.25 x $10^6$, 1 x $10^6$ or 2.5 x $10^6$ MSCs per ml were cultured in pellet cultures in standard chondrogenic conditions for 16 days. Both aggrecan and type II collagen were upregulated when the cell density increased from 0.25 x $10^6$ to 1 x $10^6$ cells, where they reached a peak in expression. In contrast, collagen type X levels were down-regulated with increasing cell density (Figure 20).


Figure 20. Effect cell density on gene expression. PCR products of aggrecan, type II and X collagen expression: MSCs in pellet cultures on day 16 treated with TGF-β1 and dexamethasone at a density of (1) 0.25 x $10^6$ cells/ml; (2) 1 x $10^6$ cells/ml; (3) 2.5 x $10^6$ cells/ml.

### 5.4. Histological evaluation of chondrogenic differentiation

For histological evaluation, pellets from TGF-β and dexamethasone-supplemented cultures were harvested on day 1 and 16. On examination of the paraffin sections stained with H & E, pellet morphology was consistent with previous observations (Johnstone *et al.* 1998). The periphery of the pellet consisted of elongated cells arranged in a few layers that have failed to differentiate, while the center region contained rounded cells characteristic of chondrocyte lacunae (Figure 21). Hypertrophic chondrocytes were not evident in pellet sections. Control pellets without added TGF-β or dexamethasone were considerably smaller and showed no evidence of matrix accumulation or chondrogenesis. Control pellets were too small to be analyzed by histology.

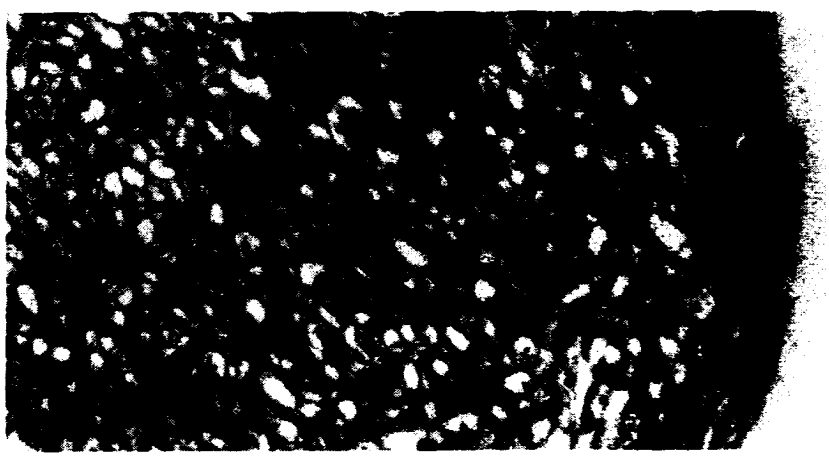

Figure 21. H&E stain of MSCs cultured in pellets in standard chondrogenic conditions for 16 days.

Safranin-O staining of paraffin sections of pellets cultured in standard chondrogenic medium stained red, indicating proteoglycan (presumably aggrecan) accumulation (Figure 22B). Histology sections were digested with Chondroitinase ABC (Sigma) to verify that the Safranin-O did not bind unspecifically. Sections treated with Chondroitinase ABC did not stain for Safranin-O (Figure 22A)

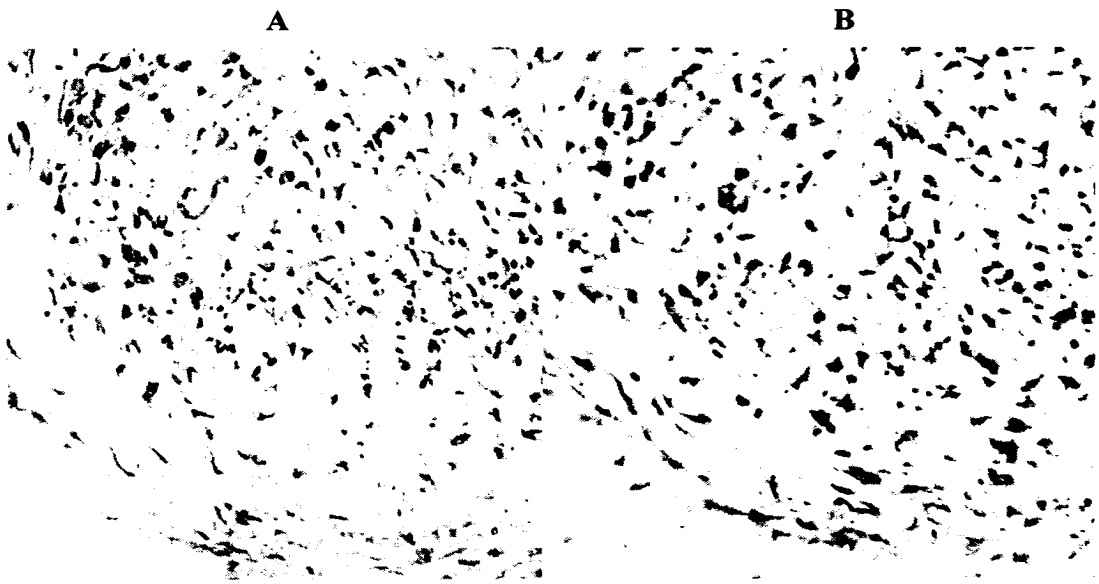


Figure 22. Aggrecan accumulation evidenced by Safranin-O stain. Paraffin sections of pellets cultured in standard chondrogenic conditions over a period of 16 days stained for aggrecan (A) after enzymatic digestion, (B) and without digestion. Sections digested with Chondroitinase ABC did not stain red.

### 5.5. Biochemical analysis

GAG accumulation in pellets was measured using a DMMB binding assay. Cells from the same donor were cultured in pellets in standard chondrogenic conditions at a density of 1 x $10^6$ cells/ml. Pellets were harvested on day 1 or day 16 of culture. Measurement of the total accumulation of GAG in the extracellular matrix of pellets showed an increase in matrix production over time (Figure 23). However, this was not statistically significant result, possibly due to the small sample number.

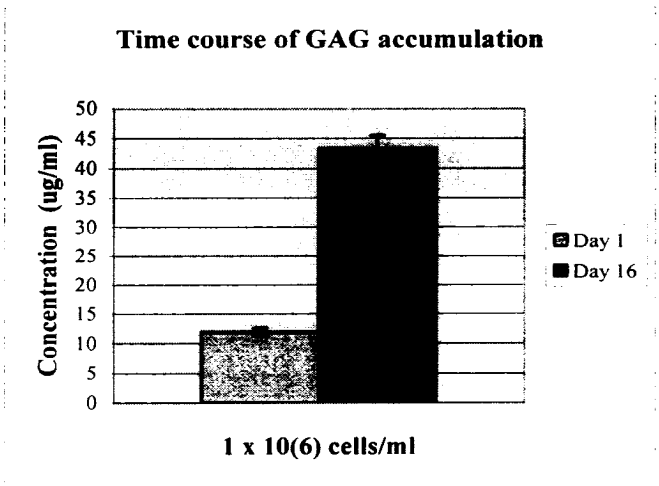


Figure 23. . GAG accumulation throughout culture period. Comparison of GAG content in pellets cultured in chondrogenic conditions for 1 or 16 days. Pellets were seeded at a density of 1 x $10^6$ cells per ml. Bars represent mean values of duplicate cultures ± standard deviation.

## 6. DISCUSSION

Symptomatic disc degeneration is believed to be a common cause of chronic low back pain. Disorders of the lumbar spine that require surgical intervention include degenerative disc disease. Current surgical treatments, namely disc excision and spinal fusion, do not address the problem of the loss of functional intervertebral disc. Our approach for the biological repair of the degenerate nucleus offers a possible alternative treatment for Thompson's grade III disc degeneration. It is based on the principle that early disc degeneration is largely confined to the nucleus, which loses its ability to resist compression and places adverse biomechanical load on the annulus, ultimately resulting in its failure also. This alternative therefore only applies to the disc when it is contained, and therefore fissures have not yet developed in the annulus. Grade IV and V degeneration would prove to be more difficult to repair biologically as it would consist of engineering an intact disc. With this model, it would not be unreasonable to assume that the restoration of nuclear function would retard further disc degeneration. Therefore, one would have to supplement the nuclear tissue with that of a healthy young adult, in which cells have a nucleus-like phenotype and can replenish the extracellular matrix.

In order to regenerate the nucleus, one must first consider the source of cells. Because there is no benign site from within the discs themselves from which autologous disc cells could be harvested, cells from a donor must be used for a source of authentic nuclear cells. If one accepts the claim that the disc is an immunologically privileged avascular structure, such allogenic cells may be suitable. However, the risk of an infective agent, such as HIV, must also be considered a factor if one wants to consider using allogenic disc cells. If autologous cells are required, then MSCs offer a potential alternative. These cells are readily available

in both the bone marrow and fat. MSCs have the potential to differentiate into many connective tissue types, including the chondrogenic, adipogenic, and osteogenic pathways (Pittenger *et al.* 1999). These autologous cells could be injected into the matrix of the degenerate nucleus to restore function, with little or no risk of an immune response by immune cell surveillance.

Because isolation of marrow aspirates in great volume causes damage and pain, it is difficult to isolate the large quantities of MSCs from the bone marrow that are required for regeneration of large injured tissues. Thus the expansion of MSCs *in vitro* is required for autologous cell transplantation. It has been shown that these cells may be isolated using standardized techniques and expanded in culture through many generations, while retaining their capacity to differentiate when exposed to appropriate signals (Barry 2001).

The problem encountered when attempting to differentiate a stem cell to a nucleus pulposus cell is how one would recognize a nucleus phenotype. Although it has some similarity to a pre-hypertrophic chondrocyte that does not express type X collagen, the nucleus cell has not yet been fully characterized. It remains unknown what differentiation pathway a stem cell must undergo to become a nucleus cell, and consequently which growth factors may be used. Although the disc cell has no unique marker of phenotype, it has been described as chondrocyte-like in the literature (Horner *et al.* 2002). However, it is known that the nucleus cell phenotype changes with age, and therefore the phenotype we are trying to obtain is that of a non-notochordal nucleus cell. Our goal was therefore to achieve a chondrogenic (nucleus-like) phenotype that would have a similar function to an authentic nucleus cell. This we defined as a tissue rich in aggrecan and type II collagen, and therefore

chondrocyte-like with the ability to produce extracellular matrix proteins to resist compression. Because we wanted a fluid rather than a firm matrix, the tissue would have a lower collagen II to aggrecan content than cartilage. It has been demonstrated in a previous study that in a normal adult disc (15 to 25 years old), the GAG (a marker of proteoglycan content) to hydroxyproline (a marker of collagen content) ratio within the nucleus is approximately 25:1. However, the GAG to hydroxyproline ratio within the same group in the hyaline cartilage of the endplate is 2.5:1 (Mwale *et al.*). We hypothesize that these ratios could serve as potential benchmarks to distinguish between the normal disc cell phenotype and hyaline cartilage (made up of chondrocyte cells). It is the higher proteoglycan to collagen ratio that provides the nucleus with its desirable gelatinous consistency rather than the firm texture of hyaline cartilage.

In our study, human MSCs were isolated from bone marrow using a previously published method of Ficoll centrifugation (Jaiswal *et al.* 1997), and expanded in FBS-supplemented culture medium (Haynesworth *et al.* 1992; Lennon, Haynesworth *et al.* 1995). Using flow cytometry, we observed that these cells showed a distribution of cell surface antigens that has been previously described for MSCs (Haynesworth *et al.* 1992; Pittenger *et al.* 1999; Majumdar *et al.* 2000). Cultures highly expressed CD73 and CD105, and were depleted of hematopoeitic markers CD34 and CD45. However, one has to note that although antibodies to several surface antigens have been used to recognize MSCs, there is still no consistent set of unique markers characteristic of these cells. Studies have also identified that bFGF stimulates the proliferation of MSCs and maintains their multilineage differentiation potential (Tsutsumi *et al.* 2001; Bianchi *et al.* 2003). Our results are consistent with the literature. Pretreatment of MSCs with bFGF resulted in the cultures reaching confluence

faster than control cells. For this reason, cultures were pre-treated with bFGF before pellet cultures.

After stem cell expansion, our first step was to attempt to induce chondrogenesis based on conditions found in the literature (Johnstone *et al. 1998;* Barry *et al.* 2001). This was done by condensing the MSCs by centrifugation, and then culturing the pellets in standard chondrogenic conditions (TGF-β1 or β3, and dexamethasone) over a period of 16 days. *In vitro* chondrogenic activity of MSCs was measured by the presence of GAG accumulation, aggrecan and type II collagen message expression in the extracellular matrix of pellets. This was verified by gene expression, histological staining and protein analysis of matrix molecules. Our results show that pellets increased in size over the time periods in culture and stained for proteoglycan (mainly aggrecan). The GAG content also seemed to increase over time in culture, although the result was not statistically significant due to small sample size. Aggrecan and type II collagen were also strongly expressed in these standard conditions. These results were consistent with the findings of previous studies. We concluded that these chondrogenic markers were maximal when the cells were exposed to these standard chondrogenic conditions. However, in these standard conditions, type X collagen message expression was inevitable. This is also consistent with what has been observed in previous studies. Many studies have characterized the chondrocyte phenotype by the expression of type X collagen message, among aggrecan and type II collagen expression (Barry *et al.* 2001; Noth *et al.* 2002). Yoo and group detected by immunostaining, as early as culture day 5, type X collagen associated with the surface of human MSCs maintained under standard chondrogenic conditions (Yoo *et al.* 1998).

Similarly Barry detected type X collagen message in the undifferentiated MSC, which was rapidly upregulated and maintained at a uniform level (Barry *et al.* 2001). The appearance of type X protein was then detected later in the differentiating cells. Although type X collagen is generally considered a component of mature hypertrophic cartilage (Cancedda *et al.* 2000), its presence among these differentiated MSCs has not been well defined. In the developing embryo, most bones are formed via a cartilage intermediate in the process of endochondral ossification. This process involves the condensation of MSCs, that ultimately differentiate to hypertrophic chondrocytes and mineralized tissue (Barry *et al.* 2001). The cells in our system seem to differentiate to their terminal phenotype, and this has been defined as chondrogenesis in these previous studies. The consequent production of hypertrophic chondrocytes by the differentiation of bone marrow-derived MSCs is therefore a component of this system.

Surprisingly, when we studied the time course of gene expression, we found that type X collagen message appeared before that for type II collagen. This is contrary to what one would expect. Since type X collagen is a marker of chondrocyte hypertrophy, and one would assume that the cells would have to go through differentiation before undergoing hypertrophy. The type X collagen protein did not show up on the Western blots with antibodies that were confirmed by enzyme linked immunosorbent assay (ELISA) to be specific for human type X collagen (Kirsch and von der Mark 1991). It remains a possibility that the antibody was not sensitive enough for the small quantities in our system. We also did not have access to a human type X collagen standard, and therefore did not have a positive control for our experiments. In our cultures, type X collagen message appears very early in the culture period in the undifferentiated cell. This observation is

consistent with that made by Barry, where they characterized the undifferentiated MSCs by the expression of type I and X collagen mRNA (Barry *et al.* 2001). However, they found that the appearance of type X collagen protein in the differentiating cell was later than both of the other collagens proteins (Barry *et al.* 2001). Since only message, and not protein, could be detected in our work, this raises the question of whether collagen type X has a serendipitous expression, but with no functional consequence. This also raises the question about the different control elements that may be involved in the regulatory expression of this gene. Several regulatory elements may play a role, including stability, processing, and transport of mRNA. In this system, type X collagen message expression may not be a good marker of chondrocyte hypertrophy.

One of the most unexpected results in our study of the time course of gene expression was that aggrecan message was present in monolayer cultures. This is contrary to what we expected and to what was found in previous studies (Johnstone *et al.* 1998; Barry *et al.* 2001). These studies have indicated that there is a rapid increase in aggrecan message after a few days and it levels out throughout the days in culture. Since stem cells do not have a need for aggrecan in the bone marrow space, as they are not required to resist compression, its presence was somewhat surprising. Our results show that aggrecan seemed to be constitutively expressed throughout the culture days, and was downregulated in control pellets (without growth factors or supplements). PCR was performed using increasing numbers of cycles, and showed that levels were in fact constant and did not plateau out. However, in some cultures supplemented with bFGF, aggrecan was downregulated with the removal of dexamethasone from the system. These observations raise the question of whether aggrecan is expressed only at the message level or also at the protein level.

Histological staining with Safranin-O showed that aggrecan is present in monolayer cultures, although its intensity increases when placed in the pellet system, as evidenced by a deeper red coloring. In the experiments described in Johnstone and group, the cells were depleted of matrix prior to pellet culture, and therefore it was concluded that the observed protein was newly synthesized. Although we based our experiments on the previous methods, the presence of aggrecan in our monolayer cultures is surprising and must be further examined. Aggrecan message may therefore not always be a good marker of chondrogenesis, unlike type II collagen that showed an increase over time in cultures supplemented with TGF-β and dexamethasone, which was consistent with previous studies.

Amongst the unexpected presence of aggrecan in monolayer cultures, there were further differences in our results from what was expected based on previous studies. First, we could not detect any difference between the effects of TGF-β1 and TGF-β3 on the chondrogenic markers. Barry and group found that TGF-β3 was the most effective in inducing chondrogenesis in human MSCs isolated from bone marrow and grown in pellet cultures (Barry *et al.* 2001). Their results showed that it produced a two-fold greater accumulation of GAG and earlier and more extensive deposition of type II collagen. Future experiments using different concentrations of each growth factor could determine their ideal concentrations in this system, and could possibly detect which growth factor is most effective at inducing chondrogenesis. Secondly, the next unexpected result was from our cell density experiment. In their experiments, Barry and group found that the cells close to the periphery of the pellets differentiated first, and differentiation proceeded in this fashion until the cells of the center were positive for type II collagen. However, in some cases,

possibly where supply of nutrients was limiting, a type II collagen-rich peripheral zone was formed around an undifferentiated central zone (Barry *et al.* 2001). Considering the results from this study, one could assume that the size of the pellet may have an effect on nutrient flow to the center of the pellet, and that increasing the pellet size would therefore limit this flow. However, our high-density pellets show an upregulation of type II collagen and aggrecan, and a downregulation of type X collagen. One explanation could be that only the cells near the periphery are undergoing differentiation due to limiting nutrient supply. However, immunostaining of sections of the pellets would be necessary to study this further.

We also found variations in our results themselves. For some donors, aggrecan expression was downregulated with the removal of dexamethasone from the system, and for others the expression remained constitutive. Control pellets also varied in their presence of type X collagen expression. These variabilities may be partly explained in the MSC population itself. This cell population may not be a homogenous population of cells. Flow cytometry analysis of these cells did not confirm a completely homogenous population. Some variability may therefore be explained in the fact that we are not certain it is a pure population of progenitor cells. We tried to control for this possibility by culturing some of the cells in monolayer supplemented with bFGF. This growth factor has been found to select for the osteo-chondrogenic progenitor cells, as well as increase their life span in culture (Tsutsumi *et al.* 2001; Bianchi *et al.*2003). However, one cannot be certain that we achieved a homogeneous population.

Different donors were used for each experiment, and therefore many clinical variables might contribute to the differences in bone marrow samples. Among these are the age and gender of the subject, recent trauma, disease, use of tobacco or other pharmaceutical agents (Muschler *et al.* 2001). These factors may have an influence on the cells ability to differentiate. In our experiments, 90% of our donors came from patients with osteoarthritis (OA). Murphy et al. found that the proliferative and chondrogenic capacity of MSCs from patients with OA were significantly reduced (Murphy *et al.* 2002). Age-related changes in the mitotic potential or differentiation of these progenitors has also been suggested (Muschler *et al.* 2001). The patients in our study varied in age, but were mostly above the age of 60.

Trends in the expression of the chondrogenic markers are always present, but the magnitude may vary. The differential expression of these markers may also be due to the culture conditions, such as passage number. It has been shown that increasing passage number may reduce the ability of MSCs to differentiate (Tsutsumi *et al.* 2001). In this study Tsutsumi *et al.* showed that the chondrogenic ability of MSCs markedly decreased when the passage number was increased from 3 to 9 subcultures, before the cells were introduced into the pellet culture system. They also showed a decrease in type X collagen message in the cultures with the highest passages. We tried to control for this parameter by limiting the number of passage to be under 5. Another question to consider is whether expansion in monolayer can induce differentiation of these cells, and explain the presence of aggrecan and type X collagen in monolayers. FBS contains a variety of growth factors that may play a role in inducing the cells in culture.

In practice, sample heterogeneity will exist if we want to use autologous cells clinically. The patients that will need this regeneration process are also the source of the stem cells that will be differentiated. Patients with OA might have cells with tendencies to follow a certain pathway, and these must therefore be identified. Our study shows that the phenotype of these cells can be modulated with growth factors and supplements. Therefore, these patients might need a particular subset of growth factors tailored to take into account the differences due to their condition. Although many conditions have been tested in this study, there remains the need to find the subset of appropriate growth factors that will give a high aggrecan to type II collagen ratio. Our results with IGF-1 suggest that one might be able to decrease type X collagen message expression, although its exact role in these cells is not known. However, the high aggrecan levels did not seem to markedly increase. Our results have demonstrated that aggrecan may not be a good marker of chondrogenesis and type X collagen message might not be a good marker of chondrocyte hypertrophy in this system.

In future studies, we need to examine the structure of the extracellular matrix produced by the stem cells cultured in the previously mentioned conditions. At present information is also missing about the production of type X collagen in correlation with the occurrence of chondrogenic markers. However, message expression may not be a good indication of the matrix composition. This can be supported by Barry *et al.* (2001) who found that type X collagen message does not necessarily correspond to type X collagen expression.

Many improvements could also be made to this system. Kavalkovich *et al.* (2002) used an alginate layer culture system to differentiate the MSCs. They found that there was a more homogeneous and rapid synthesis of cartilaginous extracellular matrix than in pellet

cultures, and it presented a more functional format as the cells could be easily isolated from the system. There have also been studies using bone morphogenetic protein (BMP)-4 transfected MSCs in alginate, that showed that this growth factor had an effect on suppressing chondrogenic hypertrophy. They found that suppression of the type X collagen gene occurred in the BMP-4 transfected cells (Steinart *et al.* 2003).

Our long-term goal is to modulate the phenotype to give high aggrecan to collagen II levels, in order to mimic the fluidity of the nucleus matrix. Our studies show that the modulation of phenotype is possible, however it remains to be established whether it can be modulated to be different than the chondrocytic phenotype. This study therefore serves as a warning regarding the differentiation of these cells and the potential difficulties to be encountered.

## 7. LIST OF ABBREVIATIONS, TABLES AND FIGURES

### 7.1. List of abbreviations

bFGF: Basic fibroblast growth factor

BM: Bone marrow

Dex: Dexamethasone

DMEM: Dulbecco's modified Eagle medium

GAG: Glycosaminoglycan

FBS: Fetal bovine serum

FITC: fluorescein-isothiocyanate

H&E: Hematoxylin and eosin

HSCs: Hematopoeitic stem cells

IGF-1: Insulin-like growth factor-1

MSCs: Mesenchymal stem cells

OA: Osteoarthritis

PE: phycoerythrin

PS: Penicillin/Streptomycin

RT-PCR: Reverse-transcriptase polymerase chain reaction

TGF-β: Transforming growth factor-β

## 7.2. List of tables

### 7.3. List of figures

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