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# Development and *in vitro* Analysis of Genipin-Cross linked Fibrin Hydrogels as Scaffolds for Human Articular Cartilage Tissue Engineering

**Emma V. Dare**

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Faculty of Graduate and Postdoctoral Studies as a
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**Abstract**

Articular cartilage is a very resilient tissue; however, it has a very limited healing capacity after damage due to osteoarthritis or injury. The objective of this project is to determine the suitability of modified fibrin hydrogels as scaffolds for articular cartilage regeneration. The chondrogenic precursor cell line, RCJ 3.1C5.18 (C5.18), was initially chosen as a test system for evaluating the effect of fibrin gel encapsulation on articular chondrogenic differentiation. Fibrin-encapsulated C5.18 cells elaborated an ECM containing type II collagen and aggrecan and exhibited increases in *collagen II* and *aggrecan* gene expression. From these experiments, it was concluded that the fibrin hydrogel scaffold had potential for articular cartilage tissue engineering. To develop the system further towards a more implantable construct, fibrin gels, derived from commercially available human fibrinogen and thrombin, were seeded with primary human articular chondrocytes. The gels were stabilized with genipin, a naturally occurring cross-linker. Genipin cross-linking significantly increased hydrogel dynamic compression and shear moduli and led to enhanced accumulation of collagen II and aggrecan within the ECM secreted by the encapsulated chondrocytes after 5 weeks. Three weeks after subcutaneous implantation of acellular fibrin into Sprague-Dawley rats, less inflammation was observed with genipin cross-linking and when the material origin was allogeneic. As a result, it was necessary to identify an autologous source of fibrin in order to avoid the issues associated with disease transmission. The CryoSeal® FS System was used, which can rapidly and reliably produce autologous fibrin glue from single units of plasma. Using the CryoSeal® fibrin glue, we found that chondrocyte viability was >90%. Gene expression analysis revealed significant increases in *collagen II* and *Sox9,* and a

significant decrease in *collagen I,* under hypoxic culture. When we compared fresh and frozen plasma-derived fibrin there was only a significant difference in mechanical properties after 7 weeks for the material derived from fresh plasma. This may be because greater amounts of collagen type I were detected in frozen compared to fresh plasma gels. Our results indicate that CryoSeal® fibrin glue derived from fresh plasma shows potential for the development of an autologous human articular cartilage tissue engineering system.

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**List of Abbreviations**

3D – three-dimensional

ACI – autologous chondrocyte implantation

ANOVA – analysis of variance

AP – alkaline phosphatase

ATP – adenosine 5'-triphosphate

BMP – bone morphogenic protein

BSA – bovine serum albumin

$Ca^{2+}$ – calcium

CBP – CREB-binding protein

CREB – cAMP response element-binding

COX – cyclooxygenase

DAPI – 4'6-diamidino-2-phenylindole

DMEM – Dulbecco's Modified Eagle's Medium

DNA – deoxyribonucleic acid

dNTP – nucleotides

DTT – dithiothreitol

ECM – extracellular matrix

FBS – fetal bovine serum

FGF – fibroblast growth factor

FIH – factor inhibiting HIF-1

FPA – fibrinopeptide A

FPB – fibrinopeptide B

FS – fibrin sealant

FSP – fresh plasma

FZP – frozen plasma

GAG – glycosaminoglycan

GAPDH – glyceraldehyde-3-phosphate dehydrogenase

$H^+$ – hydrogen

H&E – Haematoxylin and Eosin

HIF – hypoxia inducible factor

IGF – insulin-like growth factor

IL – interleukin

LO – low oxygen

MMP – matrix metalloproteases

MSC – mesenchymal stem cells

$Na^+$ – sodium

NIH – National Institutes of Health

N-O – nitric oxide

NO – normal oxygen

NSAID – non-steroidal anti-inflammatory drugs

OA - osteoarthritis

PBS – phosphate buffered saline

PFA – paraformaldehyde

PGA – apolyglycolic acid

PHD – prolyl hydroxylases

PLGA – poly(lactic-*co*-glycolic acid

rhEGF – recombinant human epidermal growth factor

RNA – ribonucleic acid

RT-PCR – reverse transcriptase polymerase chain reaction

Sox9 – SRY-box containing gene 9

TBS – tris-buffered saline

TGF – transforming growth factor

TIMP – tissue inhibitors of metalloproteases

TKA – total knee arthroplasty

TNF – tumor necrosis factor

tPA – tissue-type plasminogen activator

TPD – thrombin processing device

UV – ultraviolet

VEGF – vascular endothelial growth factor

VHL – von Hippel-Lindau tumour-suppressor protein

αMEM – alpha minimal essential medium

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# CHAPTER 1

## Introduction

### 1.1 Articular Cartilage

#### 1.1.1 Tissue Development and Structure

The complex structure of the synovial joint is formed by an arrangement of multiple and distinct tissues. The tissue that contributes the most to the extraordinary functional abilities of the joint is hyaline articular cartilage (Buckwalter and Mankin, 1998a). The mature structure of articular cartilage develops from hyaline cartilage as a result of mechanical loading. Articular cartilage is essential to normal joint function because of its ability to reduce stress and friction during movement. Articular cartilage is an incredibly durable tissue that has the ability to expand and deform its contact surfaces to reduce the effect of load by decreasing the stress on the joint (LeBaron and Athanasiou, 2000). Articular cartilage thickness is different from joint to joint and is a function of age and species (Athanasiou *et al.,* 1991).

Chondrogenesis is the formation of a cartilage intermediate, which leads to endochondral ossification. It is initiated by differentiation of mesenchymal cells from the lateral plate mesoderm to form the limbs (Olsen *et al.,* 2000). The limb develops from the primitive, proliferative, differentiating mesenchymal cells forming the blastema. Chondrogenic differentiation is regulated by Sox9 (SRY-box containing gene 9), which is expressed in condensed mesenchyme and regulates deposition of the extracellular matrix (ECM) containing collagens II, IX, XI, and aggrecan (Ng *et al.,* 1997). Sox9 might be under the control of transforming growth factor-β (TGF-β) and Bone morphogenic protein (BMP) activity (Kawakami *et al.*, 2006). The next stage is hypertrophy, where the

cellular fluid volume increases by almost twenty times, and is marked by collagen X and alkaline phosphatase activity (Goldring *et al.,* 2006). Runx2 is a transcription factor that promotes hypertrophic chondrocyte maturation, followed by osteogenesis (Enomoto *et al.,* 2000). Finally, angiogenesis is required for the replacement of cartilage with bone, which is regulated by vascular endothelial growth factor (VEGF). VEGF promotes vascular invasion. Matrix metalloproteases (MMPs) degrade the non-mineralized matrix (Colnot, 2005).

During joint formation, MSCs in the interzone (prospective joint site) differentiate into chondrocytes to form the cartilaginous skeleton and are replaced by osseous tissue during endochondral ossification. In the midst of the interzone, a cavity is formed via apoptosis (Niedermaier *et al.,* 2005). This will develop into the synovial cavity. Joint cavitation is influenced by hyaluronan synthesis and embryo movement (Dowthwaite *et al.,* 2003). Finally, the two opposing outer layers of the interzone develop into articular cartilage, defining the tips of the articulating long bones. The adult joint consists of a joint capsule that envelops the synovial cavity and articular cartilage. The inner cavity is surrounded by a synovial membrane and ligaments and tendons strengthen the joint structure (Hashimoto *et al.,* 2007). BMPs, Noggins and Wnts are expressed in the condensing mesenchyme in the interzone and are essential for joint formation. Abnormal expression of any of these morphogens leads to fusion of many of the distal digits (Storm and Kingsley 1996; Brunet *et al.,* 1998).

TGF-β signalling is very important in the formation and long-term stability of articular cartilage. Transgenic mice expressing a truncated kinase-defective TGF-β type II receptor, the articular cartilage was replaced with hypertrophic cartilage or bone, which

resembled osteoarthritic cartilage (Serra *et al.* 1997). Therefore, articular chondrocytes require TGF-β-dependent signalling and low levels of *Runx2* expression to protect their permanent phenotype (Pacifici *et al.,* 2005).

Articular cartilage is typically 75-80% water and is composed of a matrix, which is 50-73% collagen II, and 15-30% proteoglycan (consisting mostly of aggrecan but also including small amounts of decorin, biglycan, and fibromodulin; LeBaron and Athanasiou, 2000). Proteoglycans have a protein core branched with negatively charged glycosaminoglycans (GAGs). Collagen type II forms a highly cross-linked network that entraps aggrecan. This interaction between collagen and aggrecan contributes to the unique biomechanical properties of the tissue (Freemont and Hoyland, 2005). Alcian Blue and Safranin O both stain the acid GAGs of articular cartilage. Safranin O is a metachromatic dye that binds the polyanions of GAG molecules via salt linkages (Pal and Schubert 1961). Alcian Blue 8GX is a copper phthalocyanin dye that forms electrostatic bonds with the negatively charged anionic site on the polysaccharide. It is a very selective dye that stains both sulphated and non-sulphated acid GAGs and sialoglycoproteins at pH 2.5 (Spicer et al. 1967). Alcian Blue staining more accurately reflects the concentration of acid GAGs within a tissue than Safranin O (Troyer, 1980).

The chondrocytes suspended within the articular cartilage matrix occupy less than 10% of the tissue (Jeffrey *et al.*, 1991). The collagen gives the matrix tensile strength, whereas the proteoglycan assists in resistance to compression (Maroudas, 1979). The structural organization of the matrix components and cells in articular cartilage is very important for its function. It is made up of four zones (Fig. 1). The superficial layer is at the gliding surface of the joint and determines its load-bearing ability. The tangential

orientation of the collagen fibrils here gives the tissue higher tensile strength and stiffness. The chondrocytes are flattened in this zone. In the middle layer, the chondrocytes are spherical and the collagen is arranged randomly. There is much higher proteoglycan content in this zone. In the deep layer, the collagen and chondrocytes are arranged perpendicular to the subchondral plate. The calcified layer attaches the deep zone to the subchondral bone. It is an area of underlying bone growth (LeBaron and Athanasiou, 2000).

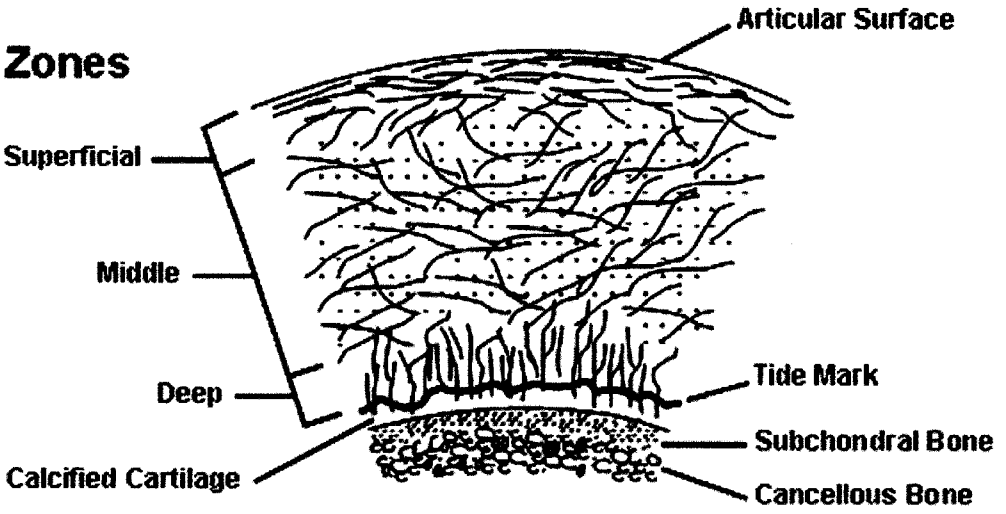


**Fig. 1. Organization of collagen fibrils throughout the zones of articular cartilage.**

Chondrocytes express several integrins, which allow them to interact with the surrounding matrix. The $\alpha_1\beta_1$ integrin is a receptor for collagen type II (Loeser 1993). Several signalling molecules, including TGF-β, increase chondrocyte adhesion to collagen II and therefore are very important for cell-matrix interactions (Loeser 1997).

Despite the tissue's remarkable durability and strength, trauma or abnormal joint loading can induce localized damage (van der Krann *et al.*, 2002). The current surgical techniques for articular cartilage restoration and regeneration, including autologous

chondrocyte implantation (ACI), have yet to result in regeneration of uniform hyaline cartilage with long term performance (Kuo *et al.,* 2006; Hunziker, 2002; Marlovits *et al.,* 2006). Cartilage lesions are classified based on extension to the subchondral bone. In partial thickness defects, subchondral bone presents a barrier between the defect and the bone marrow MSCs; partial thickness defects are unable to heal on their own (Buckwalter 1998; Hunziker 2001). In full thickness cartilage defects, contact with MSCs is available, but the gap is filled by spontaneous repair with fibrocartilaginous tissue. Fibrocartilage is a weak substitute for articular cartilage that gradually degrades over time (Shapiro *et al.*, 1993). In meniscal fibrocartilage the cell type is the fibrochondrocyte, which has properties midway between fibroblasts and chondrocytes (Nakata *et al.,* 2001). Collagen makes up approximately 78% of the tissue dry weight; type I collagen makes up 80% of the total collagen (Herwig *et al.,* 1984). In addition, GAGs only make up 2-3% of the tissue dry weight (Herwig *et al.,* 1984). As a result of these differences in ECM components, fibrocartilage and articular cartilage have very different mechanical properties. Comparison between the literature is difficult because samples are often prepared and tested differently. The compressive aggregate modulus for the human meniscus is 0.18 MPa compared to 1.18 MPa for human articular cartilage. However, the tensile modulus for the human meniscus is 130 MPa compared to 10.3 MPa for human articular cartilage (Almarza and Athanasiou, 2004).

There are several genetic differences between articular cartilage and other types of cartilage and between normal and osteoarthritic cartilage. Genome-wide expression analysis studies of chondrocytes can be useful to identify the genetic differences and to justify the selection of genes for analysis. In a study comparing normal and osteoarthritic

cartilage, *collagens I, II,* and *III* as well as *fibronectin, tenascin-C,* and *osteopontin* were elevated in moderate/severe osteoarthritic cartilage compared to normal cartilage. Genes involved in oxidative damage defence and *Sox9* were decreased compared to normal cartilage and there was no change in *aggrecan* or *decorin* (Aigner *et al.,* 2006). Therefore, simultaneous increases in the fibrillar collagens and a decrease in *Sox9* during repair would indicate a possible continued progression of the disease. In addition, *col1a1* and *col1a2* are markers of fibrochondrocytes. *Collagen II* is a marker of both articular and fibrochondrocytes; however, its expression is much higher in articular chondrocytes (Ochi *et al.,* 2003). Articular chondrocytes and cells of the nucleus pulposus (NP) in the intervertebral disc have similar genetic markers. However, a high proteoglycan-to-collagen ratio and low aggrecan-to-versican ratio can be used to distinguish NP from cartilage (Mwale *et al.,* 2005). Therefore, analysis of *collagen I, collagen II, aggrecan, versican,* and *Sox9* gene expression can be used to assess the quality of the cartilage repair tissue. Translation fidelity cannot be assumed (Mwale *et al.,* 2006), however, so protein analysis is also necessary.

### 1.1.2 Osteoarthritis

Osteoarthritis is an idiopathic disease that is characterized by articular cartilage degeneration. Cartilage matrix breakdown leads to the development of fissures, gross ulcerations, and the disappearance of the full thickness surface (Martel-Pelletier 1998). Bone changes also occur, which include osteophyte formation and subchondral plate thickening. Inflammation of the synovial membrane is often observed (Pelletier *et al.,* 1997).

The risk factors for the development of OA can be grouped into two fundamental mechanisms that relate either to adverse effects of "abnormal" loading on normal cartilage or "normal" loading on "abnormal" cartilage (Goldring and Goldring, 2007). The primary factor contributing to the "abnormal" state of cartilage is aging, although genetic factors play a role. Genetic abnormalities can result in earlier onset of OA. Twin studies have shown that the influence of genetic factors may be as high as 70% in OA (Valdes *et al.*, 2006). Polymorphisms or mutations in genes encoding ECM and signaling molecules may determine susceptibility to OA (Loughlin 2005). In addition, it has been shown that these genes may operate differently in males and females and at different body locations (Bukulmez *et al.,* 2006). Obesity, female gender, and preexisting early-stage OA contributed significantly to adverse radiographic and clinical OA outcomes (Englund and Lohmander 2004). Increased load transfer and/or altered patterns of load distribution within a joint can accelerate the initiation and progression of OA (Roos 2005). In response to traumatic injury, global gene expression is activated, resulting in increased expression of inflammatory mediators, cartilage-degrading proteinases, and stress response factors (Kurz *et al.,* 2005).

Changes in joint cartilage associated with OA include gradual proteolytic degradation of the ECM and an increase in synthesis of the matrix components by the chondrocytes (Hashimoto *et al.,* 2007). In cartilage degradation, it is known that MMP play important roles. Members of three MMP groups have been identified as being elevated in OA. They are the collagenases, the stromelysins and the gelatinases. Their activation can be induced by members of the plasminogen activation/plasmin family; urokinase and plasmin have been identified in human osteoarthritic cartilage (Martel-

Pelletier *et al.,* 1991). MMP biological enzymatic activity is controlled physiologically by tissue inhibitors of metalloproteases (TIMP) (Martel-Pelletier 1998).

Pro-inflammatory cytokines have been shown to be responsible for the catabolic processes that occur in the tissues. They are first produced in the synovial membrane and then diffuse into the cartilage. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) may be the major catabolic signaling systems involved in joint tissue destruction since blocking their activity is very effective in preventing further cartilage destruction (van de Loo *et al.*, 1995; Plows *et al.,* 1995). Inorganic free-radical nitric oxide (N-O) has also been suggested as a factor that promotes cartilage catabolism as it is found at higher levels in osteoarthritic cartilage compared to normal cartilage (Pelletier *et al.,* 1996).

In addition to cartilage degeneration, changes in the surrounding bone occur in OA. Thickening of the subchondral bone may participate in OA progression. Healing of trabecular microfractures, which occur due to repetitive loading, can generate a stiffer bone that is no longer effective at shock absorption (Radin and Rose 1986). In addition, it has been shown that osteoblasts directly influence cartilage metabolism. Primary *in vitro* cultures of osteoblasts from osteoarthritic human subchondral bone have an altered phenotype, and elevated levels of plasminogen activator and insulin-like growth factor-1 (IGF-1; Hilal *et al.*, 1998). Along with mechanical and chemical stresses, abnormal osteoblasts would accelerate subchondral bone formation, leading to enhanced mechanical pressure on overlying cartilage and resulting in further deterioration.

The most common symptom of OA is pain involving one or more joints. Morning joint stiffness is also very common. As OA progresses, prolonged joint stiffness, joint enlargement, and articular crepitation are evident (Hinton *et al.,* 2002). Treatment of OA

includes non-drug therapy, drug therapy, and surgical treatment. Non-drug therapies, including weight reduction and exercise (Miller *et al.,* 2006), muscle strengthening and aerobic conditioning by physical therapy (Thomas *et al.*, 2002), and bracing and corrective footwear (Felson *et al.*, 2000), have been effective in pain control and in slowing the progression of the disease (Hashimoto *et al.*, 2007).

For more aggressive pain and disease control, analgesics and anti-inflammatory drugs can be used. Paracetamol and Paracetamol/opiate combinations have been shown to be effective at pain control and well tolerated by many patients (Walker-Bone *et al.,* 2000). Non-steroidal anti-inflammatory drugs (NSAID) and cyclooxygenase-2 (COX-2) inhibitors have also been shown to be effective anti-inflammatory agents; however, gastrointestinal and cardiovascular side effects are possible (Pincus *et al.,* 2000). Additional relief can be obtained by topical treatment of NSAIDs or capsaicin (Moore *et al.,* 1998). Finally, intra-articular corticosteroid or hyaluronic acid injections provide temporary pain and inflammation relief (Jones *et al.,* 1995).

Surgical treatment of OA is considered after failure of non-surgical treatments. Four categories of surgical management are considered: osteotomy (bone cut for realignment), arthroscopy (minor treatment after endoscope insertion into the joint), arthrodesis (artificial induction of joint ossification), and arthroplasty (partial or total joint replacement; Felson *et al.,* 2000). For most patients, especially the elderly, total joint arthroplasty is highly successful and the therapeutic effect will last for the remainder of the patient's life. The most common reasons for arthroplasty failure are osteolysis and aseptic loosening (Jacobs *et al.,* 1994). Therefore, it is clear that a more effective, permanent solution is necessary.

## 1.2 Tissue Engineering Strategies

Autologous chondrocyte implantation was first introduced to treat patients with symptoms of articular cartilage lesions of the knee in 1987 (Brittberg *et al.,* 1994). Cartilage biopsies from patients were obtained by arthroscopy from the non-weight bearing surfaces of the chondylar cartilage. Primary chondrocyte monolayer culture was used to achieve large numbers of mature cells and the culture system was supplemented with autologous serum obtained from the patients. The cultured chondrocytes were then injected beneath a periosteal or collagen flap, covering the cartilage defect, and then sealed with fibrin glue. This technique has not been successful in regenerating uniform hyaline cartilage with long term performance (Kuo *et al.,* 2006; Hunziker, 2002; Marlovits *et al.,* 2006). The poor *in situ* healing of articular cartilage has been attributed to the inability of the chondrocytes to divide and to synthesize new ECM components (Salter *et al.,* 1992). In addition, primary chondrocytes lose their differentiated phenotype in culture if allowed to adhere to a substrate in the presence of serum (Holtzer *et al.,* 1960). However, differentiated chondrocytes maintain their phenotype if they are embedded into three-dimensional (3D) scaffolds (Horwitz and Dorfman, 1970; Guo *et al.,* 1989; Gibson *et al.,* 1984). It has also been suggested that the ECM components may act to modulate the activity of cytokines, which can signal the cells to divide and synthesize matrix. Therefore suspension of the cells into a synthetically derived matrix could allow the cells to resume their proliferative and differentiated state (Scully *et al.*, 2001).

Tissue engineering approaches to articular cartilage repair offer potentially important advantages over modern metallic and plastic joint prostheses. While total joint

replacement has been enormously successful in providing pain relief and restoring joint function for patients with debilitating arthritis, the prostheses generate wear and problematic debris, and they are not sufficiently durable for the physically active individuals (Kim and Han 2000). It would also be beneficial to successfully treat individuals at an earlier stage of disease. The search for the optimal matrix for growing articular cartilage has led to the development and testing of scaffolds made up of both natural and synthetic materials. Matrices made of polyglycolic acid, polylactic acid, poly(ethylene oxide), poly(vinyl alcohol), poly(acrylic acid), poly(propylene fumarate-co-ethylene glycol), polypeptides (all synthetic), hyaluronic acid, fibrin, collagen, gelatin, chitosan, alginate, calcium carbonate (all natural) and combinations of these compounds have been used as substrates for tissue engineering both *in vitro* and *in vivo* (van der Kraan, 2002; Drury and Mooney, 2003). These materials can form hydrogels, a class of highly hydrated polymer materials (defined as > 30% water by weight). The structural integrity of the hydrogels depends on cross-links formed between the polymer chains via physical and chemical interactions (Park and Lakes, 1992). A hydrogel is suitable for engineering articular cartilage because it can encapsulate cells, is typically degradable, forms under mild conditions, and has mechanical and structural properties similar to the ECM of many tissues (Lee and Mooney, 2001). Articular cartilage is itself a hydrogel.

There are several scaffold design variables that must be considered. For example, a suitable scaffold must be non-toxic and non-damaging to the encapsulated cells and surrounding tissues, it must allow appropriate diffusion of nutrients and wastes to and from the cells, and it must have sufficient mechanical strength for *in situ* implantation and maintenance (Lim, 1984). Materials that have been assessed for tissue engineering

have both advantages and disadvantages. Synthetic polymers allow more controlled reproducibility of physical and chemical gel properties and reduce the risk of potential bio-hazardous complications that can occur with natural polymers. However, many synthetic polymers have been shown to induce some immune/inflammatory response after implantation (Cancedda *et al.,* 2003). Moreover, many of these materials must also be cross-linked via UV exposure or reaction with hazardous chemicals (e.g. glutaraldehyde), which can be toxic to the encapsulated cells (Drury and Mooney, 2003).

There are many examples of biomaterial scaffolds that have been used for articular cartilage tissue engineering. Recently, marrow-derived MSCs were seeded into poly(lactic-*co*-glycolic acid) (PLGA) composites for cartilage regeneration. After pre-treatment with TGF-β3 and implantation into osteochondral defects in the rabbit knee, hyaline cartilage-like tissue regenerated over 12 weeks (Han *et al.,* 2008). Spiller *et al.,* (2008) developed a superporous poly(vinyl alcohol) and poly(vinyl pyrrolidone) scaffold, for seeding with chondrocytes, which was prepared using a double emulsion process. These scaffolds had a high degree of porosity and similar mechanical properties to articular cartilage. Alginate (alginic acid derived from brown algae) has also been used as a hydrogel for culture of articular chondrocytes (Tomkoria *et al.,* 2007). After 12 weeks of *in vitro* culture the mechanical properties of the constructs increased by more than 4-fold. Chitosan (polysaccharide derived from chitin) has been widely used as a scaffold for articular cartilage tissue engineering. A tri-copolymer scaffold comprised of collagen, chitosan and chondroitin sulphate was seeded with rabbit articular chondrocytes and implanted subcutaneously into nude mice (Yan *et al.,* 2007). After 12 weeks, homogenous cartilaginous tissue formed. Chitosan has also recently been used in

combination with collagen II to form a scaffold for articular cartilage tissue engineering (Shi *et al.*, 2005). When a marrow-stimulation technique was used to allow autologous blood to mix with chitosan/glycerol phosphate in the treatment of cartilage defects in the rabbit, significant repair of hyaline cartilage and subchondral bone was observed compared to controls (Chevrier *et al.*, 2007).

Another new approach in cartilage tissue engineering is the development of bi-phasic constructs for the regeneration of cartilage and bone to allow better incorporation of the material into the defect. Ito *et al.,* (2008) evaluated tissue engineered composite plugs composed of chondrocyte-seeded collagen for cartilage regeneration, and calcium hydroxyapatite ceramics as bone scaffolds. Twelve weeks after implantation into rabbits, the defects were repaired with cartilage-like tissue and good subchondral bone formation. In another study, chondrocytes were seeded directly on the surface of a calcium polyphosphate bone substitute and cultured *in vitro* (Allan *et al.,* 2007). After 8 weeks, calcified cartilage had formed adjacent to the bone substitute and hyaline-like cartilage had formed above. Despite all of the progress achieved in articular cartilage tissue engineering, no systems have been successfully translated to the clinic with implantation into human patients (Kuo *et al.,* 2006). Many of the materials are not approved for clinical use.

## 1.3 Fibrin

### 1.3.1 Material Structure and formation

Fibrinogen and fibrin play important roles in blood clotting, fibrinolysis, cellular and matrix interactions, inflammation, and wound healing. The fibrin molecule is comprised of two sets of three polypeptide chains called Aα, Bβ, and γ that are joined by

disulfide bridges within the N-terminal E domain (Henschen *et al.*, 1983). There are two outer D domains that are connected to the central E domain. Fig. 2 represents a schematic diagram of fibrin assembly and cross-linking. Thrombin mediates cleavage of fibrinopeptides A (FPA) and B (FPB) from Aα chains to expose two $E_A$ and $E_B$ polymerization sites, respectively, that also interact with platelets, fibroblasts and

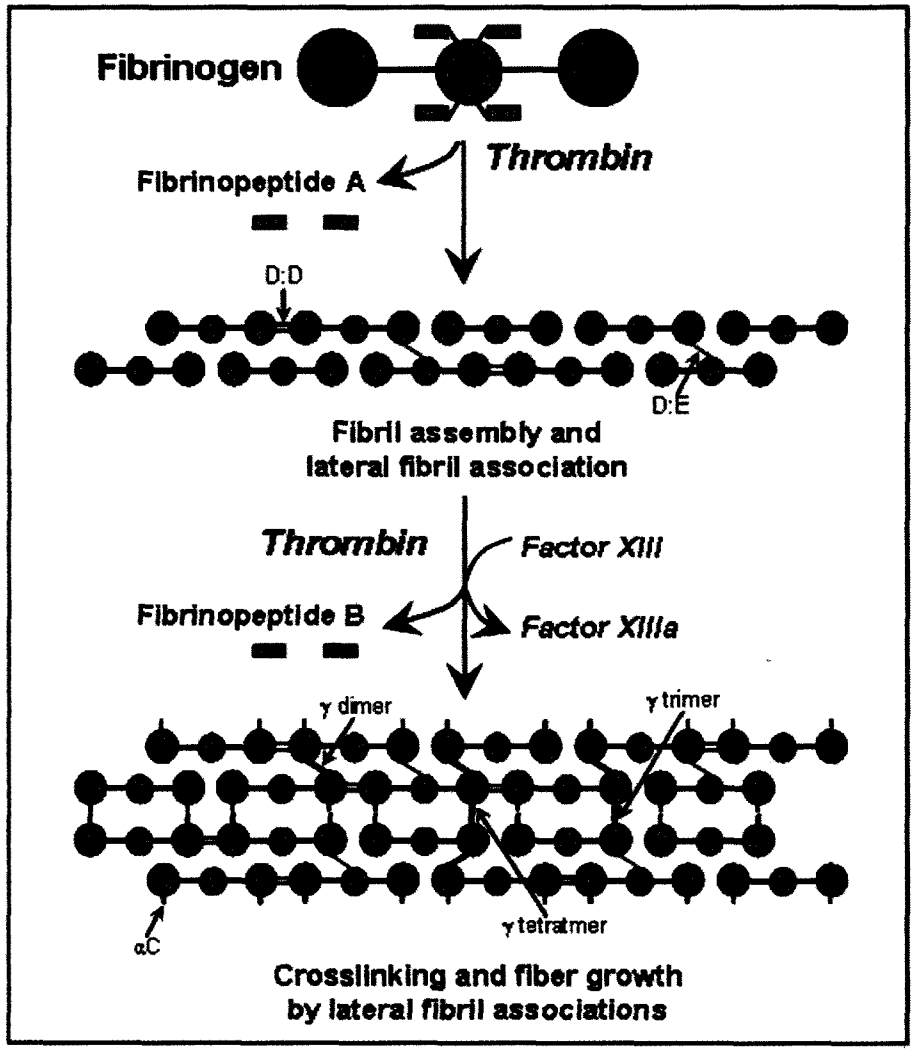


**Fig. 2. Fibrin Polymerization.** Fibrin assembly begins after FPA cleavage with D:E (green lines) and D:D (double blue lines) non-covalent interactions to form end-to-middle staggered overlapping double-stranded fibrils. After FPB cleavage, αC domains self-associate to promote lateral fibril association and fiber assembly. Factor XIIIa catalyzes γ dimer, trimer, and tetramer formation (thick blue lines), increasing the resistance of the clot to fibrinolysis.

endothelial cells (Scheraga and Laskowski 1957; Shainoff and Dardik 1983). FPB release occurs more slowly than the release of FPA (Scheraga and Laskowski 1957). Each $E_A$ site subsequently combines with a constitutive complementary binding pocket (Da) in the D domain of neighbouring molecules (Everse *et al.,* 1998). The initial $E_A$:Da associations result in alignment of fibrin molecules in an end-to-middle staggered overlapping domain arrangement (Ferry 1952). $E_B$ also interacts with a complementary Db site and also contributes to lateral fibril and fiber associations (Shainoff and Dardik 1983). In addition to its role in mediating fibrin assembly, the β chain segment of the D domain binds heparin, mediates platelet spreading, fibroblast and endothelial spreading and proliferation, capillary tube formation, and release of von Willebrand factor (Hamaguchi *et al.,* 1993; Sporn *et al.,* 1995; Ribes *et al.,* 1989).

After FPB cleavage, αC domains become available for self-association with other αC domains, leading to the promotion of lateral fibril associations and fiber assembly. In fibrinogen, the αC domains, which originate in the D domain, tend to be non-covalently tethered to the E domain (Mosesson *et al.,* 1981). Therefore, they are not available to interact until conversion to fibrin. Interactions also occur between the outer portions of the D domains (D:D). Intermolecular association between these sites is necessary for proper end-to-end alignment of fibrinogen or fibrin molecules in assembling the polymer structure (Mosesson *et al.,* 1995).

Concomitantly, factor XIII is activated by thrombin to factor XIIIa. Factor XIIIa catalyzes the formation of γ dimers (the γ chain is part of the D domain) by incorporating ε-(γ-glutamyl)lysine bridges between a donor lysine of one γ chain and a glutamine

acceptor of another γ chain (Chen and Doolittle 1971). In addition, γ trimers and γ tetramers form slowly to complete the mature network structure (Shainoff *et al.*, 1991).

Plasminogen is the enzyme that catalyzes fibrin breakdown. Plasminogen activation occurs through binding of tissue-type plasminogen activator (tPA) to fibrin at various sites followed by addition of plasminogen to form a complex (Hoylaerts *et al.,* 1982). The binding sites become exposed after non-covalent D:E interactions during fibrin fibril assembly (Yakovlev *et al.,* 2000). Proteolytic cleavage by plasmin creates more lysine binding sites, which enhance fibrinolysis by increasing plasminogen accumulation (Harpel *et al.,* 1985).

Fibrinogen contains two integrin binding sites at Aα95-98 (RGDF amino acids) and at Aα572-575 (RGDS amino acids) in the E domain (Henschen *et al.,* 1983). Many cellular interactions with fibrin and fibrinogen occur through binding to one or both of these sites. These RGD sites bind platelet $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ integrins as well as other integrins on endothelial cells and fibroblasts (Cheresh 1987; Asakura *et al.*, 1997). Chondrocytes not only express the collagen-binding $\alpha_1\beta_1$ integrin; they can also express different integrins, including $\alpha_V\beta_3$ integrin, depending on the ECM that they come into contact with, which allows better integration with the ECM (Goessler *et al.,* 2005).

### 1.3.2 Fibrin in Tissue Engineering

Fibrin has been extensively evaluated as such a scaffold for tissue engineering of cartilage (Peretti *et al.,* 2006; Chang *et al.,* 2007; Endres *et al.,* 2007). Using commercially available fibrin sealant Chang *et al.* (2007) mixed chondrocytes with the fibrin and injected the mixture into the rabbit nasal dorsa. After 8 weeks, histological staining indicated the formation of normal auricular cartilage. Higher thrombin

concentrations were found to be directly correlated with successful cartilage regeneration because of the added matrix stability. Endres *et al.* (2007) combined human adult chondrocytes with the resorbable polymer, polyglycolic acid and fibrin and implanted the constructs subcutaneously into nude rats. The regenerated tissue stained positively for articular cartilage-like ECM. In another approach, fibrin was mixed with an apolyglycolic acid (PGA) mesh to form a solid cell delivery device that was easily manipulated *in vitro* (Ameer *et al.,* 2002).

Fibrin has been used as a scaffold for cartilage regeneration in combination with many other materials including chondroitin sulphate (Wei *et al.*, 2008), polycaprolactone-based polyurethane (Eyrich *et al.,* 2007a), and calcium-phosphate (Scotti *et al.*, 2007). A tri-copolymer of gelatin, hyaluronic acid, and chondroitin-6-sulphate, when combined with fibrin, promoted ECM secretion and inhibited ECM degradation *in vitro* (Chou *et al.,* 2007). With the combination of these materials with fibrin, it would be impossible to form an autologous scaffold as they are synthetic or xenogeneically derived. A fibrin system was used for *in situ* gelation for articular cartilage regeneration, where the gel precursors were mixed and injected directly into the defective area where gelation occurred. Only limited ECM synthesis was observed after 8 weeks *in vivo* (Peretti *et al.,* 2006). This approach enhanced integration of the material into the cartilage defect, but formed overly soft gels that were prone to rapid breakdown in the presence of cells (Grassl *et al.,* 2003). A non-cytotoxic, chemical cross-linker could potentially enhance the stability of the fibrin gel and so promote long term culture and implantation of gel encapsulated cells.

Chondrocytes have been widely used as a cell source for articular cartilage tissue engineering after encapsulation into fibrin (Eyrich *et al.,* 2007a; Peretti *et al.,* 2006; Mesa *et al.,* 2006). However, many other cell types have been used for cartilage regeneration including periosteal cells (Perka *et al.,* 2000), bone marrow stromal cells (Xu *et al.,* 2005), adipose tissue-derived MSCs (Im *et al.,* 2005), and bone marrow-derived mononuclear cells (Chang *et al.,* 2008). When added to the culture media, TGF-β2 and 3 (Perka *et al.,* 2000), IGF-I (Fortier *et al.,* 2002) and fibroblast growth factor-2 (FGF-2; Dragoo *et al.,* 2003) can act as inductive cues for the fibrin glue. *BMP-2* and *IGF-I* genes have been introduced by adenovirus-mediated transfection into mesenchymal cells for culture with fibrin glue for cartilage regeneration of partial thickness lesions (Gelse *et al.,* 2003).

### 1.3.3 Autologous Fibrin Glue

Fibrin sealant offers many advantages in surgery including rapid haemostasis (Mankad and Codispoti, 2001), accelerated wound healing (Amrani *et al.,* 2001), blood loss reduction (Carless *et al.,* 2002), and protection against bacterial infection (Currie *et al.,* 2001). Fibrin sealant has also been used as a tissue engineering scaffold (Silverman *et al.,* 1999; Peretti *et al.,* 2000; Xu *et al.,* 2004; Peretti *et al.,* 2006; Chang *et al.,* 2007; Endres *et al.,* 2007). Exogenous fibrin can elicit immunological reactions, and use of very high (non physiological) concentrations of fibrin has resulted in variable success. Further improvements in fibrin hydrogels are essential if successful articular cartilage regeneration can be achieved. The most commonly used commercially available fibrin sealant is of bovine origin, which can cause immunological reactions involving formation of antibodies against bovine thrombin. These antibodies cross-react with coagulation

factor V and cause bleeding (Zehnder and Leung, 1990). With implantation of bovine or human fibrin derived from pooled plasma, there is also the concern of the risk of transmission of viral (Jackson 2001) or prion (Hunter and Houston, 2002) infections.

Buchta *et al.* (2005), compared fibrin sealants produced by commercially available CryoSeal® and Vivostat® FS (fibrin sealant) devices to the industrially produced homologous fibrin sealant Tissucol/Tisseel®. CryoSeal® fibrin glue is prepared from one unit of plasma by concentrating fibrinogen and other clotting factors via freeze/thaw cycles. Vivostat® utilizes non-physiological conversion of fibrinogen to fibrin by batroxobin, which is a snake venom protease. The fibrinogen component is made from autologous plasma. The fibrinogen and thrombin produced for the Tissucol/Tisseel® sealant are purified from either bovine or human pooled plasma units (Buchta *et al.,* 2005). It was found that the autologous fibrin sealant products contained greater batch-to-batch variability and a reduced resistance to fibrinolysis compared to the Tissucol/Tisseel® industrially produced products (Buchta *et al.,* 2005). However, the CryoSeal® FS System provides a rapid and reliable way of producing a cryoprecipitate from autologous plasma in less than one hour.

One of the major advantages to using fibrin as a scaffold for articular cartilage tissue engineering is that the material can be isolated and processed from a single unit of autologous plasma using a technique that carries minimal risk of complications. Plasma is collected through apheresis: the whole blood is collected, centrifuged, the plasma is drawn off and the remainder of the blood is returned to the patient's circulation through a closed system (Rock *et al.*, 2001). The CryoSeal® fibrin glue is then prepared from the unit of plasma. This cryoprecipitate has been found to be comparable to the commercially

available products (Rock *et al.,* 2001). Fibrin sealant can also be processed from frozen plasma. The advantage of using frozen plasma for fibrin sealant production is the greater availability of material for implantation, including for homologous recipients that cannot donate plasma or have abnormal coagulation diseases (Peyvandi and Mannucci, 1999).

### 1.3.4 Fibrin Scaffold Architecture

The architecture of a tissue engineering scaffold must be designed to insure the seeded cells are biocompatible with the scaffold. The surface chemistry and structural biocompatibility are very important (Li *et al.,* 2002). It has been shown previously that the effective pore diameters for cell ingrowth for vascular grafts are 20-60 μm and for bone ingrowth are 75-150 μm (von Recum *et al.,* 1996). However, for cell encapsulation a structure more similar to the native tissue would be more appropriate. Zhao *et al.* (2008) described the structure of a fibrin gel derived from human plasma. For the 2% fibrin gels made with 2.5 U/ml thrombin (closest comparison to CryoSeal® gels) the average pore diameter can be estimated as approximately 740 nm. However, articular cartilage has a pore size ranging from 10-150 nm (Omelianenko 1991). Therefore, the CryoSeal® fibrin scaffolds are more porous than necessary. The strength of a fibre decreases as the fibre dimensions increase (Amornsakchai *et al.,* 1993). The fibrin fibre diameter of the gels derived from plasma was approximately 100-300 nm (Zhao *et al.,* 2008), whereas the fibre diameter of the articular cartilage ECM components ranges from 20 nm at the superficial zone to 400 nm at the cartilage/bone boundary (Omelianenko 1991). Therefore, the fibre diameter of the CryoSeal® fibrin scaffolds is likely similar to the deep zone of articular cartilage. This information indicates that the structure of the CryoSeal® fibrin scaffolds may be suitable for articular cartilage tissue engineering.

## 1.4 Genipin

In this study, genipin, a naturally occurring cross-linking agent with potential medical benefits, was used to stabilize the fibrin scaffolds. Genipin is derived from geniposide (Fig. 3), which is isolated from the fruits of the plant *Gardenia jasminoides* (Tsai *et al.,* 1994). Genipin and its related iridoid glucosides (geniposide) have been widely used in herbal medicine most notably as an antiphlogistic (anti-inflammatory) compound. In an animal study it was found that the inflammatory reaction to the genipin-fixed tissue was significantly less than that to glutaraldehyde-fixed tissue (Chang *et al.,* 2001). Genipin was shown to prevent N-O production by N-O synthase through blocking nuclear factor-κB activation, thereby inhibiting transcription of N-O synthase. It was also shown to have potent antiangiogenic activity, to possess anti-inflammatory activity, and to act as a specific hydroxyl radical scavenger (Koo *et al.*, 2004). Therefore, genipin may decrease inflammation and other effects of disease after implantation into an osteoarthritic joint. Genipin has also recently been shown to exhibit neurotrophic effects by inducing neurite outgrowth through N-O and protein kinase signalling pathways (Yamazaki *et al.,* 2006; Yamazaki and Chiba 2008).

Glutaraldehyde cross-links collagen molecules by reacting its aldehyde groups with the ε-amino group of lysyl or hydroxylysyl residues (Sung *et al.*, 1999). A polymer of glutaraldehyde molecules bridges the gap intramolecularly between appropriate amino acid residues of collagen molecules (Lee *et al.,* 1994). Glutaraldehyde has been used to cross-link bovine pericardium for use as heart valve bioprostheses (Gabbay *et al.,* 1984). When genipin cross-linking was compared to glutaraldehyde it was found to be significantly less toxic than glutaraldehyde and produced no clastogenic response

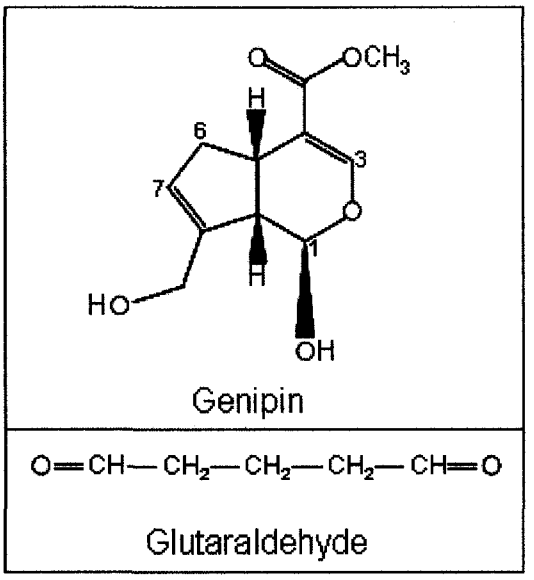


**Fig. 3. Structure of Cross-linkers.** Molecular structures of genipin and glutaraldehyde.

(mutation leading to chromosome breakage) compared to glutaraldehyde, which produced a weakly clastogenic response (Sung *et al.,* 1999; Tsai *et al.,* 2000). Very importantly, genipin has also been found to be significantly less cytotoxic and genotoxic than other chemical cross-linkers, such as glutaraldehyde, diisocyanates, and epoxides (Bedran-Russo *et al.,* 2006; Huang *et al.,* 1998; Sung *et al.,* 1998). The difference in cytotoxic activity is likely due to the structure of the molecules (Fig. 3). Glutaraldehyde has two very reactive aldehyde groups whereas genipin has an aldehyde ring structure that cross-links very differently than glutaraldehyde (Sung *et al.,* 1999). The reaction mechanism of genipin with biological tissue is still not well understood. It has been proposed that genipin reacts spontaneously with an amino acid to form a nitrogen-iridoid, which undergoes dehydration to form an aromatic monomer. Dimerization occurs at the second stage, perhaps by a radical reaction (Fujikawa *et al.,* 1988).

Genipin has been used to cross-link chitosan and/or gelatin hydrogels for tissue engineering applications (Mwale *et al.,* 2005; Moura *et al.*, 2007; Chiono *et al.,* 2007; Kuo and Ku 2007). Chitosan-gelatin scaffolds were cross-linked with genipin and modified with fibronectin. Cell proliferation and ECM production by scaffold-seeded bovine knee chondrocytes increased with increasing fibronectin concentration (Kuo and Ku, 2007). When the effect of genipin cross-linking on collagen I gels was evaluated, it was found that the mechanical properties of the gel increased with increasing genipin concentration, which was correlated with changes in fluorogenic properties of the collagen. At a concentration of genipin of 5 mM or more a significant decrease in cell viability was detected (Sundararaghavan *et al.,* 2008). Therefore, genipin has the potential to be beneficial as a cross-linking agent in articular cartilage tissue engineering at a concentration below 5 mM. Cross-linking of fibrin hydrogels by genipin for articular cartilage regeneration has not been reported previously; however, genipin has recently been used to cross-link dehydrated microporous fibrin scaffolds followed by seeding with NIH-3T3 fibroblast cells for general tissue engineering applications (Linnes *et al.*, 2007).

Factor XIIIa and genipin both compete for the primary amine sites on lysine residues in fibrin. Factor XIII is always present in fibrinogen isolated from blood and is activated by thrombin to Factor XIIIa, a transglutaminase enzyme, which then cross-links the side chains of lysine and glutamine residues in the fibrin monomers (Trumbo and Mauer 2000). The complete thrombin reaction is much faster than the complete genipin reaction (3-5 minutes vs. up to 2 hours respectively). A blue pigment results from the reaction of genipin with lysine, glycine and phenylalanine amino acids of a protein (Paik *et al.,* 2001). The associated formation of a blue colouration due to the genipin reaction is

very slow compared to cross-linking (taking up to 24 hours for complete colouration at 37°C) and may result from a separate reaction pathway (Sung *et al.* 1998; Mi *et al.* 2002), but the mechanism is not well known (Park *et al.,* 2002).

### 1.5 Physiological Conditions

It is important in the evaluation of a tissue engineering system to evaluate the cell construct in an environment that is as close to *in vivo* as possible. Mature articular cartilage is an avascular tissue that receives its nutrients, including oxygen, from the synovial fluid by diffusion and forced convection as a result of mechanical loading (McKibbin and Holdsworth, 1966). The oxygen tension of synovial fluid is approximately 7%. As a result the oxygen concentration in the deeper zones of cartilage closer to the bone-cartilage boundary can be as little as 1% (Grimshaw and Mason, 2000). As the oxygen concentration decreases, adenosine 5'-triphosphate (ATP) synthesis within the chondrocytes shifts from oxidative phosphorylation in the mitochondria to the oxygen-independent glycolysis pathway in the cytoplasm; the cells must increase their glucose intake and glycolytic rate to generate energy under hypoxia (Shapiro *et al.,* 1997). This metabolic change is shown by the fact that chondrocytes in the superficial zone of cartilage contain a higher mitochondrial volume density than cells in the deeper zones (Stockwell, 1991). The oxygen tension in the synovial fluid of osteoarthritic joints is abnormally low and as a result the cartilage environment is anoxic in the deep zones (Giatromanolaki *et al.*, 2003).

To survive this hypoxic environment, chondrocytes respond with highly conserved adaptive mechanisms (Distler *et al.,* 2004). The most important component of this hypoxic response is transcriptional activation as a result of binding of hypoxia

inducible factor-1 (HIF-1) to a hypoxia responsive element, which is present in most hypoxia inducible genes (Wang *et al.,* 1995). HIF-1 is normally degraded under normoxic conditions but under hypoxic conditions it is stabilized and can activate the transcription of several downstream target genes (Cockman *et al.,* 2000). In the presence of oxygen, prolyl hydroxylases (PHDs) catalyze the hydroxylation of HIF-1α. The hydroxylated HIF-1α subunits are then polyubiquitylated and targeted for proteasome-mediated degradation (Figure 4a-c), thus lowering cytosolic levels of the protein. An

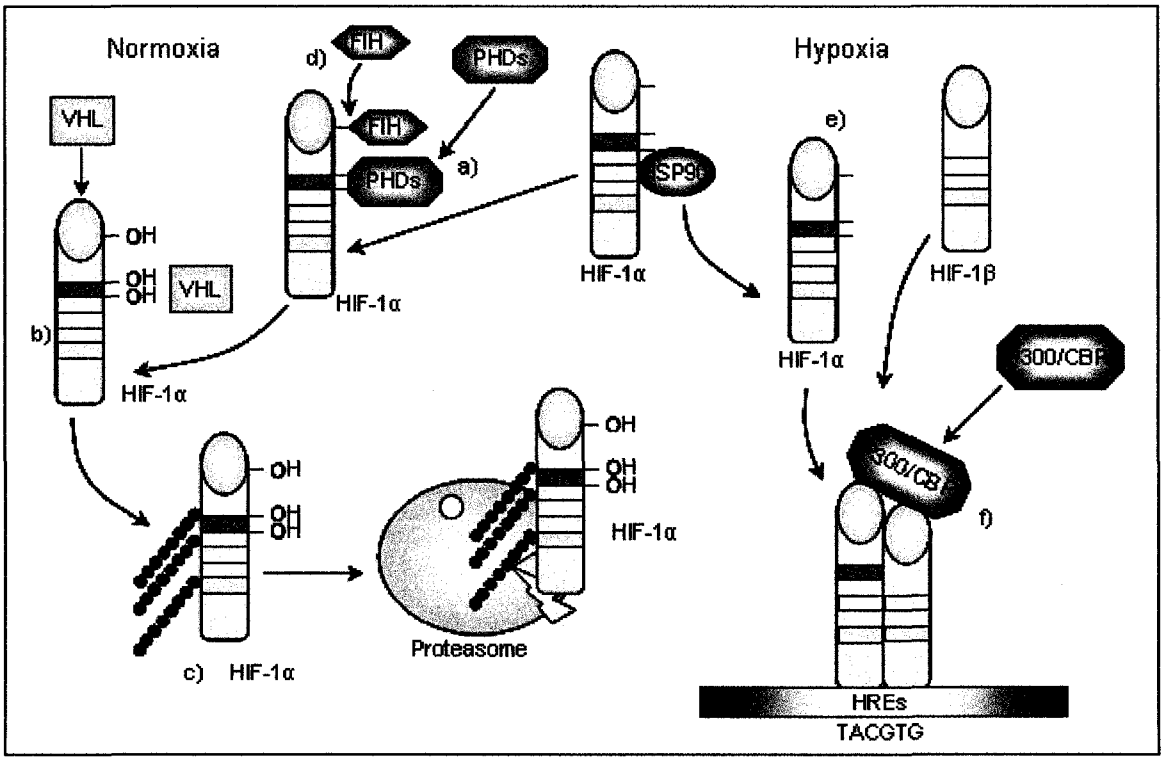


**Fig. 4. The HIF-1 Pathway. a)** Under normoxia, PHDs (blue octagons) bind to HIF-1α and catalyze hydroxylation of specific proline residues. **b)** Once hydroxylated, HIF-1α binds rapidly to the von Hippel-Lindau tumour-suppressor protein (VHL; pink rectangle), which results in its polyubiquitylation (purple spheres). **c)** HIF-1α is then targeted for proteasome-mediated degradation. **d)** An oxygen-dependent hydroxylation of a single asparagine residue within the C-terminal transactivation domain of HIF-1α is mediated by FIH and prevents the association of HIF-1α with p300/CBP. **e)** Under hypoxia, HIF-1α is stabilized, translocates to the nucleus, and dimerizes with HIF-1β. **f)** The complex associates with co-activator proteins p300/CBP to transactivate target genes containing hypoxia-responsive elements. Molecules are not drawn to scale. Adapted from Rocha (2007) *Trends Biochem Sci* **32** p. 391.

extra oxygen-dependent hydroxylation event mediated by factor inhibiting HIF-1 (FIH) takes place at the C-terminal transactivation domain of HIF-1α (Figure 4d). This modification prevents the association of HIF-1α with p300/CBP (CREB-binding protein) (Ruas and Poellinger 2005; Rocha 2007). To activate the majority of its target genes, HIF must associate with the transcriptional coactivator proteins p300 and CBP (Ruas and Poellinger 2005). However, this is not required for all HIF genes. Therefore, additional activating mechanisms or binding partners must also exist (Kasper and Brindle 2006). To date, more than 70 HIF target genes have been identified and validated in humans (Wenger *et al.,* 2005). HIFs target genes involved in cellular metabolism, cell growth and apoptosis, and restoration of the oxygen supply. Importantly, all of these target genes ensure that either the cell restores oxygen homeostasis (during which cells survive with minimal energy which is what is observed with articular chondrocytes) or that cells die due to persistent lack of energy (Rocha 2007).

Low oxygen also has an effect on ion homeostasis within the chondrocyte. Intracellular pH is maintained by chondrocytes as oxygen decreases to 5%. Chondrocytes possess many membrane transport systems that minimize changes in cytosolic composition when faced with fluctuating surroundings (Wilkins *et al.,* 2000). At lower oxygen, especially in association with disease, intracellular pH decreases significantly (Milner *et al.,* 2006). The main cause of this reduction in intracellular pH is the inhibition of acid extrusion across the plasma membrane, mediated by the amiloride-sensitive $Na^+$-$H^+$ exchanger (Milner *et al.,* 2006). It has also been shown that, at 1% compared to 20% oxygen, intracellular $Ca^{2+}$ levels are significantly elevated (White *et al.,* 2006). Because both $H^+$ and $Ca^{2+}$ are important for cell function and matrix production, these responses

have an important influence on cell function. In addition, chondrocytes increase synthesis of lactate dehydrogenase during hypoxia to metabolize lactic acid produced during anaerobic respiration (Milner *et al.,* 2006).

HIF-1 not only targets genes involved in cell metabolism and survival but it has also been proposed to target ECM genes (Pfander and Gelse 2007). HIF-2α has also been shown to target both Sox9-dependent and independent genes involved in ECM synthesis (Lafont *et al.*, 2007). Research has shown that decreasing the oxygen level during culture of tissue engineering constructs leads to an increase in collagen II and proteoglycan production, and in cell proliferation (Clark *et al.,* 1991; Domm *et al.,* 2002). *Collagen type I* expression was also delayed with low oxygen culture (Hansen *et al.,* 2001). Increased HIF-1α transcript levels have been found in osteoarthritic degenerated cartilage due to decreased oxygen in the synovial fluid (Yudoh *et al.,* 2005). In addition, enhanced gene expression of *collagen II* and other matrix components is also characteristic of osteoarthritic chondrocytes (Aigner *et al.,* 2001). This activity is generally interpreted as the cells attempting to restore degraded ECM. Recently it has been shown that N-O was induced when chondrocytes are exposed to hypoxia and this N-O inhibits reactive oxygen species-induced apoptosis. It was also shown that N-O is very important for inducing hyaluronic acid production by chondrocytes (Hashimoto *et al.,* 2006). Because oxygen tension plays such a vital role in chondrocyte function and survival, it is necessary to consider the importance of oxygen regulation and control in articular cartilage tissue engineering systems.

## 1.6 Research Summary

### 1.6.1 Rationale

Variable success has been achieved in articular cartilage tissue engineering especially in the translation of the systems to clinical use. To be suitable as a scaffold, the matrix will provide the proper environment for the cells to remain terminally differentiated, producing the characteristic ECM. The matrix will also be non-toxic to the cells during gelation and throughout culture, be biodegradable, and non-immunogenic. The matrix will also have the mechanical properties to withstand the natural forces in body. Fibrin is a structural protein that is important not only in haemostasis but also in cellular and matrix interactions and wound healing. Chondrocytes also have the ability to express integrins that allow them to interact with a fibrin scaffold (Asakura *et al.*, 1997). In addition, it is possible to isolate the components of the fibrin gel from human blood for autologous implantation and regeneration (Rock *et al.,* 2001). Therefore, fibrin was chosen as a scaffold for *in vitro* articular cartilage tissue engineering. The hypothesis is that a modified fibrin hydrogel is suitable for articular cartilage tissue engineering. To assess fibrin *in vitro* for cartilage tissue engineering several objectives must be attained.

### 1.6.2 Objectives

1) Chapter 2: To develop and assess the fibrin hydrogel for articular cartilage tissue engineering potential using the rat chondrogenic cell line RCJ 3.1C5.18.
2) Chapter 3: To evaluate the *in vitro* chondrogenic potential of a genipin modified fibrin hydrogel system with human articular chondrocytes, as they are a more feasible therapeutic cell source. The differentiation and viability of cells within the construct

will be assessed. In addition, because of its anti-inflammatory and material cross-linking properties, genipin will be evaluated as a cross-linker.

3) Chapter 4: To identified and assess an autologous method for fibrin material preparation. Human chondrocytes will be seeded into CryoSeal® fibrin glue that will be derived from single units of plasma and culture *in vitro*.
4) Chapter 4: To culture and evaluate the chondrocyte-seeded fibrin gels under more physiological (hypoxic) conditions.

If these objectives can be successfully met, the fibrin hydrogel system will be adequately assessed *in vitro* and will be ready for future design and assessment of an *in vivo* implantation system.

### 1.6.3 Research Approach

The summary of the research approach is given to direct the progression through the following chapters (collection of manuscripts).

Manuscript 1: Differentiation of a fibrin gel encapsulated chondrogenic cell line.

The chondrogenic precursor cell line, RCJ 3.1C5.18 (C5.18), was chosen as a test system for evaluating the effect of various culture conditions, including fibrin gel encapsulation, on chondrogenic cell differentiation. The C5.18 cells are derived from the fetal rat calvaria, and can differentiate into discrete 3D cartilage nodules when grown in monolayer in the presence of serum (Grigoriadis *et al.,* 1989), thereby acting as a homogenous population of chondrogenic precursors. In monolayer, it has been shown that C5.18 cells express collagen I within the nodule ECM; however, gene expression changes can be induced with culture on a type I collagen/agarose surface (McDougall *et al.*, 1996). Indeed we found that in monolayer the cells showed elevated expression of

markers for fibrocartilage differentiation (*collagen I* and *versican*) as well *collagen II*, an articular chondrogenic marker, when cultured with chondrogenic medium. High-density pellet culture of articular chondrocytes is an effective method for chondrogenic cell differentiation and matrix deposition (Xu *et al.,* 1996). Therefore, we compared the culture of C5.18 pellets cultured in chondrogenic medium to monolayer and fibrin-encapsulated cells. The cells within the chondrogenic medium-cultured pellets secreted an ECM that was comprised of type II with very little type I collagen, which indicated a trend towards a more hyaline-like chondrogenesis of the cells when compared to monolayer culture. Moreover, fibrin-encapsulated C5.18 cells, when cultured with chondrogenic medium, elaborated an ECM containing type II collagen and aggrecan. Only fibrin gel culture of C5.18 cells resulted in an increase in *collagen II* and *aggrecan* gene expression. Therefore, a more hyaline-like chondrogenic differentiation of C5.18 cells occurred with fibrin encapsulation. The results of these experiments provided evidence that the fibrin scaffold can induce chondrogenic differentiation of encapsulated cells. The gels used in this study were relatively weak and unstable. Therefore, the next step was to proceed with genipin as a cross-linker to stabilize the gels. The C5.18 cell line represented a homogeneous population of cells that were used to develop the culture and scaffold parameters; however, it was important to develop the system with human primary chondrocytes as they are a more suitable therapeutic cell source.

Manuscript 2: Genipin cross-linked fibrin hydrogels for *in vitro* human articular cartilage tissue engineered regeneration.

From the experiments described in the previous section, it was concluded that the fibrin hydrogel scaffold had potential for articular cartilage tissue engineering. The fibrin

gels used in the previous experiments were relatively weak and unstable. Therefore, to stabilize the fibrin gel, genipin, a naturally occurring cross-linker was used. To develop the system further towards a more implantable construct, commercially available human fibrinogen and thrombin were used to form the gel and were seeded with primary human articular chondrocytes. It was found that genipin added several benefits to the system. Genipin cross-linking did not significantly affect cell viability but did significantly increase the dynamic compression and shear moduli of the hydrogel material itself. Genipin cross-linking also led to enhanced accumulation of collagen II and aggrecan within the ECM secreted by the encapsulated chondrocytes after 5 weeks in culture. It is known that the relative ratio of *collagens II* to *I* can serve as an indicator of aberrant cartilage repair (Freemont and Hoyland, 2005). We found that the ratio of the change in *collagen II* versus *collagen I* gene expression increased more than 8-fold in genipin cross-linked gels at 5 weeks. Overall, these results indicated that genipin cross-linked fibrin hydrogels can be used as cell-compatible tissue engineering scaffolds for cartilage regeneration. After subcutaneous implantation of acellular fibrin scaffolds into Sprague-Dawley rats, a decrease in the amount of inflammation was observed with genipin cross-linking after 3 weeks. In addition, it was found that the species origin of the fibrin material was important. The amount of degradation and inflammation was decreased when the fibrin was allogeneic (of rat origin) when compared to xenogeneic fibrin (of human origin). While these results implicate that it is very important to implant allogeneic fibrin, because of the associated risks of infection, it was important to extend the results further and pursue the development of autologous fibrin scaffolds, where the material for implantation is derived from the recipient individual.

Manuscript 3: Comparison of fibrin sealants derived from fresh or fresh/frozen plasma as scaffolds for *in vitro* articular cartilage regeneration.

One advantage of fibrin is that there is potential for producing an autologous scaffold, which is not true for other materials such as collagen. Previous results indicated that it was important to implant allogeneic fibrin. It was then necessary to identify an autologous source of fibrin in order to avoid the issues associated with disease transmission. The CryoSeal® FS System can rapidly and reliably produce autologous fibrin glue (Rock *et al.,* 2001). The CryoSeal® product appeared to be an appropriate candidate scaffold for articular chondrocyte culture; however, the material has not previously been used as an *in vitro* cell culture system. To provide evidence that the system may be likely to succeed in the native joint environment, culture under hypoxia was undertaken. Using the CryoSeal® fibrin glue, we found that chondrocyte viability was >90% in fibrin gels derived from either fresh or frozen plasma. This was a marked improvement on the system described in the previous section, where the viability was ~50% with and without genipin cross-linking. Culture under hypoxia had a significant impact on the results. Gene expression analysis revealed that there were significant increases in *collagen II* and *Sox9,* and a significant decrease in *collagen I,* under hypoxic culture. Low oxygen culture also led to a significant increase in total GAG accumulation. We also observed differences between the fresh and frozen plasma-derived fibrin materials, in that significant changes in gel mechanical properties after 7 weeks of culture were only observed for the material derived from fresh plasma. This may be explained by the greater amount of collagen type I detected in frozen compared to fresh plasma gels, as it is known that type I collagen changes the hydration and compressive properties of

articular cartilage (Hasler *et al.,* 1999). In addition, a significant increase in *collagen I* gene expression over time was detected only in frozen plasma-derived cultures. Our results indicate that CryoSeal® fibrin glue derived from fresh plasma shows potential as a scaffold for autologous human articular cartilage regeneration.

## 1.7 Summary

Articular cartilage regeneration is important as this tissue is necessary for normal joint function and its degeneration associated with disease is a common occurrence among the aging population. Our fibrin scaffold induced chondrogenic differentiation and articular cartilage-like matrix production by chondrogenic progenitors and primary articular chondrocytes *in vitro.* The genipin cross-linked fibrin hydrogel scaffold shows promise for autologous articular cartilage regeneration.

## CHAPTER 2

The chondrogenic precursor cell line, RCJ 3.1C5.18 (C5.18), was chosen to develop chondrogenic conditions for culture of fibrin-encapsulated cells. Traditional culture conditions for the C5.18 cells include monolayer culture with medium containing high serum content (15%; Grigoriadis *et al.,* 1989; Grigoriadis *et al.,* 1996). Since articular cartilage is an avascular tissue (McKibbin and Holdsworth, 1966) and hence the cells are not exposed to blood, we hypothesized that culture of the C5.18 cells with serum-free medium containing more chondrogenic-specific growth factors would result in chondrogenic differentiation and regeneration of tissue with some properties of articular cartilage. In addition, since gene expression in C5.18 cells can be induced when they are culture on a biomaterial surface (McDougall *et al.*, 1996), they were chosen as an appropriate chondrogenic cell source for studying fibrin gel encapsulation. In the following manuscript we present and discuss the results of the comparison of basic culture versus chondrogenic fibrin gel culture of C5.18 cells in the development of a fibrin tissue engineering system.

### 2.1 Manuscript 1

**Differentiation of a Fibrin Gel Encapsulated Chondrogenic Cell Line**

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**Differentiation of a Fibrin Gel Encapsulated Chondrogenic Cell Line**

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**Abstract**

Hyaline cartilage has very limited regenerative capacity following damage. Therefore engineered tissue substitutes have been the focus of much research. Our objective was to develop a fibrin-based scaffold as a cell delivery vehicle and template for hyaline cartilage regeneration, and compare its cellular properties against monolayer and pellet culture for chondrogenic cells. The chondrogenic precursor cell line, RCJ 3.1C5.18 (C5.18), was chosen as a test system for evaluating the effect of various culture conditions, including cell encapsulation, on articular chondrogenic cell differentiation. The C5.18 cells in monolayer showed elevated expression of collagen II, an articular chondrogenic marker, but also markers for fibrocartilage differentiation (collagen I and versican) when cultured with chondrogenic medium as compared to basic maintenance medium. Pellets of C5.18 cells cultured in chondrogenic medium were histologically more organized in structure than pellets cultured in control maintenance medium. The chondrogenic medium cultured pellets also secreted an extracellular matrix that was

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comprised of type II with very little type I collagen, indicating a trend towards a more hyaline-like cartilage. Moreover, when cultured in chondrogenic medium, fibrin-encapsulated C5.18 cells elaborated an extracellular matrix containing type II collagen, as well as aggrecan, which are both components of hyaline cartilage. This indicated a more articular-like chondrogenic differentiation for fibrin encapsulated C5.18 cells. The results of these experiments provide evidence that the C5.18 cell line can be used as a tool to evaluate potential scaffolds for articular cartilage tissue engineering.

**Key Words:** Fibrin; Hydrogel; Chondrogenic Differentiation; Cartilage; Regeneration

**Running Header:** Chondrogenic Differentiation of C5.18 Cells

## INTRODUCTION

Hyaline articular cartilage is an avascular, alymphatic tissue that consists of chondrocytes encapsulated within a dense extracellular matrix (ECM). When damaged due to osteoarthritis, its regenerative potential is minimal and healing occurs with the formation of fibrocartilage (1). Fibrocartilage does not have the appropriate physical and mechanical properties to resist the forces that occur during normal wear and tear in daily articular joint function (2).

Chondrocytes are essential for maintaining the function of articular hyaline cartilage (3). They secrete the ECM components, mainly type II collagen and aggrecan, as well as matrix metalloproteinases (MMPs) for matrix remodelling (4,5). The transcription factor Sox9 regulates collagen II production and its expression is used as an early marker for chondrogenesis in hyaline cartilage formation (6,7). During abnormal fibrocartilage development in repair, chondrocytes re-enter the cell cycle and proliferate, losing *Sox9* expression, and actively transcribe *collagen I* and *versican*, rather than *collagen II* and *aggrecan* (8).

Because of the poor capacity of hyaline cartilage for regeneration, cell and tissue engineering-based therapies for repair have been intensively researched. The rat calvaria-derived RCJ 3.1C5.18 (C5.18) cell line has key cartilage progenitor properties (9). This cell line differentiates and forms discrete three-dimensional (3D) cartilage nodules when grown in monolayer (10), thereby acting as a homogenous population of chondrogenic precursors. Therefore, C5.18 cells may be a useful progenitor line for the evaluation of *in vitro* 3D scaffold systems.

Many synthetic and biological materials can be used to form 3D scaffolds for tissue engineering (11-13). The mechanical properties and micro-pore structure of the material are important to consider when the scaffold is being designed (14). The tissue engineered construct must allow for a mechanically secure and stable fixation into the host tissue (15). Computer-aided design such as free-form fabrication and rapid prototyping techniques can be used to reproducibly fabricate scaffolds from a variety of biomaterials (16). Synthetic polymer-based 3D scaffolds have been reported to enhance stem cell delivery and retention and several groups have reported promising scaffold-based tissue engineered cartilage (17-19). In a recent study, fibrin-impregnated, porous polyurethane foams were found to improve the quality and quantity of the regenerated cartilage (20) suggesting the utility of fibrin as a scaffold. Moreover, commercially available fibrin glue is a stable scaffold for *in vitro* culture of bovine articular chondrocytes, leading to development of cartilaginous tissue (21). Our objective was to investigate the feasibility of developing a natural polymer system. A fibrin-based scaffold has the advantage that all components (fibrinogen, thrombin and chondrocytes) could be harvested from the patient allowing autologous implants to eliminate issues of immunocompatibility and viral infection.

In this study we have used the C5.18 line as a stable cell source for evaluating and optimising the fibrin hydrogel for *in vitro* articular cartilage regeneration. We compared articular chondrogenic differentiation of C5.18 cells cultured in monolayer or pellet culture, with that seen after *in vitro* encapsulation into 3D fibrin gels. The chondrogenic gene expression and articular cartilage specific collagen and proteoglycan secretion from

C5.18 cells encapsulated in fibrin hydrogels suggest that this is a promising approach for tissue engineering of articular cartilage.

## MATERIALS AND METHODS

### Cell Culture

The C5.18 cells (a gift from Dr. J. Aubin, University of Toronto) were maintained in αMEM with 15% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and $10^{-7}$ M dexamethasone (hereafter referred to as maintenance medium) (Sigma, St. Louis, MO) (9). Chondrogenic differentiation medium (hereafter referred to as chondrogenic medium) consisted of DMEM (high glucose; Sigma, St. Louis, MO), $10^{-7}$ M dexamethasone, 100 μg/ml sodium pyruvate, 40 μg/ml L-proline (Sigma, St. Louis, MO), 50 μg/ml ascorbic acid 2-phosphate (Fluka, St. Gallen, Switzerland), 1x ITS-plus (0.625 μg/ml human recombinant insulin, 0.625 μg/ml transferrin, 0.625 ng/ml selenous acid, 0.125 mg/ml bovine serum albumin, 0.535 μg/ml linoleic acid; BD Biosciences, San Jose, CA), 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco BRL, Rockville, MD), and 10 ng/ml TGF-β3 (R&D Systems, Minneapolis, MN) (22).

C5.18 monolayer cultures, once confluent, were supplemented with either chondrogenic or maintenance medium. For pellet cultures, confluent C5.18 cells were trypsinized and 3 x $10^5$ C5.18 cells were added to each of 10 x 14 ml polypropylene round-bottom tubes (BD Biosciences, San Jose, CA) and centrifuged at 1500 rpm for 5 min to obtain pellets. Pellets were maintained in 0.5 ml of chondrogenic or maintenance medium.

**Fibrin Hydrogels**

Porcine fibrinogen (Sigma, St. Louis, MO) was dissolved in DMEM, and sterilized by vacuum filtration (0.45 μm-pore filter). The fibrinogen solution (100 mg/ml final) was mixed with C5.18 cells (20 x $10^6$ cells/ml final), then porcine thrombin (Sigma, St. Louis, MO) (2.2 IU/ml final) and pipetted into the wells of a 24-well plate (BD Biosciences, San Jose, CA) with a final volume of 250 μl. The solution gelled in ~5 minutes at 20°C. The final thickness of the gels was approximately 1 mm. Approximately 30 minutes after casting the gels, 500 μl of chondrogenic medium was pipetted into each well. The medium was changed every 2.5 days for up to 3 weeks in culture.

**Histochemistry**

Post-confluent cell monolayers were fixed for 10 minutes with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS). Fixed cultured were stained with 1% Alcian Blue (w/v) (BDH Chemicals Ltd., Toronto, ON) in 3% acetic acid overnight at 20°C (10).

To assess alkaline phosphatase (AP) activity, samples were washed in AP buffer (0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 0.01 M $MgCl_2$) for 10 minutes and resolved in AP buffer supplemented with 0.01% sodium deoxycholate, 0.02% NP-40, 337 ug/ml NBT (Nitro-Blue Tetrazolium Chloride; Sigma, St. Louis, MO), and 175 ug/ml BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt) (Sigma, St. Louis, MO) for 15 minutes at 20°C. Samples were washed with PBS and visualized under bright field conditions.

For cell pellet cultures or fibrin gels, samples were fixed in 4% PFA in PBS and cryosectioned. Rehydrated sections were stained with Alcian Blue for 45 minutes and AP

as described above. For Safranin O staining (Fisher, Hampton, NH), slides were rinsed in $ddH_20$, stained in 0.2 % Safranin O in 1 % acetic acid for 4 minutes and destained in running tap water for 5 minutes (23). All stained sections were 10 μm in thickness. Slides were dehydrated through an alcohol series, cleared in xylene, and mounted with a coverslip. Rat articular cartilage that was harvested from adults Sprague-Dawley rats (>6 weeks) was sectioned and stained with Alcian Blue and Safranin O as a positive control.

**Immunostaining**

Fixed (4% PFA) monolayer plates and pellet and fibrin gel sections were digested with 2% bovine testicular hyaluronidase (Sigma, St. Louis, MO) in Tris-Buffered Saline (TBS), pH 7.4 for 30 min at 20°C followed by incubation for one hour in blocking solution (TBS containing 4% FBS, 1% BSA). Sections and plates were incubated overnight at 4°C with primary antibodies to collagen II (AB2036), collagen I (AB745) or aggrecan (AB1031) (Chemicon; Temecula, CA) diluted in blocking solution 1:40 with 0.1% Triton X-100. Samples were extensively washed in TBS and then reacted with their respective Alexafluor conjugated secondary antibody (Molecular Probes, Eugene, OR) at 1:400 in blocking solution for one hour at 20°C. After extensive washing in TBS, samples were stained with DAPI (4'6-diamidino-2-phenylindole; Sigma, St. Louis, MO) 1:10,000 in PBS to visually locate cell nuclei and mounted. Adult rat articular cartilage was used as a positive control.

**Real-Time RT-PCR and Analysis**

Rat-specific primers for Real-Time RT-PCR for *glyceraldehyde-3-phosphate dehydrogenase (GAPDH), collagen II, collagen I, aggrecan, versican* and *Sox9* were obtained from SuperArray (Frederick, MD).

Trizol (Invitrogen, Carlsbad, CA) was used to extract and purify RNA from cells grown in monolayer or in pellet culture, as per manufacturer instructions and the RNeasy Fibrous Tissue Kit (Qiagen, Valencia, CA) was used to extract RNA from C5.18 cells encapsulated in fibrin gels. First strand cDNA was synthesized by combining 2 μg total RNA, 0.5 μg of Oligo dT, 0.5 μg of random hexamers (denatured at 65°C for 10 min; Promega, Madison, WI), 25 μM dNTP, 10 IU RNase inhibitor, 200 IU M-MLV Reverse Transcriptase, 0.01M DTT, and Reaction Buffer (Invitrogen, Carlsbad, CA) as per manufacturer instructions. For the PCR reaction 1X of the SYBR Green PCR Master Mix (BioRad, Hercules, CA) was combined with 1 μl of primer complex, 2 μl cDNA and $H_2O$ to a final volume of 25 μl. PCR amplification was performed using an iCycler (BioRad, Hercules, CA) with initial denaturing at 95°C for 3 minutes, 45 cycles of 95°C (30 seconds)/55°C (30 seconds)/72°C (30 seconds), and a final extension at 72°C for 10 minutes. A melt curve analysis was performed as follows: 95°C for 1 minute, 55°C for 1 minute, and 55°C for 10 seconds with an increase of 0.5°C at each successive cycle for 80 cycles.

The comparative $C_T$ method was used to analyse the results (24). C5.18 cells at confluence were used as a baseline for each gene, which was further normalized to *GAPDH* housekeeping expression. The $\Delta\Delta C_T$ was calculated with the equation: $\Delta\Delta C_T =$

$\Delta C_{T(target)} - \Delta C_{T(baseline)}$, with the baseline value obtained from C5.18 cells in monolayer at confluence. The relative gene expression level was determined by calculating $2^{(-\Delta\Delta C_T)}$.

### Statistical Analysis

Analysis of gene expression data was performed using a one-way Analysis of Variance (ANOVA) for the three treatment groups under chondrogenic conditions. The Tukey's post-hoc test was used for further analysis with $\alpha = 0.01$ unless otherwise indicated. Data was graphed using SigmaPlot version 8.0 (Systat Software Inc., Point Richmond, CA).

## RESULTS

### Effects of Chondrogenic Differentiation Medium

At confluence, cells cultured in monolayer all had a fibroblast-like morphology with no accompanying Alcian Blue staining (data not shown). C5.18 cells in post-confluent monolayer cultures formed 3D ECM-dense nodules of similar size and dimension 2 weeks after reaching confluence regardless of culture medium provided (Fig. 1). Nodules that developed under maintenance conditions showed diffuse Alcian Blue staining with higher cell density than chondrogenic cultured nodules (Fig. 1a). Chondrogenic conditions led to a more intense staining (Fig. 1b). Nodules that developed in maintenance medium exhibited higher levels of AP staining (Fig. 1c), which is more characteristic of osteogenic cells (25), compared to cells in chondrogenic medium (Fig. 1d).

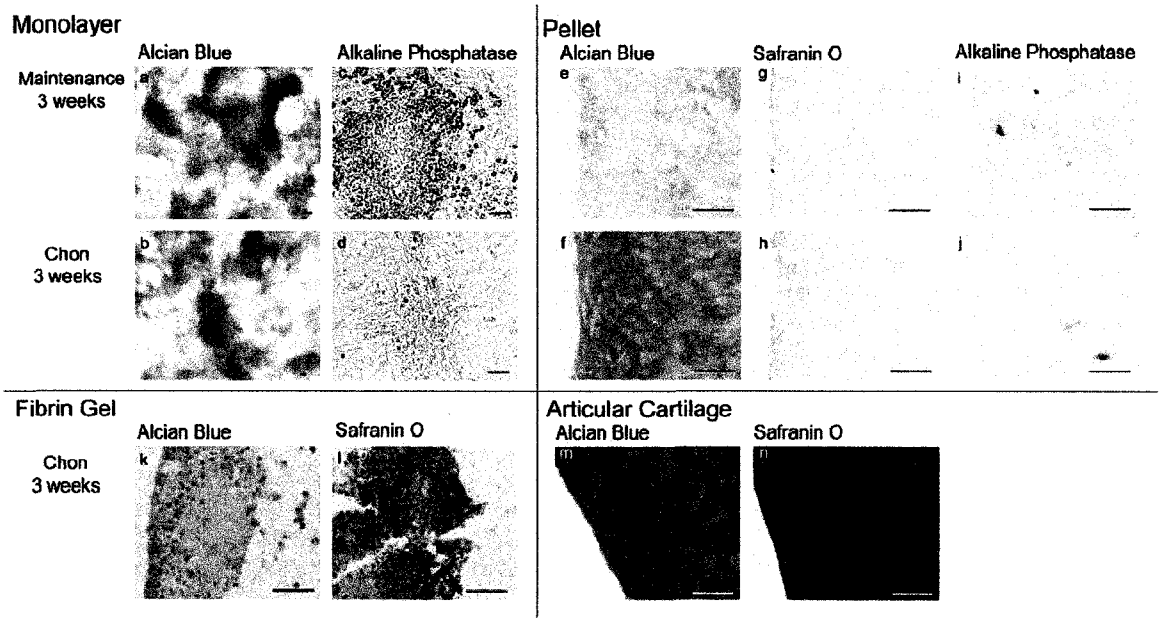


**Fig. 1.** Evaluation of differentiation of C5.18 cells after 3 weeks. Post-confluent monolayer cultured C5.18 cells cultured with chondrogenic (chon) and maintenance media were stained with Alcian Blue (a,b respectively) and for AP activity (c,d). Pellet cultures at 3 weeks (10 μm sections) were stained with Alcian Blue (e,f), Safranin O (g,h) and for AP activity (i,j) after chondrogenic or maintenance culture. Chondrogenic cultured fibrin gels (10 μm sections) were stained with Alcian Blue (k) and Safranin O (l). Positive control rat patellar articular cartilage was stained with Alcian Blue (m) and Safranin O (n). All bars 50 μm.

**Cell Differentiation and ECM Production**

Alcian Blue staining indicated that glycosaminoglycans (GAGs) were produced by cells when cultured to 3 weeks with chondrogenic medium in monolayer, in pellets, or when encapsulated within fibrin (Fig. 1b,f,k). Alcian Blue staining of pellet cultured C5.18 cells was much more intense for those cultured with chondrogenic medium (Fig. 1f) than with maintenance medium (Fig. 1e) Slightly more intense AP staining was observed for C5.18 pellets cultured in maintenance medium (Fig. 1i) as compared to chondrogenic medium (Fig. 1j). Alcian Blue staining of fibrin encapsulated C5.18 cells (Fig. 1k) was more similar to Alcian Blue stained articular cartilage staining (Fig. 1m) than were other cell cultures. Moreover, intense Safranin O staining was observed in

cultures of fibrin-encapsulated cells (Fig. 1l), in contrast to pellet cultures but similar to the positive control, rat articular cartilage (Fig. 1n). In fibrin gel culture, the cells had a rounder morphology than monolayer cultured cells, which is typical of articular chondrogenesis (Fig. 1k,l). Fibrin encapsulated cells in maintenance medium were not tested for histology or AP staining because the high serum concentration promoted rapid gel degradation.

Sections of 3D encapsulated cells were counted to measure proliferation. The average density at 24 hours was $7.05 \pm 0.65$ cells/100 $\mu m^2$ and increased after 3 weeks to $19.4 \pm 4.69$ cells/100 $\mu m^2$ (approximately 2.75-fold increase in cell number). Cell density was not analyzed for other gel formulations with different concentrations of thrombin or fibrinogen.

Immunofluorescence staining for components of hyaline cartilage matrix in cells cultured in chondrogenic medium revealed more intense staining for collagen II (Fig. 2a,d) in pellet cultured cells compared to monolayer. In contrast, collagen I was slightly more intense in monolayer cultured cells (Fig. 2c,f). The main notable difference in aggrecan staining between monolayer and pellet cultures was that it was observed within the entire pellet (Fig. 2e), whereas weaker staining of the cartilage nodules was observed in monolayer cultures (Fig. 2b).

Cells cultured within fibrin gels stained positively for collagen II (Fig. 2g) and aggrecan (Fig. 2h). The staining was a similar in intensity to the positive control rat cartilage sections. The intensity of collagen II staining in pellet cultures was greater than in fibrin gel sections. Notably, there was little collagen I staining of fibrin cultures (Fig. 2i, similar to background Fig. 2l).

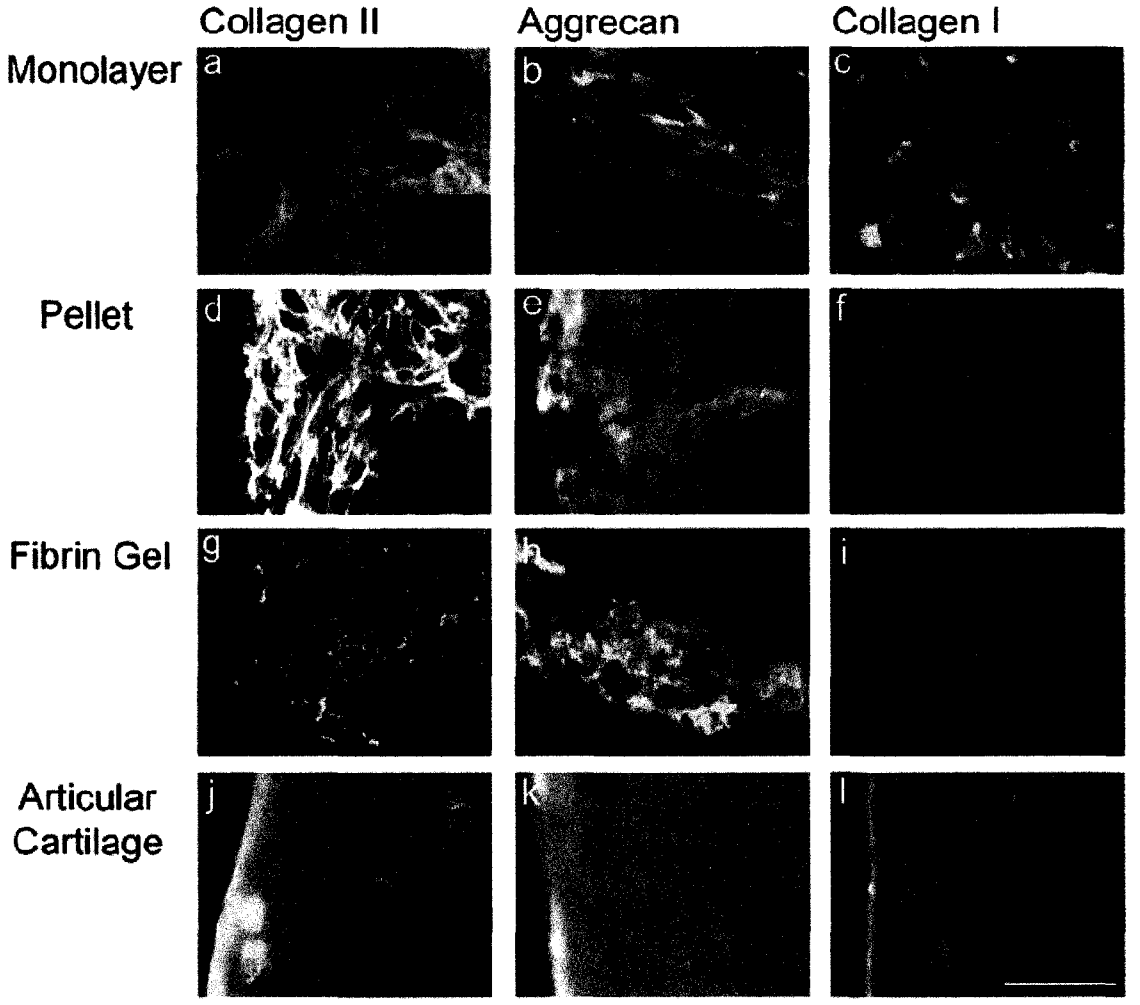


**Fig. 2.** Immunostaining of C5.18 cells. The C5.18 cells cultured in monolayer at 3 weeks in chondrogenic medium were stained for collagen II (a), aggrecan (b), or collagen I (c) primary antibodies and goat anti-rabbit Cy2 labelled secondary antibodies. Sections of C5.18 cells cultured as pellets (d,e,f) or encapsulated in fibrin gels (g,h,i) were immunostained for collagen II, aggrecan, and collagen I, respectively. Sections of rat patellar cartilage were stained as a positive control (j,k,l). All sections were also stained with secondary antibody as above. Negative controls for each tissue were stained with only the secondary antibody and DAPI to visualize nuclei (a,d,g,j; insets). Bar 50 μm.

**Gene Expression in Monolayer, Pellet and Fibrin-encapsulated Cells: Real-Time RT-PCR**

At 14 days in chondrogenic culture (Fig. 3a) expression of *collagen I* and *II*, *aggrecan*, *versican* and *Sox9* were evaluated in C5.18 cells cultured in monolayer, pellet and fibrin gel cultures. One-way ANOVA analysis revealed significant differences in

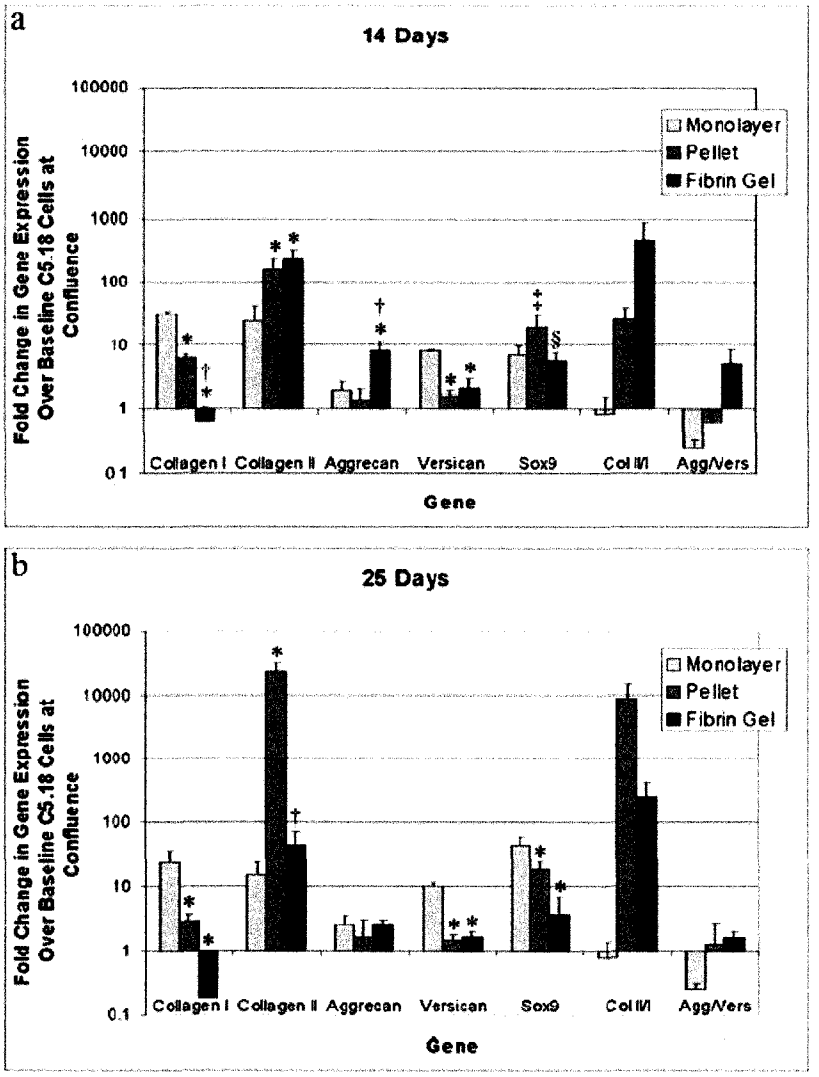


**Fig. 3.** C5.18 gene expression results. Real-Time RT-PCR analysis was performed on C5.18 cells cultured with chondrogenic medium to 14 days (a) and 25 days (b) in monolayer, pellet culture, and in fibrin gel culture using PCR primers to *collagen II*, *aggrecan*, *Sox9*, *collagen I*, and *versican* rat genes. Values are the fold-change in gene expression over baseline calculated for each gene using the comparative $C_T$ method. *Significant difference from chondrogenic monolayer cells with Tukey's post-hoc test, $\alpha = 0.01$. †Significant difference from chondrogenic pellets with Tukey's post-hoc test, $\alpha = 0.01$. ‡Significant difference from chondrogenic monolayer cells with Tukey's post-hoc test, $\alpha = 0.05$. §Significant difference from chondrogenic pellets with Tukey's post-hoc test, $\alpha = 0.05$. Error bars represent standard deviations to the means.

relative gene expression among all treatment groups [*collagen II* ($p < 0.0001$), *collagen I* ($p < 0.0001$), *aggrecan* ($p < 0.0001$), *versican* ($p < 0.0001$), and *Sox9* ($p = 0.014$)]. Tukey's post-hoc tests indicated that the *collagen I* gene expression was significantly less

and *collagen II* gene expression was significantly greater in both pellet and fibrin cultures compared to monolayer. Only fibrin gel cultured cells had significantly greater *aggrecan* expression compared to monolayer or pellet culture. Both pellet cultured and fibrin encapsulated C5.18 cells showed a significant decrease in *versican* expression compared to monolayer. Only pellet cultured C5.18 cells showed a slight but significant increase in *Sox9* gene expression compared to monolayer cultures ($\alpha = 0.05$) or fibrin gel encapsulated cells ($\alpha = 0.05$).

At 25 days in chondrogenic culture (Fig. 3b), significant differences in relative gene expression were found among monolayer, pellet and fibrin gel treatment groups for *collagen II* ($p < 0.0001$), *collagen I* ($p < 0.0001$), *versican* ($p < 0.0001$), and *Sox9* ($p < 0.0001$) by one-way ANOVA. Expression for *aggrecan* was not found to be significantly different among the 3 groups ($p = 0.18$). Tukey's post-hoc test was used for further analysis. The *collagen I* expression was significantly decreased in pellet and fibrin gel culture compared to monolayer, whereas only pellet cultured cells showed a significant increase in *collagen II* expression compared to monolayer. This increase was also significantly greater than the level observed in fibrin gel culture. Both *versican* and *Sox9* gene expression were significantly decreased in pellet and fibrin gel culture compared to monolayer culture.

The ratios of *collagen II/I* and *aggrecan/versican* were used as indices of articular chondrogenesis. The ratio of *col II/I* and *agg/vers* in confluent cultures were 1.20 and 1.06 respectively. After 25 days in monolayer culture, the ratios of *col II/I* and *agg/vers* were similar when cultured in either maintenance ($0.60 \pm 0.08$ and $0.29 \pm 0.03$ respectively) or chondrogenic conditions ($0.78 \pm 0.60$ and $0.25 \pm 0.06$ respectively). The

ratio of *col II/I* and *agg/vers* expression in pellet cultures at 25 days under maintenance conditions were 33.6 ± 2.87 and 2.43 ± 1.88, respectively, indicative of greater articular chondrogenic differentiation. However, under chondrogenic conditions, the ratios of *col II/I* dramatically increased to 8960 ± 5920, although the *agg/vers* ratio was only 1.28 ± 1.50 indicating that C5.18 pellets under chondrogenic conditions exhibited a dramatic and selective increase in *collagen II* expression. By 25 days in culture the *coll II/I* ratio was 243 ± 178 and the *agg/vers* ratio was 1.59 ± 0.39 for the fibrin gel cultured cells indicating that these conditions promoted the development of articular characteristics.

**Discussion**

A variety of cell-based approaches have been previously evaluated for tissue engineering of articular cartilage (26-28). Primary chondrocytes from human or animal origin have been utilized as a cell source for developing and comparing different scaffold materials for tissue engineering; however, there is great variability with such cells due to the age of the animal, extent of disease if present and passage number (29). Chondrogenic cell lines that are available for the study of chondrogenesis include the teratocarcinoma derived murine ATDC5 cell line and the clonal rat C5.18 line (30). Clonal cell lines exhibit a reproducible phenotype under defined cell culture conditions and differentiate in a predictable manner throughout numerous serial passages, in contrast to primary chondrocytes (9). Chondrocytes lose their differentiation phenotype in culture if allowed to adhere to a substrate in the presence of serum (31). However, if differentiated chondrocytes are embedded into artificial matrices and hence prevented from adhering to a culture dish, they maintain the articular chondrogenic phenotype (32,33). Several studies have shown that the encapsulation of chondrocytes into 3D matrices enhances

their ability to produce cartilage tissue (18,19). For example, microfracture surgery combined with implantation of a chitosan-glycerol phosphate scaffold significantly promoted hyaline tissue repair compared to control defects (17). Other groups have used fibrin for cartilage tissue engineering, but with mixed results. In particular, development of hyaline-like cartilage was insufficient (34,35).

In this study we have evaluated a natural fibrin system for controlled differentiation of encapsulated chondrogenic cells. This is a relatively simple system where fibrinogen, thrombin and cells were combined to form a robust gel, which did not require stabilization with protease inhibitors, in contrast to other formulations (36,21). We have explored the ability of a fibrin gel scaffold to support chondrogenesis of the C5.18 model cell line and formation of a cartilage-like ECM, with the ultimate goal of developing a scaffold material for repair of articular cartilage defects. When cultured in monolayer with minimal essential medium and high serum concentration, C5.18 cells develop from an undifferentiated phenotype into hypertrophic chondrocytes surrounded by a matrix that stains intensely with Alcian Blue (10). We have altered the culture conditions in order to promote articular chondrogenesis of the cells. This line has not previously been used to study *in vitro* chondrogenesis after 3D encapsulation. We assessed for the type of differentiation in the cells by looking for markers of articular (collagen II, aggrecan) versus fibrocartilage (collagen I, versican) chondrogenesis (8).

Our results indicate that aggregates of C5.18 cells, whether as nodules in post-confluent monolayers or in pellet culture, underwent differentiation into cartilage-like tissue when supplemented with an articular chondrogenic differentiation-promoting medium, which has not previously been shown. However, differences were observed in

the morphology and histochemical properties of the neo-cartilage tissue formed under these conditions.

Our AP and immunostaining results are consistent with previous reports that C5.18 cells cultured in monolayer in maintenance medium undergo hypertrophic chondrogenesis (37). With contrast, these cultures in chondrogenic medium developed nodules that were positive for markers of both hyaline (aggrecan and collagen II) and fibrocartilage differentiation (collagen I and versican) (38,39). Therefore, under chondrogenic conditions, C5.18 cells are likely a mixture of subpopulations of fibrochondrocytes and articular chondrocytes (8,38). Alternatively, the *collagen I* expressing cells may be fibroblasts or uncommitted cells (40) rather than fibrochondrocytes. This comparison confirms the efficacy of the chondrogenic medium in enhancing chondrogenesis leading to C5.18 differentiation to the chondrogenic lineage.

The C5.18 chondrogenic progenitors encapsulated in fibrin differentiated towards hyaline cartilage-like tissue. This is supported by results at 14 days in culture where only the encapsulated C5.18 cells showed a more articular chondrogenic pattern of gene expression compared to pellet and monolayer cultures with significant increases in *aggrecan* and *collagen II*. By 25 days however, a dramatic increase in *collagen II* mRNA expression was observed in pellet cultures, which was significantly greater than in fibrin gel cultures. Enhanced immunostaining for collagen type II was observed in concert with the upregulation of gene expression. There was more intense collagen II staining in pellet cultures than fibrin gels or even articular cartilage positive control samples.

Immunostaining is only a qualitative method. The collagen II epitopes may have been masked in the native cartilage samples.

While aggrecan gene expression was not significantly upregulated in the pellet culture, aggrecan proteoglycan was detected by immunostaining. This result indicates that secretion and accumulate of aggrecan in the ECM may not be sustained over time in pellet culture. The fibrin encapsulated cells did show a significant increase in aggrecan mRNA expression at the 14 day time point, compared to monolayer and pellet culture, along with greater aggrecan proteoglycan accumulation in the ECM (Fig. 3). The increase in aggrecan gene expression is accompanied by intense proteoglycan staining at 3 weeks, which suggests that aggrecan proteoglycan secretion and accumulation occurred over time. A significant decrease in *collagen I* gene expression in both pellet and fibrin cultures was observed at 25 days, and correlates with the very weak staining for collagen I in the ECM surrounding fibrin-encapsulated and pellet-cultured cells at 3 weeks in culture (Fig. 2).

It is most encouraging that fibrin gel-encapsulated C5.18 cells expressed and secreted both collagen type II and aggrecan, components of hyaline cartilage compared to monolayer or aggregated cell cultures along with significant decreases in collagen I and versican, both markers of fibrocartilage. The presence of the fibrin scaffold, therefore, enhanced chondrogenic differentiation into hyaline-like cartilage. Encapsulation of C5.18 cells in 3D fibrin hydrogels therefore represents a useful model for optimization of articular cartilage tissue engineering constructs. Our results suggest that, ultimately, an autologous fibrin-based scaffold may provide the means to deliver chondrogenic precursor cells to repair and regenerate damaged hyaline cartilage. More directly, our

results indicate that the RCJ 3.1C5.18 cell line can be used as a tool to evaluate the potential of fibrin or other materials for the development of a cartilage tissue engineering scaffold.

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# CHAPTER 3

## 3.1 Design of human construct

In the following manuscript, human fibrin gels were used as scaffolds to culture primary human articular chondrocytes. As described in Chapter 2, the chondroprogenitor C5.18 cell line underwent a more hyaline-like chondrogenesis after fibrin gel encapsulation and *in vitro* culture, indicating that fibrin had potential as a scaffold for articular cartilage tissue engineering. Therefore, we further developed the system for culture of primary human articular chondrocytes using commercially available fibrin scaffold materials derived from pooled human plasma. Human primary chondrocytes are a feasible source of cells for cartilage tissue engineering as they are the only cell type within healthy native tissue. In addition, harvesting the cells from a variety of patients with OA allowed us to test the system under more realistic conditions.

For increased strength and stability to the fibrin gel scaffold, genipin was chosen as a cross-linker and incorporated into the fibrin system. Genipin has previously been shown to increase the mechanical properties of type I collagen gels (Sundararaghavan *et al.,* 2008); however, its effect on the mechanical properties of fibrin has not previously been reported. The main function of articular cartilage is to resist compressive forces, distribute loads, and allow frictionless movement of the surfaces of the articulating joint (Brower and Hsu 1969). Therefore, the mechanical properties of the tissue engineering scaffold and regenerated tissue are very important. We evaluated the mechanical properties of the genipin cross-linked fibrin gels in shear and in compression.

In addition to its potential effects on fibrin mechanical properties, genipin was also chosen as a cross-linker because of its potential anti-inflammatory properties (Koo *et*

*al.*, 2004). As discussed in the Introduction, genipin prevents N-O production by inhibiting transcription of N-O synthase. N-O has been found at an elevated level in osteoarthritic cartilage and N-O may be a factor that leads to cartilage catabolism (Pelletier *et al.,* 1996). Therefore, any potential inhibition of inflammation as a result of genipin cross-linking may also be beneficial in the treatment of osteoarthritis. We evaluated the effects of genipin on inflammation and degradation after subcutaneous implantation of the fibrin gels. In the following manuscript, the details and results of the encapsulation of human articular chondrocytes into genipin cross-linked fibrin gels are discussed. Amplification efficiencies and melt curve analyses for the primers used for gene expression analysis are in Appendix II (Fig. 1 and 2).

## 3.2 Manuscript 2

**Genipin Cross-Linked Fibrin Hydrogels for *In Vitro* Human Articular Cartilage Tissue Engineered Regeneration**

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**Genipin Cross-Linked Fibrin Hydrogels for *In Vitro* Human Articular Cartilage Tissue Engineered Regeneration**

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**Running Head:** Fibrin hydrogels for articular cartilage regeneration

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**ABSTRACT**

Our objective was to examine the potential of a genipin cross-linked human fibrin hydrogel system as a scaffold for articular cartilage tissue engineering. Human articular chondrocytes were encapsulated into modified human fibrin gels and evaluated for mechanical properties, cell viability, gene expression, extracellular matrix (ECM) production, and subcutaneous biodegradation. Genipin, a naturally occurring compound used in the treatment of inflammation, was used as a cross-linker. Genipin cross-linking did not significantly affect cell viability but did significantly increase the dynamic compression and shear moduli of the hydrogel. The ratio of the change in *collagen II* versus *collagen I* expression increased more than 8-fold over 5 weeks as detected with Real-Time RT-PCR. Accumulation of collagen II and aggrecan in hydrogel ECM was observed after 5 weeks in cell culture. Overall, our results indicate that genipin appeared to inhibit the inflammatory reaction observed 3 weeks after subcutaneous implantation of the fibrin into rats. Therefore, genipin cross-linked fibrin hydrogels can be used as cell-compatible tissue engineering scaffolds for articular cartilage regeneration, for utility in autologous treatments that eliminate the risk of tissue rejection and viral infection.

**Key words:** Cartilage articular, Chondrocytes, human, Hydrogels, IN VITRO, Tissue engineering of cartilage and bone

**Abbreviations**

**ANOVA** - Analysis of variance

**DMEM** - Dulbecco's Modified Eagle Medium

**ECM** - extracellular matrix

**FBS** - Fetal bovine serum

**GAG** - Glycosaminoglycan

**GAPDH** - glyceraldehyde-3-phosphate dehydrogenase

**H&E** - Haematoxylin and Eosin

**PBS** - phosphate buffered saline

**PFA** - Paraformaldehyde

**RT-PCR** - Reverse-transcriptase polymerase chain reaction

**TBS** - Tris-Buffered Saline

## INTRODUCTION

Hyaline articular cartilage of synovial joints is composed of an ECM that encapsulates chondrocytes (Buckwalter and Mankin 1998). Type II collagen is the major component of the ECM, and forms a highly cross-linked network that entraps large, charged, aggregating proteoglycans, of which aggrecan is the most abundant. The interaction between collagen II and proteoglycans contributes to the unique biomechanical properties of articular cartilage (Freemont and Hoyland 2006).

Despite the remarkable durability and strength of articular cartilage, trauma, disease or abnormal joint loading can induce localized damage, which cannot be repaired (van der Kraan et al. 2002). The poor healing of damaged articular cartilage has been attributed to its avascular structure. Current surgical techniques for cartilage restoration and regeneration, including autologous chondrocyte implantation, have yet to confirm regeneration of uniform hyaline cartilage with long term performance (Hunziker 2002). This largely stems from the inferior biomechanical properties of the fibrocartilage repair tissue that results from these procedures (Marlovits et al. 2006). Collagen type I is the prominent collagen in fibrocartilage (Benjamin and Ralphs 2004); its substitution for type II collagen during repair leads to changes in cartilage hydration and compressive properties (Hasler et al. 1999). The ratio of collagen II/I can serve as an indicator of normal versus aberrant cartilage repair (Freemont and Hoyland 2006).

Primary chondrocytes lose their differentiation phenotype in culture if allowed to adhere to a substrate in the presence of serum (Holtzer et al. 1960). However, differentiated chondrocytes maintain the cartilage phenotype if cells are embedded in 3-dimenstional scaffolds (Horwitz and Dorfman 1970, Guo et al. 1989). Recent approaches

for cartilage repair and regeneration have focused on the encapsulation of donor chondrocytes (Marlovits et al. 2006). The search for the optimal matrix for tissue engineering of hyaline cartilage has led to the development and testing of scaffolds made from natural materials and synthetic materials (van der Kraan et al. 2002, Hunziker 2002). As such, fibrin has been extensively evaluated as a scaffold for tissue engineering of articular cartilage (Silverman et al. 1999, Peretti et al. 2006). Fibrin has also been successfully used to anchor orthopaedic implants into an articular cartilage defect (Nehrer et al. 2006), indicating that fibrin can lead to enhanced bonding and integration into the surgical site.

Exogenous fibrin can elicit immunological reactions, and very high (non-physiological) concentrations of fibrin have resulted in variable success. In a study where a fibrin system was used for *in situ* gelation for cartilage regeneration, limited ECM synthesis was observed after 8 weeks (Peretti et al. 2006). This approach enhanced integration of the material into the cartilage defect, but formed overly soft gels that were prone to rapid breakdown in the presence of cells (Silverman et al. 1999, Grassl et al. 2003). A non-cytotoxic cross-linker could potentially enhance the stability of the fibrin hydrogel for long term culture and implantation. Fibrin also has the potential to be used for autologous gel formation and implantation to eliminate risks associated with disease transmission (Hunziker 2002).

Genipin is a naturally occurring cross-linking agent, which is isolated from the fruits of the plant *Gardenia jasminoides* (Tsai *et al.,* 2000). It reacts with primary amine groups to fix biological tissues. Genipin is significantly less cytotoxic than other chemical cross-linkers, such as glutaraldehyde, diisocyanates, and epoxides (Bedran-

Russo et al. 2007). Genipin and its related iridoid glucoside (geniposide) have been widely used for treatments in herbal medicine (Poupon *et al.,* 2004). Genipin exhibits strong anti-inflammatory activity, perhaps by suppressing nitrous oxide formation and cyclooxygenase activity at an early stage of inflammation (Koo et al. 2006). Genipin has been used to cross-link chitosan, collagen and gelatin hydrogels for tissue engineering applications (Mi et al. 2002, Mwale et al. 2005). Cross-linking of fibrin hydrogels by genipin has not been reported previously.

In this report we demonstrate that genipin cross-linked fibrin gels support an early stage of chondrogenic differentiation where some encapsulated chondrocytes exhibit chondrogenic gene expression, leading to accumulation of an articular cartilage-like ECM. Genipin did not significantly affect cell viability, while it improved the gel mechanical properties and inhibited inflammation after subcutaneous implantation. Together, these elements meet some important tissue engineering scaffold design criteria that are desirable for articular cartilage regeneration (Hunziker 2002, Lim 1984).

## MATERIALS AND METHODS

### Cell Culture

Articular cartilage was obtained after informed consent from 4 donors with degenerative osteoarthritis undergoing a total knee arthroplasty at the Ottawa Hospital. The median patient age was 70 years. Using classification of articular cartilage damage as described by Dougados et al. (1994), only cartilage with a score of 0 or I was harvested. Cartilage of sufficient quality and quantity was available from 4 donors. The study was approved by the Ottawa Hospital's Research Ethics Board. The cartilage was minced and digested in high glucose DMEM, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma,

St. Louis, MO), and 200 μg/ml collagenase I (Gibco BRL, Rockville, MD) for 48 hours at 37°C with 5% $CO_2$ (Mackay et al. 1998). The digested cartilage was centrifuged 3 times for 5 min at 1,500 rpm and maintained in medium consisting of DMEM (high glucose), 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Sigma, St. Louis, MO). Cells were grown to confluence with medium changes every 2.5 days.

**Fibrin Hydrogels**

Human fibrinogen (Sigma, St. Louis, MO), purified from pooled human donor blood, was dissolved in DMEM and sterilized by vacuum filtration through a 0.45 μm-pore filter. The fibrinogen solution (120 mg/ml final concentration unless otherwise stated) was mixed with genipin (Wako Pure Chemical Industries, Osaka, Japan) at final concentrations of 0 to 0.18 mg/ml and allowed to chemically cross-link for one hour at 37°C. Confluent chondrocytes were trypsinized and Trypan Blue staining was used to count live cells (5 x $10^6$ live cells/ml), which were then mixed into the genipin cross-linked fibrinogen solution, followed by addition of thrombin (Sigma, St. Louis, MO; 2.2 IU/ml final concentration). Donor cells were not pooled. From each patient, cells were used to make independent gel samples (n = 4 donors). Gel cultures were fed with chondrogenic medium consisting of DMEM (high glucose), 100 nM dexamethasone, 100 μg/ml sodium pyruvate, 40 μg/ml L-proline (Sigma, St. Louis, MO), 50 μg/ml ascorbic acid 2-phosphate (Fluka, St. Gallen, Switzerland), ITS-plus (BD Biosciences, Pharmigen, San Jose, CA), 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco BRL, Rockville, MD), and 10 ng/ml TGF-β3 (R&D Systems, Minneapolis, MN) (Mackay et al. 1998, Jakob et al. 2001).

**Mechanical Testing**

Mechanical properties of fibrin gel samples were evaluated using a Mach-1™ mechanical tester (Biosyntech, Laval, PQ) and a MTS Sintech 1/G material testing machine (MTS Systems Corporation, Eden Prairie, MN). Gel disks (without cells), approximately 6 mm in thickness and 8 mm in diameter with 0 or 0.09 mg/ml genipin, were evaluated in dynamic, uniaxial, unconfined shear and compression. For gel constraint 160-grit waterproof sandpaper covered the ends of a 9.5 mm diameter load applicator and base plate.

Gel disks from each group were evaluated in dynamic compression with the Mach-1™ tester (in DMEM at 37°C) and the MTS Sintech 1/G (in DMEM at 22°C). Samples were preloaded to 1 g to establish the zero strain state, followed by cyclic loading from 0-20% compressive strain at 0.1 Hz for 20 cycles with data collected at a frequency of 10 Hz. In addition, plugs (8 mm diameter) of human tibial full-thickness cartilage samples (isolated from patient A as described in the "Cell Culture" section) were cut using an 8 mm dermal biopsy punch (Miltex, Inc., York, PA) for dynamic compression measurements with the MTS Sintech 1/G apparatus as described above.

For dynamic shear teststing, gel disks from each group were cyclically loaded with the Mach-1™ tester (in DMEM at 37°C) with an initial 5% static compressive strain offset followed by a sinusoidal displacement waveform from 0-10% shear strain applied at 0.1 Hz for 10 cycles with 10 Hz data collection frequency. Dynamic shear and compression moduli were determined by the slopes of the tangents to the stress-strain curves (average of the last 3 cycles) at 15% and 5% strain, respectively.

**Hydraulic Gel Permeability**

Flow of PBS through 100 mg/ml bovine fibrin hydrogels with 0 or 0.09 mg/ml genipin was measured as reported previously (Li et al. 2005). Briefly, each hydrogel (0.1 mm thickness) was supported on a porous glass frit and exposed to pressures generated by 10 to 100 cm columns of PBS. The hydraulic permeation coefficient ($K_s$) was calculated from the flow rates (Dillon et al. 1998). $K_s$ was found to be independent of the pressure head. The average pore size of each gel was calculated from $K_s$. The specific water content of each gel was established gravimetrically (Dillon et al. 1998).

**Live/Dead Staining**

Gels of approximately 500 μm thickness containing 5 x $10^6$ cells/ml and 0 or 0.45 mg/ml genipin, were stained after 24 hours or 1 week of culture with calcein AM and ethidium homodimer-1 (Molecular Probes, Eugene, OR). Gels were washed with PBS, compressed on a slide with a coverslip, and photographed under UV light. The number of dead and live cells were counted using Eclipse version 6.0 (Empix Imaging Inc., Cheektowaga, NY) and used to calculate the percent viability (live cells/total cell number). Tests were conducted for n = 4 patients, with one sample for each gel condition (8 samples total, ± genipin) and 6 fields counted per sample.

**Real-Time RT-PCR and Analysis**

Human-specific primers for Real-Time RT-PCR for *GAPDH*, *collagen II*, *collagen I*, *aggrecan*, and *Sox9* were obtained from SuperArray (Frederick, MD). Trizol (Invitrogen, Carlsbad, CA) was used to extract RNA from cells grown in monolayer and the RNeasy Fibrous Tissue Kit (Qiagen, Valencia, CA) was used to extract RNA from

chondrocytes fibrin-encapsulated chondrocytes. First strand cDNA was synthesized by combining 2 μg total RNA using 200 IU M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) as per manufacturer instructions. The PCR reaction mixture was prepared by combining the SYBR Green PCR Master Mix (1X; BioRad, Hercules, CA), 1 μl of primer complex, 2 μl template cDNA and $H_2O$ to a final volume of 25 μl. PCR amplification was performed using a BioRad iCycler thermocycler with the following conditions: an initial denaturing at 95°C for 3 min, 45 cycles of 95°C (30 sec)/55°C (30 sec)/72°C (30 sec), and a final extension at 72°C for 10 min. A melt curve analysis was performed by: 95°C for 1 min, 55°C for 1 min, and 55°C for 10 sec with an increase of 0.5°C at each successive cycle for 80 cycles.

The comparative $C_T$ method (Pfaffl et al. 2004) was used to analyze the results. Chondrocytes from the same donor at confluence in monolayer were used as a baseline for each gene, which was furthermore normalized to *GAPDH* housekeeping expression. The $\Delta\Delta C_T$ was calculated with the equation: $\Delta\Delta C_T = \Delta C_{T(target)} - \Delta C_{T(baseline)}$. The relative gene expression level was determined by calculating $2^{(-\Delta\Delta C_T)}$. A total of 7 fibrin gels without genipin were evaluated (one for patients A and B, two for patient C, and three for patient D) and 6 fibrin gels with genipin cross-linking (one for patients A and B, and two for patients C and D). Greater amounts of tissue were obtained from patients C and D. Therefore, an unequal number of samples for gene expression analysis were generated for each donor. For each donor, data obtained from all gel samples evaluated were averaged and analyzed statistically for each gene (at each time point n = 4 for both treatments).

## Histology

The fibrin gels at 1.5 and 5 weeks in culture were fixed with 4% PFA in 0.1 M PBS, pH 7.5. Serial cryosections of fibrin gel cross-sections were stained with 1% Alcian Blue, pH 2.5 (w/v) (BDH Chemicals Ltd., Toronto, ON) in 3% acetic acid for 45 minutes and destained twice in 3% acetic acid (Grigoriadis et al. 1989). For Safranin O staining (Fisher, Hampton, NH), slides were stained in 0.2 % Safranin O in 1 % acetic acid for 4 minutes and destained in running tap water (Yasuda et al. 2006). Alcian Blue and Safranin O both stain acid glycosaminoglycans (GAGs), although via different mechanisms (Pal and Schubert 1961). In addition to acid GAGs, Alcian Blue at pH 2.5 also stains sialoglycoproteins (Spicer et al. 1967). H&E staining was performed to assess cell morphology and tissue integrity as per standard protocols (Jumblatt and Neufeld 1983). All stained sections were 10 μm in thickness. Slides were dehydrated with alcohol, cleared in xylene, mounted with a coverslip and visualized under light microscopy. Human articular cartilage samples were stained as described above. Images are representative of 10 sections per gel for each donor (n = 4).

## Immunostaining

Serial cryosections, as prepared for histology from fibrin cultures at 1.5, 2.5 and 5 weeks, were stained for collagens I and II and aggrecan (Rukosuev et al. 1990). Briefly, sections were digested with 2% bovine testicular hyaluronidase type I-S (Sigma, St. Louis, MO) in TBS pH7.4 for 30 minutes at room temperature followed by incubation for one hour in blocking solution (TBS, 4% FBS, 1% bovine serum albumin; Sigma, St. Louis, MO). Sections were incubated overnight at 4°C with anti-rabbit primary antibodies to human collagen II (AB761), collagen I (AB745), or aggrecan (AB1031) (Chemicon;

Temecula, CA) diluted 1:40 in blocking solution with 0.1% Triton X-100 (Sigma, St. Louis, MO). Samples were washed in TBS and incubated with goat anti-rabbit Cy2-labeled secondary antibody (Amersham Biosciences; Pisctaway, NJ) at 1:200 in TBS for one hour at RT. Samples were then stained with 4,6-diamino-2 phenylindole dilactate (DAPI; Sigma, St. Louis, MO; 1:10,000 in TBS), mounted, and visualized by fluorescent microscopy. Negative controls were stained without primary antibodies. All photos, including negative controls were taken at the same exposure, gain, and offset. Samples of adult human articular cartilage were stained as positive controls. Images are representative of 10 sections per gel. Gels containing cells from individual patients (n = 4) were evaluated at each time point.

**Subcutaneous Implantation**

Acellular monospecies fibrin gels were made from either rat fibrinogen and thrombin (Sigma, St. Louis, MO) or human fibrinogen and thrombin (Sigma, St. Louis, MO) with 0, 0.09, or 0.18 mg/ml genipin. These gels were implanted into the dorsal subcutaneous tissue of adult Sprague-Dawley rats. The University of Ottawa Animal Care and Veterinary Services standards for animal care were followed. Briefly, rats were anesthetised with Isoflurane gas and gels measuring approximately 8 mm in diameter and 1 mm thickness were each inserted into 1 cm incision pockets. Wounds were closed using two surgical staples. The rats were sacrificed after 3 weeks, the implants were removed, fixed in 4% PFA, and processed for cryosectioning and staining with H&E. Stained sections were evaluated blindly by a pathologist. The presence of a fibrous capsule, observation of vascular growth, and presence of immune cells within the surrounding

tissue, capsule or implant, was noted. Serial tissue sections were used to measure extent of degradation.

### Statistical Analysis

The two-tailed, unpaired Student's t test was used to evaluate statistical significance between 0 and 0.09 mg/ml genipin groups for mechanical testing results and between 0 and 0.045 mg/ml genipin groups for cytotoxicity results. Analysis of gene expression data was performed using a one-way Analysis of Variance (ANOVA) for the three time points (at confluence, 2.5 weeks, 5 weeks) for each gene. The Tukey's post-hoc test was used for further analysis. A two-way ANOVA was also used to analyze expression of each gene with time and genipin concentration being the two variables. For all tests, when $P < 0.05$ the result was considered significant. Graphs were generated using SigmaPlot version 8.0 (Systat Software Inc., Point Richmond, CA).

## RESULTS

### Cytotoxicity

The gels were seeded initially with $5x10^6$ chondrocytes (98% viable as determined by Trypan Blue Staining). After 24 hours incubation and live/dead staining, 58.6 ± 7.4 % of cells were viable in gels with 0 genipin and 48.8 ± 8.5 % in gels with 0.045 mg/ml genipin. After one week in culture, cell viability of 58.2 ± 11.5 % and 48.7 ± 5.3 % was determined for gels with 0 or 0.045 mg/ml genipin, repectively. Cell viability with and without genipine was not significantly different. For gels with 0 genipin, the average total cell number counted for at 24 hours was 10.4 ± 3.41 cells/100 $\mu m^2$ and at 1 week was 8.69 ± 2.51 cells/100 $\mu m^2$. For gels with 0.045 mg/ml genipin, the

total cell number counted at 24 hours was 10.3 ± 2.13 cells/100 $\mu m^2$ and at 1 week was 8.47 ± 1.83 cells/100 $\mu m^2$. Therefore, total cell number did not significantly increase from 24 hours to 1 week for gels with either 0 or 0.045 mg/ml genipin.

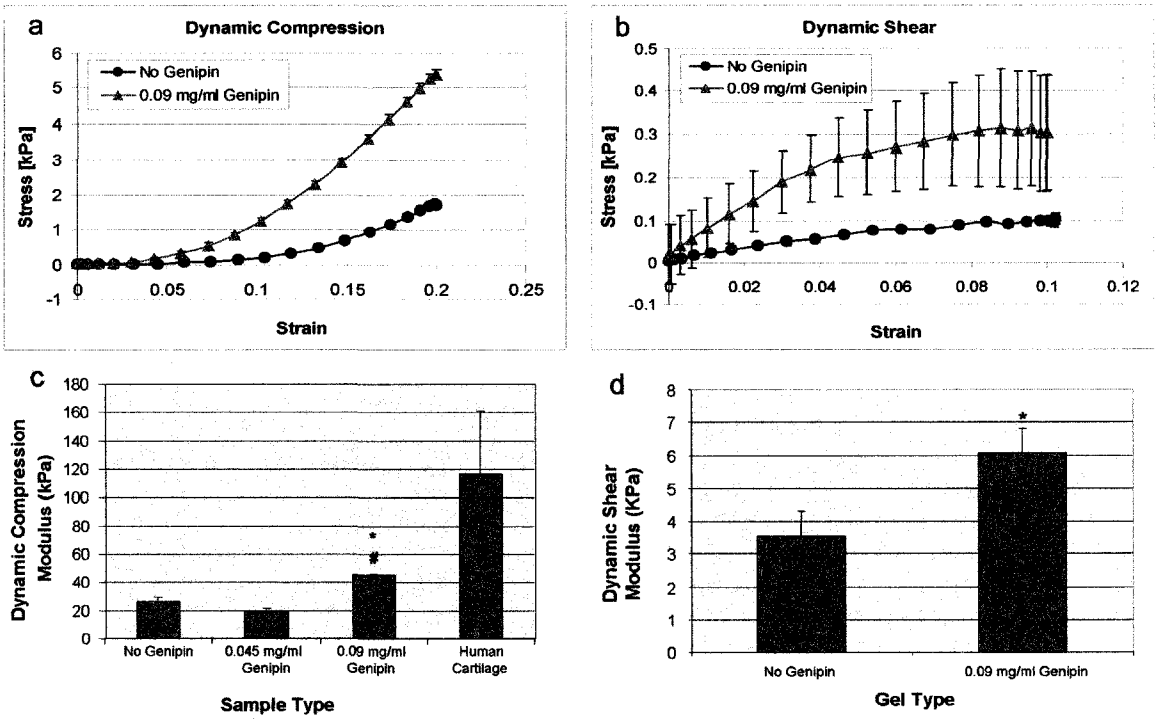


**Fig. 1: Mechanical evaluation of human fibrin hydrogels.** Representative stress vs. strain graphs of dynamic compression (a) and dynamic shear (b) tests for human fibrin hydrogels with 0 or 0.09 mg/ml genipin. Histograms show average (c) dynamic compression modulus (n = 9 tests for 0 genipin, n = 6 tests for 0.045 mg/ml genipin, n = 10 tests for 0.09 mg/ml genipin, n = 5 tests for human cartilage) and (d) average dynamic shear modulus (n = 13 tests for 0 genipin, n = 16 tests for 0.09 mg/ml genipin). * Significant difference calculated at $P < 0.05$ compared to 0 genipin; # Significant difference calculated at $P < 0.05$ compared to 0.045 mg/ml genipin. Error bars indicate standard error of the mean.

## Mechanical Properties of Fibrin Gels

Representative stress-strain curves for dynamic compression (Fig. 1 a) and dynamic shear (Fig. 1 b) are presented for fibrin gels prepared with and without genipin. The average dynamic compression modulus (Fig. 1 c) for gels cross-linked with 0.09

mg/ml genipin was 44.8 ± 1.4 kPa, which is significantly greater than the modulus for the gels with 0.045 mg/ml genipin at 18.8 ± 2.8 kPa and for gels with 0 genipin at 26.2 ± 3.2 kPa. There was no significant difference in dynamic compression modulus between gels with 0 and 0.045 mg/ml genipin. The average dynamic compression modulus for human articular cartilage was calculated as 128 ± 52.0 kPa. The average dynamic shear modulus (Fig. 1 d) for gels with 0.09 mg/ml genipin was 6.06 ± 0.77 kPa, which was significantly greater than the modulus for gels with 0 genipin at 3.54 ± 0.78 kPa.

**Hydraulic Permeability and Gel Pore Size**

Permeability coefficients ($K_s$) for fibrin alone and for fibrin cross-linked with 0.09 mg/ml genipin were (1.2 ± 0.3) x $10^{-12}$ $cm^2$ and (0.065 ± 0.02) x $10^{-12}$ $cm^2$, respectively. From the $K_s$ and measurement of water fraction in each gel, average pore diameter was calculated to be 65 ± 16 nm for genipin-free fibrin and 14 ± 5 nm for the gels with genipin cross-linking.

**Gene Expression of Encapsulated Articular Chondrocytes**

Real-Time RT-PCR analysis of the 5 genes was performed at 2.5 and 5 weeks in culture for human articular chondrocytes seeded into human fibrin gels (Fig. 2). Samples were evaluated in triplicate from independent cultures containing chondrocytes from 4 donors; results from each donor were plotted separately for each gene. After 5 weeks in culture, the ratio of *collagen II* to *I* was approximately 8.2 for gels with 0 genipin and 9.0 for gels with 0.045 mg/ml genipin. These values were normalized to chondrocytes in monolayer at confluence from the same donor.

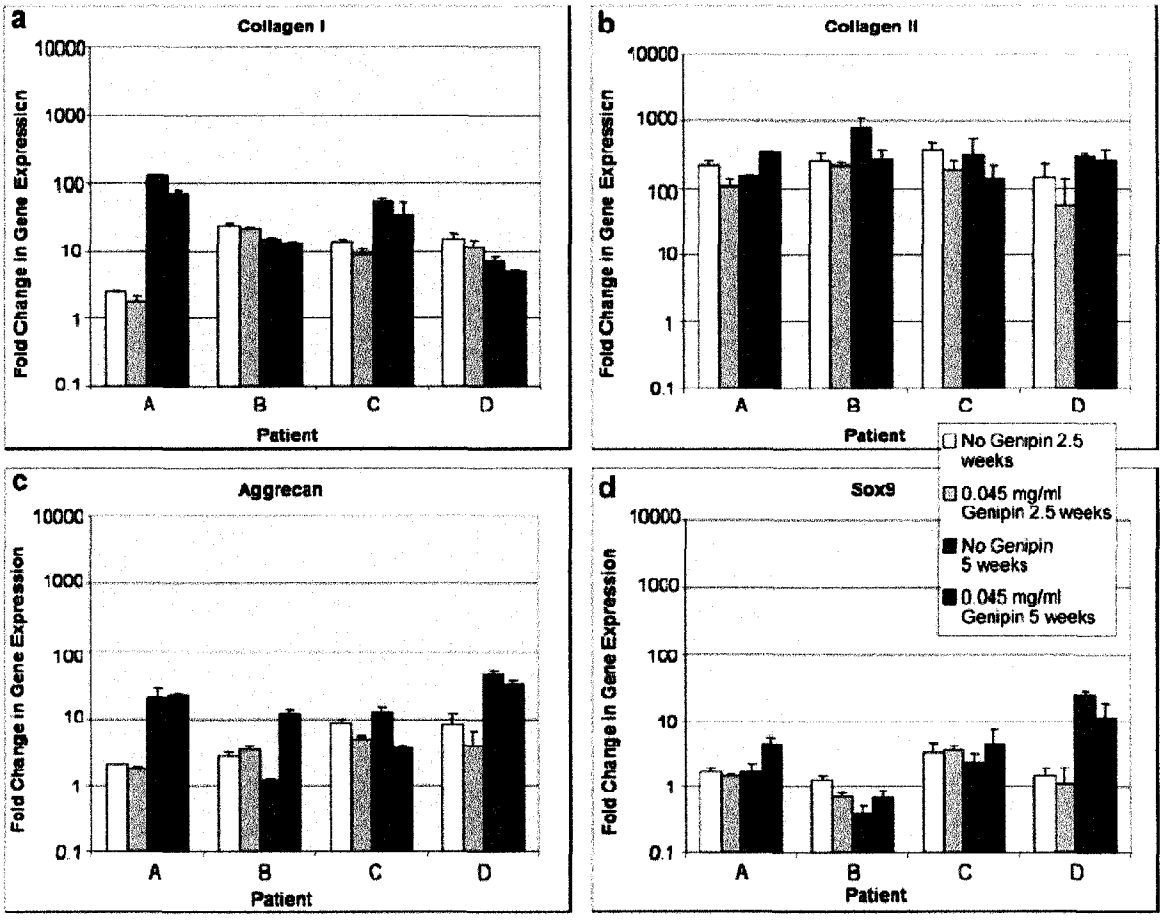


**Fig. 2: Gene expression of encapsulated chondrocytes.** Fold change in relative gene expression for *collagen I* (a), *collagen II* (b), *aggrecan* (c) and *Sox9* (d) for chondrocytes from patients A, B, C, and D seeded into fibrin gels at 2.5 weeks with 0 or 0.045 mg/ml genipin and at 5 weeks with 0 or 0.045 mg/ml genipin over baseline chondrocytes in monolayer at confluence from the same patient. The y-axis is in log-scale. Error bars indicate standard deviations to the mean (n = 4 for each treatment group).

When data for gels with genipin cross-linking from each patient (each consisting of three treatment groups with culture times of 0, 2.5 and 5 weeks) were combined (n = 4) for one-way ANOVA analysis, significant differences were found for *collagen II* and *aggrecan* ($P = 0.11$ for *collagen I*, $P = 0.0006$ for *collagen II*, $P = 0.002$ for *aggrecan* and $P = 0.16$ for *Sox9*). Further analysis with Tukey's post-hoc test revealed that cells in genipin cross-linked gels at 5 weeks exhibited significant increases in *collagen II* and *aggrecan* compared to confluence and a significant increase in *aggrecan* was detected in

genipin cross-linked gels at 5 weeks compared to 2.5 weeks. At 2.5 weeks only *collagen II* was significantly different from confluent cells.

Analysis of patient data from gels with 0 genipin (n = 4; each consisting of three treatment groups with culture times of 0, 2.5 and 5 weeks), one-way ANOVA indicated that a significant difference only occurred for *collagen II* ($P$ = 0.13 for *collagen I*, $P$ = 0.038 for *collagen II*, $P$ = 0.087 for *aggrecan,* and $P$ = 0.27 for *Sox9*). Tukey's post-hoc tests revealed that cells in uncross-linked gels at 5 weeks exhibited a significant increase in *collagen II* compared to confluent cells. No other significant gene expression changes were detected in encapsulated cells at 5 weeks compared to confluence. Cells in uncross-linked gels at 2.5 weeks had no significant gene expression changes compared to confluence.

Two-way ANOVA revealed that there was a significant increase in *collagen I* ($P$ = 0.044) *collagen II* ($P$ = 0.021) and *aggrecan* ($P$ = 0.011) gene expression between confluence, 2.5 and 5 weeks in culture. No significant differences were observed for *Sox9* gene expression between confluence, 2.5 and 5 weeks. No significant differences in gene expression were observed between uncross-linked and gels cross-linked with 0.045 mg/ml genipin for all four genes. The effect of time does not interact with the effect of genipin concentration for any of the 4 genes.

**Evaluation of ECM Production by Encapsulated Chondrocytes: Histology**

Human fibrin gels with encapsulated human chondrocytes were stable in 37°C culture, with no apparent degradation over the 5 week period. Cryosections from fibrin gels uniformly seeded with 5 x $10^6$ human chondrocytes/ml were stained with Alcian Blue and Safranin O. After 1.5 weeks there was little Alcian Blue or Safranin O staining

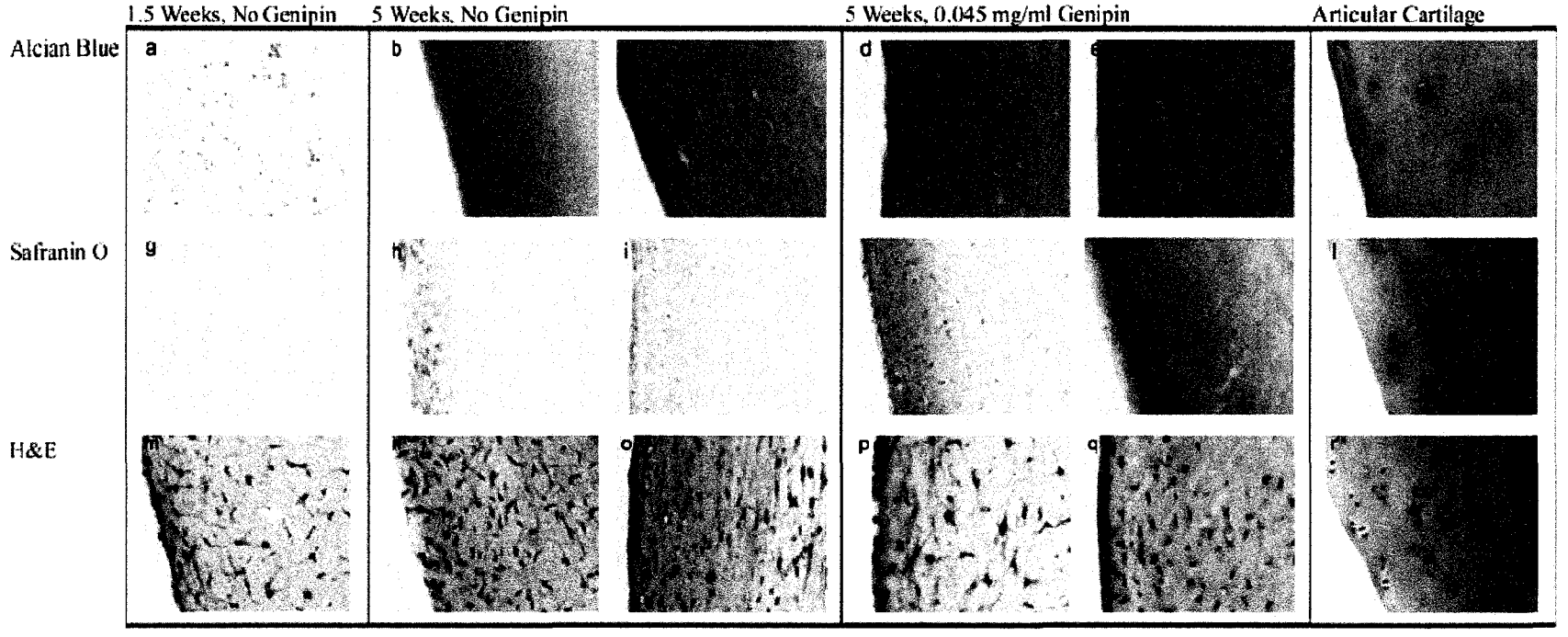


**Fig. 3: Histological evaluation of extracellular matrix development in human fibrin gels.** Serial sections of the samples were evaluated; however, they were not necessarily adjacent. Alcian Blue (a-f), Safranin O (g-l), and H&E (m-r) were used to stain 10 μm sections human fibrin gels from patient A without genipin at 1.5 weeks (a,g,m), and at 5 weeks with 0 genipin (b,h,n) and with 0.045 mg/ml genipin (d,j,p) and from patient B at 5 weeks with 0 (c,i,o) and 0.045 mg/ml genipin (e,k,q). Positive control human articular cartilage was also stained (f,l,r). Scale bar 50 μm.

(Fig. 3 a,g), which was largely intracellular. By 5 weeks in culture, ECM of gels with 0 genipin stained intensely for GAGs with Alcian Blue for independent samples seeded with cells from patients A and B (Fig. 3 b,c). There was little accompanying Safranin O staining, with some variability between the samples (Fig. 3 h,i). In 5 week 0.045 mg/ml genipin gels, there was again very intense Alcian Blue staining was observed (Fig. 3 d,e). The genipin cross-linked gels stained more intensely with Safranin O than the uncross-linked gels but, again, there was some variability between samples (Fig. 3 j,k). The encapsulated cells showed a fibroblast-like morphology (Fig. 3 m-q) compared to articular cartilage where chondrocytes appeared large and round (Fig. 3 r). In contrast to the fibrin gel constructs, human articular cartilage stained very intensely with both Alcian Blue and Safranin O (Fig. 3 f,l).

**Evaluation of ECM Production by Encapsulated Chondrocytes: Immunostaining**

Evaluation of the immunostained sections prepared from gels from patient A with 0 genipin after 1.5 weeks in culture indicated very little aggrecan (Fig. 4a), collagen II (Fig. 4h) or collagen I (Fig. 4o) staining, with mostly intracellular localization. By 2.5 weeks in culture the gels with and without genipin showed extracellular staining for aggrecan (Fig. 4b,d), collagen II (Fig. 4i,k) and collagen I (Fig. 4p,r). At 5 weeks gels both with and without genipin demonstrated a further increase in aggrecan (Fig. 4c,e) and collagen II (Fig. 4j,l) with little change in collagen I (Fig. 4q,s). The results presented in Fig. 4 from patient A are representative of immunostaining results for n = 4 patients. Negative controls (no primary antibody) are presented in Fig. 4g,n,u. Positive control human articular cartilage had intense staining for both aggrecan (Fig. 4f) and collagen II (Fig. 4m) with very little staining for collagen I (Fig. 4t).

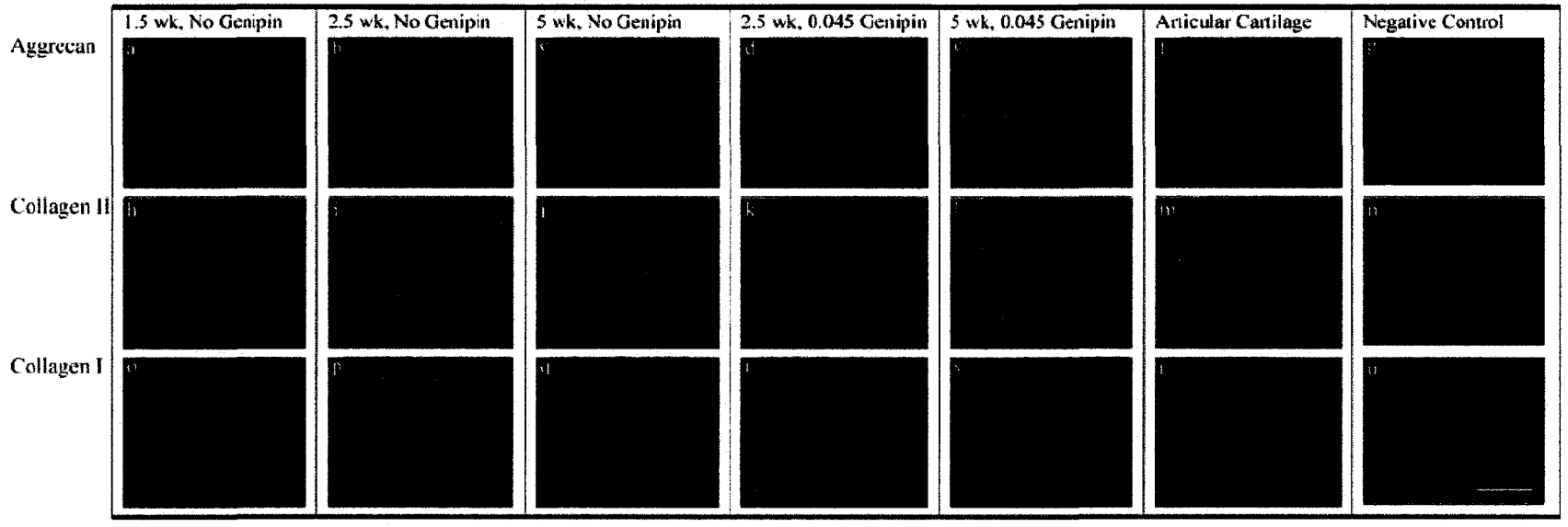


**Fig. 4: Immunostaining of extracellular matrix produced by encapsulated human chondrocytes.** Human fibrin gels (10 μm sections) from patient A with 0 genipin at 1.5 weeks (a,h,o) at 2,5 weeks (b,i,p) and at 5 weeks (c,j,q), with 0.045 mg/ml genipin at 2.5 weeks (d,k,r) and 5 weeks (e,l,s), and positive control human articular cartilage (f,m,t) were stained with rabbit anti-aggrecan (a-f), rabbit anti-collagen II (h-m), and rabbit anti-collagen I (o-t). Secondary antibody was Cy2-labelled goat anti-rabbit IgG in all cases. Negative controls were stained only with the secondary antibody (g,n,u). Sections were also stained with DAPI (blue). Scale bar 50 μm.

**Table 1.** Summary of Biocompatibility and Clearance Analysis of Fibrin Gel Implants

| | | *Capsule* | | | *Immune Cells* | | |
|---|---|---|---|---|---|---|---|
| *Animal* | *Material species*, [genipin in mg/ml]* | *Formed (Y/N)* | *Extent of Fibrosis (-/+/++ /n/a)*† | *Extent of Vascularization (-/+/++/n/a)*† | *Within or on Material (-/+/++/n/a)*† | *Within Capsule (-/+/++ /n/a)*† | *Outside of Capsule (-/+/++ /n/a)*† |
| 1 | Human, [0] | Y | +++ | + | ++ | +++ | + |
| | Human, [0.09] | Y | ++ | + | ++ | ++ | + |
| | Human, [0.18] | Y | ++ | - | + | ++ | + |
| | Rat, [0] | n/a | n/a | n/a | n/a | n/a | n/a |
| 2 | Human, [0] | n/a | n/a | n/a | n/a | n/a | n/a |
| | Human, [0.09] | Y | + | ++ | +++ | + | + |
| | Human, [0.18] | Y | ++ | - | ++ | ++ | - |
| | Rat, [0] | N | - | - | - | - | - |
| 3 | Human, [0] | n/a | n/a | n/a | n/a | n/a | n/a |
| | Human, [0.18] | Y | ++ | - | + | ++ | - |
| | Rat, [0.18] | Y | + | - | - | + | - |

*Species origin of fibrinogen and thrombin
†Extent of response determined by measurement of the distance from the implant/tissue interface to unaffected areas with the characteristics of normal tissue and vascularity; "n/a" No implant available at sacrifice for analysis

### *In vivo* Subcutaneous Biocompatibility and Clearance Assay

Extensive degradation was found in all cryosections of fibrin gel implants (Table 1). Gel dimensions diminished by 18-100 % at 3 weeks after implantation. Some gels were absent at sacrifice after 3 weeks. Genipin inhibited the degradation of the material by >29%, as assessed by comparing gel dimensions of histological sections. The general trends for impact of genipin were decreases in vascularisation, extent of fibrosis, number of immune cells, and migration of immune cells into the implant; however, in rat #2 the implant with 0.18 mg/ml genipin exhibited more fibrosis and immune cells within the capsule compared to the implant with 0.09 mg/ml. For all animals, vascularization of the fibrous capsule was less severe with increasing genipin concentration. A combination of acute and chronic aspecific inflammation with some eosinophils present was observed in all samples of human fibrin (n = 6). No granulomas were detected. In all animals there

was a reduction in the number of immune cells present within the implanted material with increasing genipin concentration. In rat #3 the implant constructed of rat fibrin with 0.18 mg/ml genipin elicited reduced fibrosis and immune cell number compared to the implant constructed of human fibrin with 0.18 mg/ml genipin. Fig. 5 shows a comparison of rat implants with 0 (Fig. 5a) and 0.18 mg/ml genipin (Fig. 5b) and human implants with 0 (Fig. 5c) and 0.18 mg/ml genipin (Fig. 5d). Note that the implants with 0.18 mg/ml (Fig. 5b,d) were still present 3 weeks after implantation.

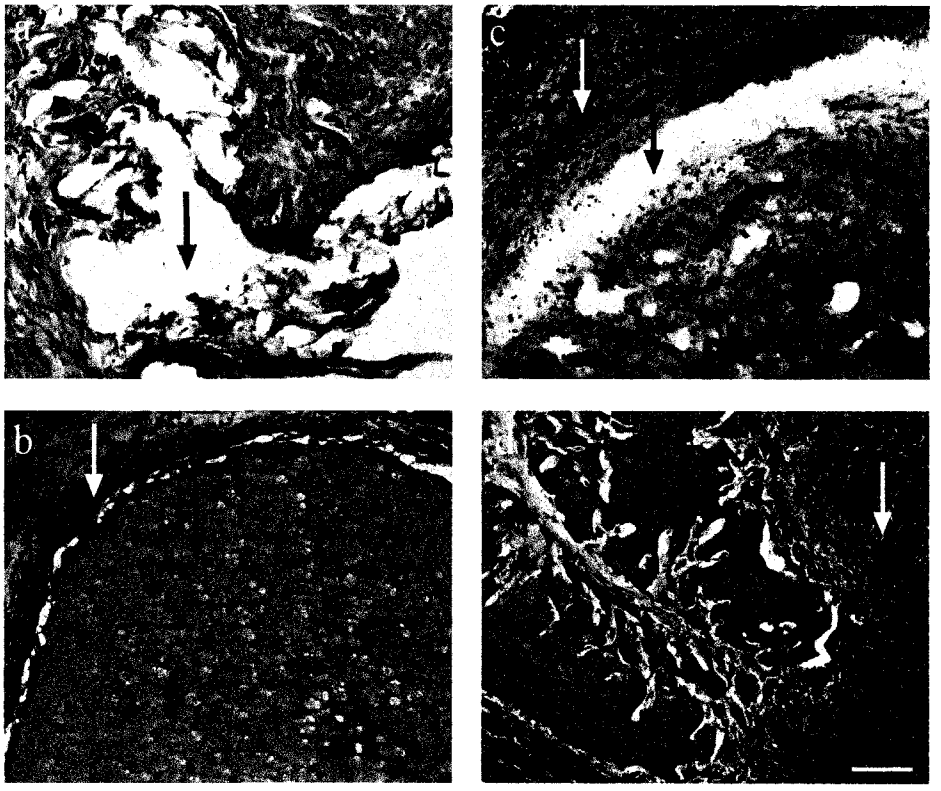


**Fig. 5: Results of *in vivo* subcutaneous biocompatibility and clearance assay.** H&E staining of sections of subcutaneous implants made with rat material with 0 genipin (a) and 0.18 mg/ml genipin (b), and with human material with 0 mg/ml genipin (c) and 0.18 mg/ml genipin removed after 3 weeks. White arrows indicate fibrous capsule layer and black arrows indicate the position of the fibrin implant. Bar 100 μm.

## DISCUSSION

The goal of this study was to evaluate the suitability of a genipin cross-linked fibrin hydrogel scaffold for *in vitro* articular cartilage regeneration. There have been reports of cartilage-like tissue formation when uncross-linked fibrin hydrogels were used for tissue engineering of articular cartilage (Peretti et al. 2006, Dare et al. 2007). Several studies used commercially available fibrin glue of bovine origin for gel formation (Mouw et al. 2005, Park et al. 2005). There have been problems reported with this approach including rapid degradation of fibrin constructs (Silverman et al. 1999). The system we have developed consists entirely of human components (fibrinogen, thrombin, cells). Over a 5 week period of *in vitro* culture, gels encapsulating chondrocytes with and without genipin cross-linking showed no degradation despite the absence of protease inhibitors such as aprotinin and 6-aminocaproic acid (Ahmed et al. 2007).

Non-toxic cross-linking is essential during gelation and subsequent cell culture in order to maintain cell viability. In our study, the cell viability *in vitro* after 24 hours and after 1 week in culture ranged from approximately 47-58%. Viability reported for other hydrogels used in cartilage tissue engineering has been variable. For example, viability of articular chondrocytes cultured in poly(ethylene glycol) under static and mechanically stimulated cultures was less than 50% even though articular cartilage-like regenerated tissue was reported (Nicodemus et al. 2007). In another study, only 26% chondrocyte viability was observed in photopolymerized gelatin gels (Hoshikawa et al. 2006). However, other groups have reported greater than 95% chondrocyte viability (Wang et al. 2003, Marsich et al. 2008). It has been shown previously that thrombin can affect cell growth by inducing apoptosis at a concentration above 1 U/ml (Zain et al. 2000, Sarker et

al. 1999), which is a likely explanation for our reduced viability following encapsulation (initially 98% viable). We observed that addition of 2.2 U/ml thrombin directly to monolayer chondrocyte cell cultures killed over 80 % of cells (data not shown). The concentration of 2.2 IU/ml was used to prepare all fibrin gel scaffolds in the study; this was selected based on the concentration present within commonly used commercially available fibrin glue (Buchta et al. 2004, Buchta et al. 2005). It has been reported that clotting time and viability in fibrin gel scaffolds were independent of fibrinogen concentration (Zhao et al. 2008). We did not detect a significant increase in total cell number per sample over one week in culture. The doubling time of monolayer-cultured adult human articular chondrocytes is approximately 84 hours (Jakob et al. 2001). Therefore, our results suggest a decrease in the rate of cell division after encapsulation.

Genipin cross-linking significantly improved the mechanical properties of the fibrin gel in both dynamic compression and shear (Fig. 1). However, in preliminary experiments we found that increasing the genipin concentration to 0.18 mg/ml did not lead to further improvements in the gel mechanical properties (data not shown). The mechanical properties of our strongest gels remain much lower than human articular cartilage (Fig. 1c). However, the purpose of the fibrin scaffold is to retain and physically support the cells as they differentiate and produce an ECM. Fetal articular cartilage has a compression modulus that is about 1/3 that of adult articular cartilage, and increases with weight-bearing activity (Williamson et al. 2001). Therefore, it is likely that the mechanical properties of our fibrin constructs would increase over time, especially if subjected to periodic mechanical stimulation (Waldman et al. 2006). In the current

formulation, our material may be suitable for treatment of low weight-bearing defects or donor site defects created during mosaicplasty (Bartha et al. 2006).

Real-Time RT-PCR confirmed that progressive and significant changes in gene expression occurred during culture of fibrin encapsulated human chondrocytes (Fig. 2). Sox9 is a marker of chondrogenesis and ongoing mesenchymal stem cell differentiation and proliferation (Lefebvre et al. 2001); it functions as a transcription factor, which regulates *collagen II* and *aggrecan* expression (Bi et al. 1999). Although we did not detect a significant increase in *Sox9* expression, we did detect an increase in both *aggrecan* and *collagen II* gene expression. The increase in *collagen II* was more than 8-fold greater than the increase in *collagen I* gene expression in gels at 5 weeks. While there was a trend of an increase in *collagen I* gene expression, this effect was not statistically significant. A tendency for increased *collagen I* could be due to tissue source and/or processing. Osteoarthritic chondrocytes are known to express elevated levels of *collagen I* in culture (Martin et al. 2001). In addition, increased *collagen I* expression occurs during dedifferentiation, which takes place during monolayer culture of articular chondrocytes (Schnabel et al. 2002; Lin et al. 2006). We speculate that the human chondrocytes that were seeded into our fibrin gels underwent dedifferentiation during harvesting and expansion. Following gel encapsulation, redifferentiation *in vitro* to a more articular phenotype during the 5 week culture period occurred, as evidenced by the significant increase in *collagen II* and *aggrecan* gene expression by encapsulated cells in our system (Freemont and Hoyland 2006, Martin et al. 2001). On the other hand, the largely fibroblast-like morphology of cells in histology indicates that the redifferentiation to fully articular chondrocytes is incomplete within 5 weeks of culture.

These changes in gene expression of the encapsulated chondrocytes are expected to lead to altered accumulation of ECM components within the fibrin gel. While we did observe significant changes in gene expression over time in culture, there were no significant differences detected when genipin cross-linked gels were compared to uncross-linked gels. We did, however, observe collagen II and aggrecan immunostaining with little staining for collagen I within the fibrin cultures. These results indicate that chondrogenic cell changes occurred within the fibrin cultures, which were not compromised by the presence of genipin. The newly synthesised matrix at 5 weeks stained intensely with Alcian Blue, while Safranin O staining was relatively weak. Alcian Blue more accurately reflects the acid GAG concentration than Safranin O (Troyer 1980). Therefore, it is not surprising that the Alcian Blue staining of our samples is more closely correlated with aggrecan immunostaining. Overall histology shows that an increase in ECM development was detected within the fibrin gel cultures over time.

Subcutaneous implants that were cross-linked with genipin exhibited reduced inflammation and enhanced stability as previously reported by Huang et al. (1998) for genipin cross-linked pericardium. These results indicate that allogeneic fibrin gels cross-linked with genipin may have desirable features as tissue engineering implants, since decreased inflammation would stabilize the fibrin gel implants. Moreover, orthopaedic implantation of such constructs could slow osteoarthritic disease progression due to inhibition of inflammation (Koo et al. 2006). However, in previous experiments we found that with 0.045 mg/ml of genipin added to the fibrin implant there was complete degradation of the material 3 weeks after subcutaneous implantation (data not shown). Therefore, the concentration of 0.045 mg/ml of genipin that we used for our cell culture

experiments would not be sufficient to inhibit inflammation and biodegradation after implantation. The level of 0.045 mg/ml genipin was chosen because cytotoxic effects have been shown only at higher concentrations (Koo et al. 2004). In the same study, a significant anti-inflammatory effect of genipin on mouse ear edema was only found with 0.09 mg/ml genipin or higher, which is confirmed by the trends observed in our experiments.

We have demonstrated that the genipin cross-linked human fibrin gel system is stable during *in vitro* culture and provides a suitable environment for articular chondrogenic gene expression, and elaboration of a cartilage-like ECM. However, 5 weeks was not adequate for complete chondrocyte differentiation and cartilage regeneration *in vitro.* Genipin cross-linking improved the mechanical properties of the gel and slowed the rate of biodegradation and inflammation of subcutaneous implants. However, we have not identified the optimal concentration of genipin in order to maximize all of the benefits for the system. Therefore, it will be important to conduct future studies with genipin, alone or in combination with other cross-linkers, to develop the most appropriate method to stabilize the fibrin hydrogels. The human fibrin gel system offers a potential patient-specific autologous scaffold for cartilage regeneration since human fibrinogen and thrombin can be isolated separately from a patient's fresh plasma, and encapsulated cells can be donated by the same patient (Rock et al. 2001). The advantage of a completely autologous approach for gel formation would be the elimination of th3e risk of tissue incompatibility and viral infection (Haisch et al. 2000) and an associated ease of transition to clinical use.

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## CHAPTER 4

### 4.1 Autologous Construct Design

The previous two manuscripts discussed the use of fibrin scaffolds derived from commercially available materials isolated from pooled plasma. We have previously shown that these fibrin scaffolds induce chondrogenic differentiation of encapsulated chodroprogenitor C5.18 cells and support growth and cartilage-like ECM by encapsulated human articular chondrocytes. Based on subcutaneous implantation results described in Chapter 3, it was evident that the species origin of the fibrin material is important to consider. Sequence homology of the fibrinogen polypeptide chains is greater than 80% between *Rattus norvegicus* and *Homo sapiens*. However, it is obvious that some of the proteins within the fibrin preparation caused an immune response. It is known that immunological reactions to components of xenogeneic fibrin glue are possible (Zehnder and Leung, 1990). Since inflammation was still evident with the implantation of allogeneic fibrin it is apparent that the fibrin source must be taken a step further. Therefore, in the following manuscript we discuss the evaluation of a system used for deriving fibrin glue from a single unit of plasma; with the potential for autologous construct formation.

The CryoSeal® FS System can rapidly and reliably produce autologous fibrin glue by processing single units of plasma donated by apheresis (Rock *et al.,* 2001). Synovial fluid is an ultrafiltrate of plasma and consists of water, electrolytes, small molecules, and glucose as well as many other nutrients (Huber *et al.*, 2000). Therefore, it is likely that the fibrin glue produced by concentrating plasma contains many of the nutrients required by chondrocytes in the native tissue environment. CryoSeal® fibrin

glue has not previously been assessed as a scaffold for *in vitro* cell culture or *in vivo* implantation as a cell carrier.

In addition to the origin of scaffold material, in Manuscript 3 we evaluated the articular cartilage regeneration system under hypoxic culture as a proof-of-principle concept. We cultured the human chondrocyte-encapsulated fibrin construct under a more physiological oxygen tension in order to gain insight into the likely success after implantation into an articulating joint. Amplification efficiencies and melt curve analyses for the primers used for gene expression analysis are in Appendix I (Fig. 1 and 2).

## 4.2 Manuscript 3

**Comparison of Fibrin Sealants Derived from Fresh or Fresh/Frozen Plasma as Scaffolds for *in vitro* Articular Cartilage Regeneration**

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**Comparison of Fibrin Sealants Derived from Fresh or Fresh/Frozen Plasma as Scaffolds for *in vitro* Articular Cartilage Regeneration**

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**ABSTRACT**

Our objective was to evaluate human CryoSeal® fibrin glue derived from single units of plasma as a scaffold for articular cartilage tissue engineering. Human articular chondrocytes were encapsulated into genipin cross-linked fibrin glue that was derived from individual units of fresh or frozen plasma using the CryoSeal® FS system. The constructs were cultured for up to 7 weeks *in vitro* under either normoxic (21% $O_2$) or hypoxic (5% $O_2$) conditions and evaluated for cell viability, gene expression, extracellular matrix (ECM) production and mechanical properties. Chondrocyte viability was >90% in fibrin gels derived from either fresh or frozen plasma. Gene expression analysis revealed that hypoxia induced significant increases in *collagen II* and *Sox9* and a significant decrease in *collagen I*. A significant increase in cartilage specific *collagen II* was detected in cultures derived from fresh plasma while frozen plasma derived cultures gave only a significant increase in more ubiquitous *collagen I*. Encapsulated chondrocytes secreted an ECM that stained with Alcian Blue after 7 weeks, which was accompanied by significant increases in total glycosaminoglycan and collagen. A significant increase in the compression modulus was only observed for gels derived from fresh plasma, which is likely explained by a greater amount of collagen type I after 7 weeks in frozen compared to fresh plasma gels as detected by Western blotting. This is the first report of cell culture with CryoSeal® fibrin glue as a scaffold for tissue engineering. Our results indicate that CryoSeal® fibrin glue derived from fresh plasma is suitable as a tissue engineering scaffold for human articular chondrocytes, and therefore should be evaluated for autologous articular cartilage regeneration.

## INTRODUCTION

Articular cartilage contributes significantly to the extraordinary functional properties of absorption of forces and distribution of load within diarthrodial joints.[1] Type II collagen is the major component by dry weight of the extracellular matrix (ECM) of articular cartilage.[2] The interaction between collagen II and proteoglycans within cartilage ECM is responsible for the unique biomechanical properties of the tissue.[3] Despite the remarkable durability and strength of articular cartilage, trauma, disease or abnormal joint loading can induce localized damage, which the body cannot fully repair.[4] Current surgical techniques for articular cartilage defect repair include marrow stimulation and autologous chondrocyte implantation, and have yet to confirm uniform hyaline cartilage regeneration with long term performance.[5,6] This largely stems from the inferior biomechanical properties of the fibrocartilage repair tissue that often results from these procedures.[7]

The search for the optimal matrix for tissue engineering of hyaline articular cartilage has led to the development and testing of scaffolds made from natural materials, including collagen, alginate, chitosan, fibrin, and synthetic materials.[8-10] Fibrin sealant offers many advantages in surgery including rapid haemostasis,[11] accelerated wound healing,[12] reduction of blood loss,[13] and protection against bacterial infections.[14] Fibrin sealant has also frequently been used as a scaffold in tissue engineering.[15-18] The most commonly used commercially available fibrin sealant is of bovine origin, which can cause immunological reactions involving formation of antibodies against bovine thrombin that cause bleeding.[19] There is also the concern of the risk of transmission of viral[20] or prion[21] infections. The CryoSeal® FS system provides rapid and reliable

cryoprecipitate production from autologous plasma. This cryoprecipitate has been found to be comparable to the commercially available cryoprecipitate.[22] A benefit of using CryoSeal® fibrin glue as a scaffold for articular cartilage tissue engineering is that potentially all the components of the implant can be isolated from the recipient patient. Fibrin sealant can also be processed from frozen plasma. The advantage of using frozen plasma for fibrin sealant production is that it could be implanted into allogeneic recipient patients that cannot donate plasma or have an abnormal coagulation diseases.[23] CryoSeal® fibrin glue has not previously been used as a scaffold for cell encapsulation and culture.

In development of an optimal scaffold for tissue engineering, it is important to evaluate the cell construct in an environment that is as close as possible to *in vivo* conditions. Mature articular cartilage is an avascular tissue that receives its nutrients, including oxygen, from the synovial fluid by diffusion and forced convection resulting from mechanical loading.[24] The oxygen tension of synovial fluid is approximately 7%.[25] As oxygen further decreases in the deeper tissue layers, ATP synthesis shifts from oxidative phosphorylation to cytoplasmic oxygen-independent glycolysis.[26] In this hypoxic environment chondrocytes rely on highly conserved adaptive mechanisms[27] including transcriptional activation by hypoxia inducible factors (HIFs).[28] HIFs not only target genes involved in cell metabolism and survival but have also been proposed to target ECM genes[29] such as *collagen type II*[30] and chondrogenic genes such as *Sox9.*[31]

The objective of our study was to evaluate human CryoSeal® fibrin glue derived from single units of plasma as a scaffold for articular cartilage tissue engineering. In this report, we demonstrate that chondrocytes encapsulated in genipin cross-linked

CryoSeal® fibrin gels expressed chondrogenic genes and secreted an ECM with cartilage-like properties, which led to an improvement in the mechanical properties of the scaffold. Superior results were obtained with fibrin gels derived from fresh plasma as compared to frozen plasma-derived fibrin. Therefore, CryoSeal® fibrin glue derived from fresh plasma is suitable as a tissue engineering scaffold for human articular chondrocytes, and should be evaluated for autologous articular cartilage regeneration.

## MATERIALS AND METHODS

### Plasma Collection

Human plasma from 7 donors (median age of 41 years) was collected by apheresis using The Trima Accel Version 5 automated blood collection system (Gambro BCT Inc., Lakewood, CO) after obtaining informed consent. Whole blood was collected, centrifuged, and plasma was removed before the remainder of the blood was returned to circulation. Two units (500 ml) were collected from each individual and split in half for processing. Each unit of plasma was treated with 36 ml of Citrate Dextrose Solution USP (ACD) Formula A (Baxter Corp., Deerfield, IL).

### Cryoprecipitate and Thrombin Production

Cryoprecipitate and thrombin were produced using the CryoSeal® FS System and the Thrombin Processing Device (TPD)$^{TM}$, respectively (Thermogenesis, Rancho Cordova, CA). In the TPD reaction chamber, 10 ml of plasma was mixed with 4 ml of thrombin reagent (7.2 mM calcium chloride, 10% ethanol), incubated for 50 minutes, agitated and incubated for an additional 10 minutes. Thrombin was harvested from the chamber, aliquoted and frozen at -80°C.[32] The remaining 240 ml of plasma was quickly

frozen to -27°C and slowly thawed over 20 minutes to 2°C. The concentrated cryoprecipitate settled in the bag and the protein-poor plasma was removed. Two repeat freeze/thaw cycles were used to enhance cryoprecipitate recovery. The cryoprecipitate was aliquoted and frozen at -80°C.[22,32]

**Cell Culture**

Articular cartilage was obtained from 7 donors with degenerative osteoarthritis undergoing total knee arthroplasty at a tertiary care teaching hospital. The median patient age was 70 years. The study was approved by the hospital's Research Ethics Board and informed consent was obtained. Grossly normal cartilage from uninvolved areas of the knee with a score of 0 or I was harvested, according to cartilage damage classification described by Dougados *et al.*[33] Cartilage was minced and digested in Dulbecco's Modified Eagle Medium (DMEM), antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml Amphotericin B), and 200 μg/ml collagenase I (Gibco BRL, Rockville, MD) for 48 hours at 37°C with 5% $CO_2$.[34] The digested cartilage was centrifuged 3 times at 1,500 rpm; chondrocytes were maintained in DMEM, 10% fetal bovine serum (FBS) and antibiotics until reaching confluence.

**Fibrin Gel Formation**

Fibrin glue processed from either fresh plasma (FSP; processed within 4 hours of collection) or from fibrin glue derived from frozen plasma (FZP; frozen within four hours of collection and stored at -20°C until processing at a later date) was used for gel formation. To prepare gels (~1.5 mm thickness), cryoprecipitate (140 μl), genipin (0.18mg/ml final) and cells (confluent chondrocytes, 5 x $10^6$ live cells/ml, after

trypsinization and Trypan blue staining) were mixed and thrombin (20 µl, from same unit as cryoprecipitate) was added to a final volume of 200 µl. Gels were cultured in transwell plates with chondrogenic medium [DMEM, 100 nM dexamethasone, 100 µg/ml sodium pyruvate, 40 µg/ml L-proline, 2 mg/ml aminohexanoic acid (AHA; Sigma, St. Louis, MO), 50 µg/ml ascorbic acid 2-phosphate (Fluka, St. Gallen, Switzerland), ITS-plus (BD Biosciences, Pharmigen, San Jose, CA), antibiotics, and 10 ng/ml TGF-β3 (R&D Systems, Minneapolis, MN)].[34,36] Chondrocytes from each donor (labelled A to G) were seeded into fibrin glue units (n = 7 donors) as described in Table 1. Previous studies have demonstrated that cryoprecipitate from plasma of healthy donors does not vary significantly.[22,32] In the absence of protease inhibitors, the fibrin gels completely degraded over 10-14 days in culture; however, the gels were fully stabilized with AHA addition to the medium. At day 1, 2.5 weeks and 7 weeks one gel/donor and gel type was divided into quarters and processed for RNA isolation, cryosectioning, papain digestion, and pepsin digestion. For each cell batch, fibrin type and time point, one gel was cultured at 37°C with 5% $CO_2$, atmospheric $N_2$ and $O_2$ (NO - normal oxygen) and another at 37°C with 5% $CO_2$, 90% $N_2$, and 5% $O_2$ (LO - low oxygen).

**Table 1: Cell source and fibrin glue source.**

| Patient | Plasma Type Used to Make Fibrin Glue for Culture* |
|---|---|
| A | FSP |
| B | FSP |
| C | FSP and FZP† |
| D | FSP and FZP† |
| E | FZP§ |
| F | FZP‡ |
| G | FZP‡ |

*All FSP gels were derived from separate plasma donors
† FZP gels with C and D were from the same donor
§ FZP gels with E from separate donor than the other FZP samples used
‡ FZP gels with F and G were from the same donor

**Live/Dead Staining**

Fibrin gels of (500 μm thickness prepared as described above) were stained after 24 hours or 2 weeks of culture with calcein AM and ethidium homodimer-1 (Molecular Probes, Eugene, OR). After 30 minutes of staining, gels were washed with PBS and photographed under UV light (n = 3 for each gel type and time point with 6 fields counted per gel). The numbers of dead and live cells were counted using Eclipse version 6.0 (Empix Imaging Inc., Cheektowaga, NY) to calculate the percent viability (live cells/total cell number).

**Real-Time RT-PCR and Analysis**

Real-Time RT-PCR primers for human *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *collagen II*, *collagen I*, *aggrecan*, *versican,* and *Sox9* were obtained from SuperArray (Frederick, MD). RNA was extracted from fibrin-encapsulated chondrocytes using the RNeasy Fibrous Tissue Kit (Qiagen, Valencia, CA). First strand cDNA was synthesized with 2 μg total RNA using 200 IU M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) as per manufacturer instructions. The SYBR Green PCR Master Mix (1X; BioRad, Hercules, CA) was used for the PCR reaction according to the manufacturer using 2 μl template cDNA and a final volume of 25 μl. PCR amplification was performed using a BioRad iCycler (BioRad, Hercules, CA) with 3 minutes denaturing at 95°C, 45 cycles of 95°C (30 seconds)/55°C (30 seconds)/72°C (30 seconds), and 10 minutes final extension at 72°C. Melt curve analysis was performed: 1 minute at 95°C, 1 minute at 55°C, and 10 seconds at 55°C with an increase of 0.5°C at each successive cycle for 80 cycles.

The comparative $C_T$ method[36,37] was used to analyse the results. Chondrocytes from the same patient and gel type at day 1 were used as a baseline for each gene, unless otherwise indicated, which was normalized to *GAPDH* housekeeping expression. Data was used to compare gene expression at day 1, and 2.5 weeks and 7 weeks, with triplicate determinations of independent cultures of 4 FSP and 5 FZP gels seeded with human articular chondrocytes (total of n = 7 patients). The $\Delta\Delta C_T$ was calculated with the equation: $\Delta\Delta C_T = \Delta C_{T(target)} - \Delta C_{T(baseline)}$. The relative gene expression level was determined by calculating $2^{(-\Delta\Delta C_T)}$. Results are plotted on a Logarithmic scale solely for the purpose of visualization of all the data together. The data calculations, including the standard deviations, are not related to the Logarithmic scale.

**Histology**

Gels at day 1, and 2.5 and 7 weeks in culture were fixed with 4% paraformaldehyde in 0.1 M PBS, pH 7.6. Serial cryosections (10 μm) were stained with 1% Alcian Blue (AB), pH 2.5 (w/v) (BDH Chemicals Ltd., Toronto, ON) in 3% acetic acid.[38] Haematoxylin and Eosin (H&E) staining was performed as per standard protocol.[39] Slides were dehydrated with alcohol, cleared in xylene, mounted with a coverslip and visualized with microscopy. Images are representative of 4 and 5 independent FSP and FZP samples, respectively at each time point.

**Biochemical Assays**

Gels were digested with papain (Sigma, St. Louis, MO; 40 μg/ml in 20 mM ammonium acetate, 1 mM ethylenediaminetetraacetic acid and 2 mM dithiothreitol) for 48 hours at 65°C. Digest aliquots were assayed for total glycosaminoglycan (GAG) with

dimethylmethylene blue (DMMB) using the Blyscan™ sulphate GAG assay (Bicolor Ltd., Carrickfergus, UK). The chloramine-T/Ehrlich's reagent assay (Sigma, St. Louis, MO) was used to determine hydroxyproline content after hydrolyzing aliquots in 6 N HCl (Sigma, St. Louis, MO) at 110°C for 18 hours.[40] Collagen ECM content was calculated assuming that hydroxyproline represented 10% of total collagen. Total GAG and hydroxyproline were normalized to DNA, which was determined using the Hoechst dye 33258 assay (Invitrogen, Carlsbad, CA).[41]

**Immunostaining**

Serial sections, cut from gels at 1 day, 2.5 and 7 weeks, were stained for collagen I, collagen II, aggrecan, Sox9, HIF-1α, $\alpha_1\beta_1$ integrin, and $\alpha_V\beta_3$ integrin. For collagens I and II staining, sections were digested with 2% bovine testicular hyaluronidase type I-S (Sigma, St. Louis, MO) in Tris-Buffered Saline (TBS) pH 7.4 for 30 minutes at room temperature (RT) followed by one hour in blocking solution (TBS, 4% FBS, 1% bovine serum albumin, 0.1% Triton X-100; Sigma, St. Louis, MO). Sections were incubated overnight at 4°C with anti-rabbit primary antibodies to collagen II, aggrecan, Sox9, collagen I (Chemicon, Temecula, CA; 1:40 in blocking solution) or anti-mouse primary antibodies to HIF-1α, $\alpha_1\beta_1$ integrin (Chemicon, Temecula, CA; 1:50 in blocking solution), $\alpha_V\beta_3$ integrin (Abcam, Cambridge, MA; 1:50 in blocking solution). Slides were washed in TBS and incubated with goat anti-rabbit Cy2-labeled secondary antibody or goat anti-mouse Cy2-labeled secondary antibody (Amersham Biosciences, Piscataway, NJ) at 1:1000 and 1:200, respectively in TBS for one hour at RT. Samples (except Sox9 staining) were stained with 0.1 μg/ml 4,6-diamino-2 phenylindole dilactate (DAPI) in TBS (Sigma, St. Louis, MO), mounted, and visualized by microscopy. Negative controls

were stained without primary antibodies. All photos were taken at the same exposure, gain, and offset. Images are representative of 4 and 5 independent FSP and FZP gel samples, respectively at each time point.

**Western Blotting**

Gel samples were homogenized with a Polytron® homogenizer (Brinkmann Instruments Inc., Westbury, NY) and lyophilized, followed by digestion with pepsin (1 mg in 0.5 M acetic acid; Sigma, St. Louis, MO) for 24-72 hours with shaking at 4°C. Digests were centrifuged at 13,000 rpm for 10 minutes and the supernatant was collected. Samples and collagen I standard (Chemicon, Temecula, CA) were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane followed by blocking with 5% BSA in PBS-tween (phosphate buffered saline with 0.1 % tween-20; Sigma, St. Louis, MO) for 1 hour at RT. Membranes were incubated overnight at 4°C with primary antibodies to collagen I (Chemicon, Temecula, CA) and β-tubulin (Abcam, Cambridge, MA) that were diluted 1:1000 and 1:2500, respectively in blocking solution. After washing with PBS-tween the secondary donkey anti-rabbit IgG – HRP conjugated antibody (Sigma, St. Louis, MO; 1:20000 in blocking solution) was incubated for 30 minutes at RT. The membrane was washed with PBS-tween and 100 mM sodium phosphate, pH 7.2 (Sigma, St. Louis, MO) followed by incubation with enhanced chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA) for 1 minute. The membrane was exposed to x-ray film for 30 seconds. For analysis of immunopositive bands, Image J software (National Institutes of Health, USA) was used to plot the number of pixels for each lane. The area under each peak (representing a band) was calculated.

Collagen I levels in each sample were determined by comparing to a standard curve. Samples were normalized to β-tubulin.

**Mechanical Testing**

Mechanical properties of fibrin gels were evaluated using a MTS Sintech 1/G material testing machine (MTS Systems Corporation, Eden Prairie, MN). Gels disks (1.5 mm thickness and 8 mm diameter) were cut with an 8 mm dermal biopsy punch (Miltex Inc., York, PA), and evaluated in dynamic, uniaxial, unconfined compression. For gel constraint during testing, the ends of the 9.5 mm diameter load applicator and base plate were covered with 160 grit waterproof sandpaper.

Gels were evaluated in dynamic compression in DMEM at 22°C. Samples were preloaded to 1 g to establish the zero strain state, followed by cyclic loading from 0-20% compressive strain at 0.1 Hz for 20 cycles with an applied linear ramp displacement waveform. The resulting data were collected at a frequency of 10 Hz. Dynamic compression moduli were determined by from the slopes of tangents to the stress-strain curves (average of the last 3 cycles) at 15% strain.

**Statistical Analysis**

The two-tailed, unpaired Student's t test was used to evaluate statistical significance for gene expression, Western blot, mechanical testing and cytotoxicity results. Analysis of gene expression data was also performed using a one-way Analysis of Variance (ANOVA). Tukey's post-hoc test was used for further analysis. Two-way ANOVA with oxygen level and gel type as the two variables was also used for gene

expression and total GAG analysis. Results were considered significant when $P < 0.05$. Graphs were generated using Excel.

## RESULTS

### Cytotoxicity

Initial cell viability within FSP and FZP gels, each seeded with 5 x $10^6$ chondrocytes was 98% (as determined by Trypan Blue Staining). After 24 hours in culture, cell viability was 90.3 ± 4.4 % in FSP gels and 87.8 ± 5.2 % in FZP gels. After two weeks in culture cell viability was 90.4 ± 2.9 % in FSP gels and 93.2 ± 2.3 % in FZP gels. No significant differences were detected between gel types or time points.

### Gene Expression Analysis

For FSP gels, chondrogenic gene expression data were compared between day 1, and NO and LO cultures at 2.5 weeks (n = 4) using one-way ANOVA analysis (Fig. 1a). Significant differences were only found for *collagen II* and *versican* ($P = 0.53$ for *collagen I*, $P < 0.05$ for *collagen II*, $P = 0.74$ for *aggrecan*, $P < 0.05$ for *versican,* and $P = 0.83$ for *Sox9*). For comparison of FSP gels (Fig. 1b) between day 1, NO and LO cultures at 7 weeks (n = 4) with ANOVA, only *versican* was significant ($P = 0.47$ for *collagen I*, $P = 0.50$ for *collagen II*, $P = 0.96$ for *aggrecan*, $P < 0.05$ for *versican,* and $P = 0.42$ for *Sox9*). Follow-up with Tukey's post-hoc tests revealed a significant increase only in *collagen II* at 2.5 weeks in LO compared to NO cultures. Significant decreases were found in *versican* in both NO and LO gels at 2.5 weeks when compared to day 1, and in both NO and LO gels at 7 weeks compared to day 1.

In comparing FZP gels between day 1, NO and LO cultures at 2.5 weeks (n = 5) with one-way ANOVA (Fig. 1c), only *collagen I* and *versican* were significantly different ($P < 0.05$ for *collagen I*, $P = 0.33$ for *collagen II*, $P = 0.56$ for *aggrecan*, $P < 0.05$ for *versican*, and $P = 0.56$ for *Sox9*). For FZP gels (Fig. 1d), there were no differences between day 1 and 7 week NO and LO cultures detected with one-way ANOVA ($P = 0.11$ for *collagen I*, $P = 0.36$ for *collagen II*, $P = 0.25$ for *aggrecan*, $P = 0.45$ for *versican*, and $P = 0.36$ for *Sox9*). Tukey's post-hoc tests revealed a significant increase only in *collagen I* in NO cultures compared to day 1. Significant decreases were found in *versican* in both NO and LO gels at 2.5 weeks compared to day 1.

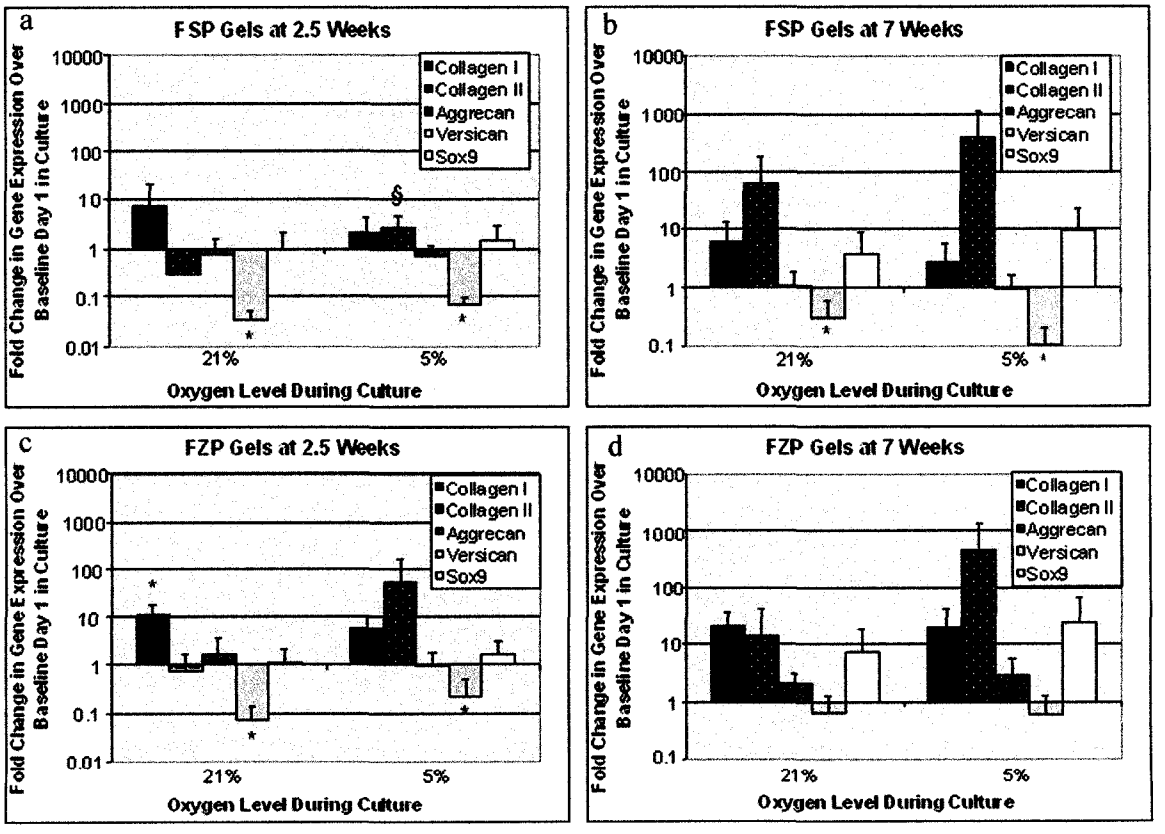


**Figure 1: Gene expression results for chondrocytes encapsulated into fibrin gels.** Fold change in gene expression of chondrogenic genes for FSP gels at 2.5 (a) and 7 (b) weeks and for FZP gels at 2.5 (c) and 7 (d) weeks using gel cultures at day 1 as baseline. * Significant difference at $P < 0.05$ compared to day 1. § Significant difference at $P < 0.05$ in LO compared to NO culture. Error bars are the standard deviation to the mean for each gene.

Two-way ANOVA was performed to determine the effect and possible interaction of oxygen level and gel type at 7 weeks. The effect of oxygen led to a significant change only for *collagen I* ($P < 0.05$) expression; however, there were no significant differences as a result of gel type. Oxygen level did not interact with gel type for any of the genes.

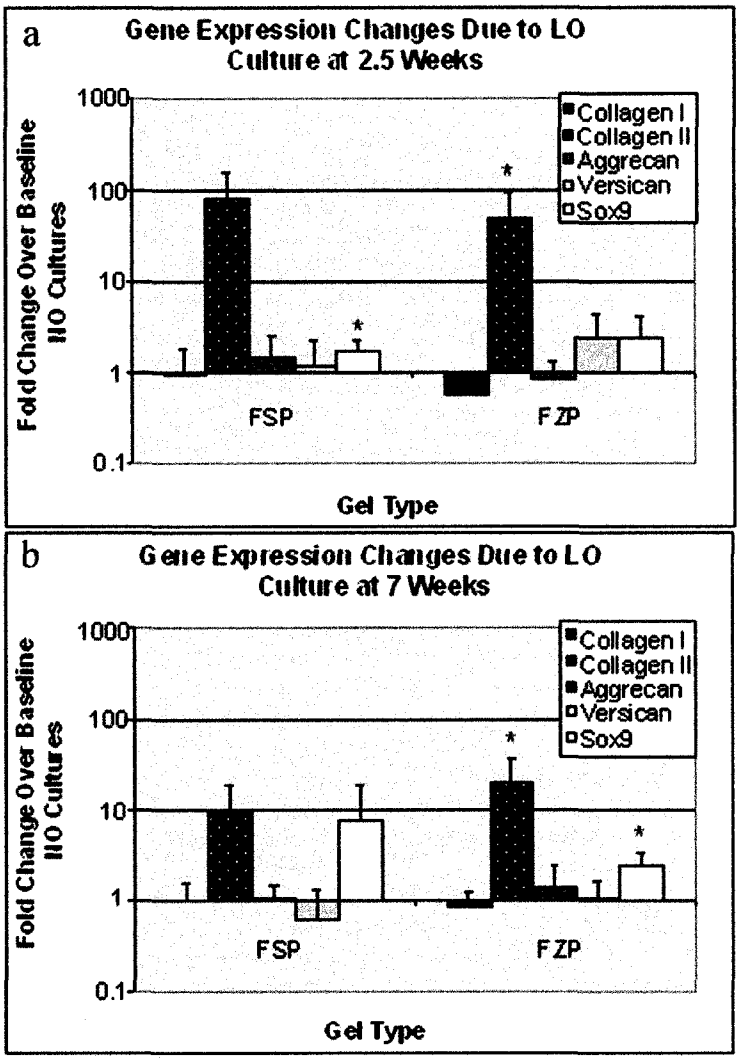


**Figure 2: Comparison of gene expression for encapsulated chondrocytes in low versus normal oxygen culture.** Fold change in gene expression of chondrogenic genes for FSP and FZP gels at 2.5 (a) and 7 (b) weeks after low oxygen culture using normal oxygen cultures as baseline. Error bars are the standard deviation to the mean for each gene. * Significant difference at $P < 0.05$ for LO compared to NO culture.

Student's t test was used to assess the effect of oxygen on the expression of each gene (Fig. 2). For these tests, NO cultures were used as the baseline for data from LO

cultures. For FSP gels at 2.5 weeks (Fig. 2a) only *Sox9* gene expression differed significantly between NO and LO culture. At 7 weeks (Fig. 2b) no significant differences were detected. For FZP gels at 2.5 weeks, LO culture only made a significant difference in *collagen II* expression. At 7 weeks significant increases were detected in *collagen II* and *Sox9* expression as a result of LO.

**Effect of Low Oxygen Culture**

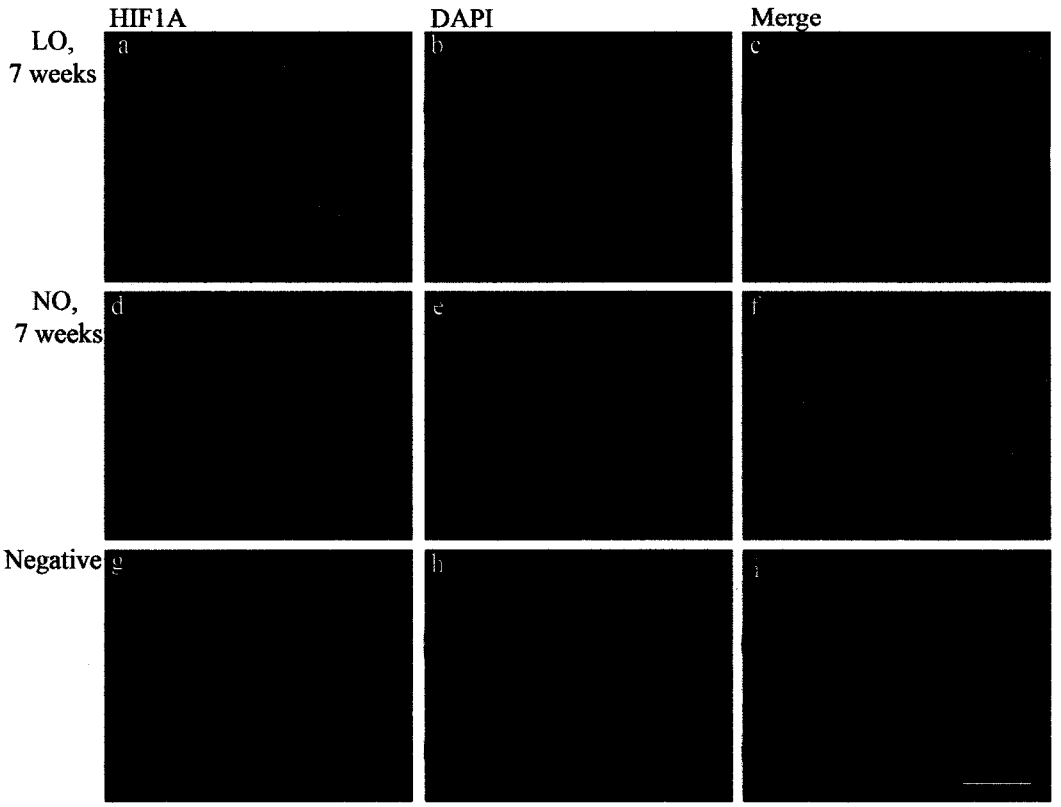


**Figure 3: HIF1A staining of chondrocytes in low and normal oxygen culture.** FSP LO (a-c) and NO (d-f) gel sections at 7 weeks in culture were stained with mouse anti-HIF1A antibody and Cy2-labeled goat anti-mouse secondary antibody (a,d,g) and DAPI (b,e,h). Negative controls were FSP LO 7 week sections stained only with secondary antibody (g) and DAPI (h). Bar 25 μm.

Cells in the FSP LO cultures at 7 weeks exhibited cytoplasmic staining for HIF-1α (Fig. 3a-c). However, cells in the corresponding NO cultures showed background levels of HIF-1α staining (Fig. 3d-f) that were similar to the negative control (Fig. 3g-i).

**Evaluation of ECM Production: Histology and Biochemistry**

Grossly, the fibrin gel samples were opaque and this opacity increased over time in culture; however, the change was not quantified. The gel dimensions were approximately 1 cm in diameter and approximately 2.2 mm in thickness. The gels were a dark blue colour as a result of the genipin cross-linking. There were no observed differences as a result of oxygen level or plasma type. By 2.5 weeks, ECM in FSP and FZP gels under both NO and LO stained weakly with AB (Fig. 4a,c,e,g). By 7 weeks, however, more intense and uniform staining was observed in LO cultures compared to those under NO, and in FZP compared to FSP cultures (Fig. 4i,k,m,o). At 2.5 weeks the chondrocytes showed mostly fibroblast-like morphology (Fig. 4b,d,f,h). However, by 7 weeks many cells appeared large and round and more similar to the expected chondrocyte morphology in native articular cartilage (Fig. 4j,l,n,p)[3]. Stained sections of cultures of chondrocytes from patient C (Fig. 4) are representative of cultures from the other 6 patients.

Total collagen was quantified using the hydroxyproline assay. A significant difference in total collagen was detected with ANOVA for FSP gels at 2.5 weeks ($P < 0.05$; Fig. 5a). Further analysis with Tukey's post-hoc tests revealed that total collagen was significantly greater in both NO and LO culture at 2.5 weeks only compared to day 1. Total collagen in FSP gels also increased significantly at 7 weeks (ANOVA; $P < 0.05$). Follow-up with Tukey's revealed that both NO and LO cultures were significantly different compared to day 1. For FZP gels at 2.5 weeks a significant difference was detected with ANOVA ($P < 0.05$; Fig. 5b). Tukey's post-hoc tests showed that collagen was significantly greater in NO 2.5-week cultures compared to day 1 and compared to

LO cultures at 2.5 weeks. However, there was no significant change in total collagen for FZP gels at 7 weeks ($P = 0.07$).

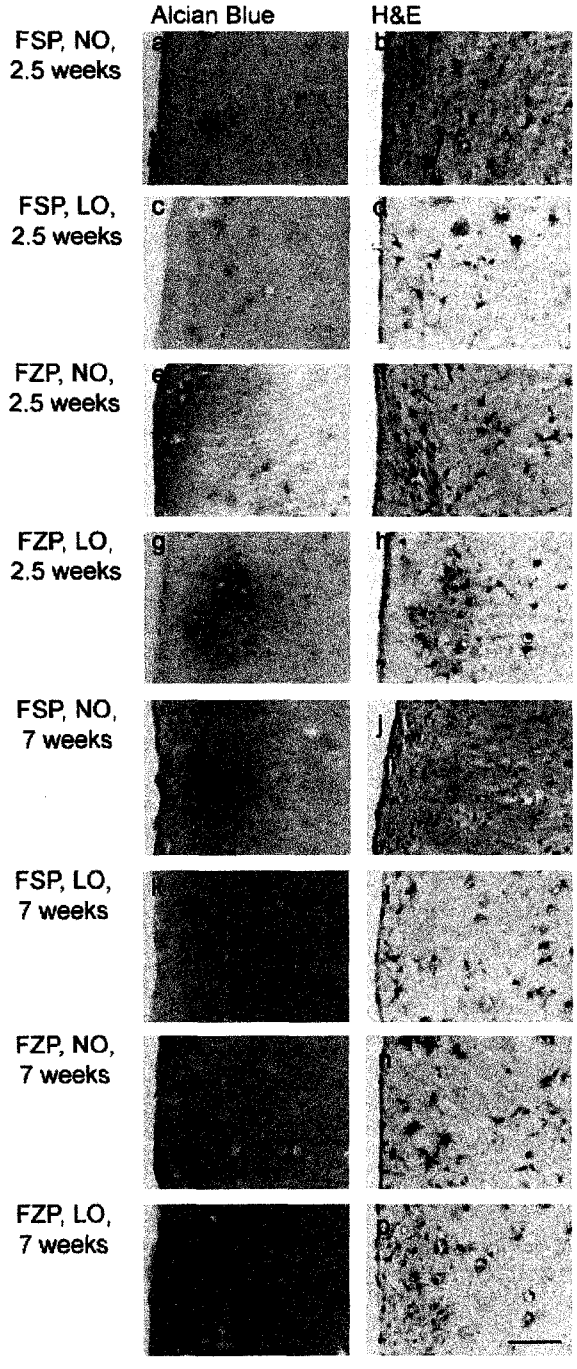


**Figure 4: Histological staining of FSP and FZP gels.** FSP gel sections from 2.5-week and 7-week NO and LO cultures were stained with AB (a,c,i,k, respectively) and H&E (b,d,j,l, respectively). FZP gel sections from 2.5-week and 7-week NO and LO cultures were also stained with AB (e,g,m,o, respectively) and H&E (f,h,n,p, respectively). Bar 50 μm.

A TWO-way ANOVA was performed to determine the effect and possible interaction of oxygen level and gel type for the samples at 7 weeks. Neither the effect of oxygen level nor gel type led to a significant change in total collagen.

The DMMB assay was used to quantify total GAG in the gels. A significant difference in total GAG was detected with ANOVA for FSP gels (Fig. 5c) at 2.5 weeks ($P < 0.05$) compared to day 1. Follow-up with Tukey's post-hoc analysis revealed that total GAG was significantly greater in both NO and LO cultures at 2.5 weeks when each

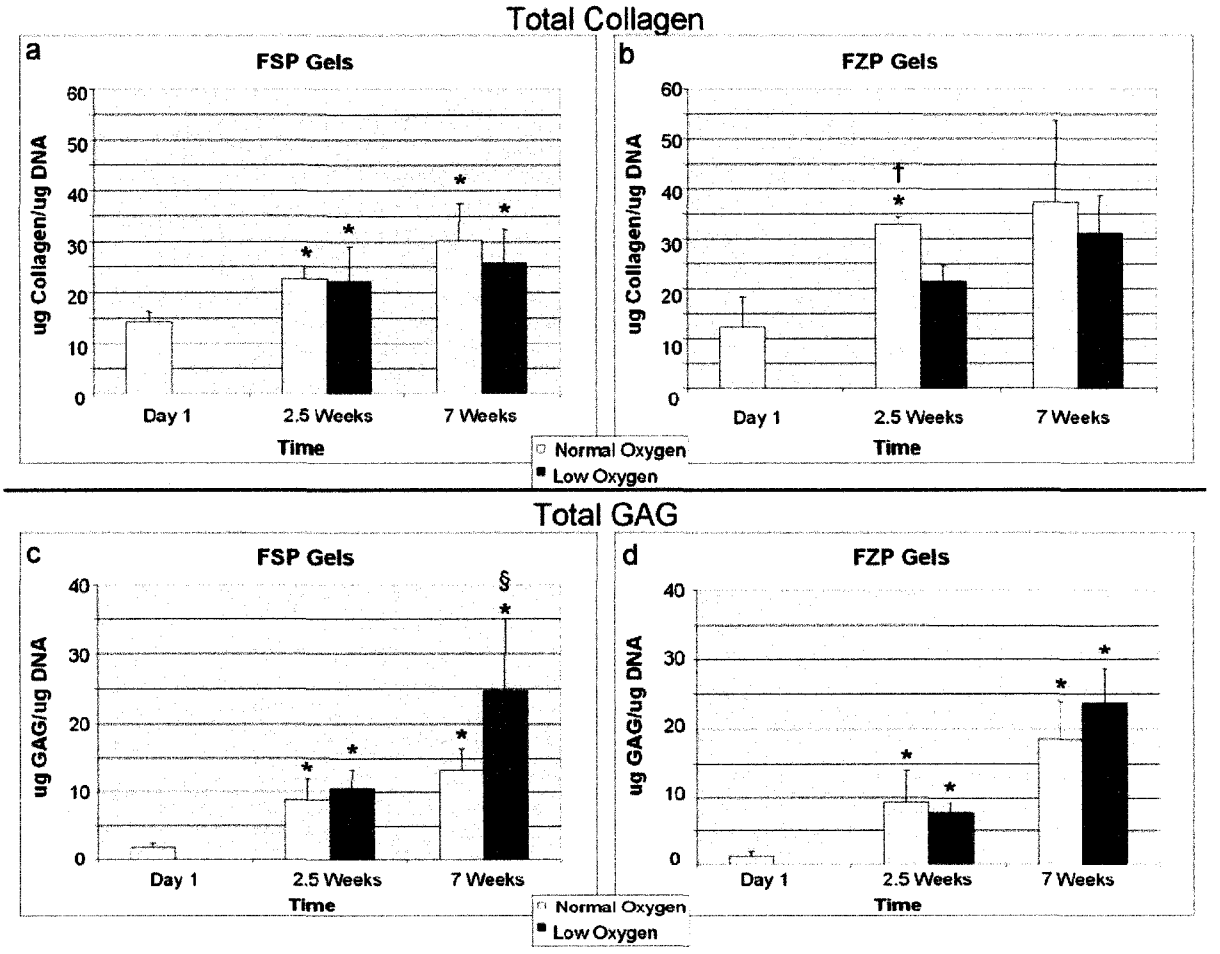


**Figure 5: Biochemical analysis of fibrin glue gels.** Total collagen and total GAG were estimated for FSP (a,c, respectively) and FZP (b,d, respectively) gels at day 1, 2.5 weeks and 7 weeks for both NO and LO culture. Error bars are standard deviations to the means. * Significant difference at $P < 0.05$ compared to day 1. § Significant difference at $P < 0.05$ in LO compared to NO culture. † Significant difference at $P < 0.05$ in NO compared to LO culture.

were compared to day 1. Total GAG also changed significantly for FSP gels at 7 weeks ($P < 0.05$). Further analysis with Tukey's tests indicated that total GAG increased significantly in NO and LO cultures at 7 weeks when compared to day 1, and that total GAG was significantly greater in LO compared to NO cultures at 7 weeks. For FZP gels (Fig. 5d) at 2.5 weeks ANOVA revealed a significant difference in total GAG ($P < 0.05$). With Tukey's analysis, total GAG was significantly greater in both NO and LO culture at 2.5 weeks when each were compared to day 1. Total GAG in FZP gels at 7 weeks was also significantly different with ANOVA ($P < 0.05$). With Tukey's tests total GAG was only significantly greater in NO and LO FZP cultures at 7 weeks when each were compared to day 1.

A Two-way ANOVA was performed to determine the effect and possible interaction of oxygen level and gel type for the samples at 7 weeks. The effect of oxygen led to a significant change in total GAG ($P < 0.05$); however, there was no significant difference as a result of gel type. The effect of oxygen did not interact with the effect of gel type.

**Evaluation of ECM Production: Immunostaining and Western Blotting**

Positive staining for aggrecan (Fig. 6a), collagen II (Fig. 6b), Sox9 (Fig. 6c) and collagen I (Fig. 6d) was observed in immunostained FSP sections after 7 weeks in NO culture. Similar staining was observed for 7-week LO FSP cultures (Fig. 6e-g). However, collagen I staining appeared reduced in LO compared to NO FSP cultures (Fig. 6h). For FZP gels at 7 weeks in NO culture, staining of all components was similar to that observed for 7-week NO FSP cultures (Fig. 6i-l). However, very little staining for collagen II (Fig. 6n) was observed in 7-week LO FZP cultures. Minimal staining was

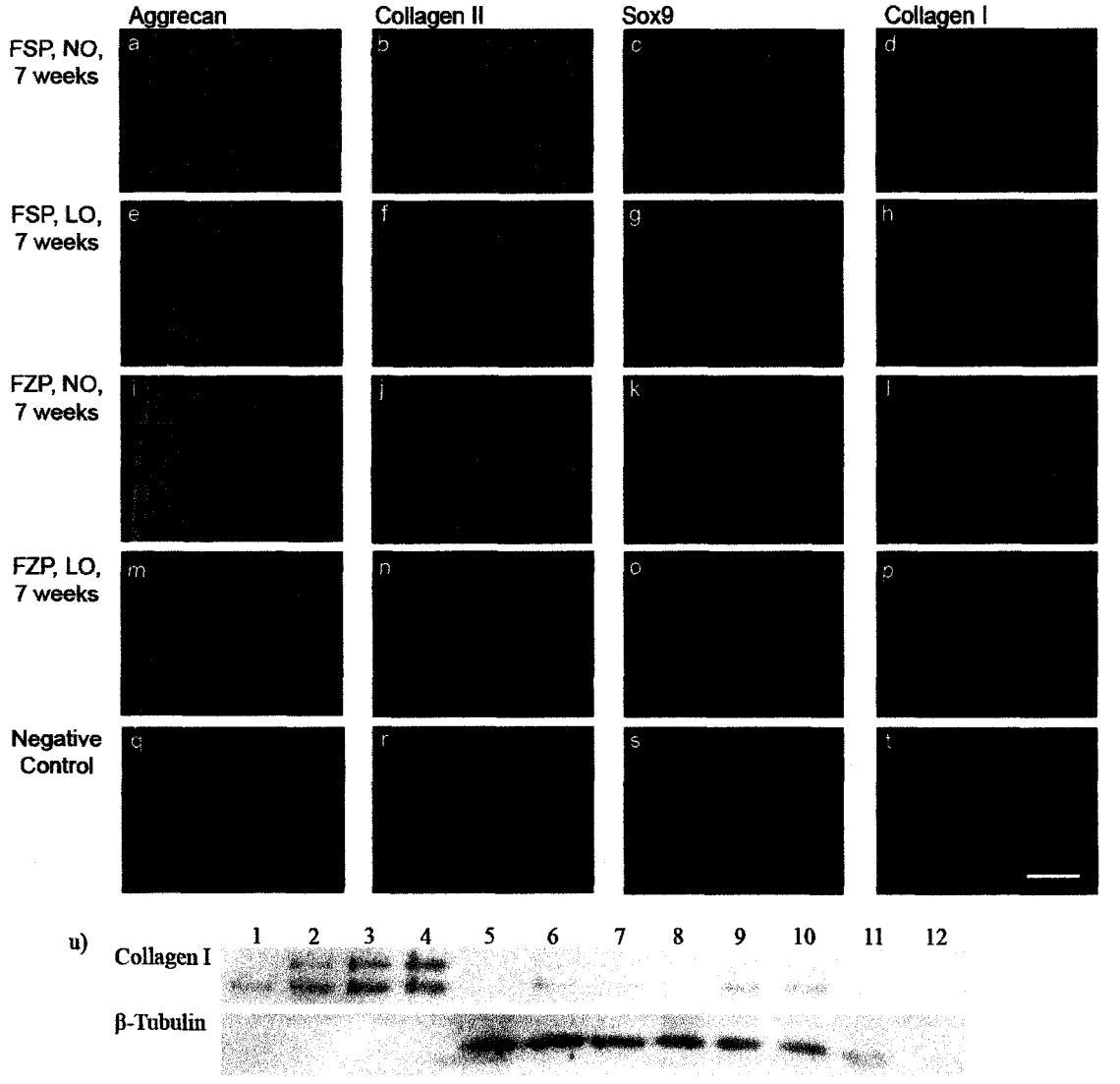


**Figure 6: Immunostaining and Western blot analysis of fibrin glue gels.** FSP gel sections from 7-week NO and LO cultures were stained with rabbit anti-aggrecan (a,e, respectively), rabbit anti-collagen II (b,f, respectively), rabbit anti-Sox9 (c,g, respectively) and anti-collagen I (d,h, respectively). FZP gel sections from 7-week NO and LO cultures were also stained with rabbit anti-aggrecan (i,m respectively), rabbit anti-collagen II (j,n respectively), rabbit anti-Sox9 (k,o, respectively) and rabbit anti-collagen I (l,p, respectively). All sections were stained with goat anti-rabbit Cy2-labeled secondary antibody. Negative controls were stained only with secondary antibody (q,r,s,t). Sections stained for aggrecan and collagen II were also stained with DAPI. Bar 50 μm. Western blots were performed on FSP and FZP gels with anti-collagen I and anti-β tubulin primary antibodies (u). Lanes are labelled as follows: 1-0.5 μg collagen I standard, 2-1 μg collagen I standard, 3-2 μg collagen I standard, 4-3 μg collagen I standard, 5-FSP day 1, 6-FSP NO 7 weeks, 7-FSP LO 7 weeks, 8-FZP day 1, 9-FZP NO 7 weeks, 10-FZP LO 7 weeks, 11-human articular cartilage, 12-acellular fibrin gel. Bar 50 μm.

observed in negative controls (Fig. 6q-t). The immunostaining results presented in Fig. 6 from patient C are representative of results from the other 6 patients. Western blots of FSP and FZP gels from patient C (Fig. 6u) indicated a significant difference in collagen I between FZP (Fig. 6u, lane 9) and FSP (Fig. 6u, lane 6) NO 7-week cultures (0.99 ± 0.10 μg and 0.58 ± 0.20 μg, respectively); however, the difference between LO FZP (Fig. 6u, lane 10) and FSP (Fig. 6u, lane 7) gels (0.94 ± 0.10 μg and 0.52 ± 0.31 μg, respectively) was not significant. There was no significant difference in collagen I between NO and LO for either FSP or FZP cultures. No collagen I was detected in either the pure fibrin glue or in articular cartilage.

**Evaluation of ECM-Chondrocyte Interactions**

To assess chondrocyte interaction with the fibrin scaffold and/or the secreted ECM, immunostaining for $\alpha_1\beta_1$ and $\alpha_V\beta_3$ integrins was performed. Some cells in the FSP LO and NO cultures at 7 weeks stained for cell membrane localization of $\alpha_1\beta_1$ integrin

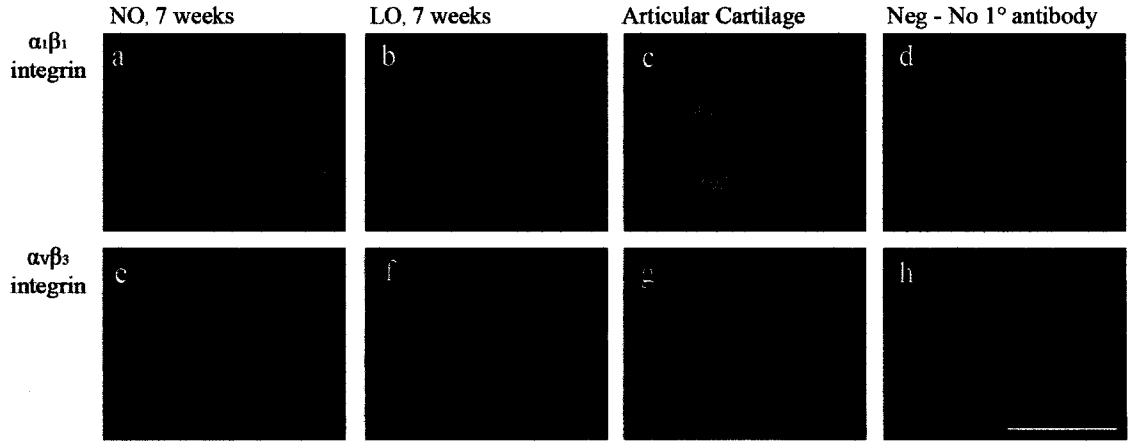


**Figure 7: Integrin staining of fibrin gel cultures.** FSP NO gel sections at 7 weeks (a,e), LO gel sections at 7 weeks (b,f) and human articular cartilage sections (c,g) were stained with mouse anti-$\alpha_1\beta_1$ integrin antibody (a-c) and anti-$\alpha_V\beta_3$ integrin antibody (e-g) and Cy2-labeled goat anti-mouse secondary antibody and DAPI (a-h). Negative controls were FSP LO 7 week sections stained only with secondary antibodies and DAPI (d,h). Bar 25 μm.

(Fig. 7a,b), which is a collagen type II receptor.[42] Cells staining for $\alpha_V\beta_3$ integrin (Fig. 7e,f), a fibrin binding receptor,[44] were also detected. Chondrocytes within articular cartilage stain positively for $\alpha_1\beta_1$ but not for $\alpha_V\beta_3$ integrin (Fig. 7c,g). Very little staining was seen in negative controls (Fig. 7d,h).

**Mechanical Properties of Fibrin Glue Cultures**

The average dynamic compression modulus (Fig. 8) for both 7-week NO and LO gels (13.5 ± 3.8 kPa and 15.2 ± 4.1 kPa, respectively) were significantly greater than the modulus determined for the FSP gels at day 1 in culture (5.2 ± 0.4 kPa). However, the average dynamic compression modulus for day 1 FZP gels (4.24 ± 0.22 kPa) did not change significantly after 7 weeks in NO or LO culture (4.7 ± 1.0 kPa and 4.6 ± 1.0 kPa, respectively).

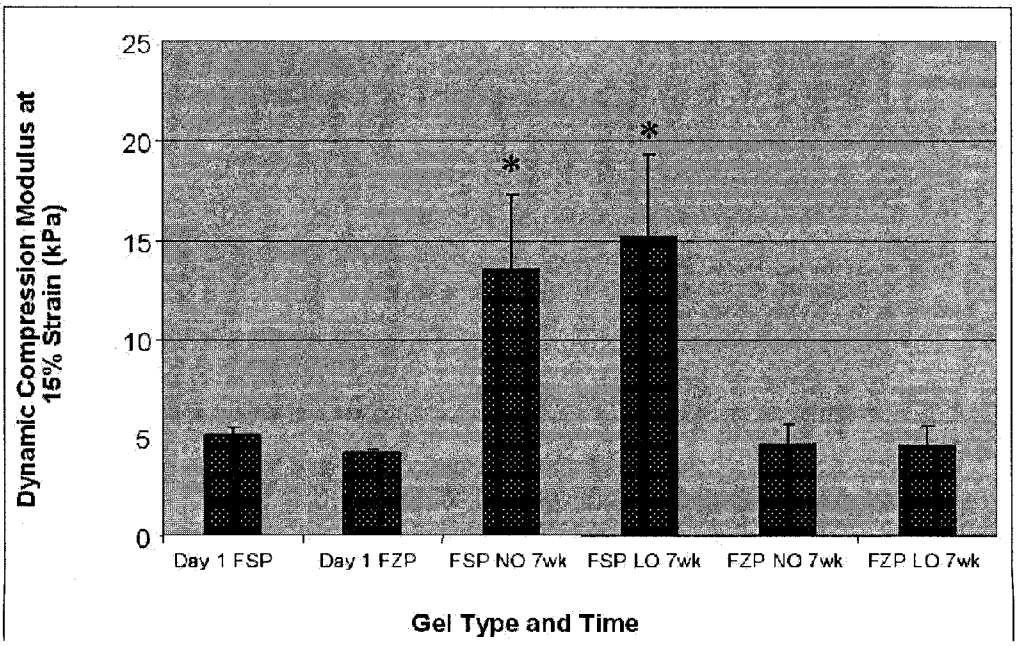


**Figure 8: Mechanical properties of fibrin glue gels.** Histogram showing average dynamic compression modulus of FSP and FZP gels under NO and LO culture over 7 weeks. * Significant difference at $P < 0.05$ compared to day 1. Error bars indicate standard error to the mean.

## DISCUSSION

The goal of this study was to evaluate the suitability of genipin cross-linked fibrin scaffolds derived from potentially autologous plasma for *in vitro* articular cartilage regeneration. Fibrin sealants have been used for articular cartilage regeneration;[15-18] however, there are several potential risks associated with implantation of fibrin derived from bovine or human pooled plasma.[19,21] Therefore, in this study we have evaluated fibrin derived from single units of plasma. In addition, CryoSeal® has not previously been used as a tissue engineering scaffold for *in vitro* culture. In addition, we added genipin to our gels. Genipin is not only valuable as a cross-linker but it also has anti-inflammatory properties and therefore, may be useful therapeutically after implantation into an osteoarthritic joint.[44]

It is important to consider the native environment of the tissue when evaluating a tissue engineering system *in vitro*. Cartilage exists normally in a hypoxic environment.[25] The hypoxia inducible factors (HIFs) are stabilized under hypoxia and can activate the transcription of several downstream target genes including ECM genes.[29] Indeed immunostaining of our samples identified HIF-1α within the cytoplasm of fibrin glue encapsulated chondrocytes cultured under LO. No HIF-1α was identified in cells cultured under NO. The hypoxic conditions did not significantly affect cell viability compared to normoxic culture. This indicates that the cells in LO were not compromised by LO culture, possibly through HIF-mediated gene regulation.[45]

The features of articular cartilage-like tissue development include increases in collagen II and aggrecan and decreases in collagen I and versican. Since collagen II and aggrecan contribute significantly to cartilage biomechanical properties, an increase in

compressive properties over time is also indicative of articular cartilage-like tissue formation.[3] Culture under LO affected ECM gene expression and secretion. It is important to note that while there were some very large changes in gene expression relative to baseline (Fig. 1 and 2) these changes were not significant due to the very large standard deviations. It is not surprising that there was some variability between samples as the chondrocytes and plasma were harvested from different patients. LO significantly increased *Sox9* and *collagen II* gene expression compared to NO culture. *Sox9* is a direct target of HIF-1α in chondrogenic differentiation of mesenchymal stem cells.[46] In turn, Sox9 is a transcription factor that regulates both *collagen II* and *aggrecan* gene expression in the chondrocyte.[47] LO culture significantly increased total GAG secretion by the encapsulated chondrocytes, compared to NO culture. These changes were also observed with histological staining. Immunostaining results indicated that levels of a number of ECM molecules were similar between NO and LO cultures but collagen I was slightly decreased in LO cultures. Therefore, it is evident that culturing human articular chondrocytes under LO helps to re-establish their articular phenotype. In some cases very little immunostaining was observed for samples that had significant changes in gene expression. Translation fidelity cannot always be assumed.[48] Therefore, there may be a delay in protein translation with respect to gene transcription. Our results provide evidence that this fibrin system may lead to more chondrogenic changes when implanted into the native joint environment. We also detected production of collagen- and fibrin-specific integrins by the encapsulated cells, which suggests that the chondrocytes are interacting both with the fibrin scaffold and with the newly synthesized ECM.[42,43] Chondrocytes can express different integrins, depending on their ECM contacts.[49]

The fibrin sealant CryoSeal® is prepared from one unit of plasma by concentrating fibrinogen and other clotting factors via freeze/thaw cycles.[22] We demonstrated that there were no significant differences in viability of articular chondrocytes between FSP and FZP gels. Therefore, freezing the plasma before processing does not affect any factors or proteins within the cryoprecipitate that are related to cell survival. Histological and biochemical results indicated that the encapsulated cells secreted cartilage-specific GAG over time in culture. We also observed that cell morphology changed over time in the cultures to become rounder and more similar to articular chondrocytes.[3] The expansion time for our cells in culture was prolonged, since the time for articular chondrocytes to reach primary confluence increases with donor age and the median age of the cartilage donors was 70 years.[50,51] Consequently, it is likely that the chondrocytes we seeded into our fibrin gels underwent dedifferentiation during harvesting and expansion. Therefore, our results indicated possible redifferentiation *in vitro* of some of the encapsulated cells to a more articular phenotype.

However, cell phenotype differences were noted between FSP and FZP encapsulated cells. Only in FZP gels was a significant increase in *collagen I* gene expression detected. Total collagen was also found to be greater in FZP gels compared to FSP gels. These results were corroborated by immunostaining and Western blotting results, which indicated that there was a greater accumulation of collagen I within the FZP gels compared to FSP gels at 7 weeks. Mechanical testing results indicated that after 7 weeks in culture only the FSP gel displayed significantly improved mechanical properties. This suggests that differences between the ECM components secreted by the

encapsulated cells affected the mechanical properties of the constructs. Compared to articular cartilage, fibrocartilage is stronger in tension but weaker in compression.[3] In addition, it is known that the substitution of collagen type I for type II during *in situ* repair leads to changes in cartilage hydration and compressive properties.[52] The differences observed between FSP and FZP gels indicate that there are alterations in the plasma components resulting from freezing the blood plasma before processing into cryoprecipitate. When considering all the results, chondrocytes encapsulated in FSP gels displayed greater chondrogenic changes that led to more articular cartilage-like tissue formation. An important next step will be to develop an animal *in vivo* orthopaedic model to fully assess autologous implants of this material for articular cartilage regeneration. An interesting animal model has been developed, which may be useful with our material. Mueller-Rath *et al.*[53] created cartilage defects in osteochondral specimens harvested from human osteoarthritic cartilage. The defects were then filled with their collagen material and chondrocytes and then the whole specimens were implanted subcutaneously into nude mice. This presents a possible method for assessment of human autologous samples with an animal model.

While our results are informative, there are some limitations to our study. Since only 7 donors were used each for plasma collection and chondrocyte harvesting, it is difficult to make reliable statistically valid conclusions. It will be beneficial to increase the sample size for future analysis. In addition, in order to make the *in vitro* culture system truly autologous, chondrocytes, plasma, and serum for cell expansion would have been better. However, these materials were not all available for us at the time of analysis. Finally, although of greater clinical relevance, chondrocytes harvested from osteoarthritic

patients are not the best source of cells for regeneration because of the effect of the disease on the gene expression of the cells.[54] Chondrocytes harvested from healthy tissue or other cell populations such as mesenchymal stem cells[48] may have led to even better results.

In conclusion, human fibrin obtained through the CryoSeal® system shows promise for articular cartilage tissue engineering, since encapsulated chondrocytes exhibited chondrogenic gene expression changes as well as cartilage-like ECM secretion, especially under hypoxic culture. However, at this time results suggest that only fibrin glue scaffolds derived from fresh plasma demonstrated significant improvement in mechanical properties over time.

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# CHAPTER 5

## General Discussion

### 5.1 Significant Scientific Contributions

As described in Chapter 1, cartilage is an avascular tissue with very limited healing capacity (Buckwalter 1998; Hunziker 2001). Many researchers have utilized 3D polymer scaffolds for the regeneration of articular cartilage (Han *et al.,* 2008; Tomkoria *et al.,* 2007; Shi *et al.*, 2005; Chevrier *et al.*, 2007). However, no previous scaffold has been proposed as an autologous scaffold for articular cartilage regeneration. Problems such as inflammation, tissue rejection, allergic reaction, and viral or prion infections are possible with material implantation (Zehnder and Leung, 1990; Jackson 2001; Hunter and Houston, 2002). Therefore, the origin of the material is important to consider. In Chapters 2-4 several important contributions to scientific research were described.

In Chapter 2 we described the results of seeding the chondroprogenitor RCJ 3.1C5.18 cells into fibrin gels. This is the first description of the culture of these cells in 3D scaffolds. McDougall *et al.* (1996) previously cultured C5.18 cells in the surface of type I collagen/agarose scaffolds. After only 7 days of culture on the biomaterial surface, they found a large increase in *collagen II* and a slight increase in *collagen I* gene expression when compared to monolayer. We observed similar findings with fibrin gel encapsulation and culture with additional increases in GAG production and *aggrecan* gene expression. We were also the first to compare the culture of these cells in maintenance medium with high serum content to serum-free chondrogenic medium. We found that culture with chondrogenic medium significantly induced articular chondrogenic changes in the C5.18 cells during monolayer and pellet culture. Therefore,

as a result of our experiments we not only developed the fibrin gel for chondrogenic culture, we were also able to contribute to development of the C5.18 cell line as a tool for studying chondrogenic differentiation.

We described the culture of human primary articular chondrocytes encapsulated within fibrin scaffolds derived from pooled plasma in Chapter 3. While genipin has been used previously to cross-link fibrin (Linnes *et al.*, 2007), this is the first manuscript to report on genipin cross-linking of fibrin scaffolds for articular cartilage tissue engineering. Cross-linking with genipin increased the mechanical properties of the fibrin gels and did not negatively affect the chondrogenesis induced after fibrin encapsulation. We also determined that the species specificity of the fibrin material is important. We found that, in rats, xenogeneic fibrin elicited a greater immune response than allogeneic fibrin after subcutaneous implantation. This indicates that the species origin of the fibrin material may not only be important for our cartilage regeneration implant but may also be important for the other applications of commercially available fibrin sealant (Amrani *et al.,* 2001; Carless *et al.,* 2002; Currie *et al.,* 2001), since they are processed from pooled plasma of either bovine or human origin.

Finally, in Chapter 4 we discussed the encapsulation of human articular chondrocytes into fibrin gels derived from single units of plasma. With these results we have evaluated the first step towards the development of an autologous articular cartilage tissue engineering system. This is the first time that the CryoSeal® fibrin sealant has been used for *in vitro* cell culture. Hyaline-like gene expression and ECM production was induced after hypoxic culture of the encapsulated cells. Chondrocytes can enter premature senescence *in vitro* and *in vivo* as a result of oxidative stress, which leads to a

decrease in the cells' ability to repair damaged tissue. However, when the oxygen level is reduced to 5% during culture, growth of the chondrocytes can be extended towards the Hayflick limit (Martin *et al.,* 2004). It is likely that there were senescent cells within our fibrin gel cultures as the chondrocytes were isolated from elderly patients with OA. Therefore, culture under hypoxia may have prevented the transition of more cells into senescence and allowed better regeneration of the tissue. Since synovial fluid is an ultrafiltrate of plasma (Huber *et al.,* 2000) it is not surprising that CryoSeal® contains many components that are favorable for chondrogenic cell culture. In addition, when we compared material derived from fresh versus fresh/frozen plasma we found that the material derived from fresh plasma was superior. This is an important result as it greatly influences the future progression of the project. It is clear that CryoSeal® material for implantation must be processed immediately from freshly collected plasma.

While there has been much progress achieved in articular cartilage tissue engineering, very few systems have been translated to the clinic with implantation into human patients (Kuo *et al.,* 2006). Therefore, in the next section it is important to discuss the strategies that should be implemented for the future progression of the fibrin tissue engineering system towards clinical use.

## 5.2 Issues for Future Consideration

### 5.2.1 Design of an *in vivo* Implantation System

In order to assess the fibrin gel system for *in situ* articular cartilage regeneration, it will be very important to design and implement an *in vivo* experimental animal implantation strategy. In the design of an animal orthopaedic model, several points must be considered. First, in choosing the species to create the model, it is important that the

anatomy be sufficiently similar to human anatomy (Rudert 2002). For example, the rabbit knee shows a gross morphological similarity to the human knee in the bone geometry, cartilage and tendons (MacLaughlin and Chiasson 1979). In addition, as in the human, regeneration of an osteochondral defect does not occur in the skeletally mature rabbit (Rudert 2002). In planning orthopaedic experimentation with the rabbit it is important to consider the housing requirements of the animal. The overall movement of the animal must not be restricted. Therefore, a large cage such as 4 $m^2$ is necessary (Rudert 2002). The age of the experimental animal is also of particular importance. As in humans, satisfactory natural repair of cartilage defects cannot be obtained, even in young rabbits (Wei *et al.,* 1997). According to many investigators, rabbits are mature by 6 months of age (Masoud *et al.,* 1986; Eggli *et al.,* 1988); however, it has been found by X-ray observation that continued epiphyseal growth occurs in 6-month-old rabbits (Rudert 2002). Therefore, to mimic adult human osteochondral healing as much as possible it will be necessary to use rabbits that are older than 6 months.

When determining the method and location for the formation of the experimental defect in the model, it is important to keep in mind the predominantly squatting position of the rabbit because: 1) the posterior part of the chondyles are almost constantly loaded (Räsänen and Messner 1996), and 2) occasional unloading of the patellofemoral groove, as in a standing human, is rather unlikely. Therefore, to achieve a loading situation comparable to the weight-bearing surface of human joints, a defect must be made in the condyle and care must be taken to reach the posterior (Wakitani *et al.,* 1994; Räsänen and Messner 1996). It is recommended to create at least one control defect per animal (Rudert 2002). The size of the defect should also be considered. The defect should be large

enough to be exposed to considerable loads, without risking fracture to the condyles. A 3-mm diameter defect has proved successful for many researchers (Freed *et al.,* 1994; Brittberg *et al.,* 1996). The thickness of adult rabbit condylar cartilage is less than 400 μm, whereas human condylar cartilage measures 2-3 mm in thickness (Athanasiou *et al.,* 1991). As a result it is difficult to create chondral defects without causing injury to the subchondral plate (Nehrer *et al.*, 1998). Therefore, the chances of experimental success are greater if full-thickness osteochondral defects are attempted rather than partial-thickness chondral defects. To create osteochondral defects, the subchondral bone must be opened. By using a hand drill, the correct depth can be established (Rudert 2002). In addition, arthritis can be chemically induced by injecting interleukin-1 into the joint (van de Loo *et al.,* 1998). Defects formed can then be assessed by the addition of the fibrin system.

Another important issue to consider is the duration of the experiment. It has been shown previously that while there may be good results after 12 weeks, degenerative changes can then begin and be observed after 48 weeks (Shapiro *et al.,* 1993). Degeneration of the repaired defect has also been observed even 12 months after the operation (Shahgaldi *et al.,* 1991). Therefore, a follow-up period of 6 months should be minimal for the first evaluation of the material. If the repair appears satisfactory, the evaluation period should be extended to ensure there is no regression. Histological staining followed by a scoring system should be used for analysis of defect repair. The O'Driscoll scoring system (O'Driscoll *et al.*, 1998) is widely used and involves histomorphological measurements after staining with H&E and Safranin O. This scoring

system includes evaluation of degenerative changes and can achieve better comparability of results in future studies than other available scoring systems (Rudert 2002).

It is also necessary to consider the possible clinical situations with which the fibrin system can be implemented in the future. In a Phase I clinical trial, usually a small number of patients are recruited (20-80) to assess the safety and tolerability of a therapy. The patients selected for these trials often have advanced disease and few existing treatment options. In the development of a Phase I clinical trial two situations are possible. One possibility is the treatment of defects in non-weight bearing articular cartilage within a joint. Defects in non-weight bearing areas do not cause as many complications to the patient; however, during a procedure called mosaicplasty, cylindrical osteochondral pieces are removed from non-weight bearing articular cartilage and transferred into debrided full-thickness defects (Hangody *et al.,* 1998). The cartilage donor sites fill with fibrocartilage during healing (Hangody *et al.,* 1997; Hangody *et al.,* 1998). Therefore, one possibility for implantation of the fibrin system is the treatment of these donor cartilage sites during mosaicplasty. For the study, plasma and chondrocytes (via a small biopsy) can be collected and processed ahead of time. During the mosaicplasty procedure some of the donor cartilage sites can be filled with the fibrin hydrogel (Fig. 1). Other control sites will be left untreated. Six months post-operative, small biopsies would be taken from the donor cartilage sites with/without the fibrin gel implants and analyzed to evaluate the efficacy of the fibrin tissue engineering system.

Although we have performed *in vitro* tests to date on the fibrin gel system it is still difficult to speculate as to whether the mechanical properties of the material are sufficient to withstand *in vivo* implantation. However, the animal studies proposed above

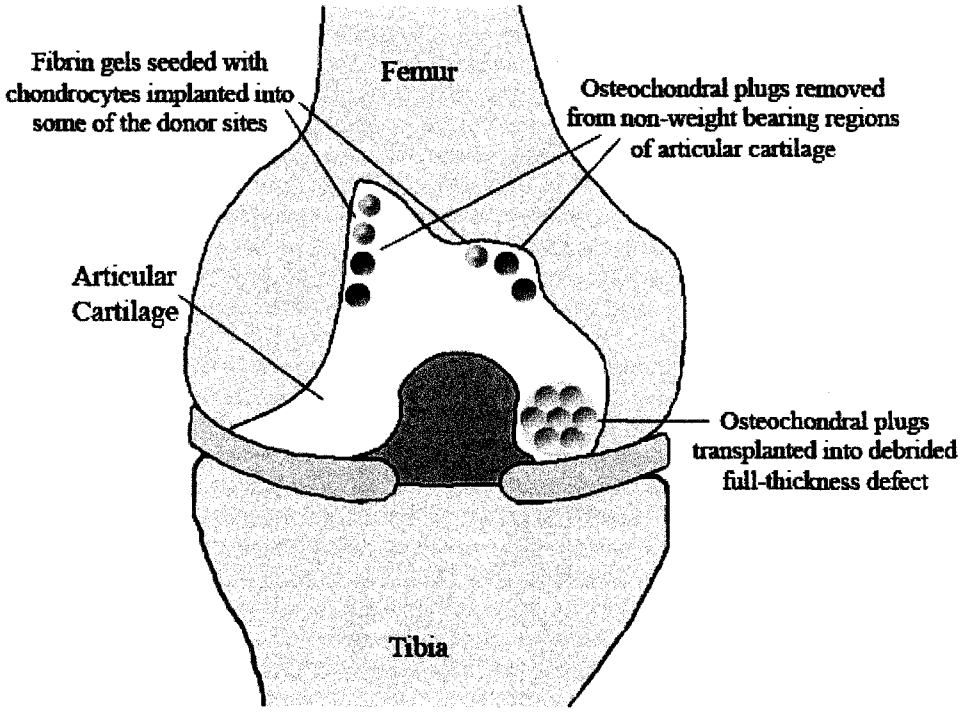


**Fig. 1. Proposed method of transplantation of fibrin gels.** Fibrin scaffolds are transplanted into non-weight bearing cartilage defects created during a mosaicplasty surgical procedure. Adapted from Redman *et al.,* (2005) *Eur Cell Mater* **9** p. 26.

would directly address that question. If reasonable success is generated from the implantation of fibrin scaffolds into weight-bearing osteochondral defects of rabbits, it will be feasible to consider extension to human patients. One possibility is a Phase I trial with treatment of patients that have no other medical options other than total knee arthroplasty (TKA). Patients that are scheduled for TKA can have a portion of their defect implanted with the fibrin gel system (after plasma collection with fibrin glue processing and cartilage biopsy with cell expansion) 6 months before the TKA surgery. Some patients will be left untreated. At the time of TKA the articular cartilage surfaces removed from the patients' knees will be collected and analyzed histologically. The ultimate surgical goal with the fibrin gel system will be the treatment of early stage cartilage defects in order to prevent the future need for total joint replacement.

### 5.2.2 Cell Source

As described in the Introduction, there are several advantages and disadvantages with using primary articular chondrocytes for articular cartilage tissue engineering. While relatively large numbers of cells are available and are easy to culture, primary chondrocytes lose their differentiated phenotype in culture if allowed to adhere to a substrate in the presence of serum (Holtzer *et al.,* 1960). Monolayer expansion is necessary to generate sufficient cell numbers. In addition, the results described in Chapters 3 and 4 were based on the culture of primary chondrocytes harvested from the joints of patients with advanced OA. The disease may have had an effect on the cells. Therefore, it is important to consider another cell source for the progression of the fibrin hydrogel system towards an *in vivo* implantation strategy.

There may be a progenitor subpopulation of articular chondrocytes that reside in the superficial zone of articular cartilage (Dowthwaite *et al.,* 2004). These cells have been identified and isolated from bovine articular cartilage (Hynes 1992; Hayes *et al.,* 2001) and have been shown to have high lineage plasticity and high colony forming ability. Therefore, they represent a possible novel solution for the treatment of articular cartilage defects. Tallheden *et al.* (2003) have also reported that culture-expanded human articular chondrocytes present similar characteristics to progenitor cells; however, they were unable to identify a specific progenitor sub-population.

Another undifferentiated cell type that has potential for the treatment of articular cartilage defects is the mesenchymal stem cell (MSC). MSCs are pluripotent stem cells that have the ability to differentiate into a variety of connective tissues (Barry 2003; Gao and Caplan 2003). Bone marrow-derived MSCs can differentiate among others into

osteoblasts, adipocytes and chondrocytes. They can be isolated from the marrow and expanded in culture, keeping their differentiating potential when cultured under appropriate conditions (Pittenger *et al.,* 1999; Reyes *et al.,* 2001). In a comparative study, the effectiveness of MSC implantation, chondrocyte implantation, periosteal grafts and mosaicplasty were evaluated in the treatment of osteochondral defects (Hui *et al.,* 2004). The results indicated that chondrocytes and MSCs had a similar effect in chondral restoration and were both superior to mosaicplasty and periosteal grafting. In another study, chondrocyte implantation was compared to MSC implantation for the treatment of full-thickness defects of rabbits. It was found that with MSCs, hyaline-like tissue was regenerated to a greater degree than with chondrocytes (Goldberg and Caplan 1995).

Treatment of chondral defects with MSCs have focused mainly on the use of marrow-derived MSCs although other tissue sources such as the synovial membrane have been explored (Redman *et al.*, 2005). A potential MSC population has also been identified within both normal and osteoarthritic human articular cartilage (Alsalameh *et al.,* 2004). It was found that there was a higher frequency of progenitor cells within osteoarthritic compared to normal cartilage. This may be because there is either local recruitment of cells from surrounding synovial tissues (Marinova-Mutafchieva *et al.,* 2000; Nishimura *et al.,*1999) or that there are MSCs residing in the diseased tissue, which can revert back to a more immature phenotype (Redman *et al.,*2005). This data provides evidence that there is promise for both intrinsic and extrinsic improvements to cartilage repair strategies. It is evident that there are a number of potential cell sources for articular cartilage tissue engineering; however, the optimal cell type has yet to be determined.

## 5.3 Conclusions

The objectives of this research project were to evaluate *in vitro* a fibrin hydrogel scaffold for articular cartilage tissue engineering by evaluating the RCJ 3.1C5.18 chondrogenic cell line and human primary chondrocytes seeded within the gel. Other goals of the project included the development of material derived from individual patients and an assessment of the impact of culturing the constructs under physiological (hypoxic) oxygen tension. The results presented in Chapters 2-4 demonstrate that the above objectives have been achieved. Genipin cross-linking exerted a positive impact on the tissue engineering constructs such as an increase in gel strength and a reduction in inflammation after subcutaneous implantation. The CryoSeal® fibrin system offers an affordable, available, low risk scaffold, which was shown to be appropriate for chondrogenic cell culture. In addition, articular cartilage-like changes in gene expression and ECM secretion were observed as a result of hypoxic culture. Overall, we have shown that the genipin cross-linked CryoSeal® fibrin glue scaffold derived from fresh plasma shows potential for the development of an autologous human articular cartilage tissue engineering system. While this represents a significant accomplishment in the field of OA and cartilage regeneration research, as described above, the continuation of the project should include the development of an animal orthopaedic model and the identification of an appropriate cell source for implantation.

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## Appendix I: Supplementary Data

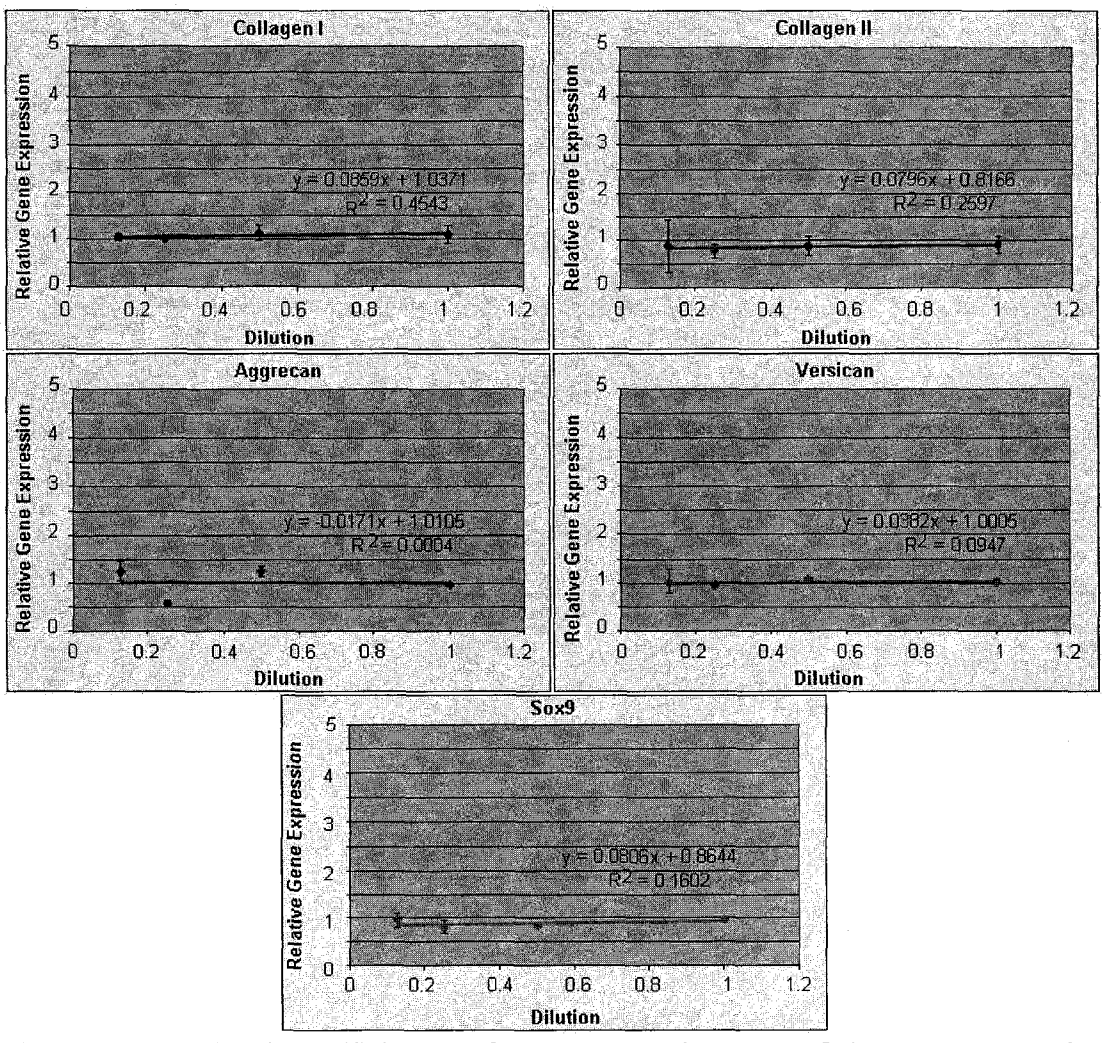


**Fig. 1. Amplification efficiencies for human primers used in gene expression analysis.** The ΔCt values are plotted versus cDNA dilution for each primer set.

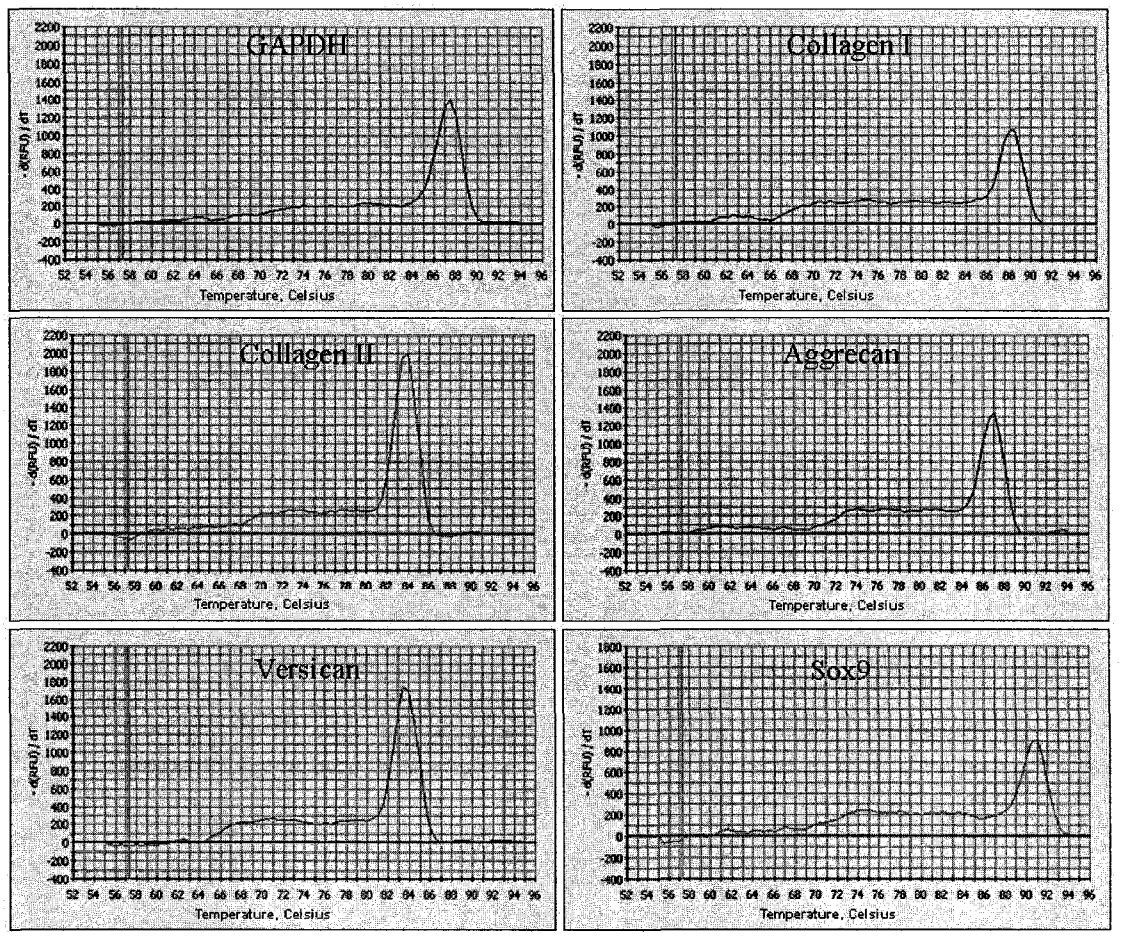


**Fig. 2. Melt curve analysis of human primers used in gene expression analysis.** Melt curve analysis for each primer set plotted as relative fluorescent units versus temperature using SYBR Green analysis.

## APPENDIX II

## Curriculum Vitae

## Emma Victoria Dare

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### Education

**Ph.D. candidate, department of Cellular and Molecular Medicine, University of Ottawa** – Expected date of convocation – 10/2008

- Supervisor: Dr. Max Hincke
- GPA of 93.3%
- Title of project: Evaluation of fibrin polymer hydrogels as scaffolds for articular cartilage tissue engineering

**B. Sc. H., major in Molecular Biology and Genetics, University of Guelph** – Date of convocation – 06/2003

- GPA of 86.25%
- 4th year research project supervisor: Dr. Andrew Bendall

### Awards

- NSERC PGSD2 Scholarship — 05/2007-present
  - Total value of $42,000
- CORS Founder's Medal for best research paper at 61st Canadian Orthopaedic Association Conference — 06/01/2006
- Ontario Graduate Scholarship — 01/2006-12/2006
  - Value of $15,000
- 2nd Best Poster Award at 11th Canadian Connective Tissues Conference — 05/2005
  - Value of $600
- Travel Award at 11th Canadian Connective Tissues Conference — 05/2005
  - Value of $250
- Honourable Mention for the Student Travel and Professional Development Award for the 30th Annual Meeting and Exhibition of the Society for Biomaterials — 04/27/2005
  - Value of $150 USD
- University of Ottawa Excellence Scholarship — 01/2005-present
  - Covers tuition each year
- Ontario Graduate Scholarship — 01/2005-12/2005
  - Value of $15,000
- Ontario Graduate Scholarship in Science and Technology — 01/2004-12/2004

- Value of $15,000
- University of Ottawa Entrance Scholarship 09/2003-08/2004
  - Value of $5,535
- University of Guelph Entrance Scholarship 09/1999-04/2000
  - Value of $2,000
- Bell Canada Scholarship 09/1999-04/2003
  - Total value of $9,000

**First Author Published Manuscripts**

1. Dare, E.V., Vascotto, S.G., Carlsson, D.J., Hincke, M.T., and Griffith, M. Differentiation of a fibrin gel encapsulated chondrogenic cell line. In J Artif Organs 2007; 30(7): 619-27.

**Co-Authored Published Reviews**

1. Ahmed T.A.E., Dare, E.V., and Hincke M.T. Fibrin: a versatile scaffold for tissue engineering applications. Tissue Eng 2008: In Press.

**First Author Submitted Manuscripts**

1. Dare, E.V., Griffith, M., Poitras, P., Kaupp, J.A., Waldman, S.D., Carlsson, D.J., Dervin, G., Mayoux, C., and Hincke, M.T. Genipin cross-linked fibrin hydrogels for human articular cartilage tissue engineered regeneration. Submitted to Cells Tissue Organs January 23rd, 2008.

2. Dare E.V., Griffith M., Poitras P., Wang T., Dervin G.F., Giulivi A., and Hincke M.T. Comparison of Fibrin Sealants Derived from Fresh or Fresh/Frozen Plasma as Scaffolds for *in vitro* Articular Cartilage Regeneration. Submitted Tissue Engineering, April 17th, 2008.

**Co-Authored Book Chapters**

1. McLaughlin C.R., Osborne R., Hyatt A., Watsky M.A., Dare E.V., Jarrold B.B., Mullins L.A. and Griffith M. Tissue Engineered Models for In Vitro Studies. In: Fundamentals of Tissue Engineering and Regenerative Medicine. Ed. U. Meyer, Springer 2008.

**First Presenter Conference Presentations**

1. Dare, E.V., Poitras, P., Dervin, G.F., Giulivi, A., Griffith, M., and Hincke, M.T. (2008) Genipin Cross-Linked Fibrin Scaffolds Derived From Autologous Human Plasma for *in vitro* Articular Cartilage Regeneration. H.K. Uhthoff Research Day, Ottawa, April 16th, 2008. (oral, presented by E. Dare)

2. Dare, E.V., Poitras, P., Dervin, G.F., Giulivi, A., Griffith, M., and Hincke, M.T. (2007) Development of cross-linked autologous human fibrin hydrogels for articular cartilage tissue engineering. 13th Canadian Connective Tissue Conference, Toronto, May 27-29th, 2007. (oral, presented by E. Dare)

3. Dare, E.V., Poitras, P., Dervin, G.F., Giulivi, A., Griffith, M., and Hincke, M.T. (2007) Development of an autologous cross-linked fibrin scaffold for human articular cartilage regeneration. H.K. Uhthoff Research Day, Ottawa, April 19th, 2007. (oral, presented by E. Dare)

4. Dare, E.V., Poitras, P., Kaupp, J., Waldman, S., Carlsson, D.J., Dervin, G.F., Griffith, M., and Hincke, M.T. (2007) Natural biodegradable fibrin hydrogels cross-linked with genipin as scaffolds for autologous human articular cartilage tissue engineering. The Orthopaedic Research Society, 53rd Annual Meeting, San Diego, CA, Feb. 10-14th, 2007 (poster, presented by E. Dare)

5. Dare, E.V., Poitras, P., Kaupp, J., Waldman, S., Carlsson, D.J., Dervin, G., Griffith, M., and Hincke, M.T. (2006) Evaluation of genipin cross-linked fibrin hydrogels for tissue engineering of human articular cartilage. The Canadian Orthopaedic Association, 61st Annual Meeting, Toronto, June 1-3rd, 2006. (oral, presented by E. Dare)

6. Dare, E.V., Poitras, P., Kaupp, J., Waldman, S., Carlsson, D.J., Dervin, G., Griffith, M., and Hincke, M.T. (2006) Regeneration of human articular cartilage with novel genipin cross-linked fibrin polymer hydrogels. 12th Canadian Connective Tissues Conference, Ottawa, May 27-29th, 2006. (oral, presented by E. Dare)

7. Dare, E.V., Poitras, P., Kaupp, J., Waldman, S., Carlsson, D.J., Dervin, G., Griffith, M., and Hincke, M.T. (2006) Human articular cartilage regeneration. H.K. Uhthoff Research Day, Ottawa, April 20th, 2006. (oral, presented by E. Dare)

8. Dare, E.V., Carlsson, D.J., Dervin, G., Griffith M., and Hincke M.T. (2005) Assessment of modified fibrin hydrogels for tissue engineering of articular cartilage. The Canadian Orthopaedic Association, 60th Annual Meeting, Montreal, June 3-5th, 2005. (poster, presented by E. Dare)

9. Dare, E.V., Poitras, P., Vascotto, S., Kaupp, J., Waldman, S., Carlsson D., Dervin G., Griffith, M., and Hincke, M. (2005) Suitability of modified fibrin hydrogels for regeneration of articular cartilage. 11th Canadian Connective Tissues Conference, Montreal, May 26-28, 2005. (poster, presented by E. Dare)

10. Dare, E.V., Carlsson, D.J., Griffith M., Dervin, G., and Hincke M.T. (2005) Evaluation of modified fibrin polymer hydrogels as scaffolds for tissue engineering of articular cartilage. Society for Biomaterials, 30th Annual Meeting and Exposition, Memphis Tennessee, April 27-30, 2005. (oral, presented by E. Dare)

11. Dare, E.V., Carlsson, D.J., Griffith M., Dervin, G., and Hincke M.T. (2005) Fibrin polymer scaffolds for tissue engineering articular cartilage. H.K. Uhthoff Research Day, Ottawa, April 21st, 2005. (oral, presented by E. Dare)

12. Dare, E.V., Lussier, B.E., Cutler, A., Carlsson, D.J., Griffith, C.M., Dervin, G. and Hincke, M.T. (2004) Evaluation of a fibrin polymer hydrogel as a scaffold for articular cartilage tissue engineering. 10th Canadian Connective Tissue Conference, Toronto, May 28-30, 2004. (poster, presented by E. Dare)

13. Dare, E., Hincke, M.T., Dervin, G., Griffith, C.M., and Carlsson D. (2003) Characterization of fibrin hydrogel scaffolds seeded with chondrogenic cells. EMK Research Day on BioInterfaces (Cartilage), McMaster University, Hamilton. October 28, 2003. (oral, presented by E. Dare)

14. Dare, E., Mahrouche, L., Hincke, M.T., Griffith C.M., Carlsson, D., and Dervin, G. (2003) Artificial articular cartilage tissue engineering. EMK Research Day. Toronto, June 20, 2003. (oral, presented by E. Dare)

**Co-Authored Presentations**

15. Hincke, M.T. Dare, E., Dervin, G., Griffith, C.M., and Carlsson D. (2004) Novel bio-synthetic matrices for articular cartilage tissue engineering. EMK Research Day. Toronto, June 27, 2004. (oral, presented by M. Hincke)

16. Hincke, M.T. Dare, E., Dervin, G., Griffith, C.M., and Carlsson D. (2004) Novel polymers for tissue engineered articular cartilage. Ottawa Hospital, Division of Orthopaedics Research Day. April 1, 2004. (oral, presented by M. Hincke)

17. Hincke, M.T. Dare, E., Dervin, G., Griffith, C.M., and Carlsson D. (2003) Novel macromolecular matrices for cartilage Tissue Engineering. EMK Research Day on BioInterfaces (Cartilage), McMaster University, Hamilton. October 28, 2003. (oral, presented by M. Hincke)

**Work Experience**

**Lecturer** 11/2007

*Introduction to Developmental Biology, University of Ottawa*

- Taught course section on vertebrate limb formation and regeneration

**Marker/Lecturer** 09/2006-12/2006

*Introduction to Developmental Biology, University of Ottawa*

- Taught course section on vertebrate limb formation and regeneration

- Marked midterms and final exam

**Summer Research Student** Summer 2003

*Cellular and Molecular Medicine, University of Ottawa*

- Supervisor: Dr. Max Hincke
- Worked on articular cartilage tissue engineering project

**Technician** Summer 2002

*Animal Health Lab, Laboratory Services, University of Guelph, Guelph, Ontario*

- Supervisors: Dr. Susy Carman and Dr. Davor Ojkic
- Lab work in veterinary diagnostic mammalian virology and avian immunology

**Avian Care Assistant** Summer 2000, 2001

*Wild Bird Care Centre, Nepean, Ontario*

- Supervisor: Steve Hamlyn
- Responsible for care and handling of all types of wild birds
- Performed examinations on over 200 birds and assisted with surgeries
- Answering phones

## Volunteer Experience

**Ontario Veterinary College: Large Animal Clinic** 2002

*Foal Watch*

- Caring for sick, newborn foals in neonatal intensive care unit

**Animal Care Worker** 2000

*Equine Research Centre, University of Guelph*

- Daily care of research animals and barn maintenance

**Languages**
English – French