Gene-Supplemented Collagen-Glycosaminoglycan Scaffolds for Nonviral Gene Delivery in Articular Cartilage Tissue Engineering

by

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ABSTRACT

Three-dimensional scaffolds and growth factors have been shown to be important for articular cartilage tissue engineering. A major problem in using recombinant proteins *in vivo*, however, is the inability to maintain therapeutic levels over prolonged times due to degradation or diffusion from the defect site. The goal of this thesis was to develop a method to employ type II collagen-glycosaminoglycan (CG) scaffolds for the nonviral delivery of the gene encoding for insulin-like growth factor (IGF)-1, as a novel means to provide a local, elevated, and prolonged release of a therapeutic growth factor via transfection of cells seeded or migrating within the scaffold. *In vitro* studies were performed to evaluate gene-supplemented CG (GSCG) scaffolds, including: 1) the type of expansion medium to use for growing chondrocytes prior to transfection, 2) methods of incorporating genes within scaffolds, 3) additional incorporation of transfection enhancers, and 4) the use of mesenchymal stem cells (MSCs) as an alternative cell source for articular cartilage tissue engineering.

The medium used during monolayer expansion not only had a significant effect on subsequent biosynthesis and chondrogenesis in CG scaffolds, but also on gene transfer to chondrocyte monolayers. The expansion medium that resulted in enhanced 3-D biosynthesis and gene transfer to cells in monolayer was used throughout the rest of the studies.

Greater plasmid retention within GSCG scaffolds was achieved by chemically cross-linking the plasmid IGF-1 (pIGF-1) to the scaffold (compared to simple plasmid absorption), and resulted in more steady and prolonged IGF-1 overexpression by seeded chondrocytes. Incorporation of a lipid transfection reagent or gelatin nanoparticles encapsulating pIGF-1 significantly enhanced gene expression. The method of gene incorporation and the type of transfection enhancer were important variables that controlled the initiation, amount, and duration of growth factor release. IGF-1 overexpression by cells successfully transfected within GSCG scaffolds also increased biosynthesis of cartilage matrix molecules and chondrogenesis. Finally, MSCs seeded into GSCG scaffolds were able to be successfully transfected and maintained IGF-1 overexpression for at least 2 weeks post-seeding.

These findings show promise in using GSCG scaffolds for providing a local, prolonged, and therapeutic release of desired growth factors using nonviral transfection methods for tissue engineering or regenerative medicine applications.

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CHAPTER 1: GENERAL INTRODUCTION

Current methods for treating articular cartilage defects (e.g. microfracture, autologous chondrocyte implantation, and osteochondral autografting) have been successful in alleviating immediate patient pain, however, long term affects of these methods have limited follow-up. Currently these methods generally result in the formation of a fibrocartilage tissue that is very different from the composition and structure of normal articular cartilage. Several studies have investigated the collagen-glycosaminoglycan (CG) scaffold as an implantable scaffold with and without autologous cells in regenerating dermis [1,2], peripheral nerve [3-5], intervertebral disc [6], and articular cartilage [7-9]. From these studies, it has been found that CG scaffolds facilitate the reparative process and result in the successful growth of various functioning tissues that are closer but not yet identical to the structure of natural tissues. Previous research has also found significant and positive affects of specific growth factors on the tissue engineering of articular cartilage particularly in enhancing proliferation of chondrogenic cells, cell differentiation, and the biosynthesis of cartilage specific matrix molecules by these cells.

A *local* and *prolonged* administration of a growth factor such as insulin-like growth factor (IGF)-1 could be a significant benefit to *in vivo* and *ex vivo* strategies for articular cartilage repair. Despite their promising reparative potential, however, administration of recombinant proteins may be hindered *in vivo* due to delivery problems related to degradation or diffusion from the defect site. Recent studies in our lab have concluded that incorporation of plasmid DNA (specifically plasmid DNA containing the luciferase reporter gene) in CG scaffolds leads to successful transfection of and gene expression by the cells seeded within the scaffold. The use of plasmid DNA instead of viruses as a means for induced gene expression is desirable as it reduces the risk of a possible immune response. Although several studies have investigated gene therapy concepts for a variety of tissue engineering applications, there has not yet been an effort to develop gene-supplemented collagen-GAG (GSCG) scaffolds for the nonviral delivery of the IGF-1 gene for enhancing the synthesis of articular cartilage *in vitro* or *in vivo*. Development of an effective method to supplement CG scaffolds with plasmid DNA encoding for desired proteins could result in a novel gene delivery system that elicits a more prolonged and localized release of growth factors: cells seeded within these GSCG scaffolds or migrating into the scaffold *in vivo* may be transfected by taking up the incorporated plasmid DNA, leading to

production of therapeutic concentrations of growth factors that can enhance the repair of articular cartilage defects.

1.1. BACKGROUND

1.1.1. COLLAGEN-GAG SCAFFOLDS FOR ARTICULAR CARTILAGE REPAIR

The use of three-dimensional (3-D) scaffolds that mimic the natural *in vivo* environment (*viz.*, extracellular matrix) of cells has been shown to facilitate the reparative process and result in the successful growth of various functioning tissues. The 3-D environment supplied by these porous scaffolds serves as a desirable structural support for seeded or migrating cells and allows for a much greater surface to volume ratio for increased cell attachment as compared to a 2-D surface. There are several requirements for a scaffold to be used as an implant for articular cartilage regeneration [10]. The scaffold needs to be biodegradable, nontoxic, able to be fixed to the defect site, facilitate cell attachment, regulate cell expression, and possess sufficient mechanical strength [11]. Natural polymers such as collagen provide a native surface to cells, since it is a major component of the natural extracellular matrix, and possesses ligands that favor cellular attachment. Collagen substrates have been shown to modify the morphology, migration, and in some cases differentiation of cells [12]. Moreover, prior studies have demonstrated that type I collagen-glycosaminoglycan (GAG) scaffolds produced by freeze-drying techniques can facilitate the regeneration of dermis and peripheral nerve [1,2,5]. Type II collagen-GAG scaffolds have also demonstrated promise for enhancing articular cartilage synthesis [7,8,13].

Studies have confirmed that the addition of cells seeded within these 3-D scaffolds enhance matrix synthesis and increase type II collagen production *in vivo* [8,14,15]. There are various cell types that may be used to enhance cartilage synthesis when seeded into scaffolds. These include articular chondrocytes and chondroprogenitor cells derived from marrow, periosteum, or perichondrium [11]. The advantage of using chondrocytes obtained from articular cartilage as a cell source is that they already express the desired phenotype for articular cartilage repair (i.e. they can synthesize cartilage matrix molecules such as type II collagen and aggrecan) [16]. Chondrocytes, however, are very limited in supply especially for autologous transplantation, and expansion in culture is necessary to obtain a number sufficient for enhanced repair. Furthermore, the site from which autologous chondrocytes are harvested (usually taken from a

minimal-load-bearing region of the joint) does not spontaneously regenerate and may pose a potential problem. To alleviate this problem of donor site morbidity, other cell sources such as bone marrow (for marrow-derived mesenchymal stem cells), which have natural regenerative capabilities and are readily accessible, have been investigated for articular cartilage repair [16,17]. The chondroprogenitor cells from marrow have been shown to be able to differentiate into a cartilage lineage when exposed to the appropriate stimuli [11] and therefore may be a more feasible cell source for articular cartilage regeneration. For this reason, both chondrocytes and marrow-derived stem cells were investigated as cell sources for culture in GSCG scaffolds.

1.1.2. ROLE OF GROWTH FACTORS

While recent studies in our lab have demonstrated the promise of implementing chondrocyte-seeded collagen-GAG scaffolds for cartilage repair, there are potential problems and significant expense associated with the need to culture a cell-seeded scaffold for several weeks prior to implantation. This focuses attention on the implementation of growth factors to accelerate cell proliferation and matrix synthesis. Numerous studies have shown the effects of various growth factors on chondrogenesis *in vivo*, and on chondrocyte proliferation, metabolism, and matrix synthesis *in vitro*. Among the most prominent growth factors investigated for articular cartilage tissue engineering are insulin-like growth factors (IGFs) [18-23], bone morphogenetic proteins (BMPs) [24-26], basic fibroblastic growth factor (bFGF, also FGF-2) [27-29] and transforming growth factor-β (TGF-β) [30-36]. The effects of growth factors in both monolayer and 3-D culture of chondrocytes have been shown to be significant. The complexity of choosing the correct combinations and doses of growth factors to obtain the optimal tissue engineered articular cartilage construct *in vitro* poses a major challenge. While there are numerous combinations of factors that can be investigated relative to the type and dose of growth factor used, studies have already demonstrated the profound benefits of certain agents for engineered articular cartilage constructs. For example, supplementation of the culture medium with IGF-1 alone has been shown to increase cell proliferation, proteoglycan synthesis, type-II collagen synthesis, and chondrogenesis, both in monolayer and in 3-D cultures [18,22,37].

In vivo, an improved histologic appearance and an increased proportion of type II collagen in full thickness cartilage defects in young mature horses was shown using fibrin polymers laden with IGF-1[38]. BMPs (specifically BMP-2 and BMP-7) have also been shown to

increase proteoglycan and matrix synthesis, maintain the chondrocyte phenotype, and stimulate cartilage formation *in vivo* in a manner similar to endochondral ossification [39]. *In vivo* studies using New Zealand White rabbits have demonstrated that full-thickness femoral osteochondral defects treated with rhBMP-2-supplemented collagen sponges displayed a greatly accelerated formation of new subchondral bone, an improved histologic appearance of overlying articular cartilage, and more type II collagen and tissue filling in the defect compared to the controls [40]. BMP-7 (also called osteogenic protein-1, OP-1) has also been shown to stimulate cartilage formation and aggrecan synthesis in subchondral defects in goats [41]. FGF-2 has demonstrated to be a potent mitogen for chondrocytes and a stimulator of matrix synthesis [39]. Furthermore, *in vivo* studies in rabbits [42] have reported that treatment of full-thickness cartilage defects with intra-articular FGF-2 can enhance differentiation of mesenchymal cells to the chondrocyte phenotype, increase proliferation of differentiated chondrocytes, and increase accumulation of type II collagen and proteoglycan.

TGF-β has been shown to be most effective in conjunction with other growth factors in eliciting its complex effects on cartilage metabolism [39]. Many findings, however, have shown contradictory effects of TGF-β supplementation in culture (*e.g.*, effects of cell proliferation and proteoglycan synthesis) due to its sensitivity to varying experimental conditions in the different studies. The more promising effects of TGF-β supplementation include increasing collagen and proteoglycan production and inhibiting matrix breakdown [39]. It has also been shown that TGF-β has a role in the regulatory network of growth factors that maintains articular cartilage in the differentiated phenotype [43] and is an important factor in inducing chondrogenesis in marrow-derived mesenchymal progenitor cells [17].

The effects of using combinations of growth factors have also been studied in monolayer and 3-D cultures and demonstrate the complex interactions and signaling events that can occur. Growth factor-supplemented media used for the expansion of chondrocytes in monolayer have been shown to directly influence the outcome of 3-D cell pellet cultures grown in a different specified serum-free medium [44]. Not only does supplementation of the expansion medium with specific growth factors affect chondrocyte proliferation, morphology, and phenotype, it also influences the chondrocytic potential or ability to redifferentiate back into a chondrocytic phenotype when re-introduced into a 3-D environment.

1.1.3. RATIONALE FOR GENE TRANSFER IN TISSUE ENGINERING

Growth factors can make significant contributions to cartilage repair procedures and tissue engineering by stimulating cell proliferation, migration, differentiation, and matrix synthesis. There are, however, major challenges faced in the direct application of human recombinant proteins in a clinical setting. Proteins are difficult to administer exogenously in accurate, sustained, and therapeutically useful amounts to sites of cartilage injury. Single bolus doses of growth factors alone *in vivo* have short half-lives as a result of degradation or diffusion from the defect sight. Various strategies including the use of polymers, pumps, and heparin, have been investigated as possible methods by which to achieve constant levels of growth factors at a given injured site; however, success remains limited [45]. Furthermore, although it is now possible to produce large quantities of these recombinant proteins for the purpose of treatment, the expense is still another unattractive feature. Delivery of a gene that could be expressed within the wound is an attractive alternative to application of the recombinant protein. Gene transfer provides the DNA that encodes for the desired protein, so that infected cells can create higher and more sustained levels of the growth factor over extended periods of time, a likely requirement for effective articular cartilage regeneration. More than one gene can be transferred and independently regulated to supply multiple growth factors to the defect site at various time points in the repair process. Furthermore, prior work has suggested that endogenously expressed proteins, induced by gene transfer, may have a more positive and more potent effect on matrix synthesis and biological activity than exogenous recombinant proteins [46].

1.1.4. GENE TRANSFER TO CELLS FOR ARTICULAR CARTILAGE TISSUE ENGINEERING

Many questions are involved in deciding the best method of gene transfer for articular cartilage repair. Such variables include: a) the cell, or cells, to be targeted (*e.g.*, chondrocytes, mesenchymal stem cells, synovial cells, etc.); b) the protein, or proteins, to be encoded; and c) the delivery vector to be employed (which is also dependent on the size of the gene encoding the growth factor). The vectors used in gene transfer procedures applied to articular cartilage repair include: viral vectors such as adenoviruses, lipid-mediated reagents such as liposomes, and naked DNA alone.

Several studies have focused on adenoviral vectors for the transduction of cells due to high infection efficiencies and ease of manufacturing. Both *in vitro* and *in vivo* studies have proved this method of infection to be beneficial to cartilage regeneration. In monolayer studies, cultured articular chondrocytes infected with an adenoviral vector containing the IGF-1 coding sequence [47] found that at an optimal adenovirus-IGF-1 concentration (100 multiplicities of infection, MOI), gene expression was detected at therapeutic concentrations for at least 28 days. This prolonged expression resulted in an 8-fold increase in matrix products secreted in the medium and an increased resistance of the cells to de-differentiation over time under serum-starved conditions. Moreover, the cells maintained a normal chondrocyte molecular phenotype compared to controls. The significant effects of using different genes separately or in combination could be seen in another monolayer study which used rabbit articular chondrocytes and compared the effects of adenoviral delivery of IGF-1, TGF-β, and BMP-2 in the absence or presence of the inflammatory cytokine, interleukin-1 (IL-1) [46]. It was found that proteoglycan synthesis was significantly stimulated by the BMP-2 (~8-fold) and IGF-1 (2-3 fold) genes separately, and the effects were additive upon co-transduction of chondrocyte monolayers. Furthermore, the IGF-1 gene most strongly stimulated collagen and noncollagenous protein synthesis. Although the addition of IL-1 decreased proteoglycan synthesis by 50-60%, IGF-1 and TGF-β genes restored proteoglycan synthesis to control levels, and BMP-2 gene transfer further elevated proteoglycan synthesis beyond control levels. It can thus be seen that finding the right genes to use individually or in combination can significantly affect the success of the final regenerated tissue. Other studies have demonstrated the successful transduction of other cell types such as mesenchymal stem cells and synovial cells in monolayer and explant cultures, for the delivery of therapeutic proteins [48].

Although the use of viral vectors has proven to be very effective in enhancing the biosynthetic activity of cells *in vitro*, much caution has to be taken when directly injecting viral particle solutions *in vivo,* due to the immunogenic nature of viruses and the possibility of transmission to other tissues and organs. This issue has been addressed in an *in vivo* study comparing the direct injection of adenoviral vectors for IGF-1 or BMP-2 to transplantation of fibroblasts infected *ex vivo* with the same vectors – with respect to virus spread, immune response, and cartilage formation [49]. Inadvertent spread of the adenoviral vector was observed in the liver, lung, and spleen in all mice that had received the vector directly, whereas spread rarely

occurred in fibroblast-mediated gene transfer. Furthermore, administering the genes via injection of *ex vivo*-infected fibroblasts limited cartilage formation to regions near the injected site, and also avoided the strong immune response that was elicited following direct application of the viral vector. *Ex vivo* methods of gene transfer to harvested cells (such as chondrocytes, fibroblasts, bone marrow cells, or synovial cells) may therefore be safer than direct injection of viral particles alone.

Nonviral methods of gene transfer are also being developed to avoid the potential problems associated with adenoviral vectors. Nonviral vector systems offer the advantages of low immunogenicity, simplicity of vector design, and relative ease of large-scale production [50]. Although transfection efficiencies for nonviral vectors have been known to be much lower than that of viral vectors, significant advances in the development of more efficient non-viral transfection reagents are emerging. Lipid–mediated gene transfer has been shown to result in the transfection of articular chondrocytes, and the maintenance of prolonged gene expression. One study implemented the lipid-mediated transfection reagent, FuGENE 6, with a hyaluronidase treatment to transfect bovine articular chondrocytes with a plasmid vector containing the cDNA for human IGF-1. Transfection efficiencies were reported to be about 41% with gene expression lasting for over 4 weeks *in vivo* [51,52]. Transplantation of the transfected chondrocytes onto the surface of articular cartilage explants led to the formation of a new tissue layer on the explant surface, which was characterized by the presence of type II collagen and proteoglycan and the absence of type I collagen, consistent with hyaline-like cartilage. Furthermore, the tissue formed by transfected chondrocytes was thicker and contained more cells than the controls. The overexpression of IGF-1 also increased DNA and glycosaminoglycan synthesis by the underlying explant cartilage chondrocytes [52]. A follow-up *in vivo* study in a rabbit model showed that chondrocytes transfected with the IGF-1 gene, encapsulated in alginate, and transplanted in osetochondral defects improved articular cartilage repair and accelerated the formation of the subchondral bone at early and later time points compared to controls [53].

1.1.5. TISSUE ENGINEERING APPROACHES INCORPORATING GENE TRANSFER

A promising approach for enhancing gene transfer and retention of genes or expressed proteins within a defect site employs 3-D scaffolds. The combination of gene therapy and tissue engineering could provide the ultimate treatment for articular cartilage defects as it involves a

supporting scaffold that can serve as a carrier for gene vectors or infected cells resulting in a sustained, prolonged, and localized delivery of therapeutic proteins *in vivo*. It has also been demonstrated that cells first seeded into 3-D scaffolds and then transfected show higher gene expression levels and longer expression times compared to 2-D transfection [54]. This observation is important in demonstrating how the 3-D environment can influence cell behavior and processes, including the transfectibility of cells.

Most studies using gene therapy and tissue engineering concepts for the regeneration of articular cartilage involve *ex vivo* infection of cells that are virally transduced [55,56] or nonvirally transfected [57] *in vitro* and then subsequently seeded into 3-D scaffolds (*e.g.*, fibrin or synthetic polymer scaffolds). Several cell types and genes have been investigated for this application including: transfection of articular chondrocytes with the IGF-1 gene [57]; transduction of periosteal stem cells with the OP-1 gene [55]; and transduction of mesenchymal cells from rib perichondrium with the BMP-2 and IGF-1 genes [56]. In all of these cases, chondrogenesis and matrix synthesis was significantly enhanced *in vivo*, following implantation of the constructs.

The disadvantage of implanting cells transfected or transduced *ex vivo* as described above is that there may be a decrease in expressed protein over time as the infected cells apoptose or migrate. It would be ideal if the scaffold could serve as a vehicle to immobilize gene vectors so that when implanted: 1) cells migrating into the scaffold and proliferating could take up the gene; and/or 2) surrounding cells could take up the genes released as the scaffold degrades. The DNA vector as well as the transiently expressed therapeutic protein would be retained within the defect site, thereby increasing the opportunity for a maximal therapeutic response [58]. With time, more endogenous cells could become infected and a prolonged release of growth factor could be maintained over the full duration of cartilage regeneration. This scaffold-based gene transfer approach could be particularly beneficial in regenerative medicine applications since *in vitro* culture would not be required (i.e. a non-cell-seeded gene-supplemented scaffold could be implanted *in vivo* for endogenous cells to infiltrate and become transfected resulting in the release of therapeutic growth factors). Several studies have investigated the use of nonviral scaffold-based gene delivery for the treatment of a variety of tissue defects. These studies are summarized in the table below with regard to the scaffold material, type and amount of plasmid, and the application.

Table 1.1 Summary of Studies Using Scaffold-Based Nonviral Gene Delivery

Publication	Scaffold	Vector	Cell/tissue
Bonadio et al. [60]	Collagen I	pMAT-1 (1-100 mg)	Bone (*in vivo*-canine)
Tyrone et al. [61]	Collagen I	pPDGF (1-3 mg)	Healing-dermal ulcer (*in vivo*-rabbits)
Berry et al. [62]	Collagen I	pFGF-2, pBDNF, pNT-3 (7.5 mg)	Retina-optic nerve (*in vivo*-rat)
Hosseinkhani et. al. [63,64]	PGA reinforced Collagen (I) sponge	Cationized-gelatin Nanoparticles pBMP-2 (2-13 mg)	MSCs (for bone)
Segura et al. [65]	HA-Collagen I hydrogel surface	Biotin/avidin bound PEI-DNA	Fibroblasts
Kushibiki et al. [66]	Cationized gelatin hydrogel	TGFβR siRNA (400 mg)	Block renal intersitial fibrosis (*in vivo*-mice kidney)
Guo et. al [59]	Chitosan-gelatin	pTGFb-1 (1 mg)	Chondrocytes
Andree et al. [67]	Fibrin (w/ keratinocytes)	pEGF (20 μg)	Wound healing, (*in vivo*-mice)
Trentin et al. [68]	Covalently immobilized in Fibrin	Peptide-DNA pEGFP (10-100 mg)	Kidney, endothelial, fibroblasts
Shea et al. [69]	Poly(lactide-co-glycolide)	pPDGF (1 mg)	Granulation tissue/blood vessels (*in vivo*-rats)
Huang et al. [70]	Poly(lactide-co-glycolide)	PEI-DNA pBMP-4 (200 mg)	Bone-cranial (*in vivo*-rats)
Jang J. et al. [71]	Poly(lactide-co-glycolide)	pVEGF (500-800 μg)	Blood vessels, subcutaneous (*in vivo*-mice)
Liang et al. [72]	Poly(lactide-co-glycolide)-electrospun	PEG-PLA Nanoparticles pb-Gal (600 mg)	MC3T3 pre-osteoblastic cell line (bone)

1.2. EXPERIMENTAL APPROACH

The main goal of this thesis was to use a nonviral scaffold-based gene delivery approach specifically developing collagen (type II)-glycosaminoglycan (CG) scaffolds incorporating the gene encoding for insulin-like growth factor (IGF)-1 to induce a localized and prolonged IGF-1 overexpression for enhancing articular cartilage tissue formation *in vitro* and ultimately *in vivo*. Plasmid containing the IGF-1 gene was used throughout the scope of this thesis and chosen on the basis of prior work that demonstrated the beneficial effects of IGF-1 on proliferation, biosynthesis, and chondrogenesis [18,19,21,22,23,48].

My preliminary studies first focused on evaluating certain medium conditions both in monolayer and in 3-D culture. Prior studies [44] have found that using a specific medium for expanding chondrocytes in monolayer has a direct effect on chondrocyte proliferation and chondrocyte redifferentiation when subsequently placed in pellet cultures and cultured in a serum-free 3-D culture medium. More importantly, the use of a serum-free medium in 3-D culture would be advantageous in eliminating the variability introduced when using fetal bovine serum (FBS) and is desirable when preparing 3-D constructs to be placeplaced *in vivo* to prevent any inherent immunogenicity associated with the addition of FBS. Furthermore, the use of a serum-free medium in my experiments would ensure that the detected IGF-1 protein in the 3-D culture medium is a reflection of successful gene transfer to cells seeded within gene-supplemented CG (GSCG) scaffolds, and not IGF-1 protein contributed by FBS.

Since the expansion medium was found to significantly influence the outcome of constructs in 3-D culture (i.e. cell pellets), it was hypothesized that the expansion medium may also have a significant effect on the ability to transfer specific genes into cells. To test this, a monolayer culture experiment was carried out using either a nonviral or viral method of transferring the IGF-1 gene into adult chondroctyes expanded in monolayer using two different types of medium. If one expansion medium demonstrated enhanced gene transfer susceptibility of cells in monolayer, then that specific medium would be commended for expansion of chondrocytes to be subsequently seeded within GSCG scaffolds to increase probable gene transfer in 3-D culture.

Once the specific media were chosen for use during monolayer expansion and subsequent 3-D culture, various methods to synthesize GSCG scaffolds were investigated. Based on the significant enhancement in gene transfer using a lipid mediated transfection reagent

(GenePorter®) in the prior monolayer culture studies, we investigated incorporating within the CG scaffolds, plasmid alone or plasmid complexed to the lipid transfection reagent. Preliminary studies eliminated the feasibility of mixing the plasmid solutions within the collagen slurry due to issues related to possible degradation of the plasmid within the acidic slurry solution and post sterilization techniques that may also compromise plasmid integrity. Methods to incorporate plasmid within the scaffolds, therefore, were focused on trying to add the plasmid after the CG slurry had been freeze-dried to form porous constructs.

Although others have successfully added plasmid to 3-D scaffolds using naked plasmid DNA alone, significant amounts of plasmid (on the order of milligram levels per scaffold) were required to see any beneficial effect. One of the goals of this thesis was to use microgram amounts of plasmid DNA per scaffold (for safety and cost reasons) that would still result in beneficial amounts of growth factor release by developing a method to better retain the plasmid within the scaffold so that not all incorporated plasmid would be released in a short period of time. This would entail more than just a mechanical entrapment of the plasmid between the collagen fibrils of the CG scaffold. One method that is commonly used in our lab as a means to chemically cross-link the CG scaffold is a carbodiimide cross-linking treatment. Upon further investigation, this carbodiimide chemical can be used to cross-link proteins to proteins and also proteins to nucleic acids. Therefore, one of the methods to incorporate the plasmid into CG scaffolds included the use of this carbodiimide treatment to cross-link plasmid DNA alone or with the transfection reagent to the collagen scaffold.

The use of a transfection reagent to enhance nonviral gene transfer is an important aspect to also minimize the amount of naked plasmid required to produce therapeutic amounts of overexpressed protein. We looked to develop gelatin nanoparticles as a potential alternate to the GenePorter transfection reagent to not only enhance gene transfer to cells, but to also allow further control over the kinetics of gene transfer and subsequent gene expression by having the ability to alter the processing parameters involved in nanoparticle synthesis. Gelatin is a natural material that seemed to be logically compatible with the collagen scaffold and has been successfully synthesized into micro- and nanoparticles for drug delivery applications. The development of gelatin nanoparticles to be used in conjunction with the CG scaffold to enhance gene transfer was, therefore, an important aspect in this thesis.

After an optimal gene transfer system was determined for chondrocytes in GSCG scaffolds, preliminary studies were undertaken to use mesenchymal stem cells (MSCs) as an alternative cell source. The goal was to evaluate the behavior of MSCs within GSCG scaffolds that were synthesized using optimal conditions for gene incorporation (determined with chondrocytes), and to evaluate the ability of MSCs to be transfected and to express the desired encoded protein when seeded into GSCG scaffolds. These studies would validate the use of GSCG scaffolds as an MSC-seeded construct or as an unseeded gene delivery vehicle to be used in conjunction with a microfracture procedure *in vivo*. In the latter scenario, a microfracture procedure in the cartilage defect would allow MSCs from the bone marrow to infiltrate the defect, after which an unseeded GSCG scaffold could be placed and serve as both a scaffold for cells to migrate and a source for the delivery of genes encoding for desired growth factors that can enhance *in vivo* regeneration.

1.2.1. SPECIFIC AIMS

Below are the specific aims of the thesis work:

1. Determine which medium conditions to use for chondrocyte expansion and 3-D culture for evaluation of GSCG scaffolds and investigate if the expansion medium can also significantly affect subsequent gene transfer to cells.
2. Develop a protocol for incorporating plasmid IGF-1 with or without a lipid-mediated transfection reagent into CG scaffolds that results in successful gene transfer to seeded chondrocytes and elevated expression of the IGF-1 protein over a prolonged time.
3. Develop gelatin nanoparticles as a means to enhance and control gene transfer to cells and evaluate the potential use of these nanoparticles in conjunction with the CG scaffold for prolonged growth factor release.
4. Evaluate the use of undifferentiated mesenchymal stem cells (MSCs) as an alternative cell source in GSCG scaffolds, for implanting a cell-seeded GSCG scaffold or an unseeded GSCG scaffold with a microfracture procedure.
5. Determine the optimal GSCG construct to be used for subsequent *in vivo* studies using a goat model.

1.2.2. *HYPOTHESES*

1. The composition of the monolayer expansion medium not only has a direct effect on biosynthesis and chondrogenesis in 3-D culture but also has an effect on gene transfer to cells.
2. Cross-linking naked plasmid DNA to the CG scaffold can result in a more prolonged delivery of genes and expression of the encoded growth factor compared to plasmid addition without cross-linking.
3. A lipid transfection reagent complexed to the pIGF-1 can be successfully incorporated and cross-linked to CG scaffolds and can result in enhanced gene transfer to seeded cells.
4. Gelatin nanoparticles can successfully enhance the transfection of chondrocytes and can be used in conjunction with a CG scaffold to produce a localized and prolonged release of encoded protein.
5. MSCs can be transfected using GSCG scaffolds and can maintain overexpression of desired proteins over prolonged times.

1.3. REFERENCES

1. Yannas IV, Lee EO,D.P., Skrabut EM, Murphy GF. Synthesis and characterization of a model extracellular matrix that induces partial regeneration of adult mammalian skin. Proc. Nat'l. Acad. Sci. USA 1989;86:933-937.

2. Yannas IV. Studies on the biological activity of the dermal regeneration template. Wound Rep. Reg. 1998;6(6):518-524.

3. Chamberlain LJ, Yannas IV, Hsu HP, Strichartz G, Spector M. Collagen-GAG substrate enhances the quality of nerve regeneration through collagen tubes up to level of autograft. Exp.Neurol. 1998 Dec;154(2):315-329.

4. Chamberlain LJ, Yannas IV, Hsu HP, Spector M. Connective tissue response to tubular implants for peripheral nerve regeneration: the role of myofibroblasts. J.Comp.Neurol. 2000 Feb 21;417(4):415-430.

5. Chamberlain LJ, Yannas IV, Hsu HP, Strichartz GR, Spector M. Near-terminus axonal structure and function following rat sciatic nerve regeneration through a collagen-GAG matrix in a ten-millimeter gap. J.Neurosci.Res. 2000 Jun 1;60(5):666-677.

6. Saad L, Spector M. Effects of collagen type on the behavior of adult canine annulus fibrosus cells in collagen-glycosaminoglycan scaffolds. J.Biomed.Mater.Res.A. 2004 Nov 1;71(2):233-241.

7. Nehrer S, Breinan HA, Ramappa A, Young G, Shortkroff S, Louie L, et al. Matrix collagen type and pore size influence behaviour of seeded canine chondrocytes. Biomat. 1997;18:769-776.

8. Lee CR, Grodzinsky AJ, Hsu HP, Spector M. Effects of a cultured autologous chondrocyte-seeded type II collagen scaffold on the healing of a chondral defect in a canine model. J.Orthop.Res. 2003 Mar;21(2):272-281.

9. Veilleux, N.H., Yannas, I.V., and Spector,M. Effect of passage number and collagen type on the proliferative, biosynthetic, and contractile activity of adult canine articular chondrocytes in type I and II collagen-glycosaminoglycan matrices in vitro. Tissue Eng 2004;10:119-27.

10. Lee CR, Spector M. Status of articular cartilage tissue engineering. Curr. Op. Orthop. 1998;9:88-93.

11. Coutts RD, Healey RM, Ostrander R, Sah RL, Goomer R, Amiel D. Matrices for cartilage repair. Clin Orthop 2001 Oct(391 Suppl):S271-9.

12. Kleinman HK, Klebe RJ, Martin GR. Role of collagenous matrices in the adhesion and growth of cells. J Cell Biol 1981 Mar;88(3):473-85.

13. Breinan HA, Martin SD, Hsu HP, Spector M. Healing of canine articular cartilage defects treated with microfracture, a type-II collagen matrix, or cultured autologous chondrocytes. J.Orthop.Res. 2000 Sep;18(5):781-789.

14. Chu CR, Coutts RD, Yoshioka M, Harwood FL, Monosov AZ, Amiel D. Articular cartilage repair using allogeneic perichondrocyte-seeded biodegradable porous polylactic acid (PLA): A tissue-engineering study. J. Biomed. Mater. Res. 1995;29(9):1147-1154.

15. von Schroeder HP, Kwan M, Amiel D, Coutts RD. The use of polylactic acid matrix and periosteal grafts for the reconstruction of rabbit knee articular defects. J. Biomed. Mater. Res. 1991;25(3):329-339.

16. Im GI, Kim DY, Shin JH, Hyun CW, Cho WH. Repair of cartilage defect in the rabbit with cultured mesenchymal stem cells from bone marrow. J Bone Joint Surg Br 2001 Mar;83(2):289-94.

17. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp.Cell Res. 1998 Jan 10;238(1):265-272.

18. Fortier LA, Lust G, Mohammed HO, Nixon AJ. Coordinate upregulation of cartilage matrix synthesis in fibrin cultures supplemented with exogenous insulin-like growth factor-I. J.Orthop.Res. 1999 Jul;17(4):467-474.

19. Huang JR, Liu SL, Song WD. Stimulation of insulin-like growth factor-I to chondrogenesis of engineering cartilage tissue. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi 2004 Jan;18(1):49-52.

20. Loeser RF, Pacione CA, Chubinskaya S. The combination of insulin-like growth factor 1 and osteogenic protein 1 promotes increased survival of and matrix synthesis by normal and osteoarthritic human articular chondrocytes. Arthritis Rheum. 2003 Aug;48(8):2188-2196.

21. Tuncel M, Halici M, Canoz O, Yildirim Turk C, Oner M, Ozturk F, et al. Role of insulin like growth factor-I in repair response in immature cartilage. Knee 2005 Apr;12(2):113-119.

22. Worster AA, Brower-Toland BD, Fortier LA, Bent SJ, Williams J, Nixon AJ. Chondrocytic differentiation of mesenchymal stem cells sequentially exposed to transforming growth factor-beta1 in monolayer and insulin-like growth factor-I in a three-dimensional matrix. J.Orthop.Res. 2001 Jul;19(4):738-749.

23. Yaeger PC, Masi TL, de Ortiz JL, Binette F, Tubo R, McPherson JM. Synergistic action of transforming growth factor-beta and insulin-like growth factor-I induces expression of type II collagen and aggrecan genes in adult human articular chondrocytes. Exp. Cell Res. 1997;237:318-25.

24. Kaps C, Bramlage C, Smolian H, Haisch A, Ungethum U, Burmester GR, et al. Bone morphogenetic proteins promote cartilage differentiation and protect engineered artificial cartilage from fibroblast invasion and destruction. Arthritis Rheum. 2002 Jan;46(1):149-162.

25. Grunder T, Gaissmaier C, Fritz J, Stoop R, Hortschansky P, Mollenhauer J, et al. Bone morphogenetic protein (BMP)-2 enhances the expression of type II collagen and aggrecan in chondrocytes embedded in alginate beads. Osteoarthritis Cartilage 2004 Jul;12(7):559-567.

26. Park Y, Sugimoto M, Watrin A, Chiquet M, Hunziker EB. BMP-2 induces the expression of chondrocyte-specific genes in bovine synovium-derived progenitor cells cultured in three-dimensional alginate hydrogel. Osteoarthritis Cartilage 2005 Jun;13(6):527-536.

27. Martin I, Vunjak-Novakovic G, Yang J, Langer R, Freed LE. Mammalian chondrocytes expanded in the presence of fibroblast growth factor 2 maintain the ability to differentiate and regenerate three-dimensional cartilaginous tissue. Exp. Cell Res. 1999;253:681-688.

28. Cucchiarini M, Madry H, Ma C, Thurn T, Zurakowski D, Menger MD, et al. Improved tissue repair in articular cartilage defects in vivo by rAAV-mediated overexpression of human fibroblast growth factor 2. Mol.Ther. 2005 Aug;12(2):229-238.

29. Veilleux N, Spector M. Effects of FGF-2 and IGF-1 on adult canine articular chondrocytes in type II collagen-glycosaminoglycan scaffolds in vitro. Osteoarthritis Cartilage 2005 Apr;13(4):278-286.

30. van der Kraan PM, Buma P, van Kuppevelt T, van den Berg WB. Interaction of chondrocytes, extracellular matrix and growth factors: relevance for articular cartilage tissue engineering. Osteoarthritis Cartilage 2002 Aug;10(8):631-637.

31. Mauck RL, Nicoll SB, Seyhan SL, Ateshian GA, Hung CT. Synergistic action of growth factors and dynamic loading for articular cartilage tissue engineering. Tissue Eng. 2003 Aug;9(4):597-611.

32. Kim SE, Park JH, Cho YW, Chung H, Jeong SY, Lee EB, et al. Porous chitosan scaffold containing microspheres loaded with transforming growth factor-beta1: implications for cartilage tissue engineering. J.Control.Release 2003 Sep 4;91(3):365-374.

33. Lee JE, Kim KE, Kwon IC, Ahn HJ, Lee SH, Cho H, et al. Effects of the controlled-released TGF-beta 1 from chitosan microspheres on chondrocytes cultured in a collagen/chitosan/glycosaminoglycan scaffold. Biomaterials 2004 Aug;25(18):4163-4173.

34. Holland TA, Bodde EW, Baggett LS, Tabata Y, Mikos AG, Jansen JA. Osteochondral repair in the rabbit model utilizing bilayered, degradable oligo(poly(ethylene glycol) fumarate) hydrogel scaffolds. J.Biomed.Mater.Res.A. 2005 Oct 1;75(1):156-167.

35. DeFail AJ, Chu CR, Izzo N, Marra KG. Controlled release of bioactive TGF-beta 1 from microspheres embedded within biodegradable hydrogels. Biomaterials 2006 Mar;27(8):1579-1585.

36. Chou CH, Cheng WT, Lin CC, Chang CH, Tsai CC, Lin FH. TGF-beta1 immobilized tri-co-polymer for articular cartilage tissue engineering. J.Biomed.Mater.Res.B.Appl.Biomater. 2006 May;77(2):338-348.

37. Makower AM, Wroblewski J, Pawlowski A. Effects of IGF-I, rGH, FGF, EGF and NCS on DNA-synthesis, cell proliferation and morphology of chondrocytes isolated from rat rib growth cartilage. Cell Biol.Int.Rep. 1989 Mar;13(3):259-270.

38. Nixon AJ, Fortier LA, Williams J, Mohammed H. Enhanced repair of extensive articular defects by insulin-like growth factor-I-laden fibrin composites. J.Orthop.Res. 1999 Jul;17(4):475-487.

39. O'Connor WJ, Botti T, Khan SN, Lane JM. The use of growth factors in cartilage repair. Orthop. Clin. N.A. 2000;31:399-410.

40. Sellers R, Peluso D, Morris E. The effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the healing of full-thickness defects of articular cartilage. J. Bone Jt. Surg. 1997;79-A:1452-1463.

41. Louwerse RT, Heyligers IC, Klein-Nulend J, Sugihara S, van Kampen GP, Semeins CM, et al. Use of recombinant human osteogenic protein-1 for the repair of subchondral defects in articular cartilage in goats. J.Biomed.Mater.Res. 2000 Mar 15;49(4):506-516.

42. Fujimoto E, Ochi M, Kato Y, Mochizuki Y, Sumen Y, Ikuta Y. Beneficial effect of basic fibroblast growth factor on the repair of full-thickness defects in rabbit articular cartilage. Arch Orthop Trauma Surg 1999;119(3-4):139-45.

43. Dounchis JS, Goomer RS, Harwood FL, Khatod M, Coutts RD, Amiel D. Chondrogenic phenotype of perichondrium-derived chondroprogenitor cells is influenced by transforming growth factor-beta 1. J Orthop Res 1997 Nov;15(6):803-7.

44. Jakob M, Demarteau O, Schafer D, Hintermann B, Dick W, Heberer M, et al. Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes enhance chondrogenesis and cartilaginous tissue formation in vitro. J Cell Biochem 2001 Mar 26;81(2):368-77.

45. Huard J, Li Y, Peng H, Fu FH. Gene therapy and tissue engineering for sports medicine. J Gene Med 2003 Feb;5(2):93-108.

46. Smith P, Shuler FD, Georgescu HI, Ghivizzani SC, Johnstone B, Niyibizi C, et al. Genetic enhancement of matrix synthesis by articular chondrocytes: comparison of different growth factor genes in the presence and absence of interleukin-1. Arthritis Rheum 2000;43(5):1156-64.

47. Brower-Toland BD, Saxer RA, Goodrich LR, Mi Z, Robbins PD, Evans CH, et al. Direct adenovirus-mediated insulin-like growth factor I gene transfer enhances transplant chondrocyte function. Hum Gene Ther 2001 Jan 20;12(2):117-29.

48. Nixon AJ, Brower-Toland BD, Bent SJ, Saxer RA, Wilke MJ, Robbins PD, et al. Insulinlike growth factor-I gene therapy applications for cartilage repair. Clin Orthop 2000 Oct(379 Suppl):S201-13.

49. Gelse K, Jiang QJ, Aigner T, Ritter T, Wagner K, Poschl E, et al. Fibroblast-mediated delivery of growth factor complementary DNA into mouse joints induces chondrogenesis but avoids the disadvantages of direct viral gene transfer. Arthritis Rheum 2001 Aug;44(8):1943-53.

50. Cohen H, Levy RJ, Gao J, Fishbein I, Kousaev V, Sosnowski S, et al. Sustained delivery and expression of DNA encapsulated in polymeric nanoparticles. Gene Therapy 2000;7(22):1896-905.

51. Madry H, Trippel SB. Efficient lipid-mediated gene transfer to articular chondrocytes. Gene Ther 2000 Feb;7(4):286-91.

52. Madry H, Zurakowski D, Trippel SB. Overexpression of human insulin-like growth factor-I promotes new tissue formation in an ex vivo model of articular chondrocyte transplantation. Gene Ther 2001 Oct;8(19):1443-9.

53. Madry H, Kaul G, Cucchiarini M, Stein U, Zurakowski D, Remberger K, et al. Enhanced repair of articular cartilage defects in vivo by transplanted chondrocytes overexpressing insulin-like growth factor I (IGF-I). Gene Ther. 2005 Aug;12(15):1171-1179.

54. Xie Y, Yang ST, Kniss DA. Three-dimensional cell-scaffold constructs promote efficient gene transfection: implications for cell-based gene therapy. Tissue Eng 2001 Oct;7(5):585-98.

55. Grande DA, Mason J, Light E, Dines D. Stem cells as platforms for delivery of genes to enhance cartilage repair. J.Bone Joint Surg.Am. 2003;85-A Suppl 2:111-116.

56. Gelse K, von der Mark K, Aigner T, Park J, Schneider H. Articular cartilage repair by gene therapy using growth factor-producing mesenchymal cells. Arthritis Rheum 2003 Feb;48(2):430-41.

57. Madry H, Padera R, Seidel J, Langer R, Freed LE, Trippel SB, et al. Gene transfer of a human insulin-like growth factor I cDNA enhances tissue engineering of cartilage. Hum.Gene Ther. 2002 Sep 1;13(13):1621-1630.

58. Chandler LA, Gu DL, Ma C, Gonzalez AM, Doukas J, Nguyen T, et al. Matrix-enabled gene transfer for cutaneous wound repair. Wound Repair Regen 2000 Nov-Dec;8(6):473-9.

59. Guo T, Zhao J, Chang J, Ding Z, Hong H, Chen J, et al. Porous chitosan-gelatin scaffold containing plasmid DNA encoding transforming growth factor-beta1 for chondrocytes proliferation. Biomaterials 2006 Oct;27(7):1095-1103.

60. Bonadio J, Smiley E, Patil P, Goldstein S. Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. Nat.Med. 1999 Jul;5(7):753-759.

61. Tyrone JW, Mogford JE, Chandler LA, Ma C, Xia Y, Pierce GF, et al. Collagen-embedded platelet-derived growth factor DNA plasmid promotes wound healing in a dermal ulcer model. J.Surg.Res. 2000 Oct;93(2):230-236.

62. Berry M, Gonzalez AM, Clarke W, Greenlees L, Barrett L, Tsang W, et al. Sustained effects of gene-activated matrices after CNS injury. Mol.Cell.Neurosci. 2001 Apr;17(4):706-716.

63. Hosseinkhani H, Inatsugu Y, Hiraoka Y, Inoue S, Shimokawa H, Tabata Y. Impregnation of plasmid DNA into three-dimensional scaffolds and medium perfusion enhance in vitro DNA expression of mesenchymal stem cells. Tissue Eng. 2005 Sep-Oct;11(9-10):1459-1475.

64. Hosseinkhani H, Inatsugu Y, Hiraoka Y, Inoue S, Tabata Y. Perfusion culture enhances osteogenic differentiation of rat mesenchymal stem cells in collagen sponge reinforced with poly(glycolic Acid) fiber. Tissue Eng. 2005 Sep-Oct;11(9-10):1476-1488.

65. Segura T, Chung PH, Shea LD. DNA delivery from hyaluronic acid-collagen hydrogels via a substrate-mediated approach. Biomaterials 2005 May;26(13):1575-1584.

66. Kushibiki T, Nagata-Nakajima N, Sugai M, Shimizu A, Tabata Y. Delivery of plasmid DNA expressing small interference RNA for TGF-beta type II receptor by cationized gelatin to prevent interstitial renal fibrosis. J.Control.Release 2005 Jul 20;105(3):318-331.

67. Andree C, Voigt M, Wenger A, Erichsen T, Bittner K, Schaefer D, et al. Plasmid gene delivery to human keratinocytes through a fibrin-mediated transfection system. Tissue Eng. 2001 Dec;7(6):757-766.

68. Trentin D, Hubbell J, Hall H. Non-viral gene delivery for local and controlled DNA release. J.Control.Release 2005 Jan 20;102(1):263-275.

69. Shea LD, Smiley E, Bonadio J, Mooney DJ. DNA delivery from polymer matrices for tissue engineering. Nature Biotech. 1999;17:551-554.

70. Huang YC, Simmons C, Kaigler D, Rice KG, Mooney DJ. Bone regeneration in a rat cranial defect with delivery of PEI-condensed plasmid DNA encoding for bone morphogenetic protein-4 (BMP-4). Gene Ther. 2005 Mar;12(5):418-426.

71. Jang JH, Rives CB, Shea LD. Plasmid delivery in vivo from porous tissue-engineering scaffolds: transgene expression and cellular transfection. Mol.Ther. 2005 Sep;12(3):475-483.

72. Liang D, Luu YK, Kim K, Hsiao BS, Hadjiargyrou M, Chu B. In vitro non-viral gene delivery with nanofibrous scaffolds. Nucleic Acids Res. 2005 Nov 3;33(19):e170.

CHAPTER 2: EFFECT OF EXPANSION MEDIUM ON *EX VIVO* GENE TRANSFER AND CHONDROGENESIS IN TYPE II COLLAGEN-GLYCOSAMINOGLYCAN SCAFFOLDS *IN VITRO*

2.1. INTRODUCTION

Several studies have demonstrated that the growth of certain cell types in three-dimensional (3-D) scaffolds for articular cartilage tissue engineering can enhance matrix synthesis and increase type II collagen production *in vivo* [1-3]. These cell types include differentiated articular chondrocytes and chondroprogenitor cells derived from marrow, periosteum, or perichondrium [4]. The advantage of using chondrocytes obtained from articular cartilage as a cell source is that they already express the desired phenotype for articular cartilage repair and they have been shown to have the ability to synthesize matrix containing type II collagen and aggrecan [5]. These cells, however, are limited in supply for autologous transplantation and expansion in culture is necessary to obtain a number sufficient for cartilage repair procedures. Yet, it has been demonstrated that articular chondrocytes lose their chondrocytic phenotype (*viz.*, the cells no longer display a rounded morphology or synthesize cartilage-specific macromolecules such as collagen type II and aggrecan) during *in vitro* expansion in monolayer [6,7].

Although studies have shown that monolayer-expanded chondrocytes can redifferentiate into the chondrocytic phenotype when introduced into a 3-D environment such as in cell pellets [8] or porous scaffolds [8,9], the greater the number of serial passages, the more fibroblast-like chondrocytes become—producing molecules such as collagen type I and versican and losing their capability to redifferentiate when put back into a 3-D environment [10]. Both the monolayer expansion medium and redifferentiation medium (used in 3-D culture) have recently been shown to directly influence the ability of chondrocytes to redifferentiate [11,12]. Not only does a specific combination of growth factors in the expansion medium affect chondrocyte proliferation and differentiation, it also influences the chondrocytic potential or ability to redifferentiate when transferred back into a 3-D environment (cell pellets) [12].

Just as selected growth factors have proven to be of importance as supplements to the media used for monolayer expansion and redifferentiation of chondrocytes, they have been

shown to be useful stimulants for chondrogenesis and enhanced biosynthesis for cartilage repair strategies. A local and prolonged administration of insulin-like growth factor-1 (IGF-1) *in vivo* or *in vitro* could significantly benefit articular cartilage tissue regeneration, as the IGF-1 recombinant protein has been shown to increase chondrocyte proliferation, proteoglycan synthesis, type-II collagen synthesis, and chondrogenesis [13]. Administration of the recombinant protein alone *in vivo*, however, may be insufficient for therapeutic results due to protein degradation or diffusion from the defect site. A promising alternative for a prolonged, localized release combines tissue engineering and gene therapy strategies involving *ex-vivo* gene transfer [14]. In this approach, cells could be transfected *in vitro* with the genes for proteins that have been shown to enhance differentiation and biosynthesis, and then immediately implanted *in vivo* so that the desired proteins can be expressed over the time course of regeneration. In using this strategy, the effect that the expansion medium has on the gene transfer susceptibility of cells has never been investigated.

Prior studies have confirmed successful IGF-1 gene transfer to chondrocytes in monolayer with both viral [15,16] and nonviral [17] techniques. These studies also demonstrated that cells overexpressing IGF-1 can enhance chondrogenesis and biosynthesis of matrix molecules in monolayer [15] or when subsequently implanted *in vivo* [16,17]. Prolonged expression of the protein from cells infected in monolayer, however, may still be limited *in vivo* due to possible apoptosis or migration of these cells from the defect site. Localization and maintenance of infected cells within the defect site may be accomplished using 3-D scaffolds onto which seeded cells can attach, migrate within, and proliferate. It has been shown that nonviral gene transfer to cells may also be enhanced by the stiffness of the substrate material upon which the cells are grown [18]. The investigators speculated that the modulation of gene uptake by cells by surface rigidity was due to the control over cell proliferation. The type II collagen-glycosaminoglycan (CG) scaffold has been shown to promote biosynthesis and proliferation of seeded adult articular chondrocytes *in vitro* [19] and improve cartilage repair *in vivo* [3,20]. Based on this prior work, the ability to transfect cells seeded within a type II CG 3-D scaffold was also investigated. Not only might the type II CG scaffold be used to promote chondrogenesis and proliferation, but it may also enhance gene transfer to cells seeded within the scaffold.

There were three objectives in the present work: 1.) To determine the effects of two different expansion media (each having different additives and growth factors) on the ability of monolayer-expanded adult canine articular chondrocytes to produce cartilage matrix molecules (*viz.*, GAG) in cell pellets or when seeded in type II CG scaffolds, 2) to determine if the expansion medium can also have an effect on the gene transfer susceptibility of adult articular chondrocytes in monolayer using the plasmid containing the IGF-1 gene, and 3) using the better of the two media, to determine the possibility of transfecting cells in 3-D culture using CG scaffolds Achieving these objectives will allow us to determine certain culture conditions that can favor the subsequent chondrogenic potential and biosynthesis of cells in 3-D culture and enhanced gene transfer to cells either in monolayer or 3-D culture.

2.2. MATERIALS AND METHODS

2.2.1. Type II Collagen Scaffolds Fabrication

Porous sheets of type II collagen were fabricated by freeze-drying a porcine cartilage-derived slurry (Geistlich Biomaterials, Wolhusen, Switzerland). Similar type II scaffolds have been reported in prior studies to have a porosity of 89 ± 2% (mean ± standard deviation) and a pore diameter of 125 ± 42 μm [21].

The collagen sheets were sterilized and cross-linked by dehydrothermal treatment [22]. Nine-mm diameter disks (~3mm thick) were punched out and additionally cross-linked by a ten minute carbodiimide treatment [23] containing an aqueous solution of 14 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 5.5 mM N-hydroxysuccinimide (EDAC; Sigma Chemical Co., St. Louis , MO). Excess EDAC was removed by rinsing in PBS. The mechanical properties of EDAC-treated collagen-GAG scaffolds have been previously reported [23].

2.2.2. Cell Isolation and Monolayer Culture

Chondrocytes were isolated from the trochleae of the knees (stifle joints) from one adult mongrel dog (2-4 yrs old). Cells from one animal were used in this study in order to eliminate

variability related to inter-animal differences. The cells were obtained using a sequential digestion of pronase (20 U/ml, 1hr) and collagenase (200 U/ml, overnight) as previously described [24]. Isolated chondrocytes were then split and suspended in either one of the following expansion media:

1) Medium 1: Dulbecco's modified Eagle's medium, DMEM/F12 (Gibco Life Technologies, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone Technologies, Logan, UT), 25 μg/ml ascorbic 2-phosphate (Wako Chemical, Osaka, Japan), and a penicillin/streptomycin/fungizone cocktail (Gibco).
2) Medium 2: High glucose DMEM (4.5 g/L D-glucose, without L-glutamine and with 1 mM sodium pyruvate) containing 10% (v/v) FBS, 0.1 mM nonessential amino acids, 10 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic (HEPES) buffer, 100 U/mL penicillin, 100 μg/mL streptomycin glutamate, and supplemented with the following growth factors (all from R&D Systems, Minneapolis, MN): 5 ng/mL of fibroblast growth factor-2 (FGF-2), 10 ng/mL of platelet-derived growth factor-ββ (PDGF-ββ), 1 ng/mL of transforming growth factor-β1 (TGF-β1).

Medium 1 employed in the present investigation was one that has been widely used as an expansion medium for growing chondrocytes in monolayer, while Medium 2 has been found in a prior study [12] to increase chondrocyte proliferation and preserve the redifferentiation potential of expanded chondrocytes when subsequently placed in pellet cultures and grown in a defined serum-free medium.

Primary chondrocytes were plated in 6-well culture plates at 250,000 cells/well for proliferation studies and in 75-cm^2 flasks at 2 million cells/flask for expansion and use in 3-D culture or in monolayer gene transfer studies. The cells were incubated at 37°C and 5% CO_2. Once cells reached confluence (P0 cells), they were trypsinized, resuspended, and re-plated to obtain P1 cells.

2.2.3. Cell Proliferation and Morphology in Monolayers

The cell counts for confluent monolayers of P0 and P1 chondrocytes were obtained by counting the cells (by hemacytometry) at confluence in 6-well plates with an initial plating density of 250,000 cells per well. The total cell number at confluence was used for comparison

in order to incorporate the effects of the media on both cell attachment onto tissue culture plates and cell proliferation.

2.2.4. Culture of Cell Pellets and Cell-Seeded Collagen Scaffolds

A portion of P0 chondrocytes from the two different expansion media cultures were either spun down to obtain cell pellets or seeded into the porous type II collagen scaffolds using a static seeding method. The culture of cell pellets is a common method used to easily assess cell behavior in 3-D culture, and is used in this study to compare results from prior work [12]. Cell pellets were obtained by spinning 5 x 10^5 cells in 1.5 ml polypropylene conical tubes at 4500 rpm for 30 seconds. For collagen type II scaffolds, scaffolds were pre-wet with serum-free medium and placed on agarose-coated wells. Two million cells were added to each scaffold by pipetting a suspension of 1 million cells (in 20 µl medium) onto each side with a 10 minute incubation period in between. By this static seeding method approximately 1.6 million chondrocytes have been found to attach to the scaffolds.

While the cells were expanded in two different culture media, the cell pellets and cell-seeded scaffolds were cultured in the same defined serum-free medium (SFM), found in previous work [12] to enhance differentiation. The SFM used consisted of high glucose DMEM (4.5 g/L D-glucose, without L-Glutamine and with 1mM Sodium Pyruvate), 0.1 mM nonessential amino acids, 10 mM HEPES buffer, 100 U/mL penicillin, 100 µg/mL streptomycin glutamate, ITS^{+1} (100x, by Sigma Chemical, St. Louis, MO), 0.1 mM ascorbic 2-phosphate, 1.25 mg/ml bovine serum albumin, 10 ng/mL of TGF-β1, and 100 nM dexamethasone. Medium was changed every 2-3 days (0.5 ml for pellets and 0.8 or 1.5 ml for scaffolds). Cultures were terminated after 2 weeks for histological evaluation and biochemical analysis. For DNA and GAG analysis, pellets and scaffolds were lyophilized and enzymatically digested using proteinase K (Roche Diagnostics, Indianapolis, IN).

2.2.5. Histology and Immunohistochemistry of Cell Pellets and Cell-Seeded Scaffolds

Cell pellets (n = 2) and cell-seeded scaffolds (n = 2) were fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, and sectioned (eight-micrometer thick) by microtomy. Sections were stained with hematoxylin and eosin to determine cell morphology and

distribution and Safranin-O for sulfated GAG. For type II collagen immunohistochemical analysis, sections were enzymatically digested by protease type XIV for 45 minutes and stained with a standard avidin-biotin complex peroxidase-based antibody staining technique (Vectastain, Vector Laboratories, Burlingane, CA). Mouse anti-chick monoclonal antibody for type II collagen was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA).

2.2.6. Monolayer Infection

P0 chondrocytes expanded in both types of media were plated in 24-well plates for gene transfer studies. At confluence, the cell monolayers were rinsed with phosphate buffered saline and incubated with either an aliquot of lipid-mediated transfection reagent (GenePorter®, Gene Therapy Systems, Inc. San Diego, CA) complexed to the plasmid encoding for IGF-1or with the adenovirus (ad)IGF-1 vector (both vectors supplied by the Center for Molecular Orthopedics, Harvard Medical School). An 8:1 (μl/μg) ratio of transfection reagent to IGF-1 plasmid per well was used for nonviral transfections. For viral transductions, 100 x 10^6 viral particles were added to each well. Three hours later, the vector solutions were removed from cultures and replaced with 0.5 ml of SFM. SFM was used during gene transfer and throughout the 2-week culture period during which medium was collected and changed every 2-3 days after infection. A sandwich ELISA kit for the human IGF-1 protein (R&D Systems) was used to detect the amount of IGF-1 in the medium (n = 3), which reflected the effect of the expansion medium on vector uptake by cells, transfection efficiency, and subsequent release of the growth factor. For the ultimate purpose of employing the monolayer-expanded cells in constructs for implantation, it is the amount of growth factor released that is the principal measure and not necessarily the transfection efficiency. It may be that only a few transfected cells are needed to produce meaningful therapeutic levels of the growth factors.

2.2.7. Nonviral Gene Transfer in Three-Dimensional Culture

Chondrocytes expanded in the medium that yielded the more favorable results in the 3-D chondrogenic assays and monolayer transfection experiments (which proved to be Medium 2) were seeded onto the type II CG scaffolds as described above. Two hours after seeding, the cells seeded in the scaffolds were transfected nonvirally by submerging the construct in a solution

containing plasmid IGF-1 (pIGF-1) complexed with the GenePorter® (GP) transfection reagent using an 8:1 ratio (v/w) of GP:pIGF-1. Four micrograms of plasmid was used to transfect cells per scaffold (n = 3). Two hours later, the cell-seeded scaffolds were transferred onto agarose-coated wells and 0.8 ml of SFM was added to each well. The controls cultured in parallel were cell-seeded scaffolds without the addition of plasmid.

Over a 2-week culture period, medium was collected from the 3-D cultures at various time points and assayed for IGF-1 using the human IGF-1 sandwich ELISA kit (R&D Systems, Minneapolis, MN). Less medium was used for these 3-D cultures (0.8 ml/scaffold versus 1.5 ml/scaffold for the chondrogenic assays) in order to concentrate the IGF-1 released in the medium. At the end of the culture period, scaffolds were lyophilized and enzymatically digested using proteinase K for DNA and GAG analysis.

2.2.8. DNA Analysis

The DNA content of cell pellets and cell-seeded scaffolds was measured using the Hoechst 33258 dye method [25] (n = 3-4). A 50 μl aliquot of the proteinase K digest mixed with 2 ml of Hoechst dye solution (10% Hoechst dye in 10 mM Tris, 1 mM Na_2EDTA and 0.1 M Na CL, pH 7.4) was assayed fluorometrically. The results were extrapolated from a standard curve established using calf thymus DNA.

2.2.9. GAG Analysis

The sulfated GAG content of cell pellets and cell-seeded scaffolds was determined by the dimethylmethylene blue (DMMB) dye assay [26] (n = 3-4). A 100 μl aliquot of the proteinase K digest was mixed with 2 ml of the DMMB dye and the absorbance at 525 nm was measured with a spectrophotometer. The results were obtained by extrapolating from a standard curve using shark chondroitin-6-sulfate. Newly accumulated GAG was determined by subtracting the unseeded values from the sample values.

2.2.10. Statistical Analysis

Data were analyzed by one, two, three-factor ANOVA, and Fisher's PLSD post-hoc testing using StatView (SAS Institute Inc, Cary, NC). Data are presented as mean ± standard error of the mean.

2.3. RESULTS

2.3.1. Proliferation of Monolayer Cultures

There were significant differences in the cell counts for P0 and P1 chondrocytes expanded in monolayer using the two types of media (Fig. 2.1). The cell counts for P0 and P1 chondrocytes grown in Medium 1 were 2.3 million ± 0.2 and 2.5 million ± 0.1, respectively, whereas, cell counts for chondrocytes grown in Medium 2 were 2.7 million ± 0.2 for P0 and 8.3 million ± 0.3 for P1 cells. P1 cells expanded in Medium 2 showed more than a 3-fold higher cell number compared to cells grown in Medium 1 after 4 days (Fig. 2.1). Two-factor ANOVA revealed a significant effect of the expansion medium ($p < 0.001$; power = 1.00) and passage number ($p < 0.001$; power = 1.00) on the proliferation of chondrocytes in monolayer culture.

2.3.2. Cell Morphology of Chondrocyte Monolayers

Morphological differences of cells grown in the two different expansion media were observed by light microscopy (Figs. 2.2a and b). Chondrocytes expanded in Medium 1 (Fig. 2.2a) were larger and cell vacuoles were more visible compared to those expanded in Medium 2 (Fig. 2.2b). Furthermore, when using Medium 2, there were a significant number of cells that had a rounded morphology (Fig. 2.2b) instead of the fibroblast-like elongated morphology that is usually seen during monolayer expansion of chondrocytes.

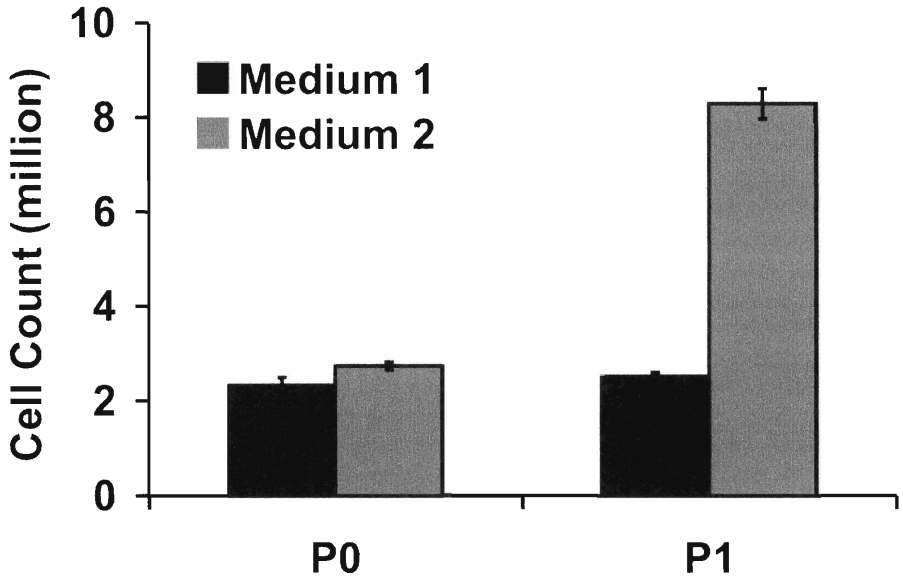


Figure 2.1 Cell counts of chondrocyte monolayers grown in Medium 1 or Medium 2. P0 cells were cultured for 120 hrs and P1 cells were cultured for 96 hours. n = 5-6; mean ± SEM.

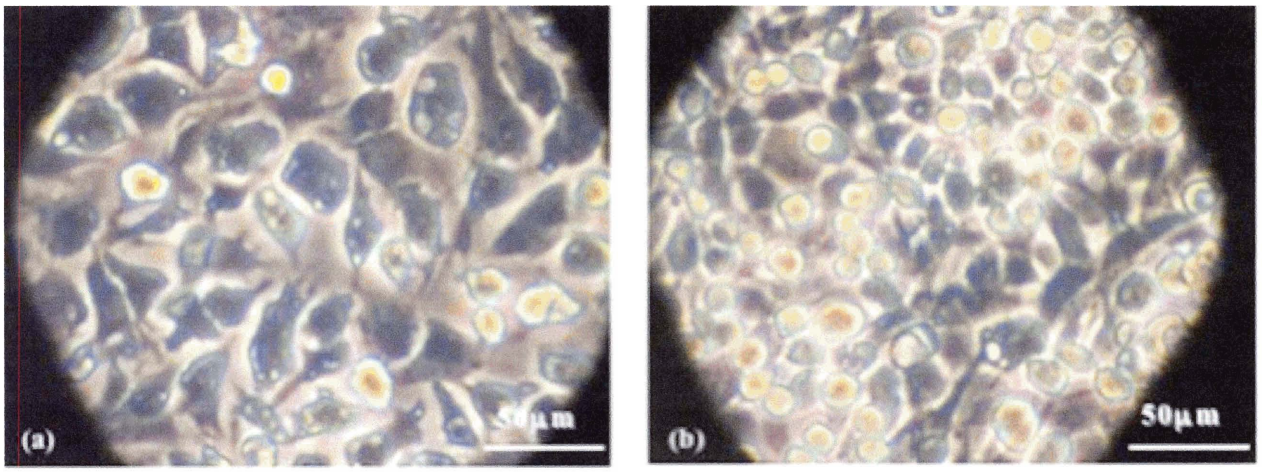


Figure 2.2 Light micrographs (phase contrast) of P1 chondrocyte monolayers grown in (a) Medium 1 or (b) Medium 2 at confluence (5 days).

2.3.3. *Appearance and Biochemical Analysis of the Cell Pellets and Cell-Seeded Collagen-GAG Scaffolds*

Over the 2-week culture period the cell pellets slightly increased in size to a final maximum diameter of approximately 1.5 mm. The cell-seeded collagen-GAG scaffolds contracted only slightly from their original diameter of 9 mm. Of note was the disc-like shape of the pellets that was maintained throughout the culture period (pellets did not contract to become more spherical in shape). There were no statistically significant differences in DNA and total GAG contents for cell pellet cultures prepared with cells expanded in either type of medium. The DNA content of cell pellets for chondrocytes expanded in Medium 1 and Medium 2 were 2.2 ± 0.1 μg and 2.4 ± 0.1 μg, respectively. The GAG content of these pellets was 84.7 ± 2.1 μg for Medium 1-expanded cells and 83.2 ± 2.8 μg for Medium 2-expanded cells.

In contrast to the results from the pellet assay, the GAG/DNA contents of the collagen-GAG scaffolds seeded with cells expanded in Medium 2 were significantly higher than values from constructs prepared with Medium 1-expanded cells (Fig. 2.3). There was also a noticeable effect of the amount of medium added per scaffold. For both types of medium, 1.5ml added per scaffold produced 40-50% higher GAG/DNA values compared to adding 0.8ml of medium per scaffold. Scaffolds seeded with cells expanded in Medium 2 showed a 20-40% increase in GAG/DNA produced compared to cells expanded in Medium 1. Two-factor ANOVA revealed a significant effect of both expansion medium and amount of medium on GAG/DNA values ($p < 0.0001$; power = 1.00).

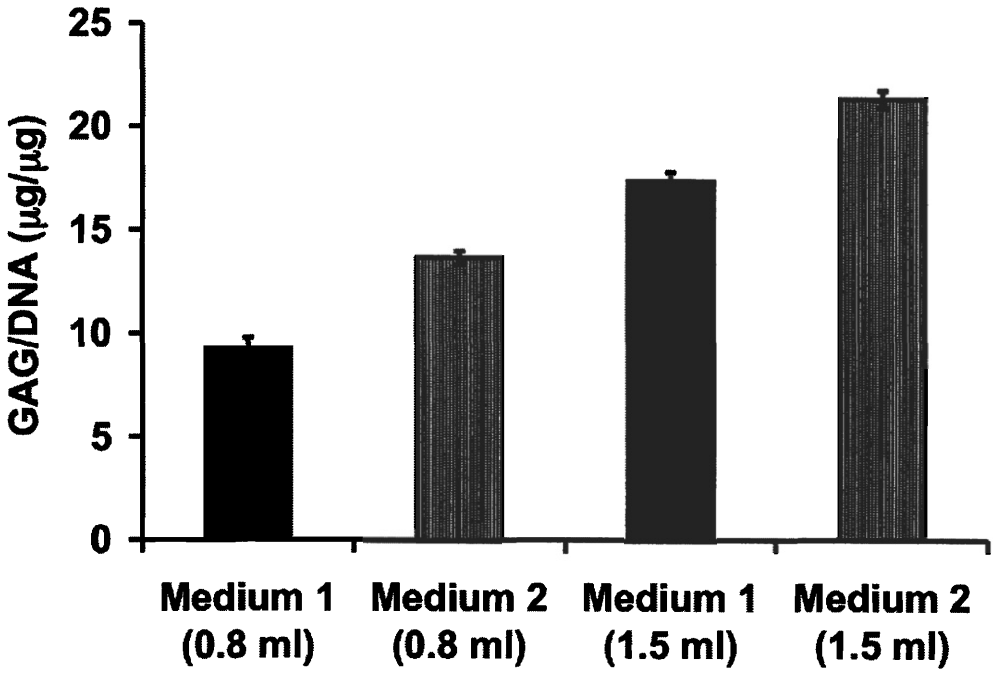


Figure 2.3 GAG/DNA content of chondrocyte-seeded CG scaffolds (2-week culture) for chondrocytes expanded in Medium 1 or Medium 2 with either 0.8ml or 1.5ml of media added per well. n=4; mean ± SEM.

2.3.4. Histology and Immunohistochemistry of 3-D Cultures

Histology revealed that chondrocytes expanded in both types of medium produced cartilaginous constructs when cultured as cell pellets for 2 weeks (Fig. 2.4). Most of the cells displayed a rounded morphology and appeared in lacunae (Fig. 2.4a and b), consistent in appearance with the distinguishing cellular features of hyaline cartilage. Cells near the surface of all pellets assumed a more elongated appearance, with a lower percentage contained within lacunae. Some areas near the pellet surface were completely devoid of cells (Figs. 2.4a and b).

A continuous extracellular matrix rich in GAG was clearly demonstrated in the histochemical sections of pellets prepared with the cells expanded in either type of media, revealed by the intense staining with Safranin O (Figs. 2.4c and d). In some pellets, however, the surface zone (approximately 50 µm thick) had a deficient number of cells and displayed little staining for GAGs (Fig. 2.4c). The cell pellet sections also showed positive immunohistochemical staining of type II collagen (Figs. 2.4e and f) for both types of expansion media. Qualitatively, there was more intensive type II collagen staining in sections of pellets prepared with cells expanded in Medium 2 (Fig. 2.4f) compared to pellets prepared with cells expanded in Medium 1 (Fig. 2.4e). Although the GAG distribution in pellets was generally

uniform throughout (except in the surface zone), the distribution of type II collagen staining generally was more intense on the outer periphery of the pellets and more diffuse in the center (Fig. 2.4g and h).

The cell density in the chondrocyte-seeded collagen-GAG scaffolds was markedly less than in the cell pellets. A lower percentage of cells in the collagen scaffolds displayed the chondrocytic morphology compared to the pellets, with no noticeable effect of expansion medium (Fig. 2.5a and b). For cell-seeded CG scaffolds, chondrocytes expanded in Medium 2 showed more areas of GAG accumulation within the scaffolds compared to cells expanded in Medium 1 (Figs. 2.5c and d). Chondrocytes expanded in both types of medium also stained positive for type II collagen in the CG scaffolds with greater staining found in areas surrounding cells (Figs. 2.5e and f). As demonstrated in the cell pellets, cells expanded in Medium 2 showed more intense staining for type II collagen in the scaffolds compared to cells expanded in Medium 1 (Figs. 2.5e and f).

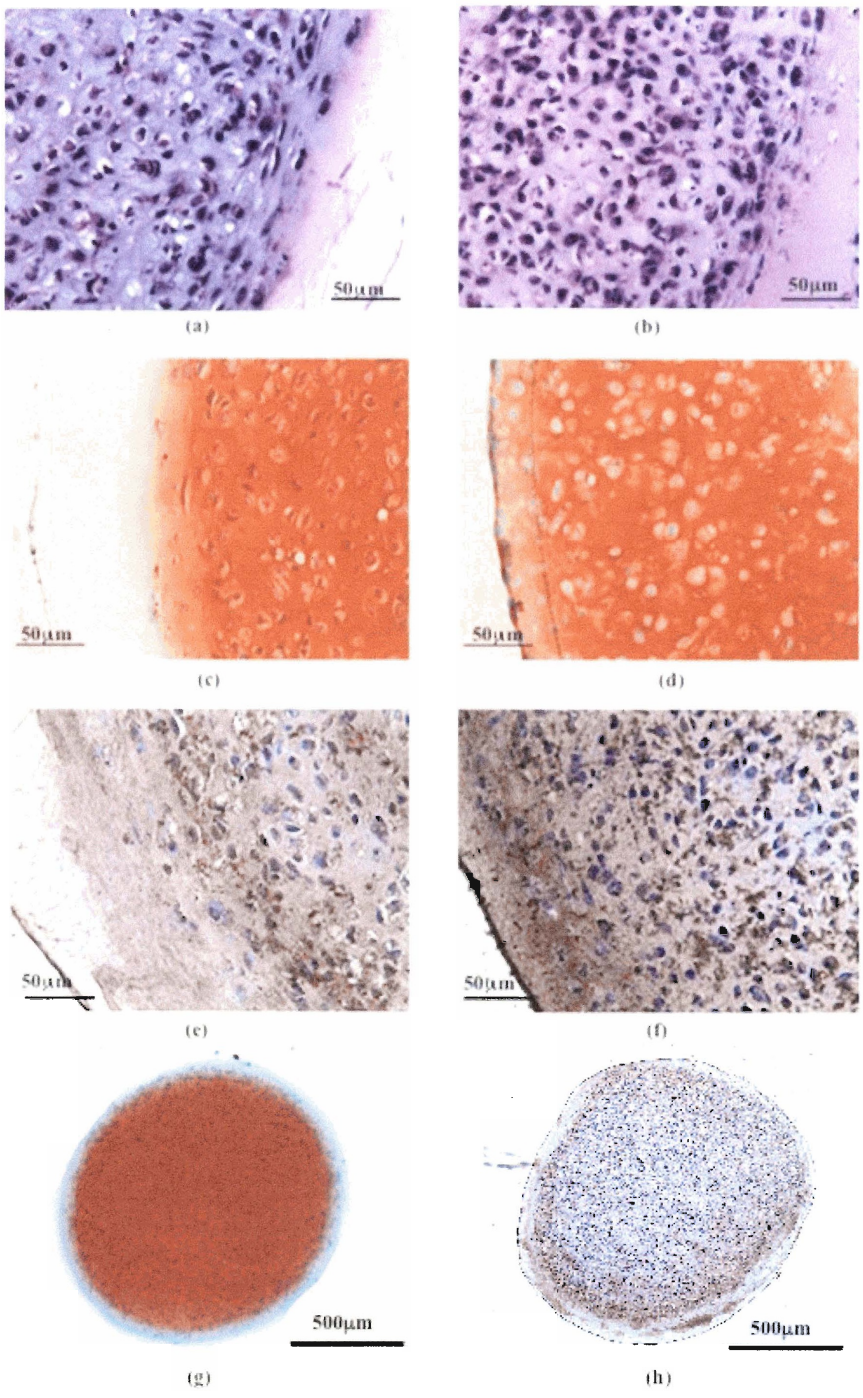


Figure 2.4 **Histology and immunohistochemistry of chondrocyte pellets (2-week culture) for cells expanded in Medium 1 (a, c, e) or Medium 2 (b, d, f) and redifferentiated in SFM. Sections were stained with Hemotoxylin and Eosin (a, b) to show cell morphology and distribution and with Safranin-O (c, d) for glycosaminoglycans (red is positive stain). Sections were also immunohistochemically stained for type II collagen (brown is positive stain) (e, f). Lower magnification micrographs of cell pellets from cells expanded in Medium 1—Safranin-O (g) and collagen type II (h).**

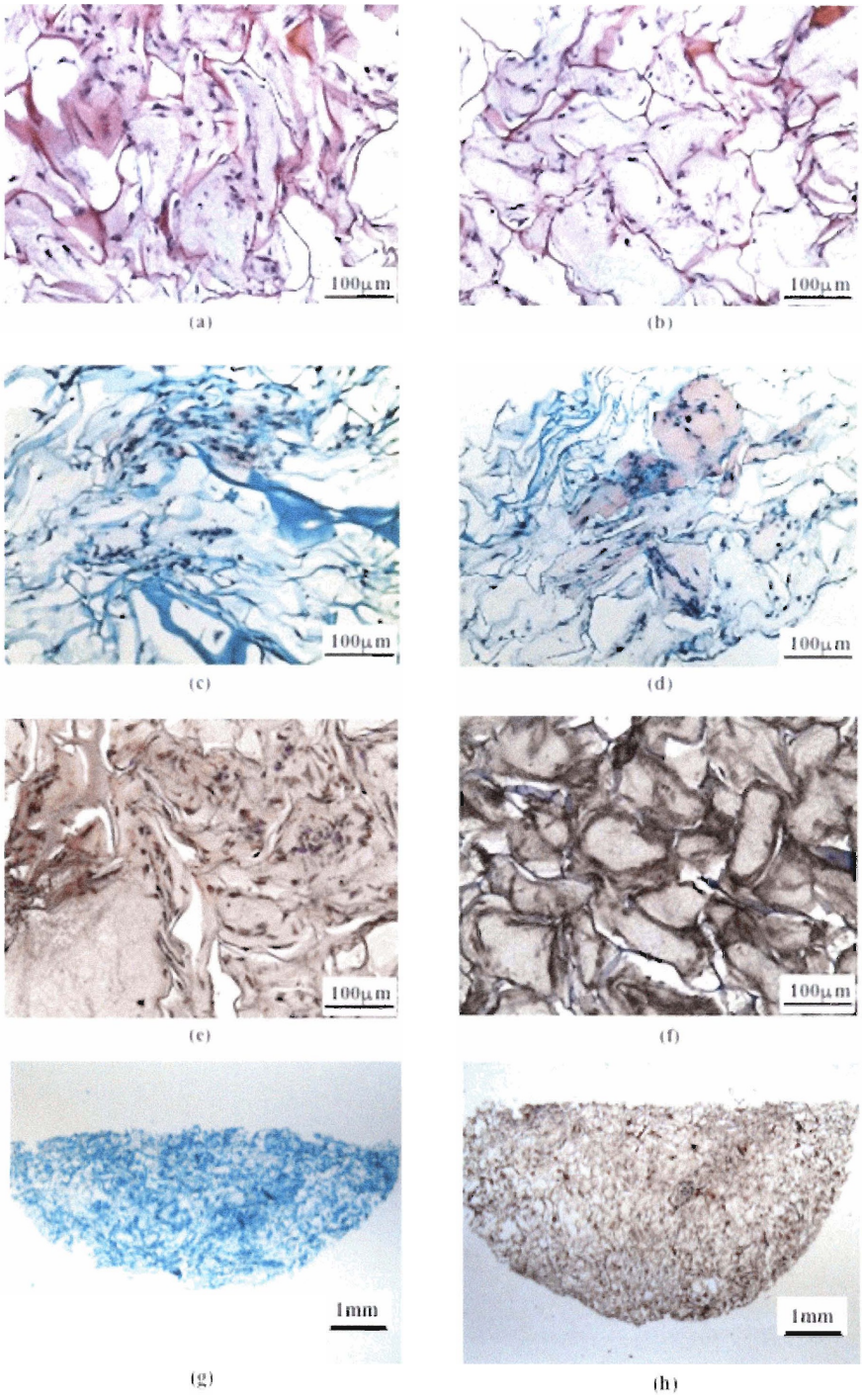


Figure 2.5 **Histology and immunohistochemistry of chondrocyte-seeded CG scaffolds (2-week culture) for cells expanded in Medium 1 (a, c, e) or Medium 2 (b, d, f) and redifferentiated in SFM. Sections were stained with Hemotoxylin and Eosin (a, b) to show cell morphology and distribution within the scaffolds and with Safranin-O (c, d) for glycosaminoglycans (red is positive stain). Sections were also immunohistochemically stained for type II collagen (brown is positive stain) (e, f). Lower magnification micrographs of scaffolds seeded with cells expanded in Medium 1—Safranin-O (g) and collagen type II (h).**

2.3.5. IGF-1 Synthesis of Infected Monolayers

Control monolayers did not display any IGF-1 release into the medium. In contrast, IGF-1 protein release reflecting gene transfer was observed for all transfected or transduced monolayers (Fig. 2.6). GP/pIGF-1 transfection of monolayers grown with both media formulations demonstrated substantially greater IGF-1 release within the first 7 days of culture (Fig. 2.6a). The peak of IGF-1 release for transfected cells expanded in the Medium 2, however, occurred earlier (day 2) than the peak release for cells expanded with Medium 1 (day 5). After the peak, IGF-1 release for these monolayers showed a general drop at day 7 followed by a gradual decrease in IGF-1 production until minimal expression was detected in the day-12 collection. Monolayers transduced with ad-IGF-1 demonstrated a different IGF-1 release profile compared to transfected monolayers (Fig. 2.6b). For both transduced monolayers, there was very little detectable IGF-1 in the medium at the day 2 collection, and both showed similar release profiles up to day 9. Thereafter, cells expanded in Medium 2 showed a noticeably higher IGF-1 release over monolayers expanded with Medium 1 (days 12 and 14). In contrast to transfected monolayers, transduced monolayers showed a significantly lower IGF-1 expression at the early collections, but maintained a more steady release of IGF-1 (no significant peak) over the two-week culture. Although infection conditions were not optimized for either type of gene transfer method in this study, the kinetics of gene expression for transfected and transduced monolayers appear to correspond with the general trend associated with these types of infection methods.

Three-factor ANOVA demonstrated significant effects of expansion medium ($P = 0.02$; power = 0.63), method of gene transfer ($P < 0.0001$; power = 1), and time ($P < 0.0001$; power = 1) on IGF-1 release in the medium over the two-week period. While the effect of expansion medium in the 3-factor ANOVA had only a moderate power (0.63), subsequent Fisher's PLSD post hoc test demonstrated a highly significant difference between Medium 1 and Medium 2 ($P < 0.0001$). In order to more fully examine statistical differences between these two types of media, 1-factor ANOVA was performed for each time point and gene transfer method comparing the two media types. Statistically significant differences in IGF-1 release between Medium 1 and Medium 2 for Geneporter® transfected monolayers occurred at day 2 ($P < 0.0001$; power = 1), day 5 ($P = 0.0005$; power = 1), and day 7 ($P = 0.0004$; power = 1). Significant differences for

transduced monolayers were seen at day 2 ($P < 0.0001$; power = 1), day 5 ($P = 0.0012$; power = 1), day 7 ($P = 0.0212$; power = 0.711), and day 14 ($P = 0.0004$; power = 1).

Of significance is that the accumulated amount of IGF-1 reached a therapeutic level (e.g., 50-100 ng/ml [13]) for both gene transfer methods over the two-week period. Moreover, this therapeutic level was reached by a nonviral gene transfer method only 5 days after transfection.

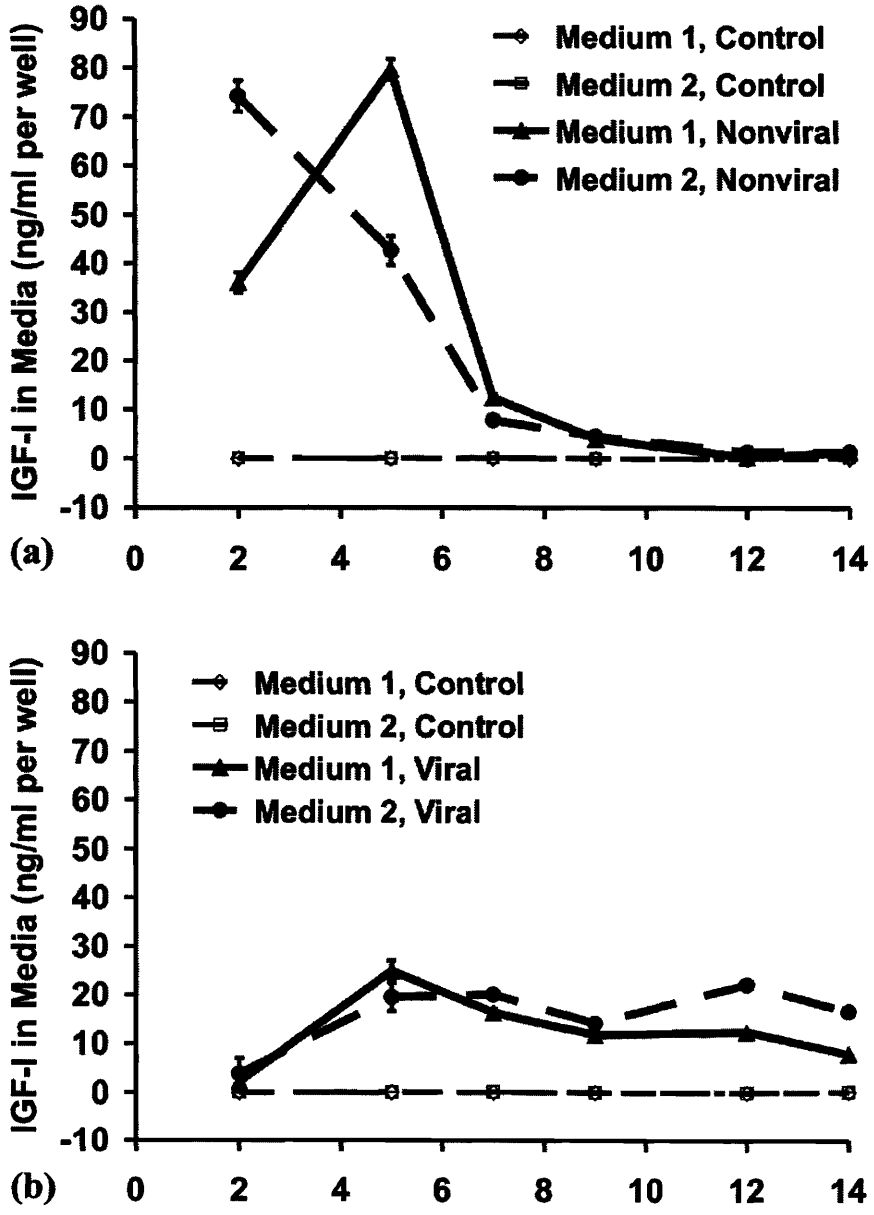


Figure 2.6 IGF-1 protein production detected in the medium over the 2-week chondrocyte monolayer culture grown in Medium 1 or Medium 2 transfected with GP/pIGF-1 complexes (a) or transduced with adIGF-1 (b). n = 3; mean ± SEM (error bars hidden by symbols). IGF-1 amounts for control cultures are on the zero axis.

2.3.6. Nonviral IGF-1 Gene Transfer in CG Scaffolds and Effects on Biosynthesis

IGF-1 gene transfer to Medium 2-expanded chondrocytes in type II CG scaffolds resulted in a substantial elevation of IGF-1 synthesis over the non-transfected control group (Fig. 2.7). Chondrocytes grown in type II CG scaffolds without treatment with the plasmid (controls) produced only minute amounts of IGF-1 in the medium with a total accumulation of about 600 pg over the 2-week culture (Fig. 2.7). There was, however, evidence of a slight increase in IGF-1 production at the end of the 2-week period for these control scaffolds. Transfected 3-D cultures showed a 35-fold higher elevation in accumulated IGF-1 collected in the medium over the 2-week culture compared to control scaffolds. For transfected cultures, there was a peak release of IGF-1 in the medium at about a week (~6 ng/ml), after which IGF-1 release started to slightly decline (Fig. 2.7).

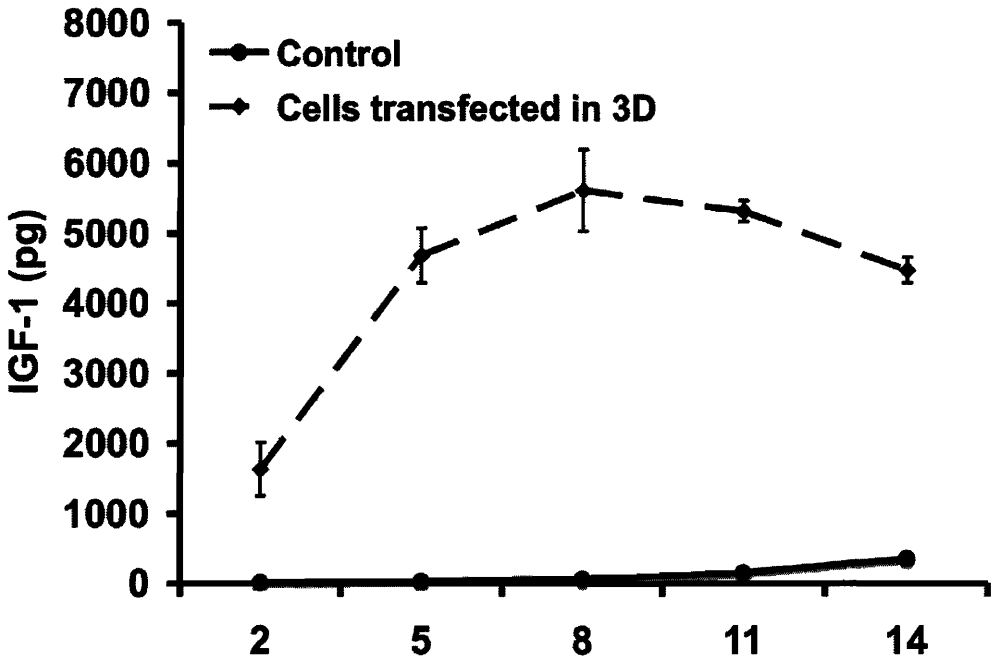


Figure 2.7 IGF-1 protein production detected in the collected media of chondrocytes expanded in Medium 2 and seeded in type II CG scaffolds, with or without subsequent transfection with GenePorter®/pIGF-1. n = 3; mean ± SEM.

IGF-1 over-expression by chondrocytes grown in type II CG scaffolds resulted in a significant increase of GAG/DNA synthesis over control scaffolds. There was a 40% higher GAG/DNA value for IGF-1 transfected cultures compared to the control group (Fig. 2.8); the control group is the same as the 0.8 ml, Medium 2 group in Fig. 2.3. One-factor ANOVA revealed a significant effect of IGF-1 over-expression on GAG/DNA ($P < 0.0001$; power = 1).

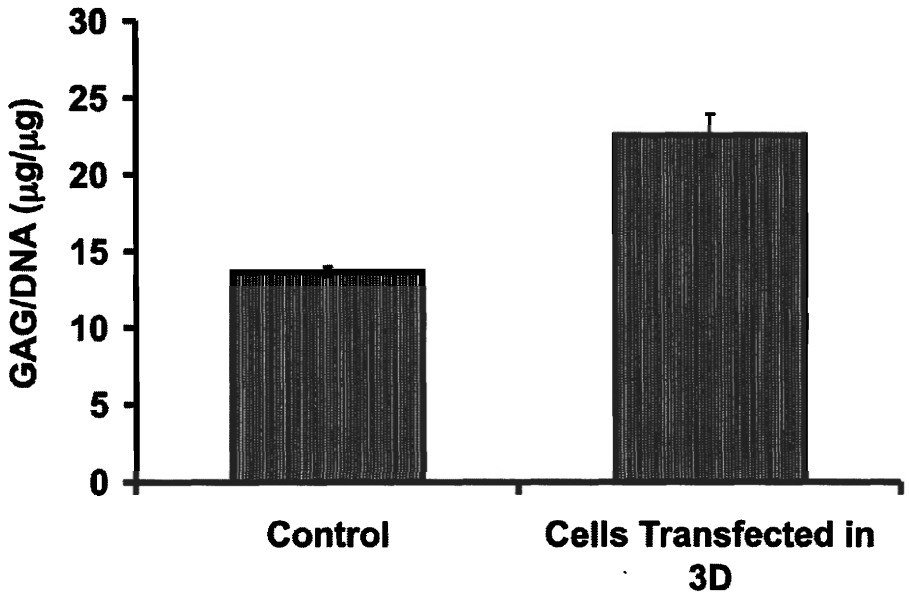


Figure 2.8 GAG/DNA (2-week cultures) of type II CG scaffolds seeded with chondrocytes expanded in Medium 2, with or without subsequent transfection with GenePorter®/pIGF-1. n = 3; mean ± SEM.

2.4. DISCUSSION

The current investigation demonstrated that the specific medium used in expanding chondrocytes in monolayer has a significant influence on GAG synthesis and chondrogenesis when chondrocytes are subsequently grown in a tissue engineering scaffold comprised of collagen (Type II)-GAG. As in previous work [12], addition of specific growth factors within the expansion medium significantly increased proliferation of chondrocytes in monolayer. The cell morphology, which is one indicator that can define a chondrocyte phenotype, was also affected. At confluence, cultures grown in Medium 2 were made up of chondrocytes that were smaller and that possessed a rounded morphology (characteristic of a chondrocytic phenotype) compared to cultures grown in Medium 1. The smaller cell size of chondrocytes expanded in the Medium 2 may be a result of a greater number of cells present at confluence. Using Medium 2 resulted in a

more than 4-fold difference in cell number for P1 cells at confluence. This may be very advantageous for decreasing the time needed to obtain a sufficient number of cells for preparation of tissue-engineered articular cartilage constructs for implantation.

Pellet cultures yielded the characteristic small diameter spheroids that contained high levels of GAG and type II collagen. The small diameters of these cell pellets, however, render these constructs undesirable for implantation. There was no significant difference in the total GAG accumulated within cell pellets for chondrocytes expanded in Medium 1 compared to Medium 2. That the total GAG values may have been maximum levels achieved in the pellet cultures, may explain why there were no detectable differences in pellets comparing the two expansion media. Despite this undetectable difference in GAG, more intense collagen type II staining was present with chondrocytes expanded in Medium 2, demonstrating greater chondrogenesis in these pellets.

There was a more prominent difference in the effects of expansion media when P1 chondrocytes were grown in CG scaffolds, compared to the pellet assay. The expansion medium demonstrated a significant effect on DNA, total GAG accumulation, and GAG per DNA content when chondrocytes were seeded in CG scaffolds and cultured in SFM for 2 weeks. Furthermore, a greater number of areas that stained for GAG were apparent in histological sections for constructs seeded with chondrocytes expanded in Medium 2. A notable finding was the significantly higher amount of total GAG accumulation and GAG staining within cell pellets compared to cell-seeded CG scaffolds. It is speculated that this difference between cell pellet and scaffold culture may be related to the effects of cell density—cell pellets having a greater concentration of cells compared to the seeded scaffolds. Prior work investigating the chondroinduction of mesenchymal stem cells in pellet cultures has also supported an association between pellet contraction, and hence increasing cell density, to chondrogenesis [27]. In the present investigation, areas within scaffolds that appeared to have a higher cell density displayed more staining for GAG. Within cell pellets, where the cell density was uniform and plentiful throughout, there was a continuous matrix rich in GAG. It is also possible that the porosity of the scaffold itself may allow for diffusion of GAGs from the scaffold into the medium, as seen in a prior study [23], making it more difficult for GAG to accumulate within the matrix. The tighter packing of cells within cell pellets may facilitate GAG accumulation.

Chondrocyte-seeded CG scaffolds also showed evidence of chondrogenesis with the positive staining for type II collagen. Like the cell pellets, there was more intense staining of type II collagen within CG matrices that incorporated cells expanded in Medium 2, suggesting that more articular cartilage-specific matrix molecules may be produced by these cells compared to ones expanded in Medium 1. The varied morphology (including the presence/absence of lacunae) indicated that there was phenotypic heterogeneity in the cell populations within CG scaffolds and pellets. Therefore, it was likely that some cells were producing non-cartilagenous matrix molecules (including collagen types other than type II). In the context of the objective to produce constructs to facilitate cartilage repair *in vivo*, future animal studies will be required to determine to what extent this heterogeneity in cell phenotype affects cartilage repair. After two weeks in *in vitro* culture, the presence of type II collagen serves as an indication that at least some of the cells are proceeding down the path of chondrogenesis.

A significant finding of this study was that viral and non-viral transgene expression levels of chondrocytes in monolayer were directly affected by the medium used to expand the cells. For transduced monolayers, the effect that the expansion medium had on IGF-1 release was not significant until the later collection periods (days 12 and 14), where IGF-1 release from cells expanded in Medium 2 had significantly higher levels than those expanded in Medium 1. For transfected cells, monolayers expanded in Medium 2 demonstrated an earlier peak in IGF-1 synthesis compared to cells expanded in Medium 1. This earlier peak in protein synthesis may indicate that the cells expanded in Medium 2 were more receptive to non-viral gene transfer. This enhanced receptiveness allowed therapeutic levels of IGF-1 to be accumulated *in vitro* sooner (in 2 days) than with cultures of chondrocytes grown in Medium 1. It is speculated that the enhanced nonviral gene transfer with Medium 2 may be due to an increased mitotic activity of the cells as reflected in the proliferation data. During cell division, the cell membrane and nuclear envelope is disrupted, and hence gene uptake and transfer within the cell nucleus may be facilitated. The greater number of cell divisions that resulted when using Medium 2 for expansion, may therefore enhance the transfection efficiency and may have resulted in the increased IGF-1 release especially at the earlier time points. It would be interesting in future studies to investigate the effects of medium composition directly on transfection efficiency with the use of reporter genes.

For *in vivo* applications, an earlier release of growth factor may be advantageous in the first stages of repair. The kinetics of growth factor release, however, would most likely require a prolonged and more sustained level to enhance biosynthesis over the regeneration process, not just a single peak of protein release at an early time point. This may require a system where genes can be incorporated within tissue engineering scaffolds to provide continuous transfection of seeded or endogenous cells *in vivo* as the scaffold degrades, to sustain growth factor release for the span of *in vivo* repair. In this study, the make-up of the medium in which chondrocytes are expanded may have a meaningful effect on gene transfer and should be considered when deciding optimal conditions for both *in vitro* and *in vivo* gene transfer.

Transfecting Medium 2-expanded chondrocytes seeded in type II CG scaffolds resulted in elevated IGF-1 synthesis when compared to controls that were not treated with the plasmid. Interestingly, chondrocytes seeded in 3-D scaffolds (without plasmid) did show some IGF-1 release in the medium, in contrast to no expression detected in monolayer control cultures. This might indicate that the cell interaction with the type II CG scaffold alone can potentially act as a stimulus for IGF-1 expression. The production of IGF-1 from control scaffolds was most noticeable at the end of the 2-week culture period. It should be noted that the actual levels of IGF-1 localized within the scaffold could be significantly higher than the concentrations detected in the medium. Future work will investigate methods to quantify IGF-1 expressed by cells that is retained within the scaffold in order to determine the minimum *local* therapeutic concentration needed to enhance chondrogenesis.

IGF-1 release kinetics resulting from cells transfected in the CG scaffolds also differed from transfected monolayer cultures. Transfected monolayer cultures showed a maximum release of IGF-1 at the beginning of culture period and decreased significantly within a week. In contrast, IGF-1 levels in the medium from transfected cell-seeded scaffolds showed a steadier release profile with a gradual increase up to about a week followed by a slight decrease at the end of the two-week period. This may indicate that using 3-D scaffolds with gene transfer methods may provide a more steady expression of desired growth factors *in vitro* and *in vivo* over prolonged times.

In this study, a significant increase in GAG biosynthesis was associated with an overexpression of IGF-1 in cultures of Medium 2-expanded cells that were transfected while growing in the scaffold. This finding is consistent with prior work demonstrating that IGF-1

stimulates chondrocyte biosynthesis [13]. Future work needs to investigate the biosynthesis of other cartilage matrix molecules and the histological features of chondrogenesis.

Of interest in the present study was the finding that the volume of medium strongly affected GAG synthesis, with a 40% increase in GAG/DNA in the cultures with 1.5 ml per scaffold compared to the cultures with 0.8 ml per scaffold. GAG/DNA values in cultures growing in 0.8 ml of medium, however, was stimulated by IGF-1 overexpression from chondrocytes transfected within CG scaffolds up to levels similar to the non-transfected cultures grown using 1.5 ml of medium. A possible reason for this finding is that the minimum amount of growth factors and nutrients needed for optimal biosynthesis in these 3-D scaffolds was not attained using just 0.8ml of medium per scaffold, but additional IGF-1 overexpression by transfected cells within the scaffolds stimulated more GAG production. This demonstrates the effectiveness of IGF-1 to stimulate GAG production in less than optimal *in vitro* culture conditions. It is speculated that IGF-1 overexpression by transfected cells may also have this beneficial effect *in vivo* where conditions are less controlled.

Although this study involved the use of cells from one animal, there is reason to believe that the major conclusions of this paper will still hold if more experiments are repeated, based on other studies involving the use of multiple canines using the same chondrocyte isolation and growth conditions presented in these studies. In similar *in vitro* studies, the coefficient of variation between different animals of this specific breed of canine was about 10% (assessing the final accumulated GAG values in similar tissue-engineered constructs after 2-weeks in culture). This inter-animal variation is smaller than the difference in the present data between experimental groups, and therefore the statistically significant differences between the groups in this study is most likely a reflection of the varying culture conditions and not from animal to animal differences.

In conclusion, this study demonstrated that the expansion medium used to grow chondrocytes has a significant effect on chondrocyte proliferation in monolayer, subsequent 3-D culture using type II CG matrices, and *ex vivo* gene transfer. Not only does Medium 2 decrease the amount of time it takes to obtain a sufficient number of cells for developing tissue-engineered articular cartilage constructs, it also enhances gene transfer kinetics and improves the production of matrix molecules and chondrogenesis when cells are seeded within CG scaffolds.

2.5. REFERENCES

1. von Schroeder HP, Kwan M, Amiel D, Coutts RD. The use of polylactic acid matrix and periosteal grafts for the reconstruction of rabbit knee articular defects. J. Biomed. Mater. Res. 1991;25(3):329-339.

2. Chu CR, Coutts RD, Yoshioka M, Harwood FL, Monosov AZ, Amiel D. Articular cartilage repair using allogeneic perichondrocyte-seeded biodegradable porous polylactic acid (PLA): A tissue-engineering study. J. Biomed. Mater. Res. 1995;29(9):1147-1154.

3. Lee CR, Grodzinsky AJ, Hsu HP, Spector M. Effects of a cultured autologous chondrocyte-seeded type II collagen scaffold on the healing of a chondral defect in a canine model. J.Orthop.Res. 2003 Mar;21(2):272-281.

4. Coutts RD, Healey RM, Ostrander R, Sah RL, Goomer R, Amiel D. Matrices for cartilage repair. Clin Orthop 2001 Oct(391 Suppl):S271-9.

5. Im GI, Kim DY, Shin JH, Hyun CW, Cho WH. Repair of cartilage defect in the rabbit with cultured mesenchymal stem cells from bone marrow. J Bone Joint Surg Br 2001 Mar;83(2):289-94.

6. Benya P, Shaffer J. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982;30:215-24.

7. Binette F, McQuaid DP, Haudenschild DR, Yaeger PC, McPherson JM, Tubo R. Expression of a stable articular cartilage phenotype without evidence of hypertrophy by adult human articular chondrocytes in vitro. J. Orthop. Res. 1998;16:207-216.

8. Stewart M, Saunders K, Burton-Wurster N, Macleod J. Phenotypic stability of articular chondrocytes in vitro: the effects of culture models, bone morphogenetic protein 2, and serum supplementation. J. Bone Miner. Res 2000;15:166-74.

9. Martin I, Vunjak-Novakovic G, Yang J, Langer R, Freed LE. Mammalian chondrocytes expanded in the presence of fibroblast growth factor 2 maintain the ability to differentiate and regenerate three-dimensional cartilaginous tissue. Exp. Cell Res. 1999;253:681-688.

10. Bonaventure J, Kadhom N, Cohen-Solal L, Ng KH, Bourguignon J, Lasselin C, et al. Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. Exp. Cell Res. 1994;212(1):97-104.

11. Yaeger PC, Masi TL, de Ortiz JL, Binette F, Tubo R, McPherson JM. Synergistic action of transforming growth factor-beta and insulin-like growth factor-I induces expression of type II collagen and aggrecan genes in adult human articular chondrocytes. Exp. Cell Res. 1997;237:318-25.

12. Jakob M, Demarteau O, Schafer D, Hintermann B, Dick W, Heberer M, et al. Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes

enhance chondrogenesis and cartilaginous tissue formation in vitro. J Cell Biochem 2001 Mar 26;81(2):368-77.

13. Fortier LA, Lust G, Mohammed HO, Nixon AJ. Coordinate upregulation of cartilage matrix synthesis in fibrin cultures supplemented with exogenous insulin-like growth factor-I. J.Orthop.Res. 1999 Jul;17(4):467-474.

14. Capito RM, Spector M. Scaffold-based articular cartilage repair. IEEE Eng.Med.Biol.Mag. 2003 Sep-Oct;22(5):42-50.

15. Brower-Toland BD, Saxer RA, Goodrich LR, Mi Z, Robbins PD, Evans CH, et al. Direct adenovirus-mediated insulin-like growth factor I gene transfer enhances transplant chondrocyte function. Hum Gene Ther 2001 Jan 20;12(2):117-29.

16. Gelse K, von der Mark K, Aigner T, Park J, Schneider H. Articular cartilage repair by gene therapy using growth factor-producing mesenchymal cells. Arthritis Rheum 2003 Feb;48(2):430-41.

17. Madry H, Kaul G, Cucchiarini M, Stein U, Zurakowski D, Remberger K, et al. Enhanced repair of articular cartilage defects in vivo by transplanted chondrocytes overexpressing insulin-like growth factor I (IGF-I). Gene Ther. 2005 Aug;12(15):1171-1179.

18. Kong HJ, Liu J, Riddle K, Matsumoto T, Leach K, Mooney DJ. Non-viral gene delivery regulated by stiffness of cell adhesion substrates. Nat.Mater. 2005 Jun;4(6):460-464.

19. Veilleux, N.H., Yannas, I.V., and Spector,M. Effect of passage number and collagen type on the proliferative, biosynthetic, and contractile activity of adult canine articular chondrocytes in type I and II collagen-glycosaminoglycan matrices in vitro. Tissue Eng 2004;10:119-27.

20. Breinan HA, Martin SD, Hsu HP, Spector M. Healing of canine articular cartilage defects treated with microfracture, a type-II collagen matrix, or cultured autologous chondrocytes. J.Orthop.Res. 2000 Sep;18(5):781-789.

21. D. Hastreiter. A collagen-GAG matrix for the growth of intervertebral disc tissue. Cambridge, MA: Massachusetts Institute of Technology; 2002.

22. Yannas IV, Lee EO,D.P., Skrabut EM, Murphy GF. Synthesis and characterization of a model extracellular matrix that induces partial regeneration of adult mammalian skin. Proc. Nat'l. Acad. Sci. USA 1989;86:933-937.

23. Lee CR, Grodzinsky AJ, Spector M. The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis. Biomat. 2001;22:3145-3154.

24. Kuettner KE, Pauli BU, Gall G, Memoli VA, Schenk RK. Synthesis of cartilage matrix by mammalian chondrocytes in vitro. I. Isolation, culture characteristics, and morphology. J Cell Biol 1982 Jun;93(3):743-50.

25. Kim YJ, Sah RL, Doong JY, Grodzinsky AJ. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. Anal Biochem 1988 Oct;174(1):168-76.

26. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986 Sep 4;883(2):173-7.

27. Kinner B, Gerstenfeld LC, Einhorn TA, Spector M. Expression of smooth muscle actin in connective tissue cells participating in fracture healing in a murine model. Bone 2002 May;30(5):738-745.

CHAPTER 3: GENE-SUPPLEMENTED COLLAGEN-GLYCOSAMINOGLYCAN SCAFFOLDS (GSCG) FOR NONVIRAL IGF-1 GENE DELIVERY IN ARTICULAR CARTILAGE TISSUE ENGINEERING

3.1. INTRODUCTION

Three-dimensional scaffolds and recombinant growth factors are effective stimulants for chondrogenesis and enhanced biosynthesis in articular cartilage tissue engineering. One major obstacle in using recombinant proteins for therapeutic delivery *in vivo,* however, is the inherent inability to maintain therapeutic levels of the cytokine for prolonged periods due to their short half-lives and the inability to contain the activity in a localized area. A promising alternative for a prolonged, localized release of growth factors combines tissue engineering and gene therapy strategies where infected cells seeded within tissue engineering scaffolds can express the desired proteins over the time course of regeneration.

Ex vivo gene transfer to cells in two-dimensional (2-D) monolayer culture for subsequent transplantation, with or without a three-dimensional (3-D) scaffold, is a common method to provide a more prolonged release of desired growth factors for tissue repair, compared to a single bolus dose of the recombinant proteins [1-4]. Prolonged expression of the protein from infected cells, however, is limited *in vivo* due to migration of these cells from the defect site or apoptosis. An alternative approach that is recently being investigated is a scaffold-based or "substrate-mediated" [5] gene transfer approach, in which tissue-engineering scaffolds are used as gene delivery vehicles to seeded cells and/or endogenous cells *in vivo*. This method could provide a continual transfection of cells and subsequent protein expression that can be achieved over extended periods of time (weeks to months).

Both viral [1,3,6] and nonviral [4,7-12] vectors have been employed to provide an elevated release of desired proteins from infected cells for tissue engineering applications. Although viral methods generally have a much higher gene transfer efficiency, especially for slowly dividing or non-dividing cells, the inherent immunogenicity of viral vectors commends nonviral methods for tissue engineering applications. Furthermore, overexpression of desired proteins in tissue engineering applications is needed only through the time period of tissue repair, and therefore

gene incorporation into the host genome, which is usually associated with viral transductions, is not required. Tissue engineering scaffolds incorporating nonviral vectors have the ability to localize the concentration of nonviral vector release within the defect area for prolonged times, which increases the probability of gene uptake by surrounding cells. Various scaffolding materials incorporating nonviral gene vectors that have been shown to provide successful prolonged and elevated expression include poly(lactide-co-glycolide) (PLGA))[10,11,13], poly(D,L-lactide)–poly(ethyleneglycol) (PLA–PEG) [11], and collagen [7,8,9,12,14].

A local and prolonged administration of insulin-like growth factor (IGF)-1 could substantially enhance cartilage repair, as the IGF-1 recombinant protein has been shown to increase chondrocyte proliferation, proteoglycan synthesis, type-II collagen synthesis, and chondrogenesis [15-20]. Although studies have shown effective IGF-1 gene transfer to cells using an *ex vivo* approach for enhancing articular cartilage tissue engineering [1,4,21], there has not yet been an investigation demonstrating IGF-1 gene transfer to cells using a substrate-mediated nonviral gene transfer approach using tissue engineering scaffolds.

In this study, gene-supplemented type II collagen-glycosaminoglycan (GAG), GSCG, scaffolds were investigated using naked plasmid DNA encoding for the IGF-1 protein, alone or with a lipid mediated transfection reagent, GenePorter®. The kinetics of plasmid release comparing two different methods of gene supplementation was investigated: 1) soaking the scaffold in the plasmid solution followed by freeze-drying, and 2) covalently linking the plasmid to the scaffold using a carbodiimide cross-linking agent. It was hypothesized that the first method would provide a rapid release of the plasmid and the second a prolonged release, thus offering a range for controlling the timing of transfection.

Adult canine articular chondrocytes were seeded into these scaffolds to assess the ability of the GSCG scaffolds to facilitate gene transfer and provide a localized, elevated, and prolonged expression of IGF-1. After two weeks in culture, the DNA and accumulated GAG contents were assessed by biochemical assays and chondrogenesis assessed by immunohistochemical staining for type II collagen. The type II collagen-GAG (CG) scaffold was used in the current work based on promising prior studies *in vitro* [22] and *in vivo* [23,24].

3.2. MATERIALS AND METHODS

3.2.1. Experimental Design

GSCG scaffolds incorporating the plasmid for IGF-1 were prepared using 2 methods (described in detail in a following section). Specimens were allocated for the evaluation of plasmid release kinetics from GSCG scaffolds using a biochemical measurement of DNA, and the assessment of the structural integrity of the released and retained plasmid by gel electrophoresis. Because of the dramatically different plasmid release kinetics resulting from leaching studies comparing the two methods of GSCG fabrication, we selected different culture conditions (*viz.*, plasmid load and cell seeding density) for evaluating chondrocyte-seeded GSCG scaffolds prepared using the two methods. These conditions were based on what we expected would be of value for future work *in vivo*. The goal of the present work was not to perform a parametric analysis of the effects of cell seeding density and plasmid load on the transfection of cells seeded within the GSCG scaffolds, but rather to determine the nonviral transfection and gene expression kinetics that results from using the two different incorporation methods with or without the addition of a lipid transfection reagent.

Chondrocyte-seeded GSCG cultures were employed to determine successful transfection of seeded chondrocytes by assessing IGF-1 release in the 3-D culture medium using ELISA and to determine the effects on biosynthesis by biochemically measuring GAG accumulation in the scaffolds over a 2-week culture period. Other samples were allocated for histological evaluation. The plasmid containing the gene encoding for the enhanced green fluorescent protein (EGFP) was implemented to allow for the imaging of transfected cells seeded within GSCG scaffolds using conventional fluorescence and confocal microscopy. Table 3.1 summarizes the experimental outline and the allocation of samples used for the aforementioned characterization techniques.

Table 3.1 Experimental Summary for *In Vitro* Evaluation of Gene-Supplemented Collagen-GAG Scaffolds

Experiment	Method of Preparing GSCG Scaffolds	Plasmid	Loading (μg)	# Cells seeded ($x10^6$)	GenePorter	Methods of Evaluation (n)
Release Kinetics/ Integrity	1 and 2	IGF-1	20	-	-	Biochemical (3) Gel Electrophoresis (1)
Chondrocyte Transfection	1	IGF-1	4	2	±	IGF-1 ELISA (3) Biochemical (4) Histochemical (2)
Chondrocyte Transfection	2	IGF-1	10	4	±	IGF-1 ELISA (6) Biochemical (4) Histochemical (2-3)
Chondrocyte Transfection	2	EGFP	10	4	±	Fluorescence (2) Confocal (2)

3.2.2. Type II Collagen-GAG Scaffolds

Porous sheets of a type II CG scaffold were fabricated by freeze-drying a porcine type II collagen-GAG slurry (Geistlich Biomaterials, Wolhusen, Switzerland). Similar scaffolds have been reported in prior studies to have a porosity of 89 ± 2% (mean ± standard deviation) and a pore diameter of 125 ± 42 μm [25]. The collagen sheets were sterilized and cross-linked by dehydrothermal treatment [26], and 8 mm diameter disks (~2 mm thick) were prepared using a dermal biopsy punch (Moore Medical, New Britain, CT).

3.2.3. Plasmid Propagation and Isolation

Multiplication of plasmids encoding for IGF-1 (pIGF-1, obtained from the Center for Molecular Orthopedics, Harvard Medical School, Boston, MA) and enhanced green fluorescent protein (pEGFP, BD Biosciences, Bedford, MA) was accomplished by heat shock transformation into *Escherichia coli* DH5α competent cells grown overnight in Luria-Bertani (LB) medium containing ampicillin and kanamycin, respectively. The pEGFP was used as a reporter gene to visualize the transfection of chondrocytes by conventional fluorescence and confocal microscopy. Plasmid was isolated and purified using a Mega QIAfilter™ Plasmid kit (Qiagen,

Valencia, CA). The absorption ratio at 260 nm and 280 nm was used to determine plasmid concentration and purity while plasmid integrity was verified by polyacrylamide gel electrophoresis. The size of pEGFP was 4.7 Kb, and pIGF-1 was 6-7 Kb.

3.2.4. Plasmid Incorporation into Collagen-GAG (CG) Scaffolds

Two methods were employed for incorporation of the pIGF-1 into CG scaffolds. Method 1 involved additional cross-linking of the DHT-treated scaffolds with a ten-minute carbodiimide treatment[27] in an aqueous solution of 14 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 5.5 mM N-hydroxysuccinimide (EDAC; Sigma Chemical Co., St. Louis , MO) at room temperature. Excess EDAC was removed by rinsing in phosphate buffered saline (PBS). A 250 μl aliquot of a diluted pIGF-1 solution containing 4 μg of pIGF-1 (at pH = 8) was added to each scaffold followed by incubation for a minimum of one hour at room temperature. This incubation in the plasmid solution allowed swelling of the collagen fibrils and absorption of the plasmid solution onto the walls/struts of the scaffold. Scaffolds were then freeze-dried to allow collapse of the collagen fibrils and entrapment of the plasmid.

The second method (Method 2) investigated to synthesize GSCG scaffolds involved placing a 60 μl aliquot of the diluted pIGF-1 solution containing 10 μg of pIGF-1 onto the DHT-treated scaffolds followed by incubation for an hour at room temperature. A 1 ml aliquot of the EDAC cross-linking solution described above was then added to each scaffold and incubated for about 30 minutes to allow cross-links to form among the collagen molecules and between the plasmid and collagen (see schematic in Fig. 3.1 for potential chemical bonds that may form between the plasmid DNA and collagen protein molecules). Excess EDAC was removed by rinsing the scaffolds in PBS for one hour. Additional GSCG scaffolds incorporating pEGFP were prepared using Method 2 for imaging analyses to demonstrate successful transfection of seeded chondrocytes over time. Control scaffolds were run in parallel and synthesized by substituting the plasmid solution with tris disodium ethylenediamine tetraacetate (TE) buffer.

For cell culture studies, plasmid DNA alone or with a GenePorter® (GP) lipid transfection reagent (Gene Therapy Systems, Inc, San Diego, CA) was incorporated within CG scaffolds using Methods 1 and 2. For the groups incorporating the GP transfection reagent with

pIGF-1, GenePorter/pIGF-1 complexes were made using a 5:1 (μl/μg) ratio of GP:plasmid and was used as the plasmid solution for incorporation within CG scaffolds as described above.

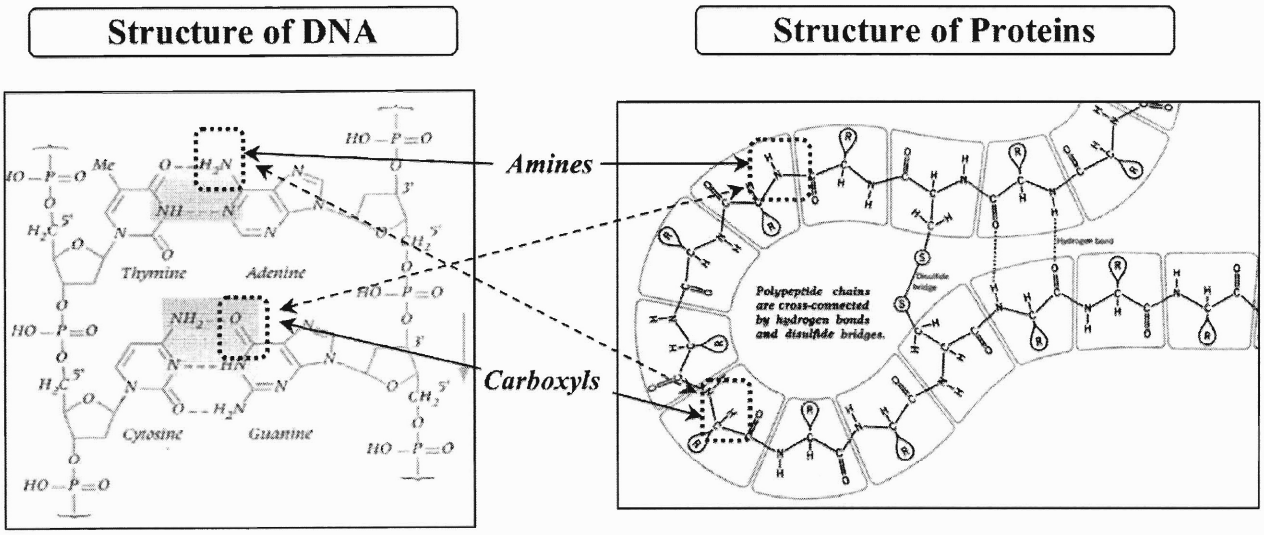


Figure 3.1 Potential peptide bonds (dotted arrows) that can form between plasmid DNA and collagen proteins during plasmid incorporation within the collagen-GAG scaffold by EDAC cross-linking.

3.2.5. Plasmid Release from CG Scaffolds and Analysis of Structural Integrity

GSCG scaffolds synthesized with a 20 μg pIGF-1 load and using the two types of incorporation methods described above, were submerged in TE buffer (pH = 8) over a 2-week period to investigate the plasmid release kinetics. At selected time points, buffer was collected from the samples and the wells were replenished with equal volumes of fresh buffer. After two weeks, GSCG scaffolds were digested overnight using Proteinase K (Roche Diagnostics, Indianapolis, IN) at 60 °C to release the plasmid remaining within the scaffolds. Plasmid released in the buffer and retained in the scaffolds was quantified by the Picogreen assay (Molecular Probes, Inc, Eugene, OR). Aliquots of the released and residual plasmid were analyzed by gel electrophoresis for comparison with the stock pIGF-1 used to synthesize the GSCG scaffolds. Samples run on the gel were digested with the restriction enzyme, XbaI (New England BioLabs, Ipswich, MA) to assess plasmid integrity.

3.2.6. Transmission Electron Microscopy of GSCG Scaffolds

Nonseeded Control and GSCG scaffolds (made using Method 2), fixed in glutaraldehyde and dehydrated in ethanol, were examined by transmission electron microscopy (TEM).

Samples were postfixed in osmium tetroxide and embedded in Epon. Images of ultrathin sections (~600 nm) of the scaffolds were obtained on a Phillips TEM (FEI EM410) at 80kV.

3.2.7. Chondrocyte Isolation and Expansion

Chondrocytes were isolated from the trochleae of both knees (stifle joints) from one adult mongrel dog (approximate age 2-4 yrs). The cells were obtained using a sequential digestion of pronase (20 U/ml, 1 hr) and collagenase (200 U/ml, overnight) as previously described[28]. Isolated chondrocytes were expanded in monolayer culture using "Medium 2" described in the previous chapter, consisting of high glucose Dulbecco's modified Eagle's medium, DMEM (4.5 g/L D-glucose, without L-glutamine and with 1mM sodium pyruvate), containing 10% (v/v) fetal bovine serum (FBS), 0.1mM nonessential amino acids, 10 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic (HEPES) buffer, 100 U/mL penicillin, and 100 μg/mL streptomycin glutamate. The medium was supplemented with the following growth factors (all from R&D Systems, Minneapolis, MN): 5 ng/mL of fibroblast growth factor-2 (FGF-2), 10 ng/mL of platelet-derived growth factor-bb (PDGF-bb), 1 ng/mL of transforming growth factor-β1 (TGF-β1). The cells were incubated at 37 °C and 5% CO_2. Once cells reached confluence, they were trypsinized, resuspended, and re-plated to obtain passage (P)1 cells for seeding into the scaffolds.

3.2.8. Nonviral Gene Transfer in GSCG Scaffolds

Two separate experiments were carried out to assess the behavior of chondrocytes seeded within the GSCG scaffolds. The first experiment used a 4 μg plasmid load per scaffold and compared the pIGF-1 treatment with and without the GP reagent prepared using Method 1 (soak and freeze-dry method). The second experiment used a 10 μg plasmid load per scaffold and compared the two different plasmid solutions using Method 2 (cross-linking the plasmid to scaffolds). After GSCG scaffolds were prepared and pre-wet in culture medium, scaffolds were dried briefly on sterile filter paper, and placed on agarose-coated 12-well tissue culture plates. For the first experiment, 2 million cells were seeded onto each scaffold by pipetting a 20 μl suspension containing half of the total amount of cells on each side of the scaffold with a 10 minute incubation period in between. By this static seeding method approximately 80% of the seeded chondrocytes have been found to attach to the scaffolds. For the second experiment, the

amount of cells seeded on to the scaffolds was increased to 4 million cells per scaffold in order to increase the rate of scaffold degradation and facilitate the release of the plasmid cross-linked to the collagen fibers. The same pipette seeding method was used in the second experiment.

Cell-seeded scaffolds were cultured in a defined serum-free medium (SFM), found in previous work[29] to enhance differentiation. The SFM consisted of high glucose DMEM (4.5 g/L D-glucose, without L-glutamine and with 1mM sodium pyruvate), 0.1 mM nonessential amino acids, 10 mM HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin glutamate, insulin-transferrin-selenium (ITS)$^{+1}$ (100x, Sigma Chemical, St. Louis, MO), 0.1 mM ascorbic acid 2-phosphate, 1.25 mg/ml bovine serum albumin, 10 ng/mL of TGF-β1, and 100 nM dexamethasone. Medium was collected and changed at various time points over a two-week culture period to assess the amount of IGF-1 released from transfected cells.

The amount of IGF-1 in the collected medium (n = 3-6) released from the cell-seeded scaffolds was detected by a sandwich ELISA kit for the human IGF-1 protein (R&D Systems, Minneapolis, MN). The IGF-1 values were reported as rates of release by dividing by the time period since the last media exchange, and as accumulated IGF-1 by summing the values of the IGF-1 in the medium samples over time. Cultures were terminated after 2 weeks for histological evaluation and biochemical analysis of the DNA and GAG contents of the constructs. For DNA and GAG analysis, scaffolds were lyophilized and enzymatically digested using proteinase K (Roche Diagnostics, Indianapolis, IN).

Chondrocytes cultured within scaffolds incorporating pEGFP were observed under fluorescence and confocal microscopy at various time points to visualize chondrocyte transfection over time. Constructs were placed on a sterile glass bottom petri dish to observe under the microscope and then returned to the tissue culture wells containing medium.

3.2.9. DNA Analysis

The DNA content of cell-seeded scaffolds was measured using the Picogreen Dye assay kit (Molecular Probes, Inc, Eugene, OR) (n = 4). The Picogreen dye was used with the reagents and standard provided according to the manufacturer instructions.

3.2.10. GAG Analysis

The sulfated GAG content of cell-seeded scaffolds after the 2-week culture period was determined by the dimethylmethylene blue (DMMB) dye assay[30] (n = 4). An aliquot of the proteinase K digest was mixed with the DMMB dye and the absorbance at 525 nm was measured with a spectrophotometer. The results were obtained by extrapolating from a standard curve using shark chondroitin-6-sulfate. Newly accumulated GAG was determined by subtracting the unseeded values from the sample values. The GAG content was normalized to the estimated volume of the cell-seeded scaffolds; reported as the GAG density.

3.2.11. Histology and Immunohistochemistry of Cell-Seeded Scaffolds

Cell-seeded scaffolds (n = 2-3) were fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, and center-cut sections (6μm thick) were stained with Safranin-O for the presence of sulfated GAG. For type II collagen immunohistochemical analysis, sections were enzymatically digested by protease type XIV for 45 minutes and stained with a standard avidin-biotin complex peroxidase-based antibody staining technique (Vectastain, Vector Laboratories, Burlingane, CA). Mouse anti-chick monoclonal antibody for type II collagen was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA).

3.2.12. Statistical Analysis

Data were analyzed by one- or two-factor analysis of variance (ANOVA), and the Fisher's protected least squares differences (PLSD) post-hoc test using StatView (SAS Institute Inc, Cary, NC). Data are presented as mean ± standard error of the mean.

3.3. RESULTS

3.3.1. IGF-1 Plasmid Release from GSCG Scaffolds—Release Kinetics and Plasmid Integrity

There was a dramatic difference in the amount of pIGF-1 initially incorporated into the GSCG scaffolds and the plasmid release profiles comparing the two different incorporation methods (Fig. 3.2). Approximately 60% of the loaded plasmid (20 μg) was incorporated in

scaffolds using Method 1 (soak and freeze-dried), whereas, ~40% was incorporated using Method 2 (cross-linking plasmid to scaffold). GSCG scaffolds synthesized by Method 1, however, released most of the incorporated plasmid (~83%) within the first two days of soaking in the TE buffer solution. Only 12% of incorporated plasmid remained in the Method 1 scaffolds at the end of the 2-week plasmid release study (Fig. 3.2). In contrast, GSCG scaffolds with plasmid cross-linked to the scaffold (Method 2) released only minute amounts of plasmid in the buffer during the leaching period, resulting in ~99% plasmid retention after 2 weeks in buffer (Fig. 3.2).

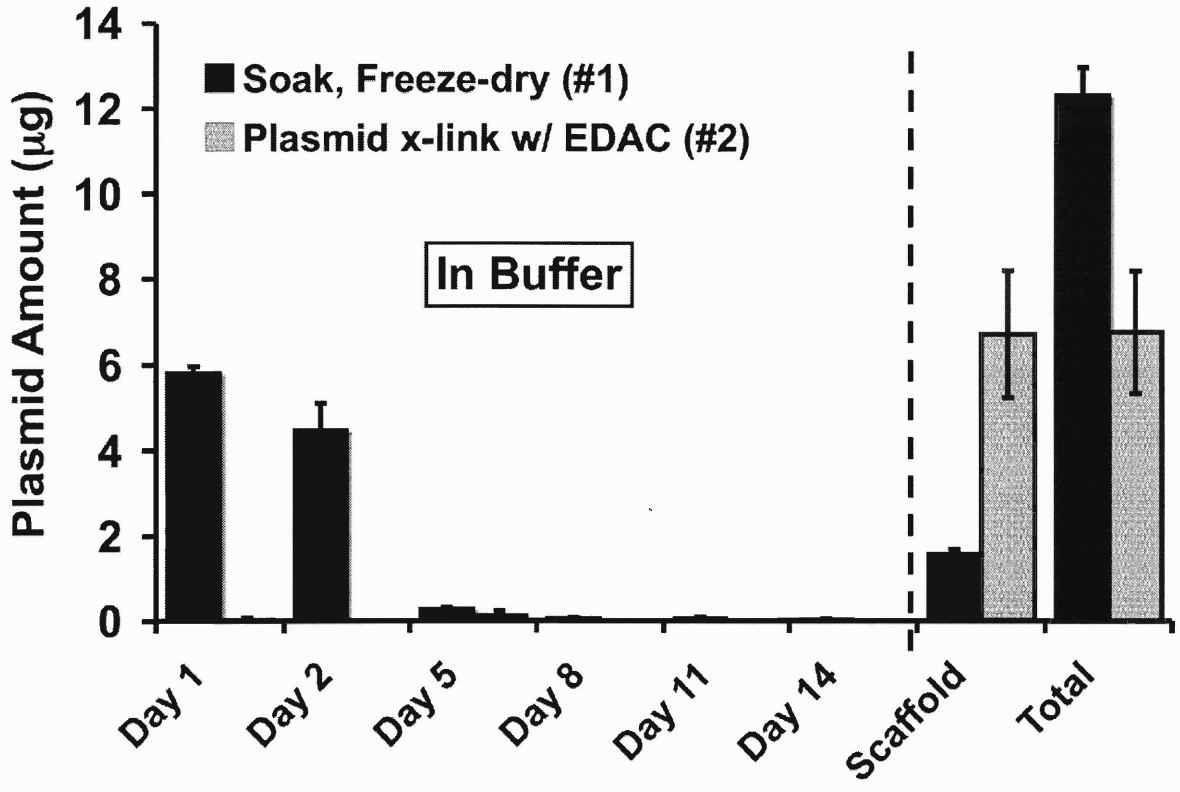


Figure 3.2 Plasmid amounts detected in the leaching buffer and remaining in the GSCG scaffolds after the 2-week leaching study (scaffolds loaded with 20µg plasmid IGF-1). n = 3; mean ± SEM.

Since most of the plasmid from GSCG scaffolds produced with Method 1 was released in the buffer, and most of the plasmid incorporated in GSCG scaffolds synthesized using Method 2 was retained in the scaffolds, plasmid released in the buffer for Method 1 samples and plasmid retained in the scaffolds for Method 2 samples were analyzed by gel electrophoresis for comparison with the original pIGF-1 stock solution. Fig. 3.3 shows the gel containing the cut

and uncut IGF-1 plasmid stock solution (columns b and c, respectively); cut and uncut plasmid released in the buffer from Method 1 samples (column d and e, respectively); and cut and uncut plasmid obtained from digesting the Method 2-synthesized GSCG scaffolds after the two week leach period (columns f and e, respectively). The plasmid released in the buffer from the Method 1-prepared samples and the plasmid retained in the scaffold for the Method 2-prepared samples retained similar migration characteristics compared to the IGF-1 plasmid stock with and without restriction enzyme digestion (Fig. 3.3), thus demonstrating that the structural integrity of plasmid released from and retained in the GSCG scaffolds was maintained.

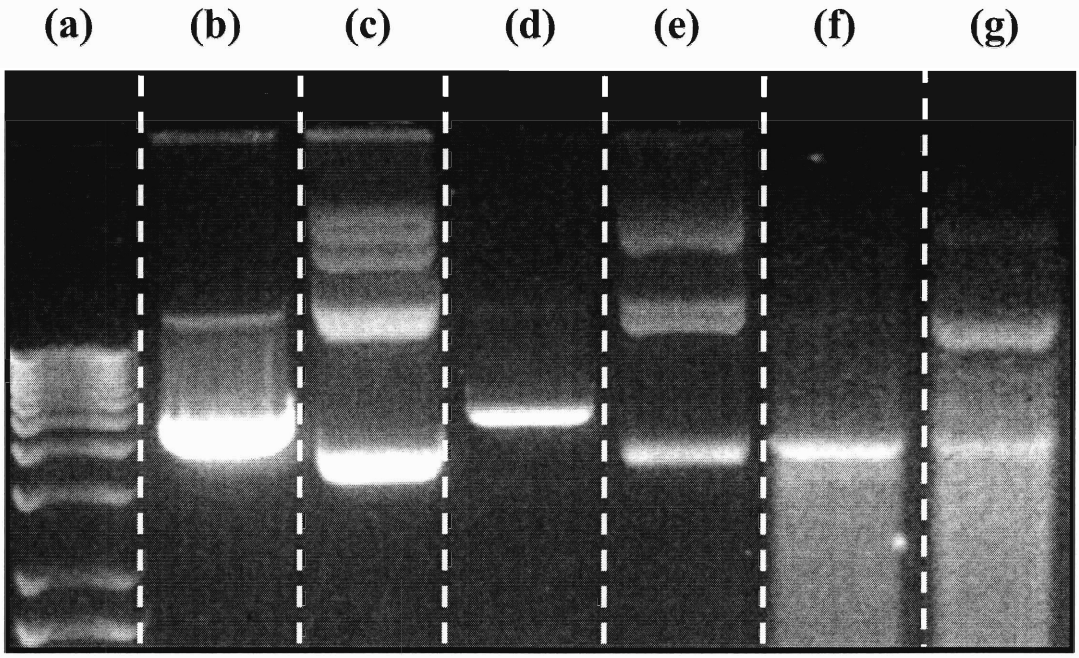


Figure 3.3 Gel electrophoresis of 1Kb DNA ladder (a); IGF-1 plasmid stock solution (b) cut and (c) uncut; plasmid released from Method 1-synthesized GSCG scaffolds (d) cut and (e) uncut; and plasmid retained within Method 2-synthesized GSCG scaffolds after the 2-week leaching study (f) cut and (g) uncut. Restriction enzyme XbaI was used to cleave the various IGF-1 plasmid samples.

3.3.2. *TEM of Unseeded GSCG Scaffolds*

Transmission electron micrographs of CG scaffolds incorporating pIGF-1 alone showed a thin darker plasmid layer lining the wall of of the scaffold strut (Fig. 3.4a) compared to no apparent dark lining in Control samples (Fig. 3.4b). Scaffolds incorporating GP/IGF complexes, on the other hand, revealed aggregation of the complexes and showed clusters of particles lining the walls of the scaffold struts (Fig. 3.4c).

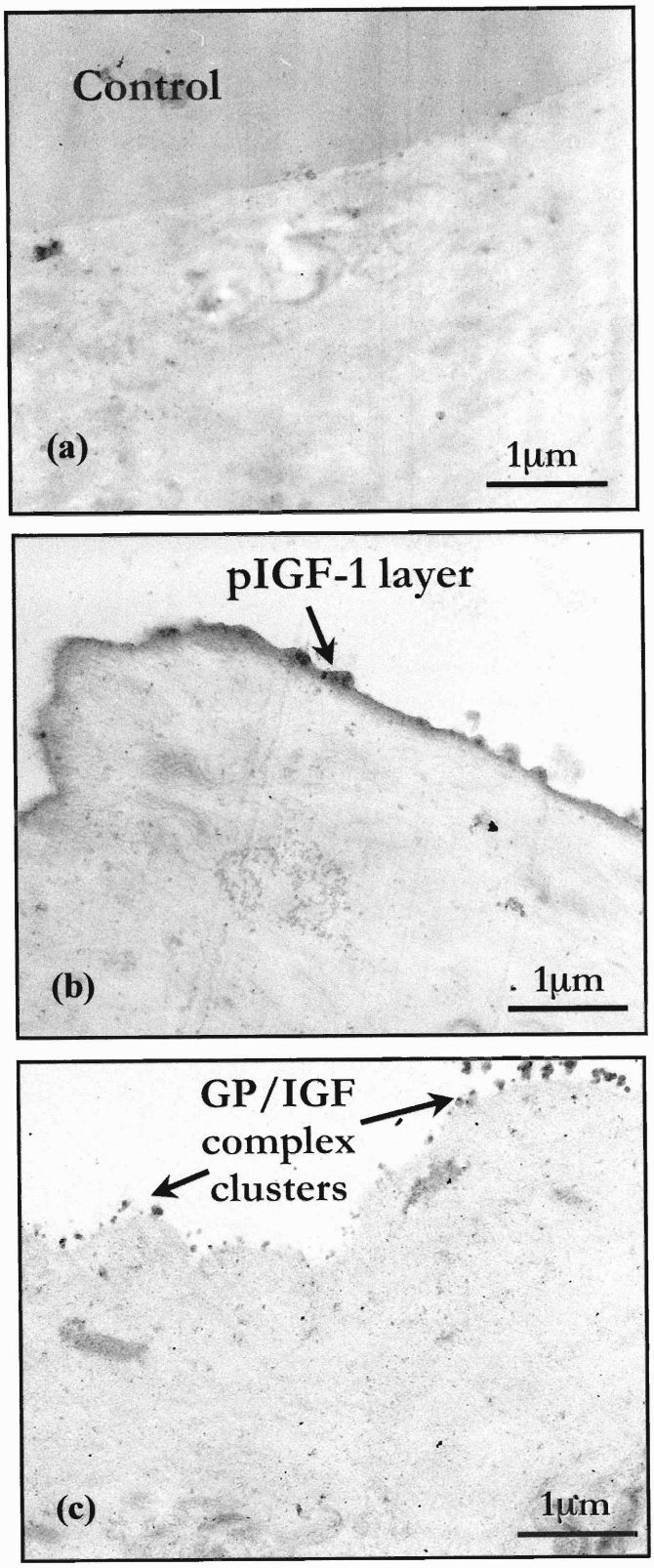


Figure 3.4 **Transmission electron micrographs of Control (a) and GSCG scaffolds incorporating pIGF-1 alone (b) or GP/IGF complexes.**

3.3.3. Nonviral Gene Transfer to Chondrocytes Seeded in GSCG Scaffolds

For GSCG scaffolds synthesized using Method 1, there was no difference in the IGF-1 release profiles for scaffolds incorporating naked pIGF-1 alone (IGF group) and the Control group (Fig. 3.5a). Incorporation of the GP transfection reagent (GP/IGF group) into the CG scaffolds, however, showed significant elevations of IGF-1 release in the medium above the controls for all media collections except for the last collection at Day 14 (Fig. 3.5a). The GP/IGF group showed a peak in IGF-1 release rate after about one week in culture (~20 pg/hr), after which there was a rapid decline from Day 11 to Day 14. There was no difference in the amount of IGF-1 detected in the Day 14 collection for the GP/IGF group compared to the control and IGF groups. After the two-week culture period, the GP/IGF group showed a 7-fold higher accumulated IGF-1 level at around 5 ng/ml versus the control and IGF groups at about 0.7 ng/ml (Fig. 3.5b). Two-factor ANOVA showed a significant effect of time ($P < 0.01$, power = 0.88) and type of gene supplementation ($P < 0.0001$, power = 1) on IGF-1 release.

GSCG scaffolds synthesized by cross-linking plasmid IGF-1 to the collagen scaffolds with (x-GP/IGF) or without (x-IGF) the transfection reagent (Method 2) resulted in noticeable elevated IGF-1 expression levels above the Controls (Fig. 3.5c). Like the GSCG scaffolds prepared using Method 1, scaffolds incorporating plasmid IGF-1 complexed to the transfection reagent produced significantly higher levels of IGF-1 compared to control scaffolds or scaffolds containing plasmid IGF-1 alone (Fig. 3.5c). Scaffolds containing the GP reagent and prepared using Method 2 (x-GP/IGF group) showed a significant increase in IGF-1 release rate between the first two collection time points, followed by a steady release between collections 2 and 4, and finally a rise in the IGF-1 release rate to about 14 pg/hr at the end of the 2-week culture period (Fig. 3.5c). There were 4.5-fold and 14.5-fold higher accumulated IGF-1 levels for the x-GP/IGF group compared to the x-IGF and Control groups, respectively (Fig. 3.5d). Two-factor ANOVA revealed a significant effect of time ($P < 0.0001$, power = 1) and type of gene supplementation ($P<0.0001$, power=1) on IGF-1 release from cell-seeded GSCG scaffolds fabricated using Method 2.

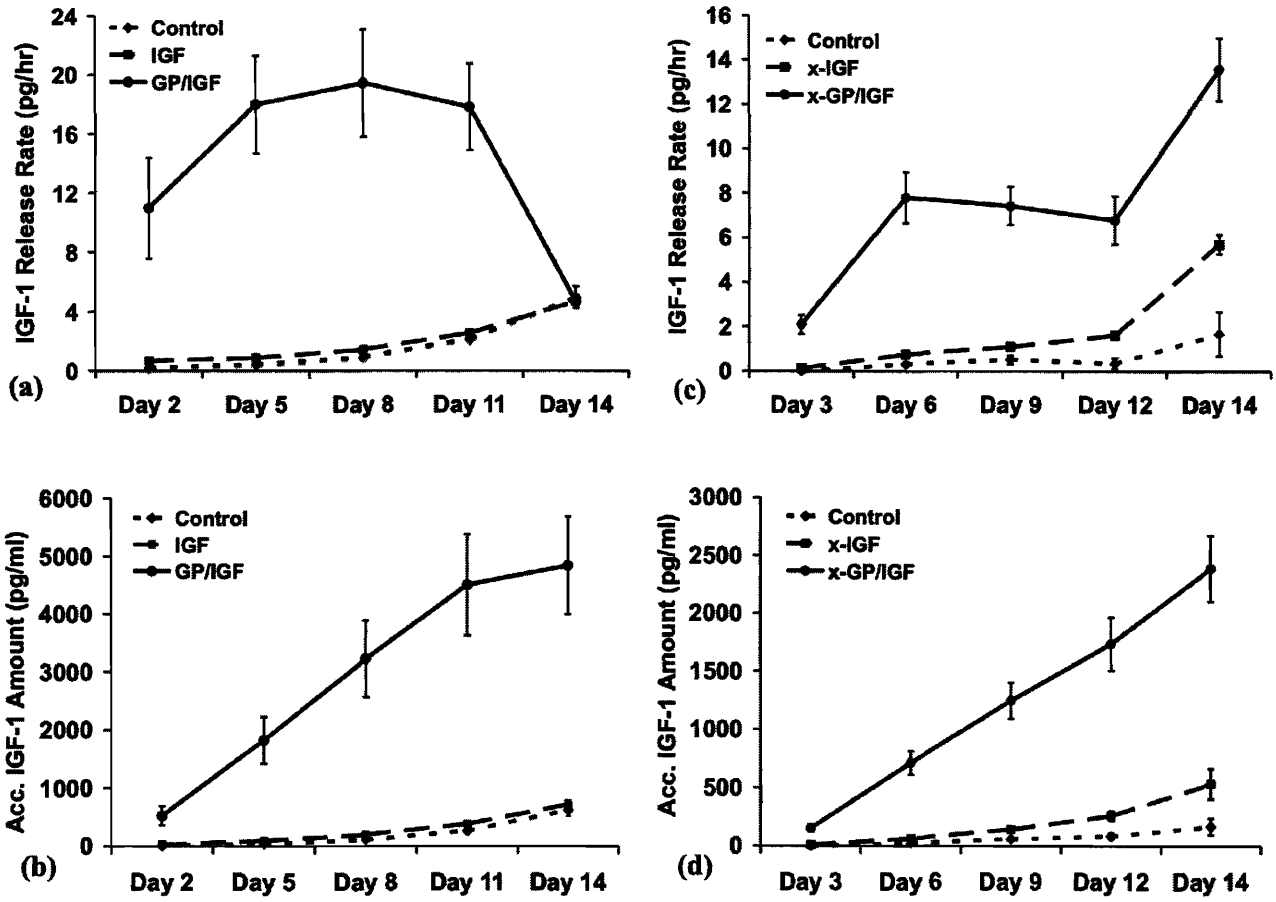


Figure 3.5 IGF-1 release rates (a, c) and accumulated IGF-1 (b, d) detected in the serum-free medium over the 2-week 3-D culture period from GSCG scaffolds synthesized using Method 1 (a, b), n = 3 and Method 2 (c, d), n = 6. mean ± SEM.

GFP expression in chondrocytes seeded within scaffolds supplemented with pEGFP with or without the transfection reagent using Method 2 was observed with fluorescence (Fig. 3.6a-c) and confocal microscopy (Fig. 3.6d). After 5 days in culture, chondrocytes seeded in Control scaffolds (no plasmid) did not show any evidence of GFP expression (Fig. 3.6a). Chondrocytes seeded in scaffolds supplemented with pEGFP alone (Fig. 3.6b) or with the GP transfection reagent (Fig. 3.6c) displayed evidence of transfection, with a significantly higher number of cells fluorescing in scaffolds incorporating the plasmid with the transfection reagent. Evidence of continued transfection (i.e. presence of fluorescing cells in the scaffold) was still apparent up to 2 months in the scaffolds. Confocal microscopy of GSCG scaffolds containing the plasmid/transfection reagent complex seven days after seeding showed that only a small percentage of cells in the scaffold were transfected at that time point (Fig. 3.6d).

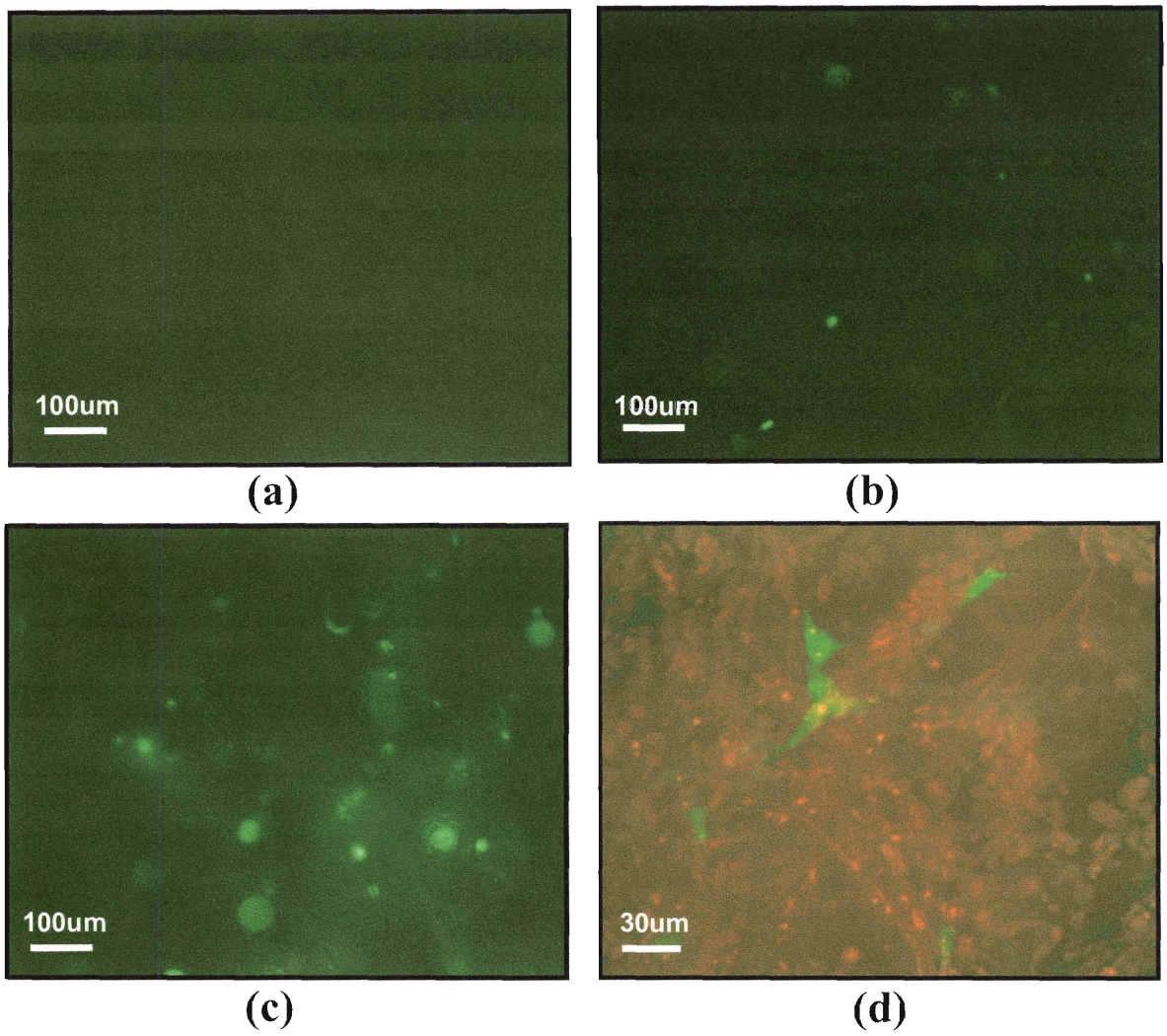


Figure 3.6 Fluorescent microscopy images of GSCG scaffolds synthesized using method 2 (a) without any supplementation (control), (b) with incorporation of pEGFP alone, and (c) with incorporation of pEGFP and the GenePorter ® transfection reagent after 5 days in 3-D culture. Confocal image of GSCG scaffold incorporating pEGFP and the GenePorter ® transfection reagent using Method 2, after 7 days in culture (d).

3.3.4. Biochemical Analysis of Cell-Seeded GSCG Scaffolds

Control scaffolds and scaffolds incorporating IGF-1 plasmid alone synthesized using Method 1 were similar in DNA content after two weeks in culture with ~10 μg DNA/scaffold (Fig. 3.7a). For the GP/IGF group created using Method 1, however, the DNA content was significantly lower, at about half the amount of DNA found in the IGF and Control groups (Fig. 3.7a). In contrast, GSCG scaffolds incorporating plasmid IGF-1 with the transfection reagent using Method 2 showed a 25% higher DNA content (~21 μg/scaffold) compared to the x-IGF and Control scaffolds (Fig. 3.7b). One-factor ANOVA showed a significant difference between

the GP/IGF group versus the IGF or Control groups ($P < 0.0001$, power = 1) and the x-GP/IGF group versus the x-IGF or Control groups ($P < 0.0001$, power = 1).

There was no significant difference in the accumulated GAG densities at the end of the 2-week culture period for Method 1-synthesized GSCG scaffolds and its controls, with GAG densities of about 1 $\mu g/mm^3$ for all groups (Fig. 3.7c). For Method 2-synthesized scaffolds, there was a significant elevation (~2-fold increase) in accumulated GAG density in the GSCG samples compared to the Controls ($P < 0.03$, power = 0.7). There was no significant difference in accumulated GAG density between the x-IGF and x-GP/IGF groups (Fig. 3.7d).

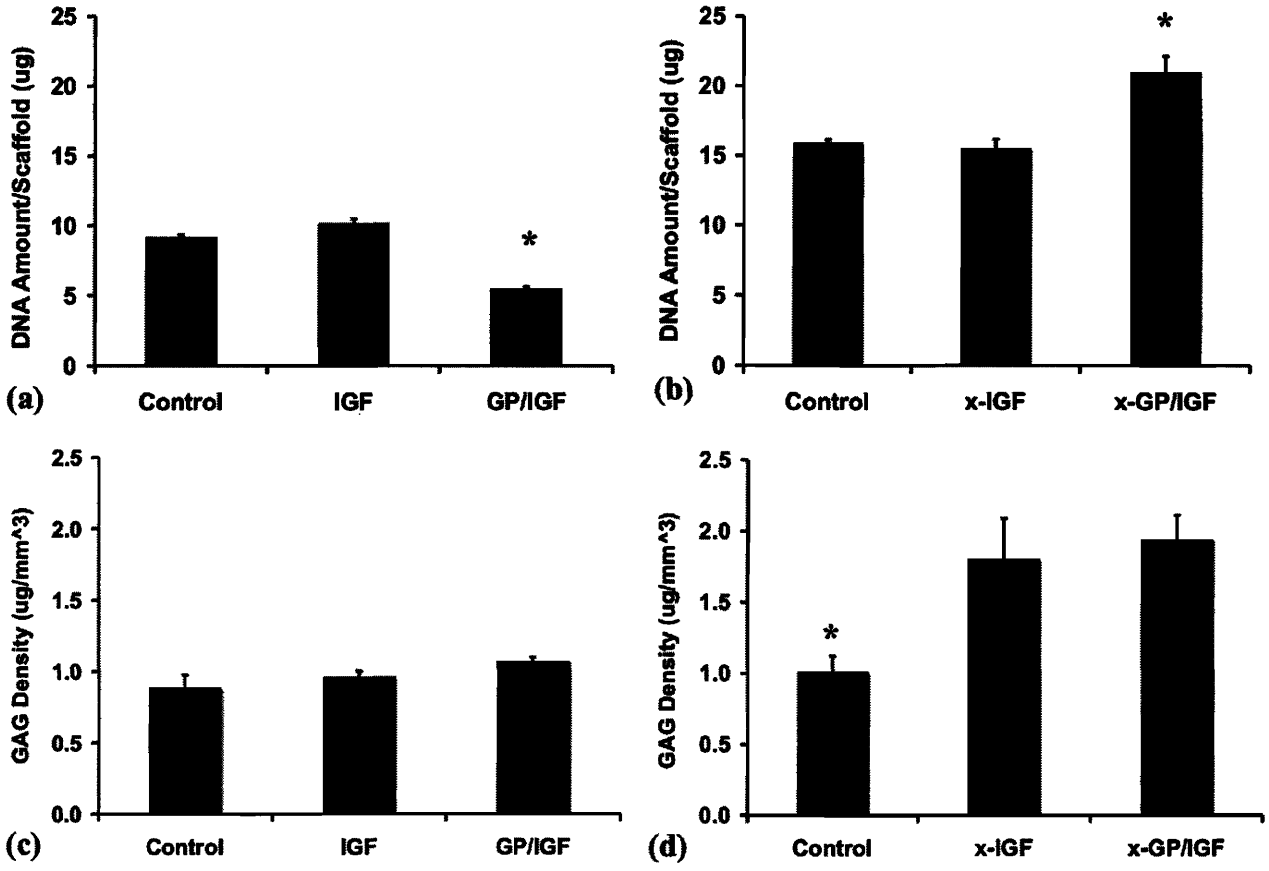


Figure 3.7 **DNA contents (a, b) and accumulated GAG densities (c, d) measured at the end of the 2-week 3-D culture period in GSCG scaffolds synthesized using Method 1 (a, c) and Method 2 (b, d). n = 3-4; mean ± SEM.**

3.3.5. Histology and Immunohistochemistry of 3-D Cultures

Histochemical results for the Controls and Method 1-synthesized GSCG scaffolds (with or without the transfection reagent) were all similar with regard to the amount of tissue formation, Safranin-O staining, and type II collagen staining. Most of the cells in these scaffolds were elongated fibroblast-like cells with evidence of some tissue formation in the pores. There was very little staining for GAG (Fig. 3.8a and c) or collagen type II (Fig. 3.8b and d) in the synthesized tissue and a significant amount of the scaffold was still present after the 2-week culture period.

Scaffolds prepared in the experiment using Method 2 to synthesize GSCG constructs, on the other hand, showed a greater amount of tissue formation even for Control scaffolds (Figs. 3.9 and 3.10). For all groups in this experiment, there were some cells present in the constructs that displayed a rounded chondrocytic morphology and were located in lacunae (see arrows in Fig. 3.10). The x-IGF and the x-GP/IGF groups showed more tissue formation, GAG staining and collagen type II staining compared to the Control group (Figs. 3.9 and 3.10). For the Control and x-IGF groups, more tissue formation was present at the outer faces of the constructs. The x-GP/IGF group showed the most tissue formation throughout the whole construct, number of cells with chondrocyte-like features, Safranin-O staining, and type II collagen staining (Figs. 3.9e and f; 3.10e and f) compared to the other groups. There was still evidence of the collagen scaffold still present for all groups (stained green in Fig. 3.9) after 2-weeks in 3-D culture.

Areas of histological sections that stained for GAG generally seemed to correlate with the areas that stained for type II collagen (Figs. 3.9 and 3.10), with more intense staining for GAG and type II collagen in areas with greater tissue synthesis. Within these areas, there was also a smaller amount of residual scaffold (indicating a greater degree of scaffold degradation), a higher cell density, and more cells displaying a chondrocytic phenotype (Figs. 3.9 and 3.10).

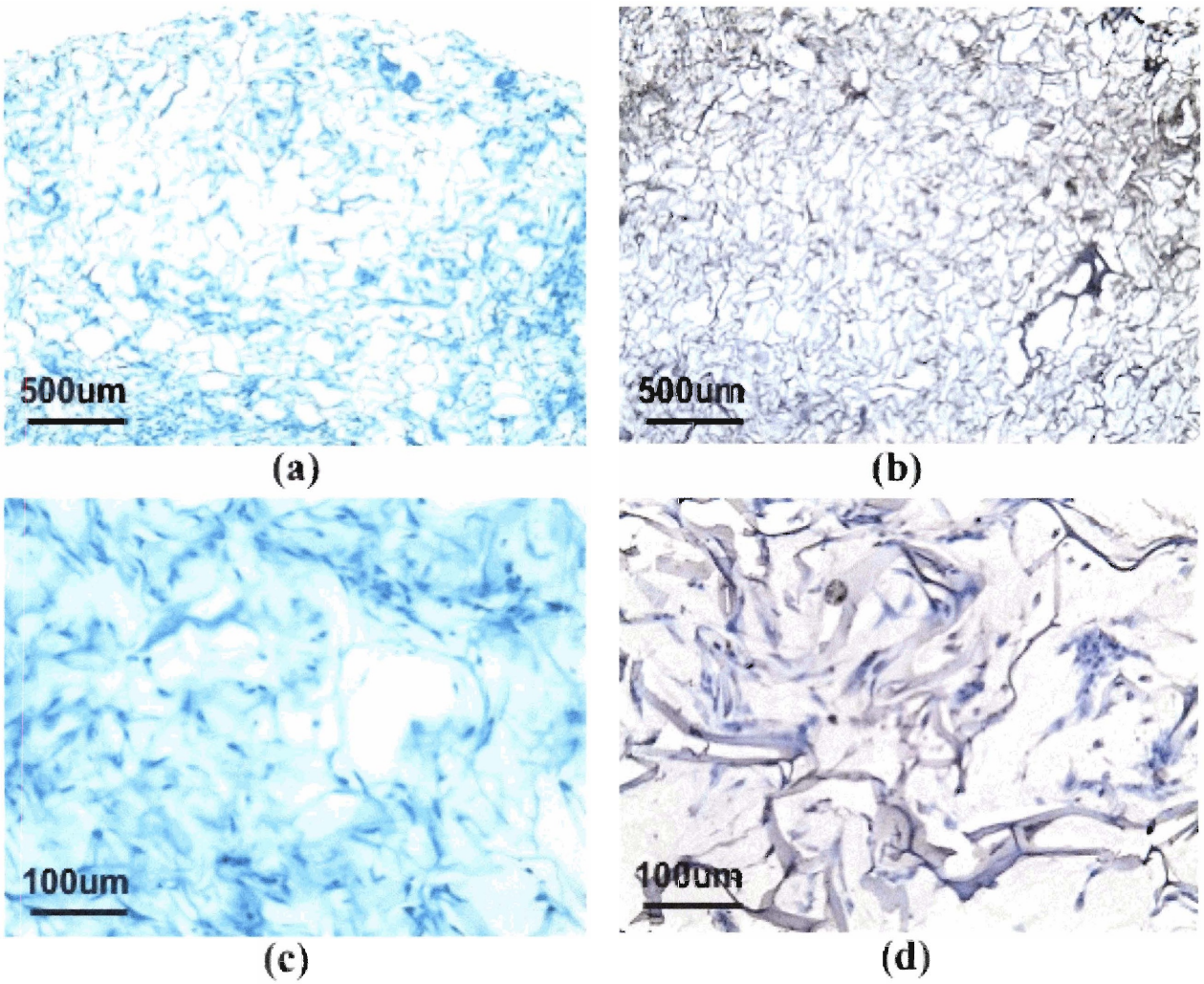


Figure 3.8 **Typical Safranin-O stain for GAG (a, c) and immunohistochemical stain for type II collagen (b, d) from chondrocyte-seeded Control scaffolds and GSCG scaffolds synthesized using Method 1 (with or without the GP reagent) after the 2-week culture period. 2 million cells per scaffold were seeded onto the scaffolds. Red is a positive stain for the presence of GAG and brown is a positive stain for the presence of type II collagen.**

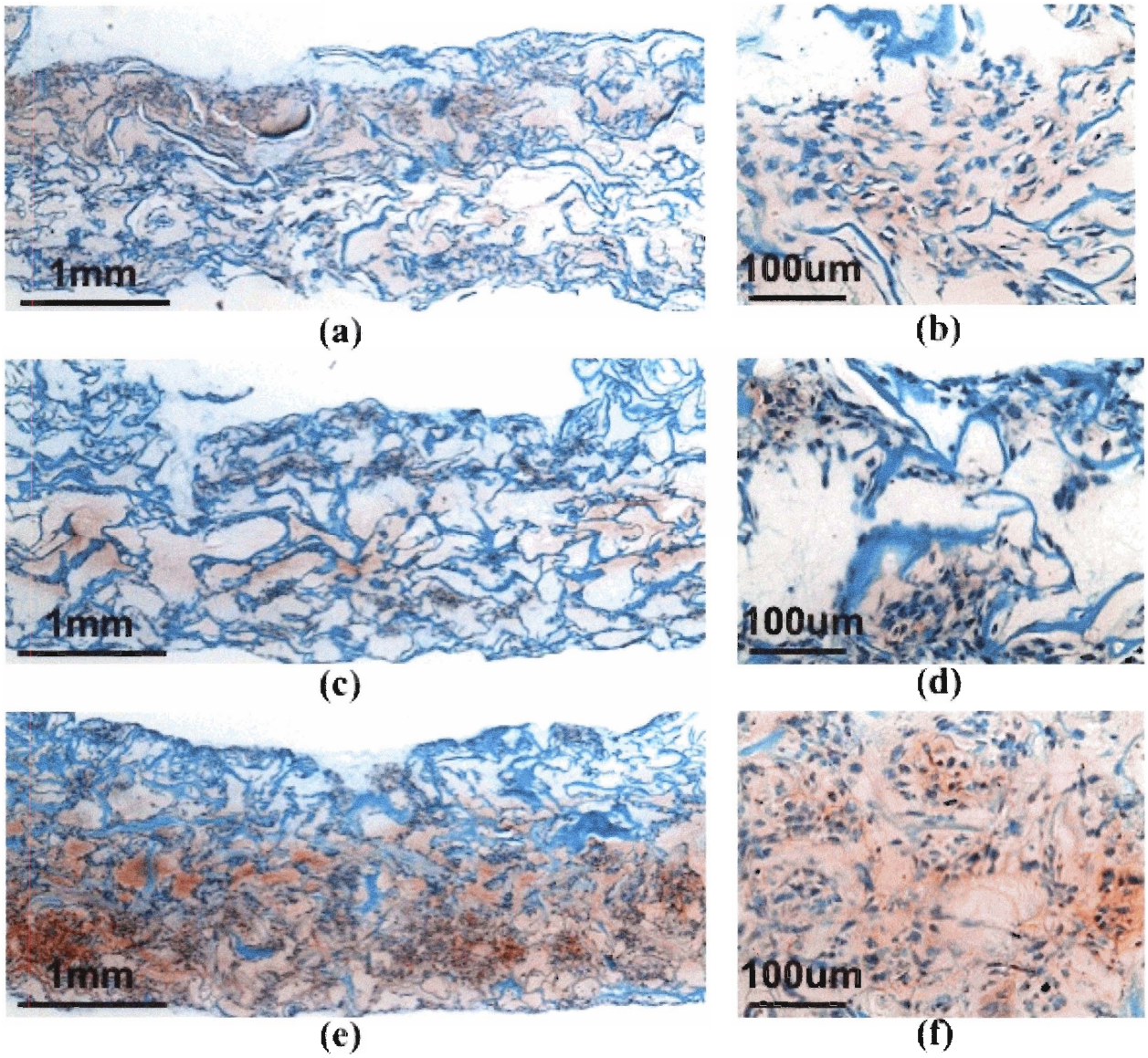


Figure 3.9 Safranin-O stain for GAG (red is positive stain) of scaffolds synthesized using Method 2 for Controls (a, b) and GSCG scaffolds incorporating IGF-1 plasmid alone (c, d) or the GP/IGF complexes (e, f) after 2-weeks in 3-D culture. 4 million cells were seeded onto the scaffolds.

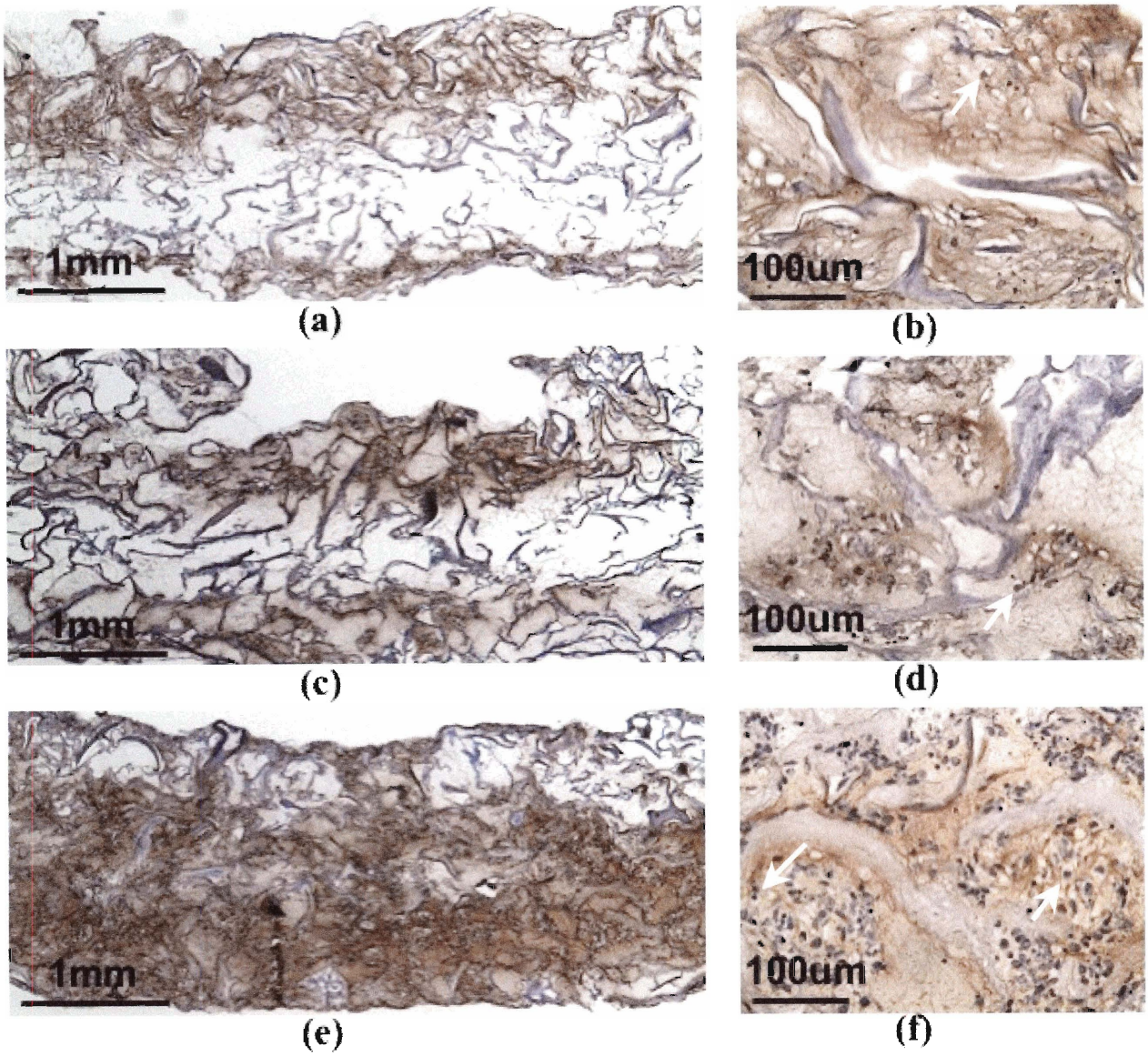


Figure 3.10 **Immunohistochemical stain for collagen type II (brown is positive stain) of scaffolds synthesized using Method 2 for Controls (a, b) and GSCG scaffolds incorporating plasmid IGF-1 alone (c, d) or the GP/IGF complexes (e, f) after 2-weeks in 3-D culture. 4 million cells were seeded onto the scaffolds.**

3.4. DISCUSSION

A notable finding of this study was that covalently linking as little as 10 μg of plasmid DNA to a type II collagen-GAG scaffold can result in the prolonged overexpression of the encoding growth factor, thus allowing (nonviral) gene transfer to be successfully combined with tissue engineering principles. Prior work using naked plasmid DNA incorporated within scaffolds have also demonstrated elevated levels of encoded protein production, but most of these studies required the use of significantly higher plasmid loads [7,31-35] (on the order of milligram levels per scaffold) compared to the current study. Control over plasmid release rates from GSCG scaffolds and incorporating a lipid transfection reagent (compared to the use of naked plasmid DNA alone) significantly improved prolonged expression and increased gene transfer to seeded cells.

The kinetics of IGF-1 plasmid release from these scaffolds can be modified using different incorporation methods. Two approaches were investigated to synthesize GSCG scaffolds and resulted in a dramatic variation in plasmid release kinetics over a 2-week leaching study. Plasmid incorporation by submerging scaffolds in a plasmid solution followed by a freeze-drying step resulted in a faster release of plasmid DNA, with about 82% of incorporated plasmid released within the first two days in buffer. It is likely that this passive release was due to the weak mechanical entrapment of the plasmid between the collagen fibrils, which resulted from the swelling and collapsing of the collagen fibrils during the supplementation and subsequent freeze-drying procedure. Once these scaffolds were re-hydrated in solution, the collagen fibrils swelled again and released the entrapped plasmid at a rate that was dependent on collagen swelling and diffusion kinetics. The 12% plasmid retained in these GSCG scaffolds after the 2-week culture may have been due to plasmid that was more securely entangled within the collagen network, and which would only be released upon scaffold degradation. Chemically cross-linking the plasmid to the scaffold, on the other hand, resulted in 99% retention of incorporated plasmid in the scaffold after the 2-week leach study. Although only 40% of loaded plasmid was actually incorporated into the scaffold using this procedure (as opposed to 60% incorporation with Method 1), the fact that most of the plasmid resisted passive release and remained within the scaffold commends this method of supplementation for prolonged release. In this case, incorporated plasmid would only be released upon scaffold degradation, and

therefore transfection and subsequent gene expression could occur as long as residual scaffold remains over the time course of tissue repair. Of importance was that the integrity and functionality of the pIGF-1 released in buffer or remaining in GSCG constructs was preserved, indicating that the plasmid structure was not significantly altered by the interaction with the collagen scaffold.

The kinetics of plasmid release from GSCG scaffolds had a direct effect on the amount and kinetics of IGF-1 protein synthesized by seeded and subsequently transfected chondrocytes. Interestingly, chondrocytes placed in a scaffold without any plasmid supplementation also showed production of IGF-1 that was released in the medium after about one week in 3-D culture, albeit in very low amounts. This IGF-1 expression by non-transfected chondrocytes may have been stimulated by the cellular interaction with the type II collagen-GAG scaffold. For Method 1-synthesized scaffolds where most of the incorporated plasmid was released within the first 2 days in solution, there was no overexpression of IGF-1 over the controls for scaffolds containing naked plasmid IGF-1 alone. With the transfection reagent, there was a significant elevation of IGF-1 detected in the medium over control scaffolds, however, IGF-1 release rates reached a maximum after about a week and then declined to control levels at the end of the 2-week culture period. This peak and decline in IGF-1 release may indicate that transfection of the seeded cells may have only occurred near the beginning of the culture period. The decline to control IGF-1 levels at the end of the 2-week period was evidence that GP/IGF complexes most likely were no longer present in the scaffold at the end of the two week culture period, and that the transient expression of earlier transfected cells had faded.

Unlike GSCG scaffolds made using Method 1, scaffolds created by cross-linking naked plasmid IGF-1 alone demonstrated noticeable elevations in IGF-1 released in the medium over controls. As opposed to a fast release of plasmid only at the beginning of the culture period as displayed by Method 1-synthesized GSCG scaffolds, it is speculated that cross-linking the plasmid alone to the collagen scaffold (x-IGF group) may have allowed for a continued presence and concentration of plasmid as the scaffold was degraded by the seeded cells. This prolonged concentration of plasmid may have increased the probability of cellular uptake of the plasmid leading to subsequent gene expression. Cells seeded in GSCG scaffolds incorporating both the plasmid and transfection reagent using Method 2 resulted in increased release rates up to one week, followed by a steady release of IGF-1, and finally a spike in the IGF-1 release rate within

the last 2 days of the 2-week culture. This IGF-1 release profile may indicate the continued transfection of seeded cells throughout the culture period (and further on) as the scaffold degrades. It is important to note that the actual levels of IGF-1 localized within the scaffold could be significantly higher than the concentrations detected in the medium. Future work will need to investigate methods to quantify IGF-1 produced by transfected cells that is retained within the scaffold in order to determine the minimum *local* therapeutic concentration required for enhanced biosynthesis.

Visual evidence of prolonged gene expression using GSCG scaffolds was demonstrated by incorporating the EGFP gene using Method 2. The presence of fluorescing cells within GSCG scaffolds, resulting from successful gene transfection, continued up to about 2 months in 3-D culture. Although confocal imaging demonstrated that only a small percentage of cells showed gene expression at any given time-point, a high transfection efficiency may not be required to produced local therapeutic levels of desired growth factors. These findings prove that not only can these GSCG scaffolds maintain both plasmid integrity and functionality, but they can also facilitate gene transfer to seeded cells resulting in prolonged and elevated gene expression.

Of interest is the effect of the plasmid and the lipid transfection reagent on the resulting cell number (reflected in the DNA measurements) at the end of the 2-week culture period. Of note is the lower DNA content for the GP/IGF group in the Method-1 prepared scaffolds and the elevated DNA content for the x-GP/IGF group in the Method-2-fabricated GSCG scaffolds. These results seem to indicate that supplementing collagen scaffolds with the GP/IGF complexes using Method 1 may interfere with initial cell attachment. It is speculated that the lipid/plasmid complexes may have coated the walls of the collagen scaffold, which could have masked some of the ligands present on the collagen fibrils preventing integrin-mediated cellular attachment directly to the scaffold walls. Cells that were seeded onto these scaffolds may have effectively associated with the lipid/plasmid "coating" but then could have become detached once the collagen fibrils swelled and released the weakly attached complexes. On the other hand, chemical cross-linking these lipid/plasmid complexes to the collagen scaffold may still have resulted in the association of cells with the lipid/plasmid complexes incorporated into the scaffold, but detachment of cells could have been prevented due to the stronger chemical bond between the lipid/plasmid complexes and the collagen fibrils. Future work using electron

microscopy may be needed to further understand the cellular interactions with these GSCG scaffolds.

Although the DNA content that was measured in the scaffolds may have included the plasmid remaining from gene supplementation of the scaffolds as well as the nuclear material from the cells, measurements of plasmid DNA loaded into GSCG scaffolds with the transfection reagent (data not presented) showed that when plasmid was complexed to the transfection reagent it could not be detected by the Picogreen assay. The presence and retention of the GP/IGF complexes within the scaffolds was indirectly determined through functional assays (i.e., gene expression of IGF-1 or GFP proteins), which did indicate that plasmid was present within the scaffolds. It is speculated that the lipid transfection reagent may have prevented the Picogreen dye from interacting with the plasmid DNA, leading to the lack of detection. The majority of the DNA content reported for the GSCG groups that incorporated the GP/IGF complexes, therefore, was most likely a reflection of the number of cells present at the end of the 2-week culture period.

The effect of IGF-1 overexpression of cells seeded in GSCG scaffolds on biosynthesis was assessed by the accumulated GAG density amounts and histochemical analysis after 2-weeks in 3-D culture. For scaffolds synthesized by Method 1, there was no significant difference among the three groups with regard to accumulated GAG density and histochemical staining. All of these scaffolds showed minimal staining, if any, for Safranin-O or type II collagen. GSCG scaffolds synthesized by Method 2, however, showed a much higher amount of tissue formation, more GAG synthesis, and the presence of type II collagen, even for the controls, compared to scaffolds fabricated with Method 1. This difference in histogenesis may have been due to an insufficient cell-seeding density used in the Method 1-synthesized scaffolds (2 million cells/scaffold). Even though IGF-1 was significantly elevated in the GP/IGF group, its effects on biosynthesis and chondrogenesis may not have been apparent if the cell number was not high enough to result in a measurable difference in tissue formation. Prior studies [36,37] have demonstrated the importance of cell density on biosynthesis and chondrogenesis in 3-D culture. Future work needs to investigate if increasing the cell seeding number in GSCG scaffolds supplemented using Method 1 will show the effect of IGF-1 overexpression on biosynthesis and chondrogenesis.

In contrast to the experiment using the Method 1-prepared scaffolds, there was a noticeable difference in tissue formation among the groups in the cultures using the cross-linking method of gene-supplementation. In this study, 4 million cells were seeded per scaffold. The effect of elevated IGF-1 levels over the control group for the x-IGF and x-GP/IGF groups was reflected in the higher accumulated GAG densities. Both of the groups with plasmid supplementation showed an almost two-fold increase in accumulated GAG density over controls. Although there was a slightly higher GAG density for the x-GP/IGF group over the x-IGF group, this difference was not significant. The difference in GAG synthesis was more apparent in the histological staining for Safranin-O, where most tissue formation and GAG synthesis was present in the x-GP/IGF group. Scaffolds incorporating plasmid IGF-1 with the transfection reagent also showed the most collagen type II staining and the highest number of cells located in lacunae and displaying a rounded chondrocyte-like morphology. The x-IGF group also displayed a greater degree of histogenesis, GAG synthesis, and type II staining compared to the Control group. Of note in the histological results is the association among cell density, degree of scaffold degradation, and amount of tissue formation and chondrogenesis. These findings demonstrate that IGF-1 overexpression by cells transfected when seeded within GSCG scaffolds can result in enhanced biosynthesis and chondrogenesis.

In conclusion, a more prolonged release of plasmid from GSCG scaffolds can be successfully accomplished by covalently linking the plasmid to the CG scaffold with a carbodiimide cross-linking treatment. The kinetics of IGF-1 plasmid release has a direct effect on gene expression and IGF-1 release over time from adult canine articular chondrocytes seeded within GSCG scaffolds. Furthermore, incorporating a lipid transfection reagent in conjunction with the plasmid DNA significantly increases gene transfer and subsequent protein synthesis. A local, elevated, and prolonged overexpression of IGF-1 by transfected cells seeded within GSCG scaffolds (with or without a transfection reagent) can result in enhanced cartilage formation.

3.5. REFERENCES

1. Brower-Toland BD, Saxer RA, Goodrich LR, Mi Z, Robbins PD, Evans CH, et al. Direct adenovirus-mediated insulin-like growth factor I gene transfer enhances transplant chondrocyte function. Hum Gene Ther 2001 Jan 20;12(2):117-29.

2. Gelse K, von der Mark K, Aigner T, Park J, Schneider H. Articular cartilage repair by gene therapy using growth factor-producing mesenchymal cells. Arthritis Rheum 2003 Feb;48(2):430-41.

3. Grande DA, Mason J, Light E, Dines D. Stem cells as platforms for delivery of genes to enhance cartilage repair. J.Bone Joint Surg.Am. 2003;85-A Suppl 2:111-116.

4. Madry H, Kaul G, Cucchiarini M, Stein U, Zurakowski D, Remberger K, et al. Enhanced repair of articular cartilage defects in vivo by transplanted chondrocytes overexpressing insulin-like growth factor I (IGF-I). Gene Ther. 2005 Aug;12(15):1171-1179.

5. Jang JH, Houchin TL, Shea LD. Gene delivery from polymer scaffolds for tissue engineering. Expert Rev.Med.Devices 2004 Sep;1(1):127-138.

6. Doukas J, Chandler LA, Gonzalez AM, Gu D, Hoganson DK, Ma C, et al. Matrix immobilization enhances the tissue repair activity of growth factor gene therapy vectors. Hum.Gene Ther. 2001 May 1;12(7):783-798.

7. Bonadio J, Smiley E, Patil P, Goldstein S. Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. Nat.Med. 1999 Jul;5(7):753-759.

8. Goldstein SA. In vivo nonviral delivery factors to enhance bone repair. Clin.Orthop.Relat.Res. 2000 Oct;(379 Suppl)(379 Suppl):S113-9.

9. Scherer F, Schillinger U, Putz U, Stemberger A, Plank C. Nonviral vector loaded collagen sponges for sustained gene delivery in vitro and in vivo. J.Gene Med. 2002 Nov-Dec;4(6):634-643.

10. Jang JH, Shea LD. Controllable delivery of non-viral DNA from porous scaffolds. J Control Release 2003 Jan 9;86(1):157-68.

11. Luu YK, Kim K, Hsiao BS, Chu B, Hadjiargyrou M. Development of a nanostructured DNA delivery scaffold via electrospinning of PLGA and PLA-PEG block copolymers. J.Control.Release 2003 Apr 29;89(2):341-353.

12. Fu H, Hu Y, McNelis T, Hollinger JO. A calcium phosphate-based gene delivery system. J.Biomed.Mater.Res.A. 2005 Jul 1;74(1):40-48.

13. Shea LD, Smiley E, Bonadio J, Mooney DJ. DNA delivery from polymer matrices for tissue engineering. Nature Biotech. 1999;17:551-554.

14. Samuel RE, Lee CR, Ghivizzani S, Evans CH, Yannas IV, Olsen BR, et al. Delivery of plasmid DNA to articular chondrocytes via novel collagen-glycosaminoglycan matrices. Human Gene Therapy 2002;13:791-802.

15. Fortier LA, Lust G, Mohammed HO, Nixon AJ. Coordinate upregulation of cartilage matrix synthesis in fibrin cultures supplemented with exogenous insulin-like growth factor-I. J.Orthop.Res. 1999 Jul;17(4):467-474.

16. Huang JR, Liu SL, Song WD. Stimulation of insulin-like growth factor-I to chondrogenesis of engineering cartilage tissue. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi 2004 Jan;18(1):49-52.

17. Loeser RF, Pacione CA, Chubinskaya S. The combination of insulin-like growth factor 1 and osteogenic protein 1 promotes increased survival of and matrix synthesis by normal and osteoarthritic human articular chondrocytes. Arthritis Rheum. 2003 Aug;48(8):2188-2196.

18. Tuncel M, Halici M, Canoz O, Yildirim Turk C, Oner M, Ozturk F, et al. Role of insulin like growth factor-I in repair response in immature cartilage. Knee 2005 Apr;12(2):113-119.

19. Worster AA, Brower-Toland BD, Fortier LA, Bent SJ, Williams J, Nixon AJ. Chondrocytic differentiation of mesenchymal stem cells sequentially exposed to transforming growth factor-beta1 in monolayer and insulin-like growth factor-I in a three-dimensional matrix. J.Orthop.Res. 2001 Jul;19(4):738-749.

20. Yaeger PC, Masi TL, de Ortiz JL, Binette F, Tubo R, McPherson JM. Synergistic action of transforming growth factor-beta and insulin-like growth factor-I induces expression of type II collagen and aggrecan genes in adult human articular chondrocytes. Exp. Cell Res. 1997;237:318-25.

21. Nixon AJ, Brower-Toland BD, Bent SJ, Saxer RA, Wilke MJ, Robbins PD, et al. Insulinlike growth factor-I gene therapy applications for cartilage repair. Clin Orthop 2000 Oct(379 Suppl):S201-13.

22. Veilleux, N.H., Yannas, I.V., and Spector,M. Effect of passage number and collagen type on the proliferative, biosynthetic, and contractile activity of adult canine articular chondrocytes in type I and II collagen-glycosaminoglycan matrices in vitro. Tissue Eng 2004;10:119-27.

23. Breinan HA, Martin SD, Hsu HP, Spector M. Healing of canine articular cartilage defects treated with microfracture, a type-II collagen matrix, or cultured autologous chondrocytes. J.Orthop.Res. 2000 Sep;18(5):781-789.

24. Lee CR, Grodzinsky AJ, Hsu HP, Spector M. Effects of a cultured autologous chondrocyte-seeded type II collagen scaffold on the healing of a chondral defect in a canine model. J.Orthop.Res. 2003 Mar;21(2):272-281.

25. D. Hastreiter. A collagen-GAG matrix for the growth of intervertebral disc tissue. Cambridge, MA: Massachusetts Institute of Technology; 2002.

26. Yannas IV, Lee EO,D.P., Skrabut EM, Murphy GF. Synthesis and characterization of a model extracellular matrix that induces partial regeneration of adult mammalian skin. Proc. Nat'l. Acad. Sci. USA 1989;86:933-937.

27. Lee CR, Grodzinsky AJ, Spector M. The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis. Biomat. 2001;22:3145-3154.

28. Kuettner KE, Pauli BU, Gall G, Memoli VA, Schenk RK. Synthesis of cartilage matrix by mammalian chondrocytes in vitro. I. Isolation, culture characteristics, and morphology. J Cell Biol 1982 Jun;93(3):743-50.

29. Jakob M, Demarteau O, Schafer D, Hintermann B, Dick W, Heberer M, et al. Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes enhance chondrogenesis and cartilaginous tissue formation in vitro. J Cell Biochem 2001 Mar 26;81(2):368-77.

30. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986 Sep 4;883(2):173-7.

31. Tyrone JW, Mogford JE, Chandler LA, Ma C, Xia Y, Pierce GF, et al. Collagen-embedded platelet-derived growth factor DNA plasmid promotes wound healing in a dermal ulcer model. J.Surg.Res. 2000 Oct;93(2):230-236.

32. Berry M, Gonzalez AM, Clarke W, Greenlees L, Barrett L, Tsang W, et al. Sustained effects of gene-activated matrices after CNS injury. Mol.Cell.Neurosci. 2001 Apr;17(4):706-716.

33. Hosseinkhani H, Inatsugu Y, Hiraoka Y, Inoue S, Shimokawa H, Tabata Y. Impregnation of plasmid DNA into three-dimensional scaffolds and medium perfusion enhance in vitro DNA expression of mesenchymal stem cells. Tissue Eng. 2005 Sep-Oct;11(9-10):1459-1475.

34. Hosseinkhani H, Inatsugu Y, Hiraoka Y, Inoue S, Tabata Y. Perfusion culture enhances osteogenic differentiation of rat mesenchymal stem cells in collagen sponge reinforced with poly(glycolic Acid) fiber. Tissue Eng. 2005 Sep-Oct;11(9-10):1476-1488.

35. Guo T, Zhao J, Chang J, Ding Z, Hong H, Chen J, et al. Porous chitosan-gelatin scaffold containing plasmid DNA encoding transforming growth factor-beta1 for chondrocytes proliferation. Biomaterials 2006 Oct;27(7):1095-1103.

36. Mauck RL, Seyhan SL, Ateshian GA, Hung CT. Influence of seeding density and dynamic deformational loading on the developing structure/function relationships of chondrocyte-seeded agarose hydrogels. Ann.Biomed.Eng. 2002 Sep;30(8):1046-1056.

37. Moretti M, Wendt D, Dickinson SC, Sims TJ, Hollander AP, Kelly DJ, et al. Effects of in vitro preculture on in vivo development of human engineered cartilage in an ectopic model. Tissue Eng. 2005 Sep-Oct;11(9-10):1421-1428.

CHAPTER 4: DELIVERY OF PLASMID IGF-1 TO CHONDROCYTES VIA CATIONIZED GELATIN NANOPARTICLES

4.1. INTRODUCTION

It has become increasingly clear that a number of tissue engineering applications would benefit from the administration of selected growth factors in conjunction with a biomaterial scaffold [1-4]. One approach being investigated for prolonged overexpression of growth factors by cells *in vivo* is the incorporation of gene vectors into 3-D scaffolds [5-7]. In an effort to implement the safest methodology for wedding gene therapy and tissue engineering, several studies have focused on the incorporation of non-viral vectors directly into the scaffold [8,9]. An alternative approach is to encapsulate plasmid DNA containing the gene encoding for the selected growth factor into nanoparticles, which can be injected or implanted along with or bound to a scaffold prior to its implantation. The benefit to using nanoparticles as gene delivery vehicles is the capability to easily direct and control gene expression kinetics by altering various processing parameters used to make nanoparticles. A wide array of materials are undergoing investigation as nanoparticle delivery vehicles for plasmid DNA. Because our own work has employed collagen-based scaffolds [10,11], we were prompted to investigate gelatin nanoparticles for this application. Gelatin, the water-soluble molecular chain resulting from the heat dissolution and partial hydrolysis of collagen, has been investigated for the production of nanoparticles for drug delivery for almost three decades [12,13].

While gelatin nanoparticles were initially introduced as carriers for small molecular weight drugs, [14,15] they were later investigated as carriers for peptides [16]. More recently gelatin nanoparticles have been studied as delivery vehicles for DNA [17-19]. Issues related to the use of gelatin nanoparticles for these applications include size distribution [19], charge [20], stability, and reproducibility. Methods for producing gelatin nanoparticles [21] include solvent evaporation techniques [22], water-in-oil emulsion [23,24], complex coacervation [17], and an emulsifier-free emulsion method [16]. While all of these methods can be employed to incorporate small molecules into gelatin nanoparticles, a few are particularly useful for incorporating large molecules such as plasmid DNA. The advantage of complex coacervation, which is the separation caused by

interaction of two oppositely charged colloids, is that it is simple and quick, and leads to the condensation of plasmid DNA [17].

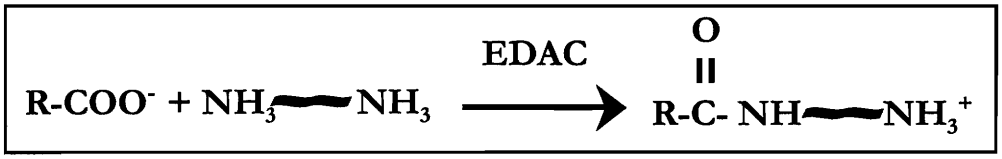
Gelatin is ideal for nanoparticle formation due to its versatility to be chemically modified and cross-linked to meet specific controlled release needs. In one case, thiolated gelatin nanoparticles were developed to release the incorporated molecules in a highly reducing environment [25]. In other studies, DNA-containing poly(ethylene glycol)-modified (PEGylated) gelatin nanoparticles were synthesized in order to develop systemically administered non-viral gene therapy vectors for solid tumors [26,27]. Still other applications benefited from the modification of the surface of gelatin nanoparticles by covalent attachment of biotin-binding proteins, enabling the binding of biotinylated drug targeting ligands by avidin-biotin-complex formation [28-30]. Antibody modified gelatin nanoparticles have also been used as drug carrier systems to target nanoparticles to specific cell types [31]. Of particular interest is the modification of the charge (increased positivity) of gelatin by cationization [19]. Cationized gelatin nanoparticles have been used for a myriad of applications [18,32-35] that benefit from the increased positive charge on the gelatin nanoparticles. As delivery vehicles for plasmid DNA, positively charged gelatin nanoparticles could be capable of condensing DNA and favoring interactions with the negatively charged cell membrane to facilitate endocytosis.

The objective of the present study was to investigate the use of gelatin nanoparticles for non-viral delivery of plasmid DNA encoding for insulin-like growth factor (IGF)-1 into adult articular chondrocytes *in vitro*. The ultimate goal would be to use such nanoparticles along with or incorporated into collagen scaffolds [10,11] for cartilage tissue engineering. IGF-1 was implemented in the study on the basis of prior *in vitro* and *in vivo* studies that have demonstrated its favorable effects on chondrogenesis. Supplementation of culture medium with IGF-1 alone has been shown to increase cell proliferation, proteoglycan synthesis, type-II collagen synthesis, and chondrogenesis, both in monolayer and in three-dimensional cultures [36-41]. In addition, the combination of IGF-1 and osteogenic protein (OP)-1 promotes increased survival of and matrix synthesis by normal and osteoarthritic human articular chondrocytes [42]. I*n vivo,* fibrin polymers laden with IGF-1 resulted in improved histologic appearance and increased proportion of type II collagen in full-thickness cartilage defects in young mature horses [43]. Moreover, *ex vivo* gene transfer of a human IGF-1 cDNA into chondrocytes was found to enhance cartilage tissue engineering both *in vitro* [44] and *in vivo* [45].

4.2. MATERIALS AND METHODS

4.2.1. Preparation of Cationized Gelatin

Gelatin (porcine skin; G2625, Sigma-Aldrich, Inc., St. Louis, MO) was chemically modified by grafting amino groups to carboxyl groups as previously reported [33-35] using a carbodiimide chemical treatment:



Briefly, 2.5 g gelatin was dissolved in 0.1 M phosphate-buffered solution (pH 5.0), to which were added 7.9 ml ethylenediamine (Sigma-Aldrich, Inc.) and 1.34 g 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, EDAC (Sigma-Aldrich, Inc.). The pH was immediately adjusted to 5.0 with 5-6 N hydrochloric acid. The mixture was stirred at room temperature for 16-18 hours, dialyzed for 48 hours in distilled water, and then freeze-dried to obtain the cationized gelatin.

4.2.2. Preparation of Plasmid DNA

The plasmids encoding for IGF-1 (pIGF-1) and enhanced green fluorescent protein (pEGFP) were amplified in *Escherichia coli* host strain DH5α, and purified by column chromatography with the QIAfilter plasmid Mega kit (QIAGEN Inc.-USA, Valencia, CA) according to the manufacturer's protocol. The plasmid for EGFP was used as a reporter gene to visualize the transfection of chondrocytes by fluorescence microscopy. The antibiotics used to select pIGF-1 and pEGFP transformed cells were ampicilin and kanamycin, respectively. The size of the pEGFP was 4.7 Kb and the pIGF-1 was between 6 and 7 Kb in size. The yield, purity, and integrity of the prepared plasmids were evaluated with an ultraviolet spectrophotometer and by gel electrophoresis.

4.2.3. Incorporation of Plasmid IGF-1 into Cationized Gelatin Nanoparticles

Cationized gelatin-plasmid IGF-1 nanoparticles (CGPIN) were prepared by complex coacervation, in which separation is caused by the interaction of two oppositely charged colloids. The cationized gelatin stock solution (80 mg/ml) was made by dissolving 0.24 g cationized gelatin in 3 ml distilled water. The solution was then filtered (pore size = 22 μm) for sterilization. Working solutions with different cationized gelatin concentrations were diluted with sterilized water. The pIGF-1 and pEGFP working solutions (200 μg/ml) were prepared with sterile filtered 50 mM sodium sulfate (Fisher Scientific, Hampton, NH). Aliquots (100-150 μl) of the cationized gelatin and pIGF-1 or pEGFP solutions were heated separately at 55°C for 30-45 min. Equal volumes of the solutions were quickly mixed and vortexed for 60 sec. Nanoparticles, which were prepared with either the pIGF-1 or the pEGFP, were used without further purification. The nanoparticles containing pEGFP were prepared with cationized gelatin using a gelatin:plasmid weight ratio of 250:1 whereas a variety of ratios were investigated when making nanoparticles encapsulating pIGF-1. As a control group, nanoparticles were also synthesized using the original (non-cationized) gelatin material.

4.2.4. Environmental Scanning Electron Microscopy

Environmental scanning electron microscopy (ESEM, XL30, FEI/Philips, Hillsboro, OR) was used to investigate the size and shape of the nanoparticles. Samples were prepared by placing 1 μl of the nanoparticle suspension onto glass slides and air drying. The air-dried samples were then observed directly under ESEM, without the need to coat the samples with a conducting layer as required for conventional SEM.

4.2.5. Determination of Nanoparticle Size Distribution

The particle size distribution was determined by a dynamic light scattering technique, performed at 25°C using a Brookhaven 200SM goniometer, a BI-9000AT digital auto-correlator, and Spectra-Physics Argon laser operating at 514 nm (Brookhaven Instruments Corporation, Holtsville, NY). The measured scattering intensities were analyzed by software provided by Brookhaven.

4.2.6. Zeta Potential

The zeta potential of the nanoparticles, with different weight ratios of gelatin to plasmid were measured with a Brookhaven Zeta Plus apparatus (Brookhaven Instruments Corporation). The electrophoretic mobility was determined at 25°C, and the zeta potential calculated.

4.2.7. Gene Transfer to Adult Articular Chondrocytes in Monolayer Using Gelatin Nanoparticles Incorporating pEGFP and pIGF-1

Chondrocytes were isolated from the trochleae of both knees (stifle joints) from one adult mongrel dog (approximate age 2-4 yrs) using a sequential digestion of pronase (20 U/ml, 1 hr) and collagenase (200 U/ml, overnight). The cells were expanded in number in monolayer culture with medium containing Dulbecco's modified Eagle's medium (DMEM, high glucose 4.5% without L-glutamine), 0.1mM nonessential amino acids, 10mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic (HEPES) buffer, 100 U/mL penicillin, 100μg/mL streptomycin glutamate, 10% FBS (Invitrogen Corporation, Carlsbad, CA), and a mixture of the following growth factors (R&D Systems, Minneapolis, MN): TGF-β1 (1 ng/ml), FGF-2 (5 ng/ml) and PDGF-bb (10 ng/ml). At confluence, cells were trypsinized and either plated onto glass bottom microwell dishes (MatTek Corporation, Ashland, MA) for transfection with nanoparticles incorporating pEGFP or into 24-well tissue culture plates for transfection with nanoparticles incorporating pIGF-1. Passage 0 cells were seeded in both types of dishes at a density of $5x10^4$ cells per well (density of 30,000 cells/cm^2) and were allowed to expand overnight to about 80-90% confluence (about 100,000 cells) before transfection.

For transfection with gelatin nanoparticles incorporating either plasmid, the medium was removed and replaced with a 250 μl suspension of nanoparticles diluted in a serum-free medium. The serum-free medium consisted of DMEM (high glucose 4.5% without L-glutamine), 0.1 mM nonessential amino acids, 10 mM HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin glutamate, ITS^{+1} (100x, by Sigma Chemical, St. Louis, MO), 0.1mM ascorbic acid 2-phosphate, 1.25 mg/ml bovine serum albumin, 10 ng/mL of TGF-β1, and 100 nM dexamethasone. Five hours later, the nanoparticle solution was removed and replaced with fresh serum-free medium (500 ul/well) that did not contain nanoparticles. Based on an average nanoparticle diameter and a gelatin density of about 1 g/ml, we estimated that the number of nanoparticles added to the

monolayer chondrocytes was on the order of 10^{12}. Assuming that the cultures to which the nanoparticles were added contained approximately 100,000 cells, the number of nanoparticles per cell was estimated to be 10^{7}.

Monolayers that were treated with cationized gelatin nanoparticles incorporating pEGFP at a weight ratio of 250:1 and a plasmid amount of 10 μg per well were examined by transmitted fluorescence microscopy 48 hours after transfection in order to visualize successful gene transfer to cells using these nanoparticles.

4.2.8. IGF-1 Expression as a Function of Plasmid Loading in the Nanoparticles

For IGF-1 transfected monolayers, the effects of cationized gelatin:IGF-1 ratio and plasmid amount added to each well were investigated. Five different cationized gelatin:IGF-1 ratios were investigated (by weight): 150:1, 200:1, 250:1, 300:1, and 400:1. For these groups, a constant plasmid load of 10 μg per well was used. In experiments in which plasmid amount was the main variable, the five different plasmid amounts used were 2, 5, 8, 10, and 12 μg of plasmid per well, at a constant cationized gelatin to pIGF-1 weight ratio of 250:1. Control conditions consisted of pIGF-1 only (10 μg) or no treatment (just added serum free medium). Nanoparticles synthesized using unmodified (original) gelatin at a gelatin:IGF-1 ratio of 250:1 was also included as an experimental condition to deterimine the effect of cationization on nanoparticle characteristics and transfectibility. The serum-free medium from IGF-1 transfected cultures was collected at 144 hrs after transfection and assessed for the presence of IGF-1 protein (n = 4) with a human IGF-1 sandwich ELISA kit (R&D Systems, Minneapolis, MN).

4.2.9. Collagen-GAG (CG) Scaffolds Seeded with Monolayer Transfected Chondrocytes

Porous sheets of type II collagen were fabricated by freeze-drying a porcine cartilage-derived slurry (Geistlich Biomaterials, Wolhusen, Switzerland). The collagen sheets were sterilized and cross-linked dehydrothermally by placing the sheets in a vaccum oven at 105 °C for 24 hours. Nine-mm diameter disks (~2mm thick) were punched out using a dermal punch (Moore Medical, New Britain, CA) and additionally cross-linked by a 10 minute carbodiimide treatment containing an aqueous solution of 14 mM 1-ethyl-3-(3-dimethylaminopropyl)

carbodiimide hydrochloride and 5.5 mM N-hydroxysuccinimide (EDAC; Sigma Chemical Co., St. Louis , MO). Excess EDAC was removed by rinsing in PBS.

Using CGPIN synthesized with a gelatin:plasmid weight ratio of 250:1, chondrocyte monolayers were transfected and subsequently seeded in CG scaffolds approximately 24 hours after transfection to assess the duration of IGF-1 overexpression in 3-D culture and the effects on biosynthesis. Four million transfected cells were seeded onto each scaffold by pipetting a 20 μl suspension containing half of the total amount of cells on each side of the scaffold with a 10 minute incubation period in between. By this static seeding method approximately 80% of the seeded chondrocytes have been found to attach to the scaffolds. Cell-seeded scaffolds were cultured in the serum-free medium described above. Medium was collected and changed every 2-3 days over a 14-day culture period. The amount of IGF-1 in the medium (n = 6) was detected by a sandwich ELISA kit for the human IGF-1 protein (R&D Systems). Cultures were terminated after 2 weeks and scaffolds were lyophilized and enzymatically digested using proteinase K (Roche Diagnostics, Indianapolis, IN) for DNA and GAG biochemical analysis.

4.2.10. DNA Analysis

The DNA content of cell-seeded scaffolds was measured using the Picogreen Dye assay kit (Molecular Probes, Inc, Eugene, OR) (n = 4-6). The Picogreen dye was used with the reagents and standard provided according to the manufacturer instructions.

4.2.11. GAG Analysis

The sulfated GAG content of cell-seeded scaffolds was determined by the dimethylmethylene blue (DMMB) dye assay [46] (n = 4-6). An aliquot of the proteinase K digest was mixed with the DMMB dye and the absorbance at 525 nm was measured with a spectrophotometer. The results were obtained by extrapolating from a standard curve using shark chondroitin-6-sulfate. Newly accumulated GAG was determined by subtracting the unseeded values from the sample values.

4.2.12. Statistical Analyses

Data were analyzed by one or two-factor analysis of variance (ANOVA), and the Fisher's protected least squares differences (PLSD) post-hoc tests to determine the significance in the difference between selected groups using StatView (SAS Institute Inc, Cary, NC). Data are presented as mean ± standard error of the mean.

4.3. RESULTS

4.3.1. Morphology of the Cationized Gelatin-pIGF-1 Nanoparticles

ESEM (Fig. 4.1) revealed different sizes and morphologies of the pIGF-1-containing nanoparticles prepared with cationized gelatin versus regular gelatin (using the same gelatin:plasmid weight ratio of 250:1). The cationized gelatin nanoparticles (Fig. 4.1a and b) generally appeared to be of spherical shape less than 200 nm in diameter. In contrast, the original gelatin formed micro-scale particles with spherical and ellipsoid shapes (Fig. 4.1c and d), with the average diameter (and long axis) appearing to be from 10 μm to greater than 20 μm. For both the cationized and non-cationized gelatin:plasmid preparations, the nanoparticles and microparticles, respectively, generally appeared to be of uniform size.

4.3.2. Particle Size Distribution

Dynamic light scattering revealed that the size of the cationized gelatin nanoparticles ranged from 7 nm to 387 nm, with an average diameter of 172 nm. The original gelatin particles displayed a wider size range from 139 nm to 1.8 μm (Fig. 4.2).

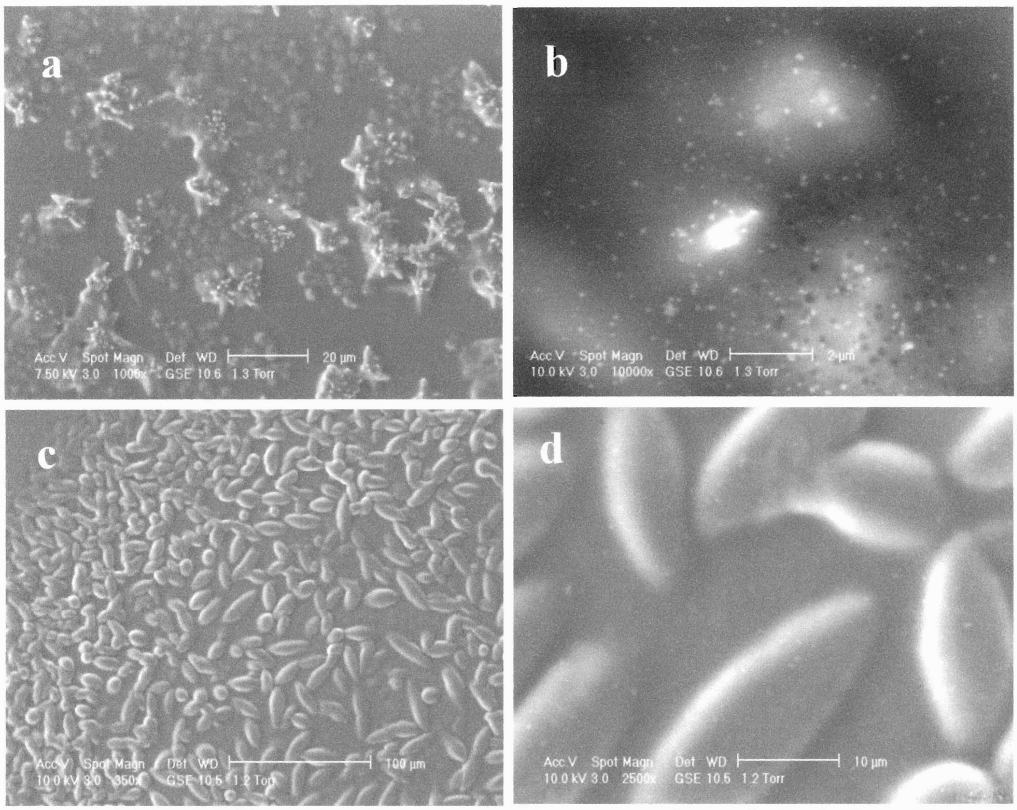


Figure 4.1 **Environmental scanning electron microcopy images of cationized gelatin-pIGF-1 nanoparticles (a, b) and non-cationized (original) gelatin-pIGF-1 microparticles (c, d), using a gelatin-plasmid weight ratio of 250:1.**

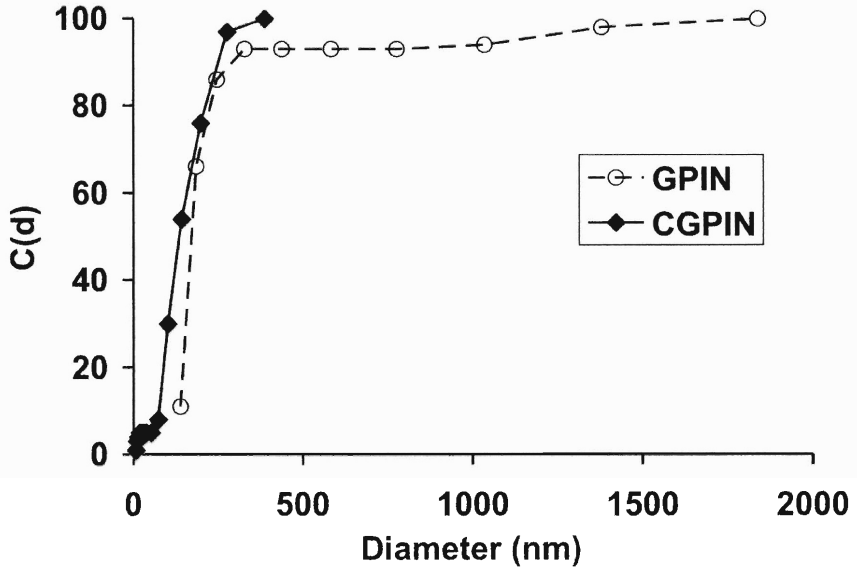


Figure 4.2 **Particle size distribution of cationized gelatin-pIGF-1 nanoparticles (CGPIN) and original gelatin-pIGF-1 particles (GPIN). Cumulative percentage of nanoparticles, C(d), plotted against particle size. Nanoparticles were made with cationized gelatin and original gelatin at a weight ratio of 250:1.**

4.3.3. Surface Charge of Gelatin Nanoparticles

The zeta potential (Fig. 4.3) of the naked plasmid IGF-1 solution was -48 ± 2 mV (mean ± standard error for 5 runs of the same sample). When the plasmid was coupled with different amounts of the positively charged cationized gelatin (+18 ± 0.7 mV for cationized gelatin alone), the zeta potential went from a negative value to a positive value and increased by approximately 50-58 mV. Interestingly, with the increasing cationized gelatin:plasmid weight ratio, the surface charge of the nanoparticles did not show a significant change. Cationized gelatin pIGF-1 nanoparticles displayed a 4-fold higher positive charge compared to particles made with the unmodified gelatin (GPIN) using a 250:1 gelatin to plasmid weight ratio.

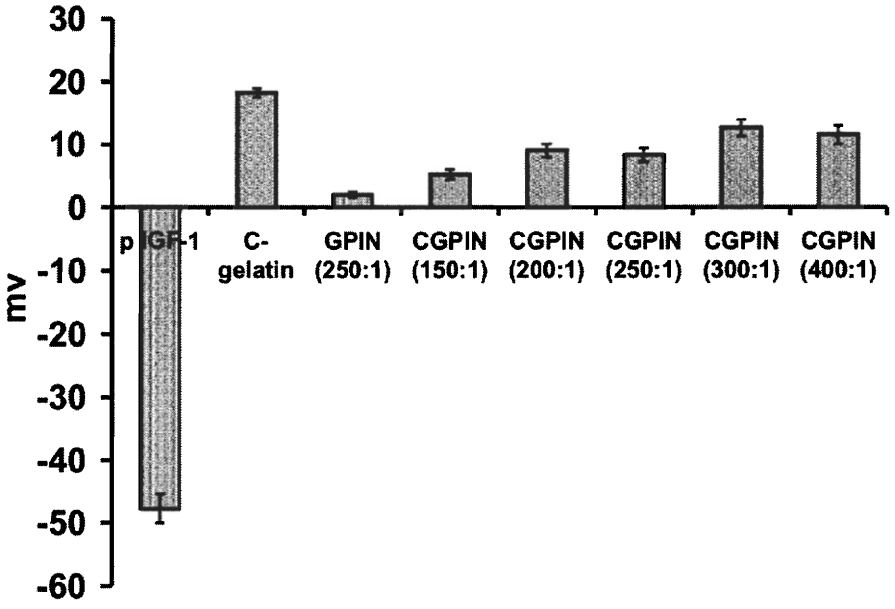


Figure 4.3 Zeta potential of naked IGF-1 plasmid (1st bar), cationized gelatin (2nd bar), original gelatin complexed to pIGF-1 at a 250:1 weight ratio (3rd bar), and cationized gelatin complexed with different amounts of pIGF-1 to yield the following gelatin:plasmid weight ratios: 150:1, 200:1, 250:1, 300:1, and 400:1.

4.3.4. Fluorescence Microscopy of the Chondrocytes Transfected with the Nanoparticles Containing pEGFP

There was no noticeable fluorescence from the cells treated with the nanoparticles prepared with the original (non-cationized) gelatin particles containing the pEGFP (Fig. 4.4a). In contrast numerous cells in the group transfected with the cationized gelatin-pEGFP nanoparticles were found to fluoresce indicating successful transfection and gene expression (Fig. 4.4b).

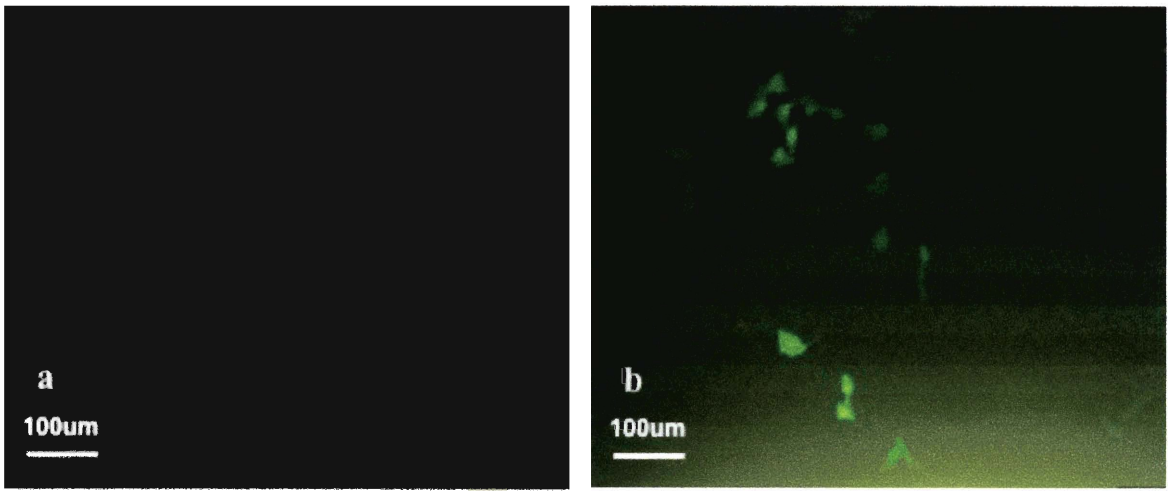


Figure 4.4 Fluorescence microscopy of chondrocytes transfected with nanoparticles incorporating pEGFP 48hrs after transfection using original gelatin (a) or cationized gelatin (b).

4.3.5. IGF-1 Gene Expression in Transfected Chondrocyte Monolayers

The coefficients of variation for the IGF-1 levels recorded from all but two of the nine experimental groups, varying gelatin to plasmid weight ratios and plasmid loading (n = 4), were from 10-30%. In two of the groups (10μg loading with a gelatin:plasmid ratio of 400:1 and 10 μg loading with 250:1) the coefficients were around 60% owing to an outlier in each that was about twice the mean value for the group. It was decided to omit the elevated data point in each of the two groups, reducing the sample size from 4 to 3, but taking a more conservative approach to the analysis of the data (i.e. omitting the outliers decreased the detected levels of protein).

There was a clear effect of varying the weight ratio of gelatin to pIGF-1 on gene transfer and subsequent IGF-1 release in the medium (Fig. 4.5a). Optimal IGF-1 expression was recorded for gelatin to plasmid weight ratios of 200-300:1. There was a 5-fold elevation in the amount of IGF-1 produced from the group treated with nanoparticles synthesized at a weight ratio of 250:1 compared to the control group that was treated with pIGF-1 alone (Fig. 4.5a). One-factor ANOVA revealed a significant effect of the weight ratio of cationized gelatin to pIGF-1 on the amount of IGF-1 synthesized by the cells ($p < 0.0002$; power = 1). Fisher's PLSD post-hoc testing demonstrated that all plasmid ratios had statistically significant elevations of IGF-1 production over the control condition treated with plasmid only (i.e. no incorporation in nanoparticles, $p < 0.04$). Among the gelatin:plasmid weight ratios, there was no statistically

significant difference between the 200:1, 250:1, and 300:1 groups, but there was a significant difference comparing these groups with the 150:1 group ($p < 0.03$), and comparing the 250:1 and 300:1 groups versus the 400:1 group ($p < 0.02$).

There was also a notable effect of varying the amount of plasmid added to each well on IGF-1 produced by the transfected chondrocytes (Fig. 4.5b). There was a gradual increase in IGF-1 expression with increasing plasmid load. Linear regression analysis demonstrated a correlation between IGF-1 expression and plasmid load ($R^2 = 0.65$). One-factor ANOVA showed a significant effect of plasmid amount added per well on IGF-1 expression ($p < 0.0001$; power = 1). Post-hoc testing revealed that there was a statistically significant elevation in IGF-1 produced for all plasmid loads except the 2 μg load when compared to the control group. The 12 μg load showed a statistically significant elevation of IGF-1 expression above the plasmid load groups of 8 μg or less ($p < 0.02$). The 10 μg load showed a significant elevation above plasmid loads of 5 μg or less ($p < 0.01$). There was, however, no significant difference between the 5 and 8 μg plasmid load groups or the 10 and 12 μg groups.

The difference between using unmodified (non-cationized) gelatin and cationized gelatin nanoparticles for the transfection of the chondrocytes was clearly demonstrated (Fig. 4.5c). There was a 5-fold elevation in the amount of IGF-1 produced by transfected cells when using the cationized gelatin nanoparticles (Fig. 4.5c). ANOVA showed a statistically significant difference in the IGF-1 expression between the groups ($p < 0.003$; power = 0.99).

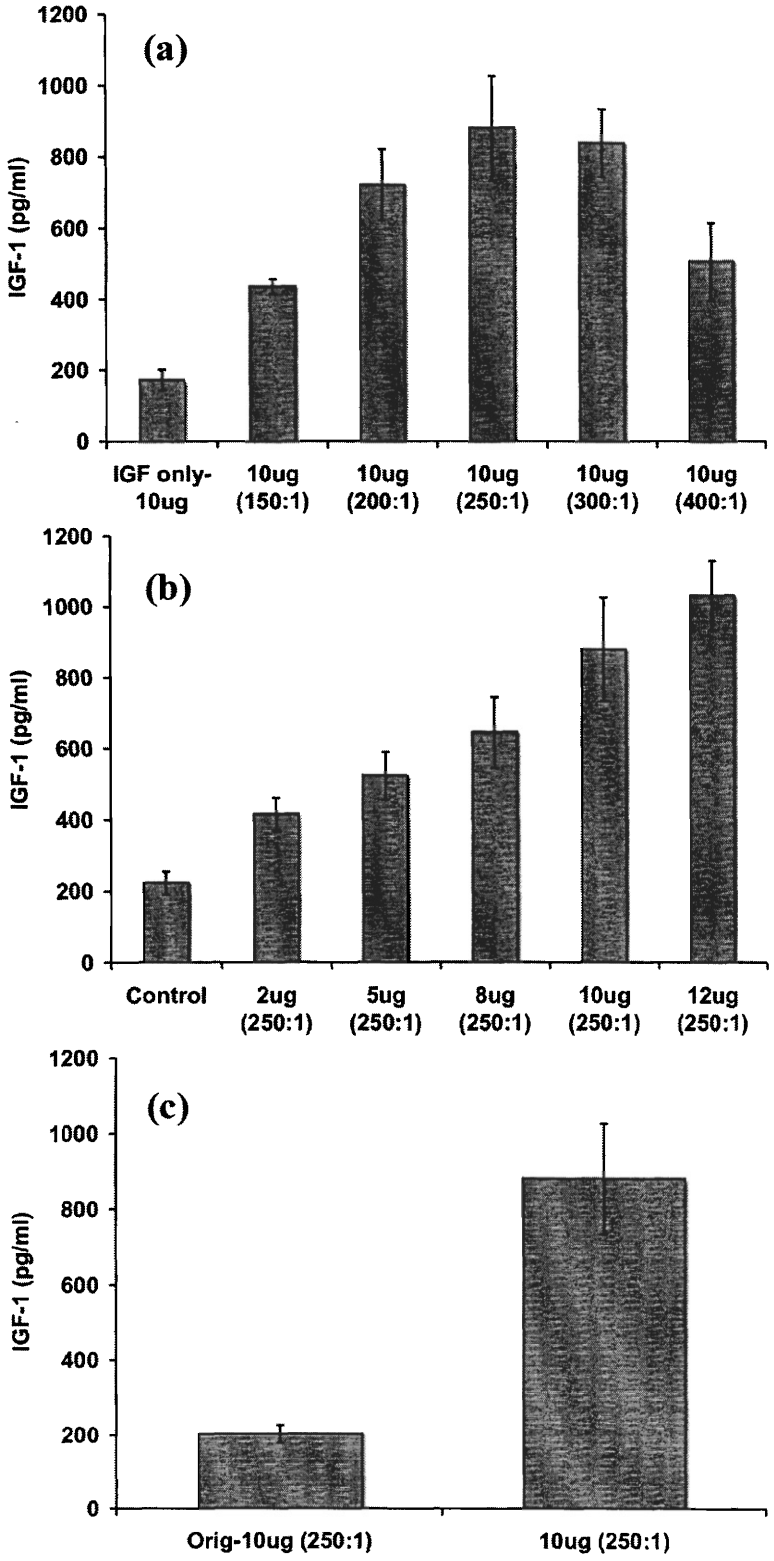


Figure 4.5 IGF-1 protein released into the medium from monolayer chondrocytes 144 hours after treatment with gelatin-plasmid IGF-1 nanoparticles: varying the cationized gelatin to pIGF-1 weight ratio using a 10μg plasmid load (a); varying plasmid load using a 250:1 weight ratio (b); and comparing transfection using original gelatin particles vs. cationized gelatin nanoparticles (c). n = 3-4; mean ± SEM.

4.3.6. IGF-1 Gene Expression of Transfected Chondrocytes Seeded in CG Scaffolds and Effects on Biosynthesis

Scaffolds seeded with chondrocytes that were transfected in monolayer using gelatin/pIGF-1 nanoparticles (CGPIN group) showed a clear elevation of IGF-1 release above the Control (no treatment, medium only) and Gelatin (gelatin alone, no plasmid) groups for all media collections (Fig. 4.6). The CGPIN group showed a steady IGF-1 release of about 2200 pg/ml every 3 days over the course of the 12-day period. There was no noticeable amount of IGF-1 released in the media for the Control or Gelatin groups. Two-factor ANOVA verified a significant effect of transfection with gelatin/pIGF-1 nanoparticles on IGF-1 release from cell-seeded scaffolds ($P < 0.0001$, power = 1) but no significant effect of time over the 12-day culture period.

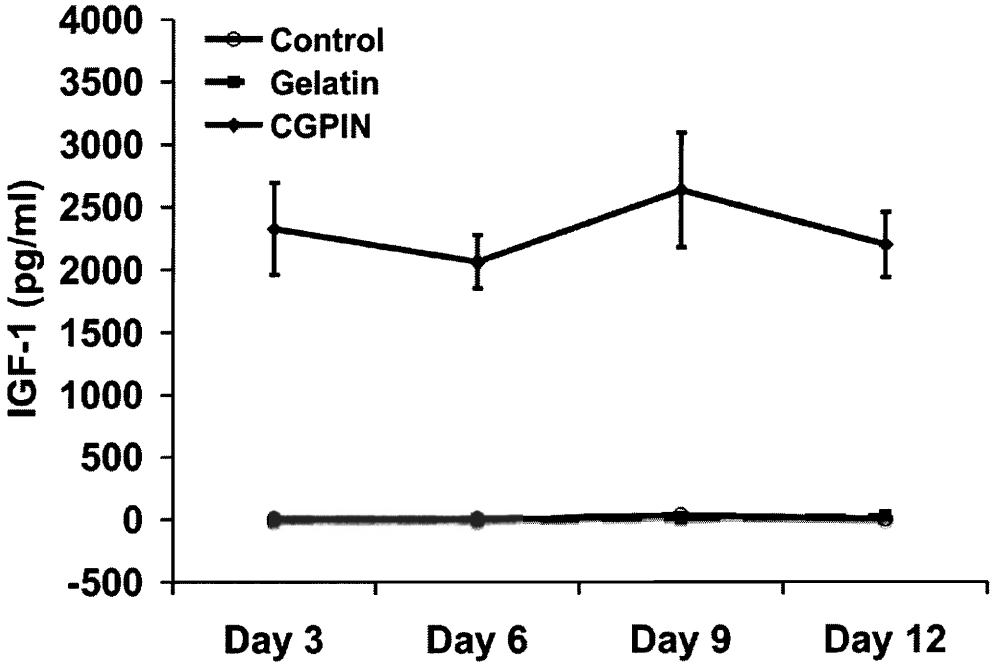


Figure 4.6 IGF-1 released in medium from control cell-seeded scaffolds and chondrocytes transfected in monolayer with CGPIN (gelatin:plasmid IGF-1 of 250:1) and subsequently seeded in CG scaffolds. n = 6; mean ± SEM.

There was a slight, significant elevation in DNA content (Fig. 4.7) at the end of the culture period for the Control group compared to the other groups ($P < 0.03$, power = 0.7). GAG and DNA analysis showed the highest level of accumulated GAG per DNA for the CGPIN group, with a 60% higher level than the Control group and a 40% higher GAG/DNA level than

the Gelatin group. Constructs seeded with cells treated in monolayer with gelatin alone (Gelatin group) also showed a 38% higher GAG/DNA content than the Control group. ANOVA showed a significant effect of monolayer treatment on the resulting accumulated GAG/DNA content at the end of the 3-D culture period ($P < 0.001$, power = 1).

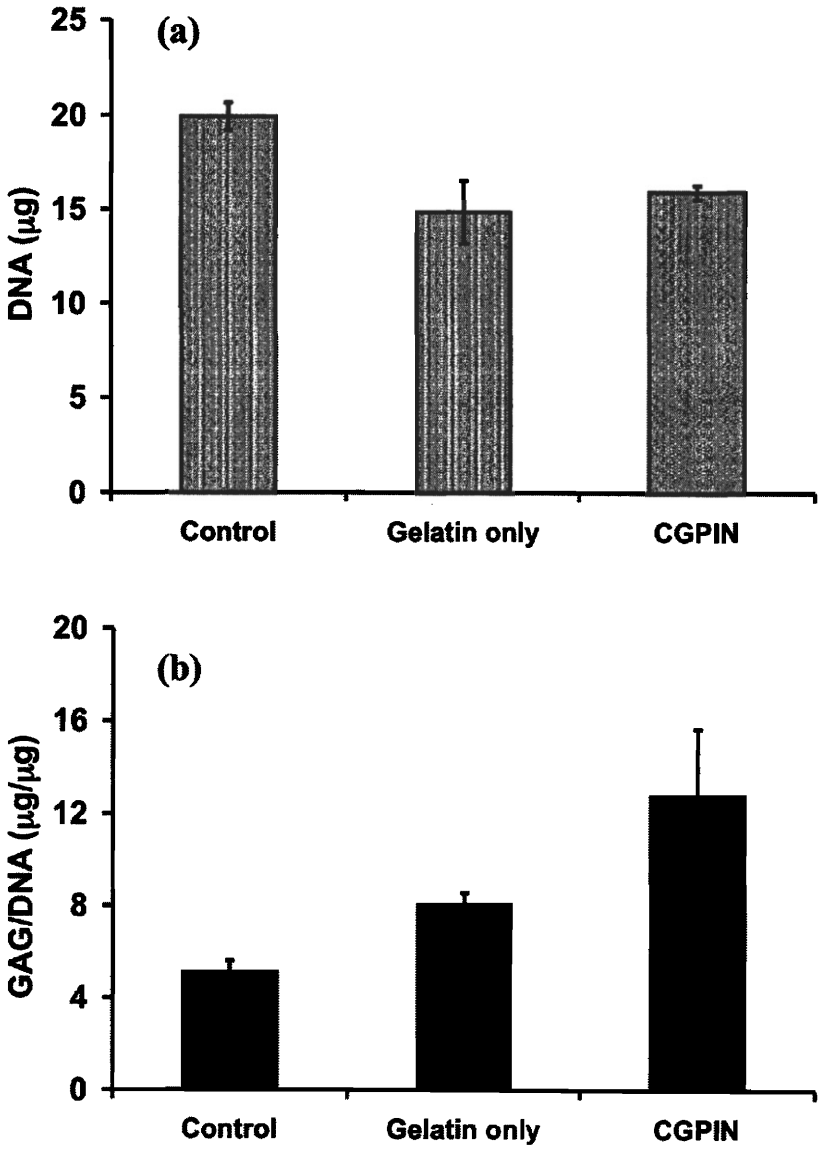


Figure 4.7 **DNA contents (a) and accumulated GAG/DNA (b) measured at the end of the 3-D culture period in CG scaffolds seeded with untreated chondrocyte monolayers (Control), chondrocytes treated with gelatin alone (Gelatin only), or chondrocytes transfected in monolayer with CGPIN at a gelatin:plasmid IGF-1 of 250:1 (CGPIN). n = 4; mean ± SEM.**

4.4. DISCUSSION

The results of the present study demonstrated the benefits of altering the charge of gelatin through cationization, with respect to its use as a delivery vehicle for plasmid IGF-1 for non-viral gene transfer to chondrocytes. Increasing the positive charge of gelatin enabled it to condense the pIGF-1 such that smaller (nanometer-sized) particles could be produced. When complexed with the plasmid, unmodified gelatin tended to form particles of substantially larger size and broader size range. The change in the charge of the gelatin nanoparticles with cationization was demonstrated in the zeta potential measurements, with the cationized nanoparticles displaying a 4-fold higher positive charge than particles synthesized using the original gelatin.

Of interest were the findings demonstrating the difference in the functionality of the cationized and non-cationized gelatin particles as transfection agents for the plasmids encoding EGFP and IGF-1. Virtually no fluorescence was detected in chondrocytes treated with the marker gene, EGFP, incorporated in the unmodified gelatin particles, while many cells were transfected with the pEGFP using the cationized gelatin nanoparticles. This difference was also demonstrated by the 5-fold difference in expression of IGF-1 between groups treated with the cationized gelatin-pIGF-1 versus the non-cationized-pIGF-1 particles indicating that there are important functional differences imparted by charge modification, perhaps owing to a difference in particle size or surface charge. A higher positive surface charge may increase interactions with the negatively charged cellular membrane and a smaller particle diameter may increase probable entry into to cell, resulting in enhanced gene expression. Additional studies will be required to provide a deeper understanding of the transfection mechanisms that are responsible for the differences in the behavior of the cationized and non-cationized particles.

The present findings also revealed an optimal cationized gelatin:pIGF-1 weight ratio range for transfecting adult articular chondrocytes, with maximum IGF-1 expression recorded for weight ratios of 200-300:1. The surface charge of the nanoparticles made with the varying weight ratios, however, may not have been a significant factor influencing gene transfer to chondrocytes as the surface charge did not seem to change significantly using different weight ratios. It will be useful in future work to evaluate other potential mechanisms related to the ability of these nanoparticles to transfect cells, such as particle size and morphology and the kinetics of plasmid release from nanoparticles prepared with other gelatin:plasmid weight ratios.

The current investigation demonstrated a nearly linear increase in IGF-1 production by cells with increasing plasmid load applied to the cultures when using cationized gelatin nanoparticles. Based on an estimated cell number in each well of about 100,000 cells and using a cationized gelatin:IGF-1 weight ratio of 250:1, one would need about 50 pg of incorporated plasmid within cationized gelatin nanoparticles per cell for meaningful gene expression. The approximately 1 ng of IGF-1 collected in the first 144 hours after transfection (using a 250:1 cationized gelatin:pIGF-1 and a 10 μg plasmid load) is well below the minimum therapeutic levels generally found *in vitro* to elicit a response from chondrocytes using the recombinant IGF-1 as a medium supplement. However, this 1 ng level was achieved after only 5 hours of incubation of the cells with the nanoparticles. Moreover, in a contained defect *in vivo,* small levels of overexpressed IGF-1 concentrated locally may still be able to achieve therapeutic results.

The effect of localized and elevated levels of IGF-1 expression on biosynthesis was demonstrated by comparing CG scaffolds seeded with untreated chondrocytes and chondrocytes transfected in monolayer with CGPIN. These results showed that IGF-1 overexpression above the control groups can be maintained when transfected chondrocytes are seeded within CG scaffolds for up to about 2 weeks in 3-D culture. The CGPIN group showed very steady IGF-1 release levels with no evidence of decreasing IGF-1 overexpression at the end of the 12-day culture period. The elevated levels of IGF-1 for the CGPIN group resulted in a 60% greater GAG/DNA content than the Control group after 12-days in 3-D culture. Surprisingly, constructs containing chondrocytes treated with gelatin alone (without plasmid) in monolayer also resulted in a significantly higher GAG/DNA content (38% higher) verses the Control group even though there was no elevation in IGF-1 expression for this group compared to the Control. This finding seems to indicate that gelatin alone can affect chondrocytes in monolayer and may induce elevations in biosynthesis when these cells are subsequently grown in CG scaffolds.

Few studies have yet investigated the mechanisms by which gelatin nanoparticles gain entry into cells. One recent transmission electron microscopy study [23] provided evidence of the endocytosis of gelatin nanoparticles by fibroblasts. While the nanoparticles in the cytoplasm of the cells appeared to disrupt the F-actin and beta-tubulin cytoskeleton, there was no evidence of toxicity. Additional work is necessary to more completely understand the mechanisms by which

gelatin nanoparticles are endocytosed, and the contained plasmid released and incorporated into the cell nucleus.

In conclusion, the present work demonstrates that gelatin nanoparticles can be synthesized to incorporate the plasmid IGF-1 and successfully transfect expanded chondrocytes in monolayer culture. Chemical modification of gelatin by cationization for nanoparticle synthesis, varying the cationized gelatin to plasmid weight ratio, and varying the amount of plasmid added to the cells all significantly affect resulting gene expression and growth factor release kinetics. Furthermore, chondrocytes transfected with pIGF using cationized gelatin nanoparticles are able to maintain steady IGF-1 overexpression when subsequently seeded within CG scaffolds for up to two weeks in 3-D culture, and show enhanced biosynthesis. These findings warrant additional study of the implementation of cationized gelatin nanoparticles incorporating growth factor plasmids in conjunction with scaffolds to facilitate tissue engineering.

4.5. REFERENCES

1. Vasita R, Katti DS. Growth factor-delivery systems for tissue engineering: a materials perspective. Expert Rev.Med.Devices 2006 Jan;3(1):29-47.

2. Tabata Y. Tissue regeneration based on growth factor release. Tissue Eng. 2003;9 Suppl 1:S5-15.

3. Whitaker MJ, Quirk RA, Howdle SM, Shakesheff KM. Growth factor release from tissue engineering scaffolds. J.Pharm.Pharmacol. 2001 Nov;53(11):1427-1437.

4. Babensee JE, McIntire LV, Mikos AG. Growth factor delivery for tissue engineering. Pharm.Res. 2000 May;17(5):497-504.

5. Partridge KA, Oreffo RO. Gene delivery in bone tissue engineering: progress and prospects using viral and nonviral strategies. Tissue Eng. 2004 Jan-Feb;10(1-2):295-307.

6. Jang JH, Houchin TL, Shea LD. Gene delivery from polymer scaffolds for tissue engineering. Expert Rev.Med.Devices 2004 Sep;1(1):127-138.

7. Capito RM, Spector M. Scaffold-based articular cartilage repair. IEEE Eng.Med.Biol.Mag. 2003 Sep-Oct;22(5):42-50.

8. Samuel RE, Lee CR, Ghivizzani S, Evans CH, Yannas IV, Olsen BR, et al. Delivery of plasmid DNA to articular chondrocytes via novel collagen-glycosaminoglycan matrices. Human Gene Therapy 2002;13:791-802.

9. Bonadio J, Smiley E, Patil P, Goldstein S. Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. Nat.Med. 1999 Jul;5(7):753-759.

10. Lee CR, Grodzinsky AJ, Hsu HP, Spector M. Effects of a cultured autologous chondrocyte-seeded type II collagen scaffold on the healing of a chondral defect in a canine model. J.Orthop.Res. 2003 Mar;21(2):272-281.

11. Breinan HA, Martin SD, Hsu HP, Spector M. Healing of canine articular cartilage defects treated with microfracture, a type-II collagen matrix, or cultured autologous chondrocytes. J.Orthop.Res. 2000 Sep;18(5):781-789.

12. Kreuter J. Nanoparticles and nanocapsules--new dosage forms in the nanometer size range. Pharm.Acta Helv. 1978;53(2):33-39.

13. Marty JJ, Oppenheim RC, Speiser P. Nanoparticles--a new colloidal drug delivery system. Pharm.Acta Helv. 1978;53(1):17-23.

14. Vandervoort J, Ludwig A. Preparation and evaluation of drug-loaded gelatin nanoparticles for topical ophthalmic use. Eur.J.Pharm.Biopharm. 2004 Mar;57(2):251-261.

15. Leo E, Arletti R, Forni F, Cameroni R. General and cardiac toxicity of doxorubicin-loaded gelatin nanoparticles. Farmaco 1997 Jun-Jul;52(6-7):385-388.

16. Li JK, Wang N, Wu XS. Gelatin nanoencapsulation of protein/peptide drugs using an emulsifier-free emulsion method. J.Microencapsul. 1998 Mar-Apr;15(2):163-172.

17. Truong-Le VL, August JT, Leong KW. Controlled gene delivery by DNA-gelatin nanospheres. Hum.Gene Ther. 1998 Aug 10;9(12):1709-1717.

18. Kushibiki T, Tomoshige R, Fukunaka Y, Kakemi M, Tabata Y. In vivo release and gene expression of plasmid DNA by hydrogels of gelatin with different cationization extents. J.Control.Release 2003 Jun 24;90(2):207-216.

19. Zwiorek K, Kloeckner J, Wagner E, Coester C. Gelatin nanoparticles as a new and simple gene delivery system. J.Pharm.Pharm.Sci. 2005 Feb 3;7(4):22-28.

20. El-Shabouri MH. Positively charged nanoparticles for improving the oral bioavailability of cyclosporin-A. Int.J.Pharm. 2002 Dec 5;249(1-2):101-108.

21. Farrugia CA, Groves MJ. Gelatin behaviour in dilute aqueous solution: designing a nanoparticulate formulation. J.Pharm.Pharmacol. 1999 Jun;51(6):643-649.

22. Coester CJ, Langer K, van Briesen H, Kreuter J. Gelatin nanoparticles by two step desolvation--a new preparation method, surface modifications and cell uptake. J.Microencapsul. 2000 Mar-Apr;17(2):187-193.

23. Gupta AK, Gupta M, Yarwood SJ, Curtis AS. Effect of cellular uptake of gelatin nanoparticles on adhesion, morphology and cytoskeleton organisation of human fibroblasts. J.Control.Release 2004 Mar 5;95(2):197-207.

24. Cascone MG, Lazzeri L, Carmignani C, Zhu Z. Gelatin nanoparticles produced by a simple W/O emulsion as delivery system for methotrexate. J.Mater.Sci.Mater.Med. 2002 May;13(5):523-526.

25. Kommareddy S, Amiji M. Preparation and evaluation of thiol-modified gelatin nanoparticles for intracellular DNA delivery in response to glutathione. Bioconjug.Chem. 2005 Nov-Dec;16(6):1423-1432.

26. Kaul G, Amiji M. Tumor-targeted gene delivery using poly(ethylene glycol)-modified gelatin nanoparticles: in vitro and in vivo studies. Pharm.Res. 2005 Jun;22(6):951-961.

27. Kaul G, Amiji M. Cellular interactions and in vitro DNA transfection studies with poly(ethylene glycol)-modified gelatin nanoparticles. J.Pharm.Sci. 2005 Jan;94(1):184-198.

28. Wartlick H, Michaelis K, Balthasar S, Strebhardt K, Kreuter J, Langer K. Highly specific HER2-mediated cellular uptake of antibody-modified nanoparticles in tumour cells. J.Drug Target. 2004;12(7):461-471.

29. Langer K, Coester C, Weber C, von Briesen H, Kreuter J. Preparation of avidin-labeled protein nanoparticles as carriers for biotinylated peptide nucleic acid. Eur.J.Pharm.Biopharm. 2000 May;49(3):303-307.

30. Coester C, Kreuter J, von Briesen H, Langer K. Preparation of avidin-labelled gelatin nanoparticles as carriers for biotinylated peptide nucleic acid (PNA). Int.J.Pharm. 2000 Mar 10;196(2):147-149.

31. Balthasar S, Michaelis K, Dinauer N, von Briesen H, Kreuter J, Langer K. Preparation and characterisation of antibody modified gelatin nanoparticles as drug carrier system for uptake in lymphocytes. Biomaterials 2005 May;26(15):2723-2732.

32. Zillies J, Coester C. Evaluating gelatin based nanoparticles as a carrier system for double stranded oligonucleotides. J.Pharm.Pharm.Sci. 2005 Feb 2;7(4):17-21.

33. Aoyama T, Hosseinkhani H, Yamamoto S, Ogawa O, Tabata Y. Enhanced expression of plasmid DNA-cationized gelatin complex by ultrasound in murine muscle. J.Control.Release 2002 Apr 23;80(1-3):345-356.

34. Fukunaka Y, Iwanaga K, Morimoto K, Kakemi M, Tabata Y. Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation. J.Control.Release 2002 Apr 23;80(1-3):333-343.

35. Hosseinkhani H, Aoyama T, Ogawa O, Tabata Y. Ultrasound enhancement of in vitro transfection of plasmid DNA by a cationized gelatin. J.Drug Target. 2002 May;10(3):193-204.

36. Chiyokura YE-. Effects of IGF-I, IGF-II, and PDGF on the proliferation of rabbit articular chondrocytes in culture. Journal of Orthopaedic Science 1996 01//;1(1):59-63.

37. Sah RL, Chen AC, Grodzinsky AJ, Trippel SB. Differential effects of bFGF and IGF-I on matrix metabolism in calf and adult bovine cartilage explants. Arch.Biochem.Biophys. 1994 Jan;308(1):137-147.

38. Fortier LA, Lust G, Mohammed HO, Nixon AJ. Coordinate upregulation of cartilage matrix synthesis in fibrin cultures supplemented with exogenous insulin-like growth factor-I. J.Orthop.Res. 1999 Jul;17(4):467-474.

39. Worster AA, Brower-Toland BD, Fortier LA, Bent SJ, Williams J, Nixon AJ. Chondrocytic differentiation of mesenchymal stem cells sequentially exposed to transforming growth factor-beta1 in monolayer and insulin-like growth factor-I in a three-dimensional matrix. J.Orthop.Res. 2001 Jul;19(4):738-749.

40. Makower AM, Wroblewski J, Pawlowski A. Effects of IGF-I, rGH, FGF, EGF and NCS on DNA-synthesis, cell proliferation and morphology of chondrocytes isolated from rat rib growth cartilage. Cell Biol.Int.Rep. 1989 Mar;13(3):259-270.

41. Verschure PJ, Van Noorden CJ, Van Marle J, Van den Berg WB. Articular cartilage destruction in experimental inflammatory arthritis: insulin-like growth factor-1 regulation of proteoglycan metabolism in chondrocytes. Histochem.J. 1996 Dec;28(12):835-857.

42. Loeser RF, Pacione CA, Chubinskaya S. The combination of insulin-like growth factor 1 and osteogenic protein 1 promotes increased survival of and matrix synthesis by normal and osteoarthritic human articular chondrocytes. Arthritis Rheum. 2003 Aug;48(8):2188-2196.

43. Nixon AJ, Fortier LA, Williams J, Mohammed H. Enhanced repair of extensive articular defects by insulin-like growth factor-I-laden fibrin composites. J.Orthop.Res. 1999 Jul;17(4):475-487.

44. Madry H, Padera R, Seidel J, Langer R, Freed LE, Trippel SB, et al. Gene transfer of a human insulin-like growth factor I cDNA enhances tissue engineering of cartilage. Hum.Gene Ther. 2002 Sep 1;13(13):1621-1630.

45. Madry H, Kaul G, Cucchiarini M, Stein U, Zurakowski D, Remberger K, et al. Enhanced repair of articular cartilage defects in vivo by transplanted chondrocytes overexpressing insulin-like growth factor I (IGF-I). Gene Ther. 2005 Aug;12(15):1171-1179.

46. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986 Sep 4;883(2):173-7.

CHAPTER 5: SCAFFOLD-BASED NONVIRAL IGF-1 GENE DELIVERY USING COLLAGEN-GAG SCAFFOLDS INCORPORATING TRANSFECTION ENHANCERS

5.1. INTRODUCTION

The combination of nonviral gene delivery and tissue engineering strategies via gene incorporation within 3-D scaffolds is an effective means to provide a prolonged release of desired growth factors for enhancing tissue regeneration. Previous chapters have demonstrated that the GenePorter® (GP) lipid mediated transfection reagent and cationized gelatin nanoparticles can be used to facilitate transfer of the gene encoding for insulin-like growth factor (IGF)-1 to adult canine articular chondrocytes, and that IGF-1 overexpression by transfected cells can result in enhanced biosynthesis in type II collagen-GAG (CG) scaffolds *in vitro*. The objective of this study was to evaluate the use of these transfection enhancers in combination with the type II CG scaffold with regard to the ability to successfully transfect seeded goat chondrocytes and the ability to control subsequent IGF-1 release kinetics. In order to investigate potential techniques for varying vector release from the scaffolds, two methods of incorporating the GP/IGF complexes or cationized gelatin pIGF-1 nanoparticles (CGPIN) were employed. The first method (Method 1) involved cross-linking half of the total amount of plasmid IGF-1 (5 μg) to the scaffold and then adding the second half of the load (5 μg) by absorption into scaffold, whereas, the second method (Method 2) involved cross-linking the full plasmid load (10 μg) to the scaffold. During the 2-week 3-D culture period, scaffold contraction was monitored and media was collected at various time points to detect IGF-1 released in the medium. At the end of the culture period, scaffolds were allocated for biochemical and histological analysis to assess the effects on biosynthesis and chondrogenesis.

5.2. MATERIALS AND METHODS

5.2.1. Type II Collagen-GAG Scaffolds

Porous sheets of a type II CG scaffold were fabricated by freeze-drying a porcine type II collagen-GAG slurry (Geistlich Biomaterials, Wolhusen, Switzerland). Similar scaffolds have been reported in prior studies to have a porosity of 89 ± 2% (mean ± standard deviation) and a pore diameter of 125 ± 42 μm [1]. The collagen sheets were sterilized and cross-linked by dehydrothermal treatment [2], and 8 mm diameter disks (~2 mm thick) were prepared using a dermal biopsy punch.

5.2.2. Plasmid Propagation & Isolation

Multiplication of plasmid encoding for IGF-1 (pIGF-1) was accomplished by heat shock transformation into *Escherichia coli* DH5α competent cells grown overnight in Luria-Bertani (LB) medium containing ampicillin. Plasmid was isolated and purified using a Mega QIAfilter™ Plasmid kit (Qiagen, Valencia, CA). The absorption ratio at 260 nm and 280 nm was used to determine plasmid concentration and purity while plasmid integrity was demonstrated by polyacrylamide gel electrophoresis. The size the pIGF-1 was 6-7 Kb.

5.2.3. Preparation of Cationized Gelatin

Gelatin (from porcine skin; G2625, Sigma-Aldrich, Inc., St. Louis, MO) was chemically modified to increase the overall positive charge by grafting amino groups to carboxyl groups as previously reported[3-5]. Briefly, a 2% gelatin solution (w/v) was made using 0.1 M phosphate-buffered solution (pH 5.0). Ethylenediamine (Sigma-Aldrich, Inc.) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC, Sigma-Aldrich, Inc.) were added to obtain a gelatin solution consisting of 6% (v/v) ethylenediamine and 1% (w/v) EDAC. The pH was immediately adjusted to 5 with 5-6 N HCl. The mixture was stirred at room temperature for 16-18 hours, dialyzed for 48 hours in distilled water, and then freeze-dried to obtain the cationized gelatin.

5.2.4. Cationized Gelatin Plasmid IGF-1 Nanoparticle Synthesis

Cationized gelatin plasmid IGF-1 nanoparticles (CGPIN) were prepared by a complex coacervation method where separation into nanoparticles is caused by the interaction of two oppositely charged colloids. An 8% aqueous cationized gelatin stock solution (w/v) was made and sterile filtered. Working solutions of cationized gelatin were obtained by dilution in sterilized water. The plasmid IGF-1 working solution (200 μg/ml) was prepared with sterile filtered 50 mM sodium sulfate (Fisher Scientific, Hampton, NH). 100-150 μl of the cationized gelatin and pIGF-1 solutions were heated separately at 55 °C for 30-45 min. Equal volumes of the solutions were quickly mixed and vortexed for 60 seconds. Nanoparticles were used without further purification. The nanoparticles containing pIGF-1 were prepared with a cationized gelatin to plasmid weight ratio of 250:1.

5.2.5. Incorporation of GP/IGF Complexes or Cationized Gelatin/pIGF-1 Nanoparticles (CGPIN) into CG Scaffolds

To investigate a scaffold-based gene transfer approach to provide a prolonged release of nonviral vectors *in vitro* or *in vivo*, GP/IGF complexes or CGPIN were incorporated into the scaffolds using two methods that would result in a varied vector release profile. The first method (Method 1) used to synthesize GSCG scaffolds involved placing a 60 μl aliquot of the plasmid solution containing 5 μg of plasmid onto the DHT-treated scaffolds followed by incubation for an hour at room temperature. A 1 ml aliquot of an aqueous carbodiimide solution [6] consisting of 14 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 5.5 mM N-hydroxysuccinimide (EDAC; Sigma Chemical Co., St. Louis, MO) was added to the scaffold and incubated at room temperature for about 30 minutes to allow cross-links to form among the collagen molecules and between the plasmid and collagen. Excess EDAC was removed by rinsing the scaffolds in PBS. Scaffolds were then briefly dried on filter paper and a 30 μl aliquot of plasmid solution containing another 5 μg of pIGF-1was added to each scaffold and incubated for about an hour before seeding chondrocytes. This method of incorporation provided a total load of 10 μg of pIGF-1 (complexed to the lipid transfection reagent or the cationized gelatin nanoparticles) some of which were cross-linked and not cross-linked to the CG scaffold.

The second method (Method 2) used to synthesize GSCG constructs involved cross-linking the full plasmid load to the scaffold. A 60 μl aliquot of the plasmid solution containing

10 μg of plasmid onto the DHT-treated scaffolds followed by incubation for at least an hour at room temperature. A 1 ml aliquot of the EDAC solution was added to the scaffold and incubated at room temperature for about 30 minutes. Excess EDAC was removed by rinsing the scaffolds in PBS. Below is a summary of the various conditions investigated in this study.

Table 5.1 Experimental Conditions: CG Scaffolds Supplemented with GP/IGF Complexes or Cationized Gelatin pIGF-1 Nanoparticles

Sample Label	Method of Preparing GSCG Scaffolds	Total Loading (μg)	Supplementation	Methods of Evaluation (n)
GenePorter (x-link & soak)	1	10	GP/IGF	Contraction (12) IGF-1 ELISA (12) Biochemical (6) Histochemical (6)
GenePorter (x-link)	2	10	GP/IGF	Contraction (12) IGF-1 ELISA (12) Biochemical (6) Histochemical (6)
Nanoparticles (x-link & soak)	1	10	CGPIN	Contraction (12) IGF-1 ELISA (12) Biochemical (6) Histochemical (6)
Nanoparticles (x-link)	2	10	CGPIN	Contraction (12) IGF-1 ELISA (12) Biochemical (6) Histochemical (6)

5.2.6. Swelling Ratio Analysis

The swelling ratio (i.e. inverse of the cross-link density) was determined for unseeded control scaffolds and scaffolds containing GP/IGF complexes or CGPIN to determine if gene incorporation during the carbodiimide treatment affects the degree of scaffold cross-linking (n = 4). Scaffolds were immersed in a 90 °C water bath for 2 minutes and placed in between sheets of filter paper (Whatman No. 1, Fisher Scientific, Pittsburgh, PA). A one-kilogram weight was placed on top of the filter paper for 20 seconds after which the weight was taken off and the scaffold was removed and weighed to obtain the wet weight (WM). Scaffolds were then completely dried by placing them in a 105 °C oven overnight. The dry weight (DM) was then

determined for each scaffold and the swelling ratio was calculated according to the following equation:

$$\textit{Swelling ratio} = [(DM/r_c) + ((WM-DM)/r_{water})] \times r_c/DM$$

where $r_c = 1.32$ g/cm^3 (density of collagen) and $r_{water} = 1.00$ g/cm^3 (density of water). The inverse of the swelling ratio was then calculated to obtain the cross-link density.

5.2.7. Chondrocyte Isolation and Expansion

Chondrocytes were isolated from the trochleae of both knees (stifle joints) from six adult Spanish goats (4-5 yrs old). The cells were obtained using a sequential digestion of pronase (20 U/ml, 1hr) and collagenase (200 U/ml, overnight) as previously described [7]. Isolated chondrocytes were expanded in monolayer culture using a medium consisting of high glucose Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L D-glucose, without L-glutamine and with 1 mM sodium pyruvate), containing 10% (v/v) fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 10 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic (HEPES) buffer, 100 U/mL penicillin, and 100 μg/mL streptomycin glutamate. The medium was supplemented with the following growth factors (all from R&D Systems, Minneapolis, MN): 5 ng/mL of fibroblast growth factor-2 (FGF-2), 10 ng/mL of platelet-derived growth factor-bb (PDGF-bb), 1 ng/mL of transforming growth factor-β1 (TGF-β1). The cells were incubated at 37 °C and 5% CO_2. Once cells reached confluence, they were trypsinized, resuspended, and re-plated to obtain passage (P)1 cells.

5.2.8. Cell Seeding GSCG Scaffolds

Four million cells were seeded onto each scaffold by pipetting a 20 μl suspension containing half of the total amount of cells on each side of the scaffold with a 10-minute incubation period in between. By this static seeding method approximately 80% of the seeded chondrocytes have been found to attach to the scaffolds. Cell-seeded scaffolds were cultured in serum-free medium consisted of DMEM (high glucose 4.5% without L-glutamine), 0.1 mM nonessential amino acids, 10 mM HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin

glutamate, ITS^{+1} (100x, by Sigma Chemical, St. Louis, MO), 0.1 mM ascorbic acid 2-phosphate, 1.25 mg/ml bovine serum albumin, 10 ng/mL of TGF-β1, and 100 nM dexamethasone. Medium was collected and changed at various time points over a two-week culture period. At each medium exchange, the diameter of each scaffold was measured to monitor any change in scaffold size (n = 12; 2 scaffolds per goat). The amount of IGF-1 in the collected medium (n = 12; 2 scaffolds per goat) was detected by a sandwich ELISA kit for the human IGF-1 protein (R&D Systems). Cultures were terminated after 2 weeks for histological evaluation and biochemical analysis. For DNA and GAG analysis, scaffolds were lyophilized and enzymatically digested using proteinase K (Roche Diagnostics, Indianapolis, IN).

5.2.9. DNA Analysis

The DNA content of cell-seeded scaffolds was measured using the Picogreen Dye assay kit (Molecular Probes, Inc, Eugene, OR) (n = 6). The Picogreen dye was used with the reagents and standard provided according to the manufacturer instructions.

5.2.10. GAG Analysis

The sulfated GAG content of cell-seeded scaffolds after the 2-week culture period was determined by the dimethylmethylene blue (DMMB) dye assay [8] (n = 6). An aliquot of the proteinase K digest was mixed with the DMMB dye and the absorbance at 525 nm was measured with a spectrophotometer. The results were obtained by extrapolating from a standard curve using shark chondroitin-6-sulfate. Newly accumulated GAG was determined by subtracting the unseeded values from the sample values.

5.2.11. Histology and Immunohistochemistry of Cell-Seeded Scaffolds

Cell-seeded scaffolds (n = 6) were fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, and sectioned (six-micrometer thick) by microtomy. Sections were stained with Safranin-O for the presence of sulfated GAG. For type II collagen immunohistochemical analysis, sections were enzymatically digested by protease type XIV for 45 minutes and stained with a standard avidin-biotin complex peroxidase-based antibody staining technique (Vectastain, Vector Laboratories, Burlingame, CA). Mouse anti-chick

monoclonal antibody for type II collagen was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA).

5.2.12. Statistical Analysis

Data were analyzed by one or two-factor analysis of variance (ANOVA), and the Fisher's protected least squares differences (PLSD) post-hoc test using StatView (SAS Institute Inc, Cary, NC). Data are presented as mean ± standard error of the mean.

5.3. RESULTS

5.3.1. Effect of Gene-Supplementation on Cross-link Density

Swelling ratio results demonstrated that the Control group was the least cross-linked among the groups and that GSCG scaffolds incorporating CGPIN had the highest amount of cross-links (Fig. 5.1). There was about a 15% and a 30% higher cross-link density for scaffolds containing GP/IGF complexes and CGPIN, respectively, compared to the Control group. ANOVA showed a significant effect of gene incorporation on the resulting cross-link density ($P < 0.002$, power = 1). Post hoc analysis revealed a significant difference in the density of cross-links for the Control group versus the other groups ($P<0.04$, power=1), the GenePorter (x-link & soak) group verses the Nanoparticles (x-link & soak) and Nanoparticles (x-link) groups ($P < 0.02$, power = 1), and the GenePorter (x-link) group compared to the Nanoparticles (x-link & soak) group ($P < 0.04$, power = 1).

5.3.2. Cell-Mediated Contraction of GSCG Scaffolds

GSCG scaffolds incorporating GP/IGF complexes using either method of incorporation showed the most contraction, with about a 30% decrease in diameter at the end of the 2-week culture. There was no noticeable decrease in size for scaffolds incorporating CGPIN and the Control group showed a slight 10% decrease in diameter. Two-factor ANOVA revealed a significant effect of type of gene-supplementation ($P < 0.0001$, power = 1) and time ($P < 0.0001$, power = 1) on scaffold contraction.

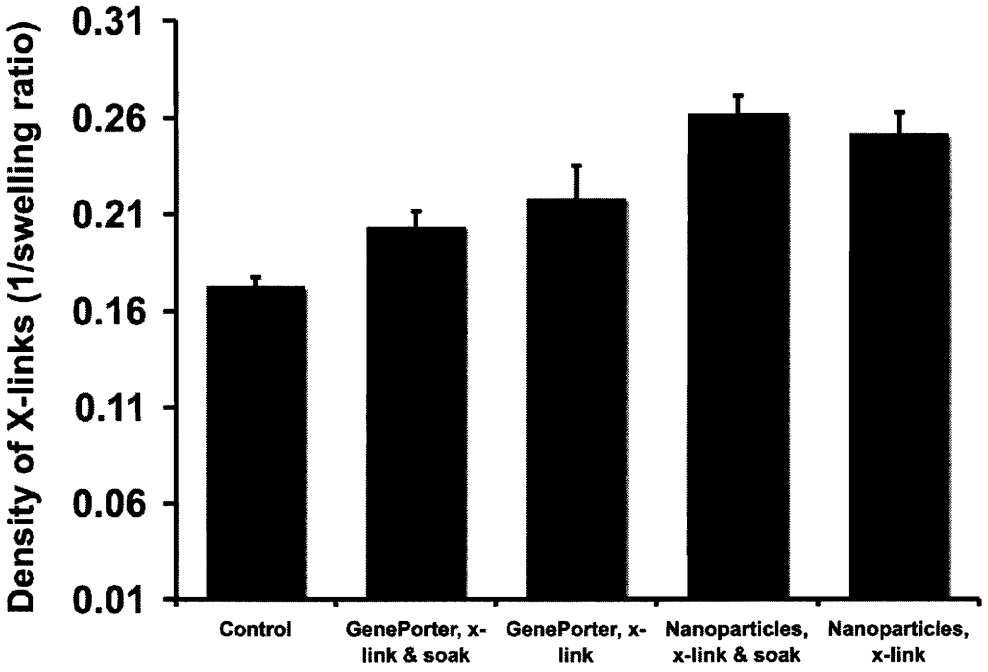


Figure 5.1 Cross-link density (1/swelling ratio) of unseeded control and GSCG scaffolds. n = 4; mean ± SEM.

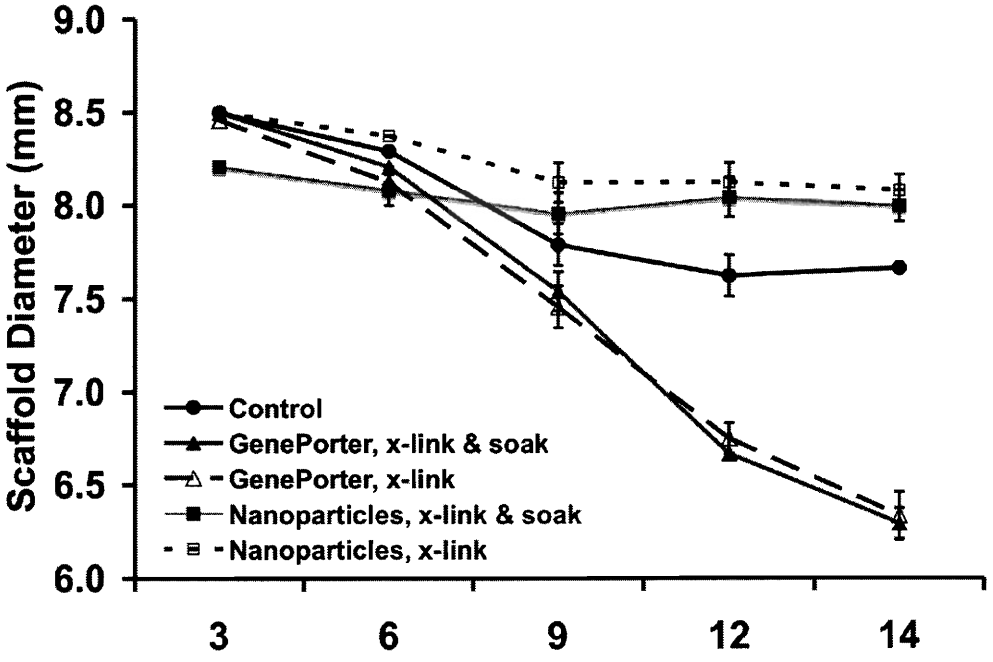


Figure 5.2 Contraction of cell-seeded Control and GSCG scaffolds over the 2-week 3-D culture period. n = 12; mean ± SEM.

5.3.3. IGF-1 Release in 3-D Culture Medium

There was an obvious elevation in IGF-1 protein released in the medium for all GSCG scaffolds containing either the GP/IGF complexes or CGPIN above the Control group except for the Nanoparticles (x-link) group (Fig. 5.3a). In all groups, there seemed to be a peak in IGF-1 release after about a week in culture. The GenePorter (x-link & soak) group displayed the most IGF-1 release in the 3-D culture medium for all time points. Two-factor ANOVA revealed a significant effect of time ($P < 0.03$, power = 0.7) and type of gene-supplementation on IGF-1 release in the medium ($P < 0.0001$, power = 1). Post hoc analysis confirmed a significant difference in IGF-1 release for the Controls versus the GenePorter (x-link & soak) group ($P < 0.0001$, power = 1), GenePorter (x-link) group ($P < 0.0002$, power = 0.984), and Nanoparticles (x-link & soak) group ($P < 0.01$, power = 1). The GenePorter (x-link & soak) group showed significantly higher IGF-1 release over all the other groups ($P < 0.0001$, power = 1) and there was a significant difference between the Nanoparticles (x-link & soak) group compared to the Nanoparticles (x-link) group ($P < 0.01$, power = 1).

Over the 2-week culture period, the total accumulated IGF-1 detected in the medium revealed a 50-fold higher level for the GenePorter (x-link & soak) group and a 14-fold higher level for the Nanoparticles (x-link & soak) group compared to the Control group (Fig. 5.3b). There was an 11-fold difference between the Nanoparticles (x-link & soak) and Nanoparticles (x-link) groups and a 5-fold difference between the GenePorter (x-link & soak) and GenePorter (x-link) groups. Statistical differences between groups for total accumulated IGF-1 were similar to the analysis of the IGF-1 release data.

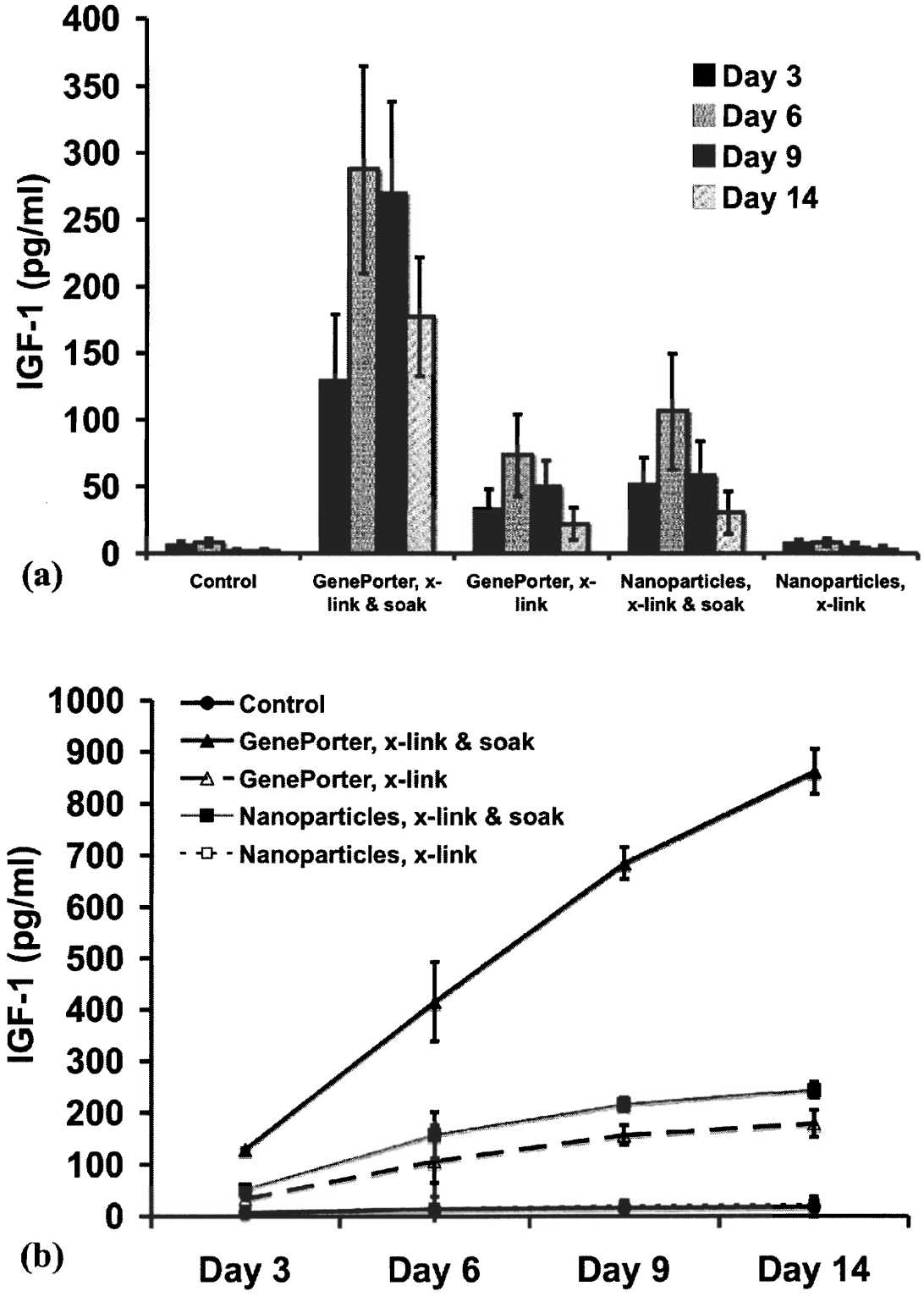


Figure 5.3 IGF-1 detected in each media collection (a) and total accumulated IGF-1 (b) over the course of the 2-week 3-D culture period for Control and GSCG scaffolds incorporating GP/IGF complexes or CGPIN. n = 12; mean ± SEM.

5.3.4. Biochemical Analysis of Cell-Seeded GSCG Scaffolds

DNA content at the end of the 2-week culture period showed noticeably lower values for GSCG scaffolds supplemented with the GP/IGF complexes (Fig. 5.4a). There was about a 70% lower DNA content for the GenePorter (x-link & soak) and GenePorter (x-link) groups and a 23% decrease for the Nanoparticles (x-link & soak) group compared to the Control group. ANOVA and post hoc analysis revealed a significant effect of gene supplementation conditions on final DNA content after 2 weeks in culture ($P < 0.0001$, power = 1) with the GenePorter (x-link & soak) and GenePorter (x-link) groups having a significantly lower DNA content compared to the rest of the groups ($P < 0.005$, power = 1). Although there was no significant difference in DNA content between the Nanoparticles (x-link) and Control groups, there was a significant difference between the Nanoparticles (x-link) and Nanoparticles (x-link & soak) groups ($P < 0.03$, power = 1).

The GAG/DNA values for CG scaffolds supplemented with CGPIN were similar to the Controls after the 2-week culture period (Fig. 5.4b). GSCG scaffolds containing the GP/IGF complexes, on the other hand, showed 64% and 47% higher GAG/DNA content for the GenePorter (x-link & soak) and GenePorter (x-link) groups, respectively, over the Controls. ANOVA and post hoc analysis confirmed a significant effect of gene supplementation condition on GAG/DNA content ($P < 0.0005$, power = 1) and showed significant differences between the GenePorter (x-link & soak) group verses the Control, Nanoparticles (x-link & soak), and Nanoparticles (x-link) groups ($P < 0.0006$, power = 1), and the GenePorter (x-link) group versus the Nanoparticles (x-link & soak) and Nanoparticles (x-link) groups ($P < 0.04$, power = 1).

5.3.5. Histology and Immunohistochemistry of 3-D Cultures

Histochemical staining for all conditions showed most tissue formation in areas surrounding greater cell concentrations located mostly in the center of the scaffolds (Figs 5.5 and 5.6). In general, there were many cells that displayed a rounded morphology in all sample conditions, however, cells displaying a rounded morphology *and* located in lacunae were evident only in GSCG scaffolds containing GP/IGF complexes. All sample conditions showed light staining for Safranin-O, especially in areas where more tissue was formed (Fig. 5.5). CG scaffolds containing the GP/IGF complexes possessed more areas of high cell densities, tissue

formation, and GAG staining compared to the Control or scaffolds incorporating CGPIN. Type II collagen immunohistochemical staining revealed type II collagen deposition mostly on the struts of the scaffolds (Fig. 5.6). Type II staining was most evident in scaffolds containing GP/IGF complexes and least evident for scaffolds containing CGPIN (Fig. 5.6). There was no obvious difference comparing the two methods of gene incorporation regarding the resulting cellular distribution, morphology, and histochemical staining (i.e. GenePorter (x-link & soak) group was similar to GenePorter (x-link) group and Nanoparticles (x-link & soak) group was similar to Nanoparticles (x-link) group). For all conditions, a significant amount of residual scaffold was present after two-weeks in culture.

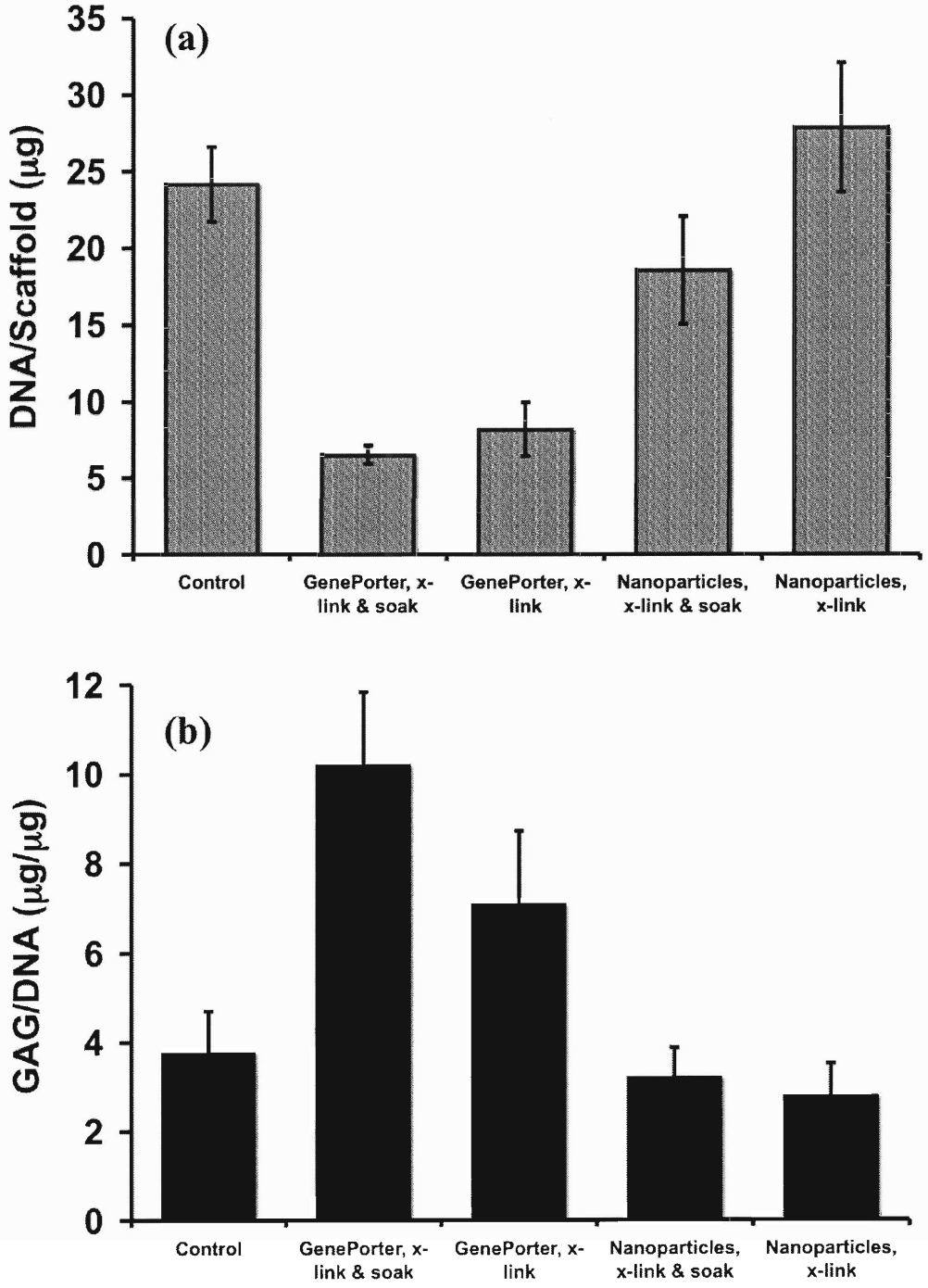


Figure 5.4 DNA (a) and GAG/DNA (b) content for Control and GSCG scaffolds incorporating GP/IGF complexes or CGPIN at the end of the 2-week 3-D culture period. n = 6; mean ± SEM.

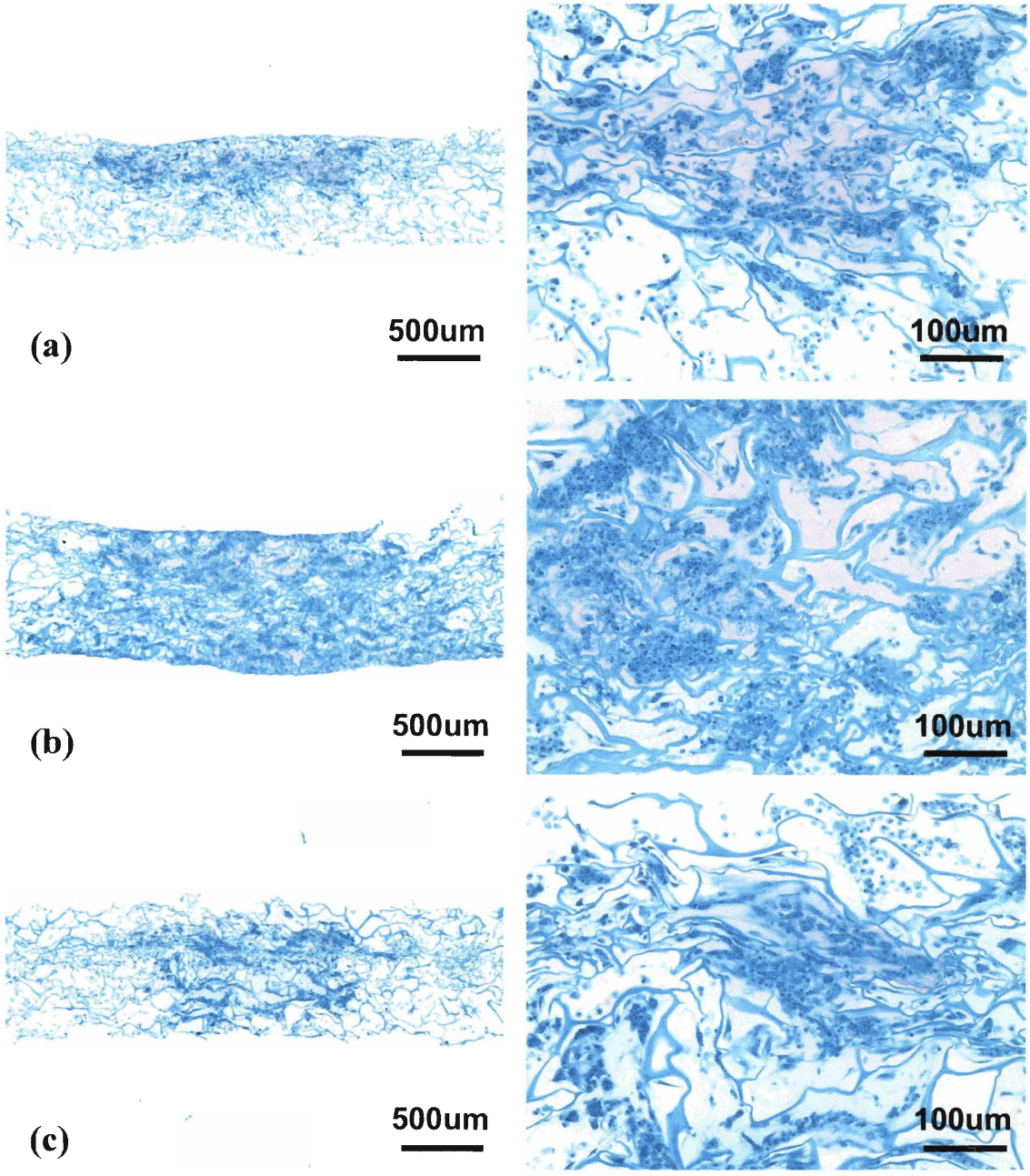


Figure 5.5 Safranin-O staining (red/pink is a positive stain) for Control (a) and GSCG scaffolds incorporating GP/IGF complexes (b) or CGPIN (c) after 2 weeks in 3-D culture.

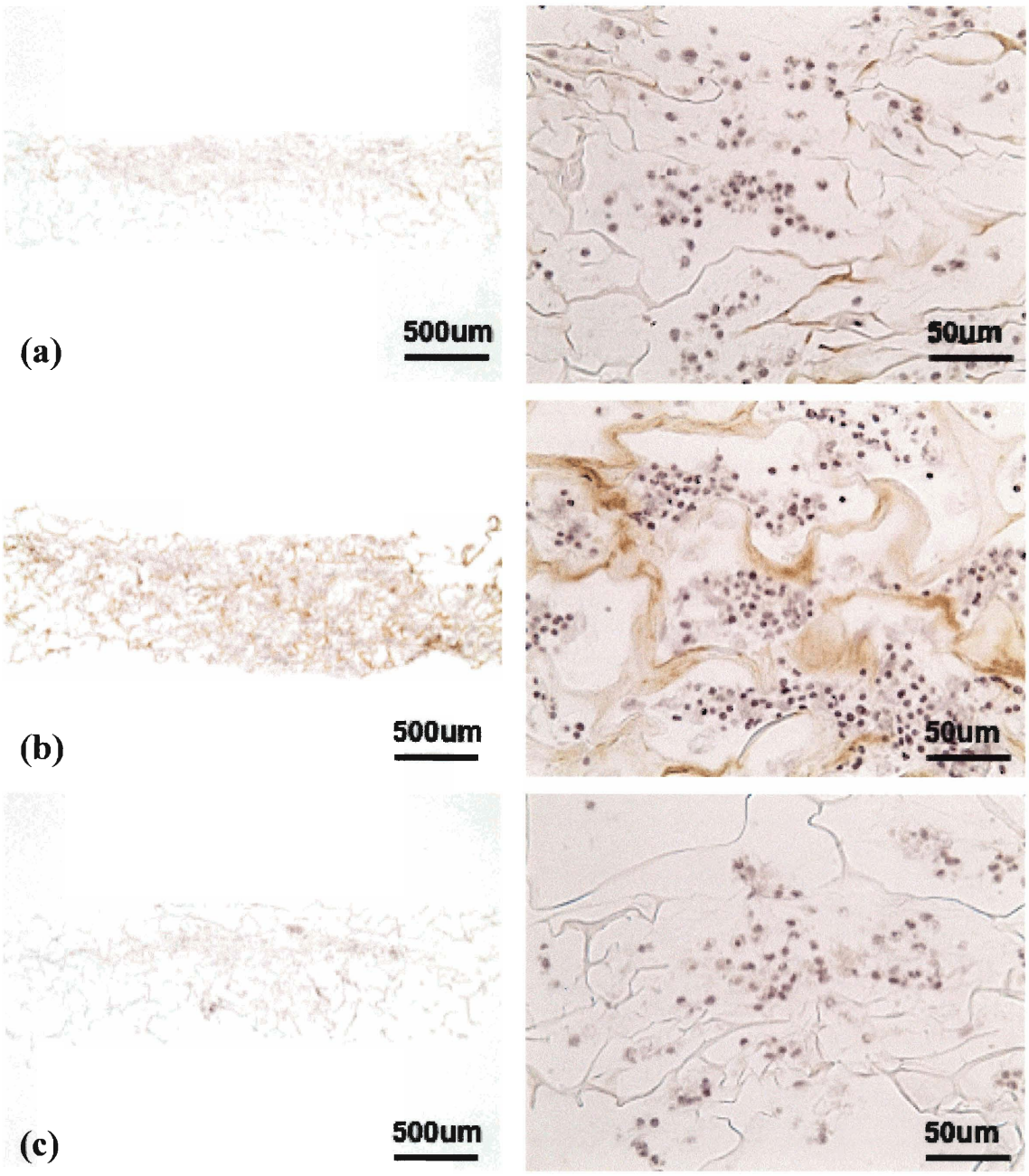


Figure 5.6 **Type II collagen immunohistochemical staining (brown is a positive stain) for Control (a) and GSCG scaffolds incorporating GP/IGF complexes (b) or CGPIN (c) after 2 weeks in 3-D culture.**

5.4. DISCUSSION

Of significance in this study was that both GenePorter/pIGF-1 (GP/IGF-1) complexes and cationized gelatin pIGF-1 nanoparticles (CGPIN) incorporated within CG scaffolds could result in successful overexpression of IGF-1 by seeded goat articular chondrocytes. Prior chondrocyte monolayer studies have shown that GP/IGF complexes have a higher transfection efficiency than the CGPIN, and therefore the greater IGF-1 overexpression that resulted from cells seeded within scaffolds incorporating the GP/IGF complexes was expected. The fact that cells seeded within scaffolds incorporating CGPIN resulted in elevated levels released in the medium over the Control scaffolds shows promise in using this approach as an alternative method to control gene transfer and to direct the start, amount, and duration of growth factor release for articular cartilage repair. Further studies in developing GSCG scaffolds using CGPIN is necessary to find the best nanoparticle synthesis conditions and incorporation methods for optimal gene transfer to seeded cells.

An interesting finding in this study is the effect of gene supplementation on cell-mediated contraction of the scaffolds. Cell-mediated contraction can be affected by the scaffold cross-linking properties (stiffness and degradation), smooth muscle actin (SMA) expression of cells, or cell remodeling of the extracellular matrix (ECM). Although the greatest amount of scaffold contraction occurred in scaffolds supplemented with the GP/IGF complexes, the cross-link density for this group was actually higher than the Control group. It is, therefore, speculated that the cause of the increased cell-mediated contraction for scaffolds incorporating the GP/IGF complexes is not due to scaffold cross-linking, but rather on either stimulated SMA activity or increased remodeling of the matrix that may have been induced by these complexes. Future work should further investigate this contraction phenomenom to understand the mechanism of contraction induced by the GP/IGF complexes.

GSCG scaffolds incorporating the gelatin nanoparticles, on the other hand, posessed the highest cross-link density and least scaffold contraction over the two-week period. This elevated scaffold stiffness may be due to the increased number of potential cross-link sites present when the gelatin nanoparticles are added to the collagen scaffold. The carbodiimide cross-linking agent not only could have cross-linked the gelatin nanoparticles to the collagen scaffold, but it may have also created cross-links between the gelatin molecules, resulting in a stiffer gel within the CG scaffold. This higher cross-link density may have also been the cause of the lower IGF-1

overexpression for scaffolds containing CGPIN compared to scaffolds with the GP/IGF complexes (for both Method 1 and Method 2). That is, a higher cross-link density would have made it more difficult for seeded cells to degrade the scaffold, and therefore may have resulted in a decreased release rate of incorporated CGPIN and a subsequently lower rate of gene transfer. Furthermore, as mentioned before, the transfection efficiency of CGPIN has been shown to be lower than that of the GP/IGF complexes in monolayer studies and therefore would also explain the difference in the amount of IGF-1 released from scaffolds synthesized using Method 1 (where half of the plasmid load was absorbed and not cross-linked to the scaffolds). Future *in vitro* experiments need to investigate other formulations for synthesizing CGPIN for optimal transfection efficiencies and other methods of incorporation within CG scaffolds to control vector release rates and subsequent IGF-1 expression kinetics over prolonged times.

As anticipated, cross-linking half of the plasmid load and absorbing the other half within the scaffolds resulted in higher levels of IGF-1 compared to cross-linking the full plasmid load. Although after one week in 3-D culture there seemed to be a peak in IGF-1 release from all sample conditions, it remains to be seen if further breakdown or degradation of the scaffold would result in another "wave" of vector release and a subsequent increase in IGF-1 production. Histological results did confirm an abundance of residual scaffold left after 2-weeks in culture, and therefore a significant amount plasmid that is cross-linked to the scaffold may still be present.

Of note is the dramatically lower DNA content for the scaffolds containing GP/IGF complexes compared to the other groups at the end of the two-week culture period. It is speculated that the significant decrease in scaffold size may have contributed to cell apoptosis and a decrease in the number of live cells at the end of 2-weeks. Further studies involving cell attachment assays need to verify that this difference in DNA content was not due to initial effects on cell attachment. Prior work using adult canine chondrocytes seeded within GSCG scaffolds incorporating GP/IGF complexes (using Method 2) did not display this decrease in DNA content below the Control conditions, however, they also did not show as much scaffold contraction at the end of 2 weeks.

Although the DNA content was significantly lower for scaffolds incorporating the GP/IGF scaffolds, the GAG/DNA content was the highest for these groups especially for the GenePorter (x-link & soak) group. This elevated GAG/DNA level for the GenePorter (x-link &

soak) group could be the result of the higher production of IGF-1 by seeded chondrocytes relative to the other groups. The fact that there was no noticeable increase in GAG/DNA content for the Nanoparticles (x-link & soak) group compared to the Nanoparticles (x-link) group, despite the higher release of IGF-1 for the Nanoparticles (x-link & soak) group, may indicate that scaffold conditions (e.g. degradation properties) may not have been ideal to reflect an enhanced biosynthetic response that was expected from elevated IGF-1 production. This may also be the case when comparing the results from the GenePorter (x-link) and Nanoparticles (x-link & soak) groups. These groups demonstrated similar IGF-1 release levels over the two-week period; however, the GenePorter (x-link) group displayed a significantly higher GAG/DNA amount compared to the Nanoparticles (x-link & soak) group. The most obvious difference between these two groups is the amount of scaffold contraction over the course of the 2-week culture. Prior studies [9,10] have shown that cell density has a significant effect on biosynthesis and chondrogenesis within 3-D scaffolds. The much higher contraction of the GenePorter (x-link) group may have increased the cell density within the scaffold, which may have also contributed to the increase in biosynthesis. Future work should further investigate the individual influences of scaffold contraction and IGF-1 expression on enhanced biosynthesis within these GSCG scaffolds.

Histochemical evaluation revealed that for all conditions, there was a majority of cells displaying a rounded morphology. Although many cells were rounded, the presence of lacunae was difficult to assess since not much tissue was evident, especially for the Control and CGPIN scaffolds. There was not an even cellular distribution for most of the samples, with sections of the scaffold (particularly the center) having a denser cell population, most likely due to the pipette seeding method. Greater cell densities were evident for samples containing GP/IGF complexes, most likely the result of greater scaffold contraction. The fact that a greater amount of histogenesis was demonstrated for GSCG scaffolds containing GP/IGF complexes supports the significant effect that cell density can have on biosynthesis and chondrogenesis. It is difficult to assess the result of the type II collagen staining since most staining was present on the struts of the scaffold. The fact that there was more collagen type II staining on the struts for GSCG scaffolds containing the GP/IGF complexes, may indicate that cells within the GP/IGF complexes did produce more type II collagen but it was mostly deposited on the scaffold walls due to insufficient tissue synthesis within the scaffold pores. The lack of tissue formation

(especially for the Control and CGPIN scaffolds) and the amount of residual scaffold left after 2-weeks in culture may indicate that the cross-linking conditions were not ideal for sufficient biosynthesis. Future studies need to investigate the behavior of these goat articular chondrocytes within GSCG scaffolds using other cross-linking conditions.

In conclusion, incorporation of GP/IGF complexes or CGPIN within CG scaffolds has a significant effect on cell-mediated contraction of the scaffolds and IGF-1 overexpression of seeded chondrocytes. The initiation, amount, and duration of IGF-1 release can be potentially controlled using different nonviral vector formulations and gene incorporation methods. IGF-1 overexpression resulting from successful transfection of chondroctyes seeded within GSCG scaffolds can result in enhanced biosynthesis, but is also dependent on scaffold contraction and degradation characteristics. This study commends further investigation using nonviral gene transfer agents incorporated within CG scaffolds for a prolonged and localized delivery of growth factors in articular cartilage tissue engineering.

5.5. REFERENCES

1. D. Hastreiter. A collagen-GAG matrix for the growth of intervertebral disc tissue. Cambridge, MA: Massachusetts Institute of Technology; 2002.

2. Yannas IV, Lee EO,D.P., Skrabut EM, Murphy GF. Synthesis and characterization of a model extracellular matrix that induces partial regeneration of adult mammalian skin. Proc. Nat'l. Acad. Sci. USA 1989;86:933-937.

3. Aoyama T, Hosseinkhani H, Yamamoto S, Ogawa O, Tabata Y. Enhanced expression of plasmid DNA-cationized gelatin complex by ultrasound in murine muscle. J.Control.Release 2002 Apr 23;80(1-3):345-356.

4. Fukunaka Y, Iwanaga K, Morimoto K, Kakemi M, Tabata Y. Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation. J.Control.Release 2002 Apr 23;80(1-3):333-343.

5. Hosseinkhani H, Aoyama T, Ogawa O, Tabata Y. Ultrasound enhancement of in vitro transfection of plasmid DNA by a cationized gelatin. J.Drug Target. 2002 May;10(3):193-204.

6. Lee CR, Grodzinsky AJ, Spector M. The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis. Biomat. 2001;22:3145-3154.

7. Kuettner KE, Pauli BU, Gall G, Memoli VA, Schenk RK. Synthesis of cartilage matrix by mammalian chondrocytes in vitro. I. Isolation, culture characteristics, and morphology. J Cell Biol 1982 Jun;93(3):743-50.

8. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986 Sep 4;883(2):173-7.

9. Mauck RL, Seyhan SL, Ateshian GA, Hung CT. Influence of seeding density and dynamic deformational loading on the developing structure/function relationships of chondrocyte-seeded agarose hydrogels. Ann.Biomed.Eng. 2002 Sep;30(8):1046-1056.

10. Moretti M, Wendt D, Dickinson SC, Sims TJ, Hollander AP, Kelly DJ, et al. Effects of in vitro preculture on in vivo development of human engineered cartilage in an ectopic model. Tissue Eng. 2005 Sep-Oct;11(9-10):1421-1428.

CHAPTER 6: NONVIRAL IGF-1 GENE TRANSFER TO MESENCHYMAL STEM CELLS VIA GENE-SUPPLEMENTED COLLAGEN-GLYCOSAMINOGLYCAN (GSCG) SCAFFOLDS

6.1. INTRODUCTION

Over recent years, there has been a tremendous increase in research directed towards the use of adult bone marrow-derived or mesenchymal stem cells (MSCs) for the purposes of tissue engineering. The *in vitro* expansion of chondrocytes that is essential to obtain sufficient cell numbers for articular cartilage tissue engineering applications, and the resulting donor site morbidity associated with the use of autologous chondrocytes, commends MSCs as a more favorable cell source; as these progenitor cells have a natural ability to regenerate and are more readily available *in vivo*. The simplest procedure for using MSCs as a cell source to enhance articular cartilage regeneration *in vivo* is with a microfracture procedure, in which small holes (~1 mm in diameter) are punctured through the subchondral bone to allow blood and bone marrow containing MSCs to infiltrate the defect. Prior *in vivo* studies in our lab comparing microfracture treatment alone, microfracture with a type-I1 collagen scaffold placed in the defect, and a type-ll scaffold seeded with cultured autologous chondrocytes demonstrated that the greatest amount of defect filling (% of cross-sectional area of the original defect filled with reparative tissue) after 15 weeks was found in the dogs whose defects were treated with microfracture and implantation of the type II collagen scaffold [1]. Tissue filling in all of the defects, however, was predominantly fibrocartilage.

To improve the quality of tissue formation *in vivo* to a more hyaline-like cartilage tissue using the microfracture procedure in conjunction with a type II collagen scaffold, it may be advantageous to use various regulators to induce differentiation of MSCs toward the chondrogenic phenotype. Several studies have demonstrated successful differentiation of MSCs into a chondrogenic phenotype under specific *in vitro* culture conditions [2-5]. Of interest is that both type II collagen [6] and insulin-like growth factor (IGF)-1[7-9] have also been shown to have potent chondrogenic effects on MSCs.

Using a scaffold-based gene delivery approach, the objective of this study was to employ a collagen (type II)-GAG (CG) scaffold incorporating the gene encoding for IGF-1 to determine

if seeded MSCs could be successfully transfected and provide an elevated and prolonged release of IGF-1. Previous chapters have demonstrated that these gene-supplemented CG (GSCG) scaffolds can successfully transfect seeded chondrocytes leading to elevated, localized, and prolonged delivery of IGF-1 *in vitro*. It has also been shown that gene transfer in CG scaffolds can be enhanced by the incorporation of a GenePorter® (GP) transfection reagent complexed to the plasmid IGF-1 (GP/IGF). In this study, two methods of incorporating the GP/IGF complexes into CG scaffolds (investigated in the previous chapter) were employed. The first method (Method 1) involved cross-linking half of the total amount of GP/IGF complexes (5μg plasmid load) to the scaffold and then adding the second half of the load (also containing 5μg) by solution absorption into scaffold. The second method (Method 2) involved cross-linking the full plasmid load (10 μg) to the scaffold. During the 2-week 3-D culture period, media was collected at various time points to detect IGF-1 overexpression. At the end of the culture period, scaffolds were assayed for DNA and accumulated GAG content. The presence of GAG and total collagen was also assessed histochemically.

6.2. MATERIALS AND METHODS

6.2.1. Type II Collagen-GAG Scaffolds

Porous sheets of a type II CG scaffold were fabricated by freeze-drying a porcine type II collagen-GAG slurry (Geistlich Biomaterials, Wolhusen, Switzerland). Similar scaffolds have been reported in prior studies to have a porosity of 89 ± 2% (mean ± standard deviation) and a pore diameter of 125 ± 42 μm [10]. The collagen sheets were sterilized and cross-linked by a dehydrothermal treatment [11], and 8 mm diameter disks (~2 mm thick) were prepared using a dermal biopsy punch (Moore Medical, New Britain, CA).

6.2.2. Plasmid Propagation & Isolation

Multiplication of plasmid encoding for IGF-1 (pIGF-1) was accomplished by heat shock transformation into *Escherichia coli* DH5α competent cells grown overnight in Luria-Bertani (LB) medium containing ampicillin. Plasmid was isolated and purified using a Mega QIAfilter™ Plasmid kit (Qiagen, Valencia, CA). The absorption ratio at 260 nm and 280 nm

was used to determine plasmid concentration and purity while plasmid integrity was demonstrated by polyacrylamide gel electrophoresis. The size the pIGF-1 was 6-7 Kb.

6.2.3. Incorporation of GenePorter/plasmid IGF-1 (GP/IGF) Complexes into CG Scaffolds

Plasmid IGF-1 (pIGF-1) was complexed to a lipid mediated transfection reagent GenePorter ® (GP) (Gene Therapy Systems, Inc., CA) using a 5:1 (µl of GenePorter:µg of pIGF-1) ratio. GP/IGF complexes were incorporated into the scaffolds using two methods that would result in varied release kinetics of the vectors from the CG scaffolds. The first method used to synthesize GSCG scaffolds, GenePorter (x-link & soak) group, involved placing a 60 µl aliquot of the GP/IGF plasmid solution containing 5 µg of plasmid onto the DHT-treated scaffolds followed by incubation for an hour at room temperature. A 1 ml aliquot of an aqueous carbodiimide solution [12] consisting of 14 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 5.5 mM N-hydroxysuccinimide (EDAC; Sigma Chemical Co., St. Louis , MO) was added to the scaffold and incubated at room temperature for about 30 minutes. Excess EDAC was removed by rinsing the scaffolds in PBS. Scaffolds where then briefly dried on filter paper and 30 µl aliquot of GP/IGF solution containing another 5 µg of pIGF-1was added to each scaffold and incubated for another hour before seeding chondrocytes. This method of incorporation provided a total plasmid load of 10 µg (complexed to the lipid transfection reagent) some of which were cross-linked and not cross-linked to the CG scaffold.

The second method (Method 2) used to synthesize GSCG constructs involved cross-linking the full plasmid load to the scaffold. A 60 µl aliquot of the plasmid solution containing 10 µg of plasmid onto the DHT-treated scaffolds followed by incubation for at least an hour at room temperature. A 1 ml aliquot of the EDAC solution was added to the scaffold and incubated at room temperature for about 30 minutes. Excess EDAC was removed by rinsing the scaffolds in PBS.

6.2.4. Mesenchymal Stem Cell (MSC) Isolation and Monolayer Expansion

Mesenchymal stem cells (MSCs) were isolated from heparinized bone marrow aspirates obtained from the iliac crest of six adult Spanish goats (4-5 yrs old). The cells were isolated using a Ficoll-Paque PLUS gradient (GE Life Sciences, NJ). Isolated cells were plated and expanded in monolayer culture using a medium consisting of low glucose Dulbecco's modified

Eagle's medium, DMEM (Invitrogen), containing 10% (v/v) fetal bovine serum (FBS), and 1% penicillin/streptomycin. The cells were incubated at 37°C and 5% CO_2. MSCs were grown through two subcultures to obtain Passage 2 cells.

6.2.5. Monolayer Transfection of Mesenchymal Stem Cells (MSCs)

For comparison with transfection in 3-D culture, cells were transfected in monolayer culture to be subsequently seeded in CG scaffolds. For monolayer transfection, cells were expanded to 80-90% confluence in 150 cm^2 tissue culture flasks (about 3 million cells per flask) and transfected with the GenePorter transfection reagent complexed to pIGF-1 at a 5:1 ratio (µl of GP:µg pIGF-1) according to the manufacturer's instructions for transfection of adherent cells. 15 µg of pIGF-1 was added per flask. Approximately 24 hrs later, cells were trypsinized, counted, and seeded into CG scaffolds.

6.2.6. Cell Seeding CG Scaffolds, IGF-1 Detection in the Medium, and Scaffold Contraction

Two million MSCs were seeded onto each scaffold by pipetting a 20 µl suspension containing half of the total amount of cells onto each side of the scaffold with a 10 minute incubation period in between. Cell-seeded scaffolds were cultured in a serum-free medium containing high glucose Dulbecco's modified Eagle's medium, DMEM (high glucose 4.5% without L-glutamine), 0.1mM nonessential amino acids, 10mM HEPES buffer, 100 U/mL penicillin, 100 µg/mL streptomycin glutamate, ITS^{+1} (100x, by Sigma Chemical, St. Louis, MO), 0.1mM ascorbic acid 2-phosphate, 1.25mg/ml bovine serum albumin, 10ng/mL of TGF-β1, and 100nM dexamethasone. Medium was collected and changed at various time points over a two-week culture period and the amount of IGF-1 in the collected medium (n=11-12; 2 scaffolds per goat) was detected by a sandwich ELISA kit for the human IGF-1 protein (R&D Systems). At each medium exchange, the diameter of each scaffold was measure to monitor any change in scaffold size (n=12; 2 scaffolds per goat). Cultures were terminated after 2 weeks for histological evaluation and biochemical analysis. For DNA and GAG analysis, scaffolds were lyophilized and enzymatically digested using proteinase K (Roche Diagnostics, Indianapolis, IN).

6.2.7. DNA Analysis

The DNA content of cell-seeded scaffolds was measured using the Picogreen Dye assay kit (Molecular Probes, Inc, Eugene, OR) (n=6). The Picogreen dye was used with the reagents and standard provided according to the manufacturer instructions.

6.2.8. GAG Analysis

The sulfated GAG content of cell-seeded scaffolds after the 2-week culture period was determined by the dimethylmethylene blue (DMMB) dye assay [13] (n=6). An aliquot of the proteinase K digest was mixed with the DMMB dye and the absorbance at 525 nm was measured with a spectrophotometer. The results were obtained by extrapolating from a standard curve using shark chondroitin-6-sulfate. Newly accumulated GAG was determined by subtracting the unseeded values from the sample values.

6.2.9. Histological Analysis of Cell-Seeded Scaffolds

Cell-seeded scaffolds (n=6) were fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, and sectioned (six-micrometer thick) by microtomy. Sections were Safranin-O stained to detect the presence of sulfated GAG. For type II collagen immunohistochemical analysis, sections were enzymatically digested by protease type XIV for 45 minutes and stained with a standard avidin-biotin complex peroxidase-based antibody staining technique (Vectastain, Vector Laboratories, Burlingame, CA). Mouse anti-chick monoclonal antibody for type II collagen was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Based on the lack of immunohistochemical staining for type II collagen, a Mason's Trichrome stain was also performed to assess the presence of total collagen.

6.2.10. Statistical Analysis

Data were analyzed by one or two-factor analysis of variance (ANOVA), and the Fisher's protected least squares differences (PLSD) post-hoc test using StatView (SAS Institute Inc, Cary, NC). Data are presented as mean ± standard error of the mean.

6.3. RESULTS

6.3.1. Cell-Mediated Contraction of GSCG Scaffolds

There was a notable decrease in scaffold diameter over the course of the 3-D culture for all MSC-seeded scaffolds (Fig. 6.1). Controls (non-transfected MSCs seeded in scaffolds) displayed the least amount of contraction with a 30% reduction in diameter after the 2-week culture period. The GenePorter (x-link) group demonstrated a 38% size reduction, while the Monolayer Transfected and GenePorter (x-link & soak) groups displayed the most contraction, with a 44% decrease in diameter. Two-factor ANOVA revealed a significant effect of transfection condition ($P < 0.004$, power = 0.9) and time ($P < 0.0001$, power = 1) on scaffold contraction. Fisher's PLSD post hoc test showed significant differences between the Control group versus the Monolayer Transfected and GenePorter (x-link & soak) groups and the Monolayer Transfected group versus the GenePorter (x-link) group.

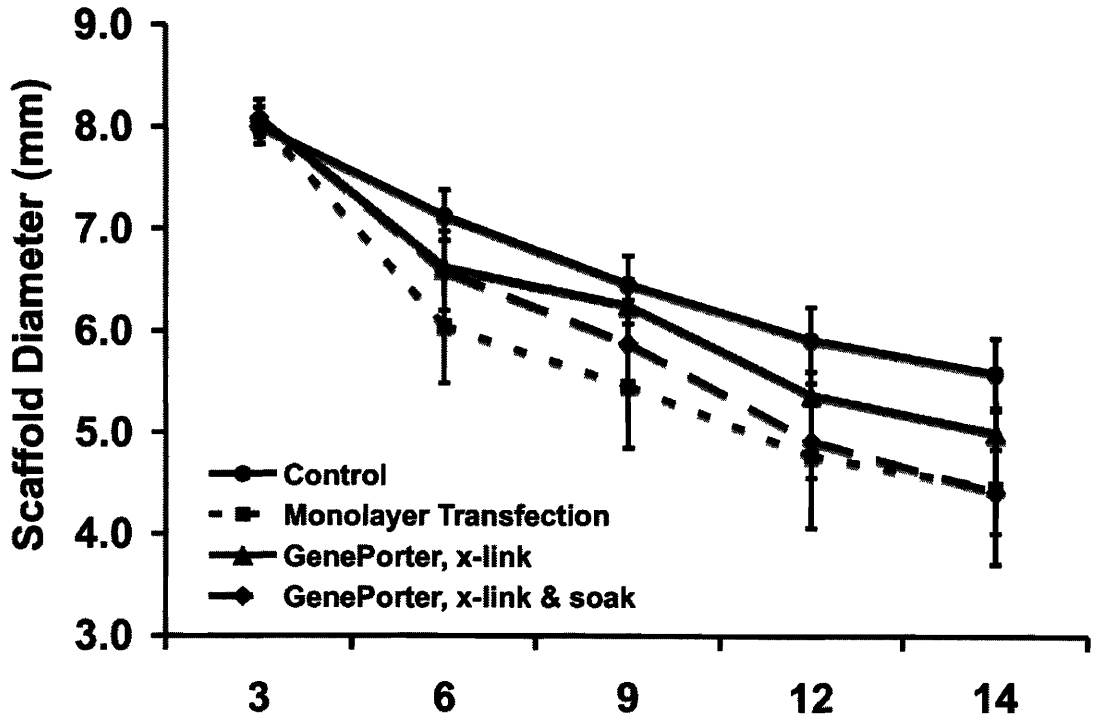


Figure 6.1 Diameter change of MSC-seeded CG scaffolds over the 2-week 3-D culture period. n = 11-12; mean ± SEM.

6.3.2. IGF-1 Release in 3-D Culture Medium

The GenePorter (x-link & soak) group and the Monolayer Transfected group showed a noticeable elevation in IGF-1 expression over the Control group (Fig. 6.2). At the end of the 2-week period, total accumulated IGF-1 for the GenePorter (x-link & soak) and Monolayer Transfected groups was 11-fold and 2-fold higher, respectively, than the Control group (Fig. 6.2b). For all conditions, there seemed to be a trend in the kinetics of IGF-1 release in the medium with a peak expression occurring at the Day 12 collection (Fig. 6.2a). Two-factor ANOVA revealed a significant effect of time ($P < 0.0001$, power = 1) and transfection condition on IGF-1 release in the medium ($P < 0.0001$, power = 1). Post hoc analysis confirmed a significant difference in IGF-1 release for the GenePorter (x-link & soak) group versus all other groups ($P < 0.0001$, power = 1).

6.3.3. Biochemical Analysis of Cell-Seeded GSCG Scaffolds

DNA content at the end of the 2-week culture period for the GenePorter (x-link & soak) and the Monolayer Transfected groups showed noticeably lower values compared to the Control group (Fig. 6.3a). There was a 54% and 30% lower DNA content for the GenePorter (x-link & soak) and the Monolayer Transfected groups, respectively, compared to Controls. ANOVA and Fisher's PLSD post hoc results revealed a significant effect of transfection condition on final DNA content after 2 weeks in culture ($P < 0.0008$, power = 1) with significant differences between the GenePorter (x-link & soak) group versus the Control and GenePorter (x-link) groups ($P < 0.001$, power = 1) and between the Monolayer Transfected group versus the GenePorter (x-link) group. GAG/DNA values for the Control, Monolayer Transfected, and GenePorter (x-link) groups were all similar (Fig. 6.3b). The GenePorter (x-link & soak) group displayed about a 2-fold higher GAG/DNA value above all other conditions. ANOVA and post hoc analysis confirmed a significant effect of transfection condition on GAG/DNA content ($P < 0.0015$, power = 0.97) and showed significant differences between the GenePorter (x-link & soak) group versus all the other groups ($P < 0.003$, power = 0.97).

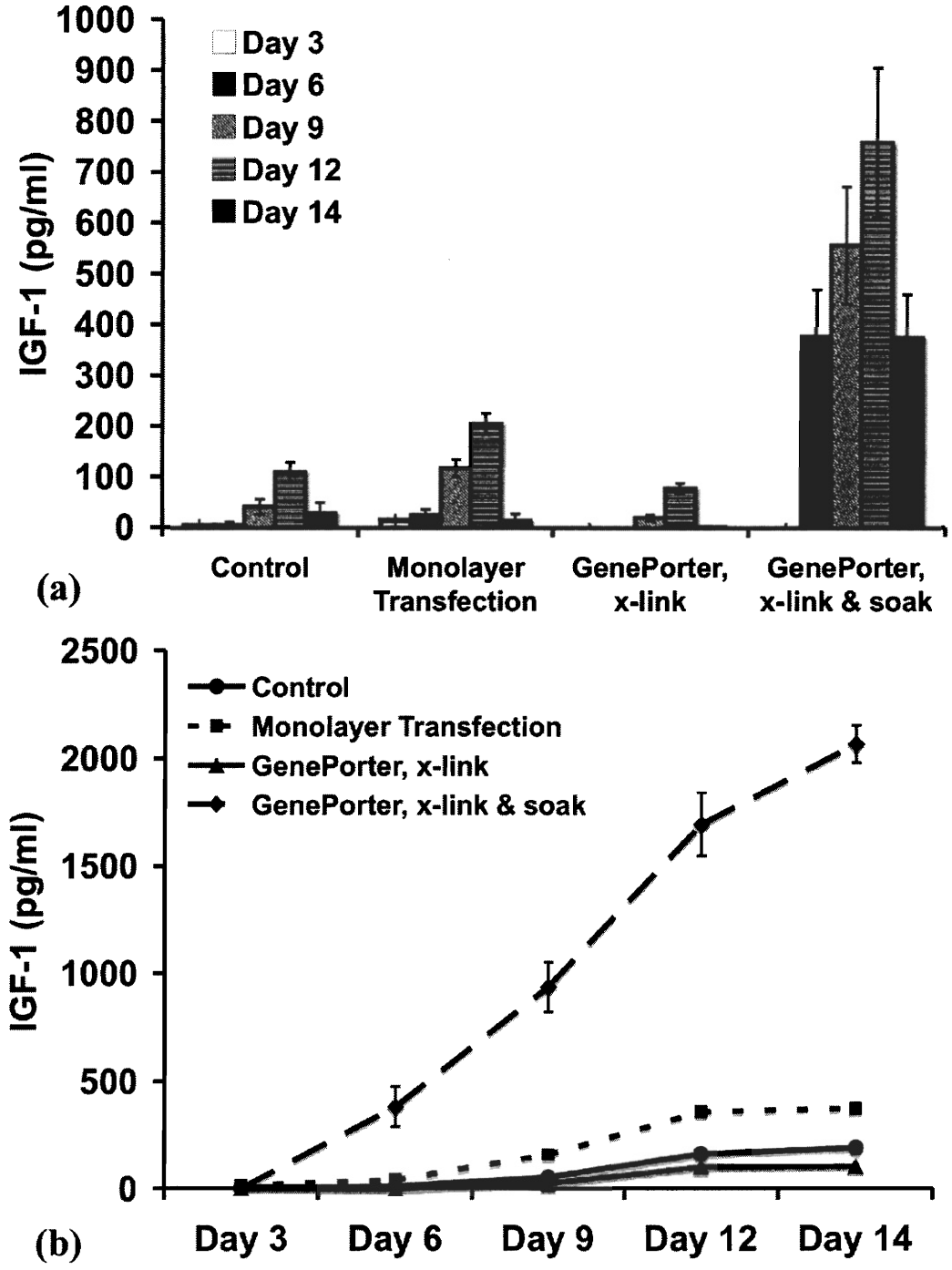


Figure 6.2 IGF-1 detected in the medium at each time point from MSC-seeded constructs (a) and total accumulated IGF-1 (b) over the course of the 2-week 3-D culture period. n = 11-12; mean ± SEM.

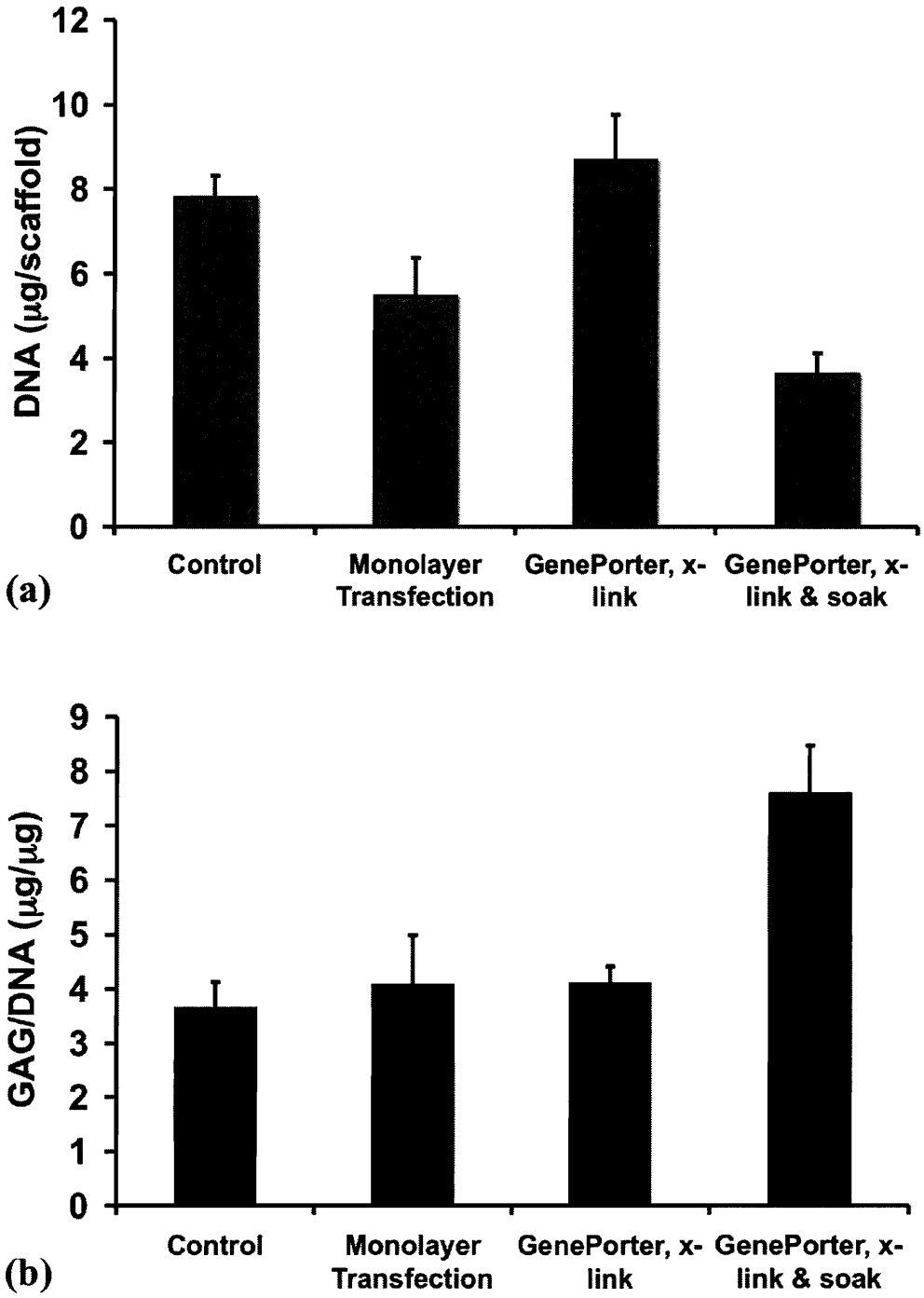


Figure 6.3 DNA (a) and GAG/DNA (b) contents of MSC-seeded scaffolds at the end of the 2-week 3-D culture period. n = 6; mean ± SEM

6.3.4. Histological Analysis of 3-D Cultures

Histochemical staining showed a concentration of MSCs at the periphery of the scaffold for Control, Geneporter (x-link) and Geneporter (x-link & soak) groups (Figs. 6.4 and 6.5). In these scaffolds, there was an absence of cells and tissue formation in the mid-region of the cross-sectional slices. Some of these scaffolds demonstrated a concavity that was observed to be facing up in the tissue culture wells when the cultures were ended after 2 weeks. The Monolayer Transfected group showed the most contraction over time and a high cell concentration in the middle of the scaffold (Fig. 6.5). Vertical cross-sections could not be obtained from the Monolayer Transfected group due to the high deformation of these constructs. Safranin-O staining revealed very little GAG in all of the constructs with only lightly pink regions in between some of the scaffold pores (Fig. 6.4). Tissue that lightly stained for GAG between pores was not dense and had a "string-like" appearance. For the Monolayer Transfected group, most of the tissue and scaffold were contracted and areas staining positive for Safranin-O had a denser appearance. Some samples in the Monolayer Transfected group and the GenePorter (x-link & soak group) showed cells that appeared to be rounded and in lacunae (see arrow on 6.4b).

The Masson's trichrome stain (Fig. 6.5) more clearly distinguished remnants of the remaining scaffold and newly synthesized collagen. Type II collagen immunohistochemical analysis revealed that the synthesized collagen did not seem to be made up of type II collagen as there was very little, if any, positive staining. The Monolayer Transfected group showed the greatest amount of degradation of the scaffold with very little residual scaffold left in the high cell populated middle region. The stages of scaffold degradation can be seen more clearly in the Monolayer Transfected scaffolds were the degrading scaffold displayed a more "foamy" appearance (see arrow on Figure 6.5b) as opposed to the more glassy and opaque appearance of a non-degraded scaffold strut (see arrow on Figure 6.5c). Collagen synthesized from seeded cells displayed a smoother tissue appearance surrounding the cells and had a less opaque blue staining compared to the undegraded scaffold struts (see arrow on Figure 6.5d). All regions that were more populated by cells (on the periphery of the scaffold) for the Control, Geneporter (x-link) and Geneporter (x-link & soak) groups showed more collagen synthesis in between the cells, in contrast to the middle cross-sectional region of the scaffold where most nondegraded scaffold was apparent. The GenePorter (x-link & soak) group seemed to have more collagen synthesis at

the top and bottom of the scaffold that penetrated more deeply into the scaffold compared to the Control and GenePorter (x-link) groups.

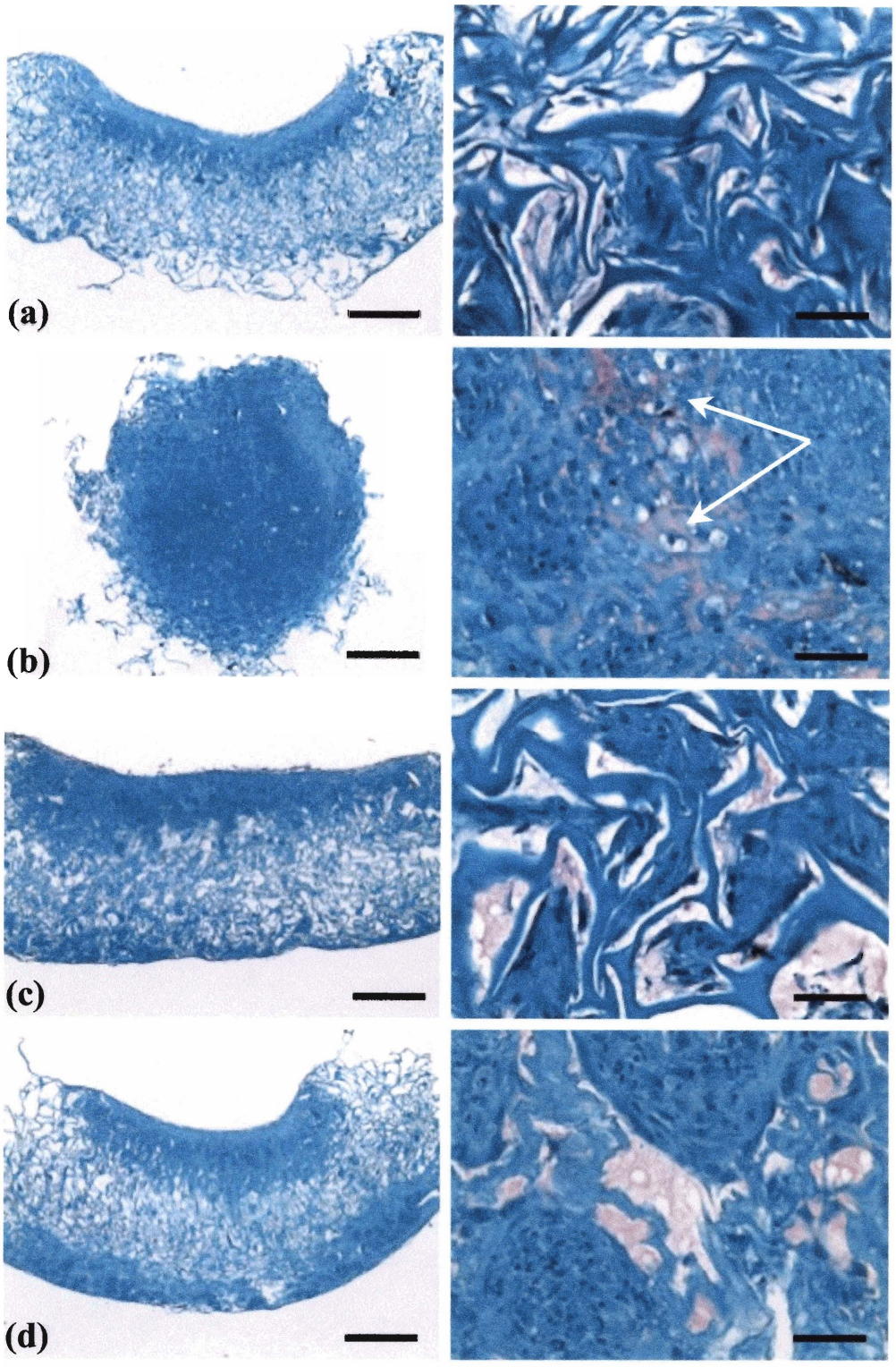


Figure 6.4 **Safranin-O stain for GAG of MSC-seeded scaffolds after 2 weeks in 3D culture (a) Control group, (b) Monolayer transfected group, (c) GenePorter, x-link group, (d) GenePorter, x-link & soak group. Scale bars: 1st column is 500 μm and 2nd column is 50 μm. Red is a positive stain for GAG.**

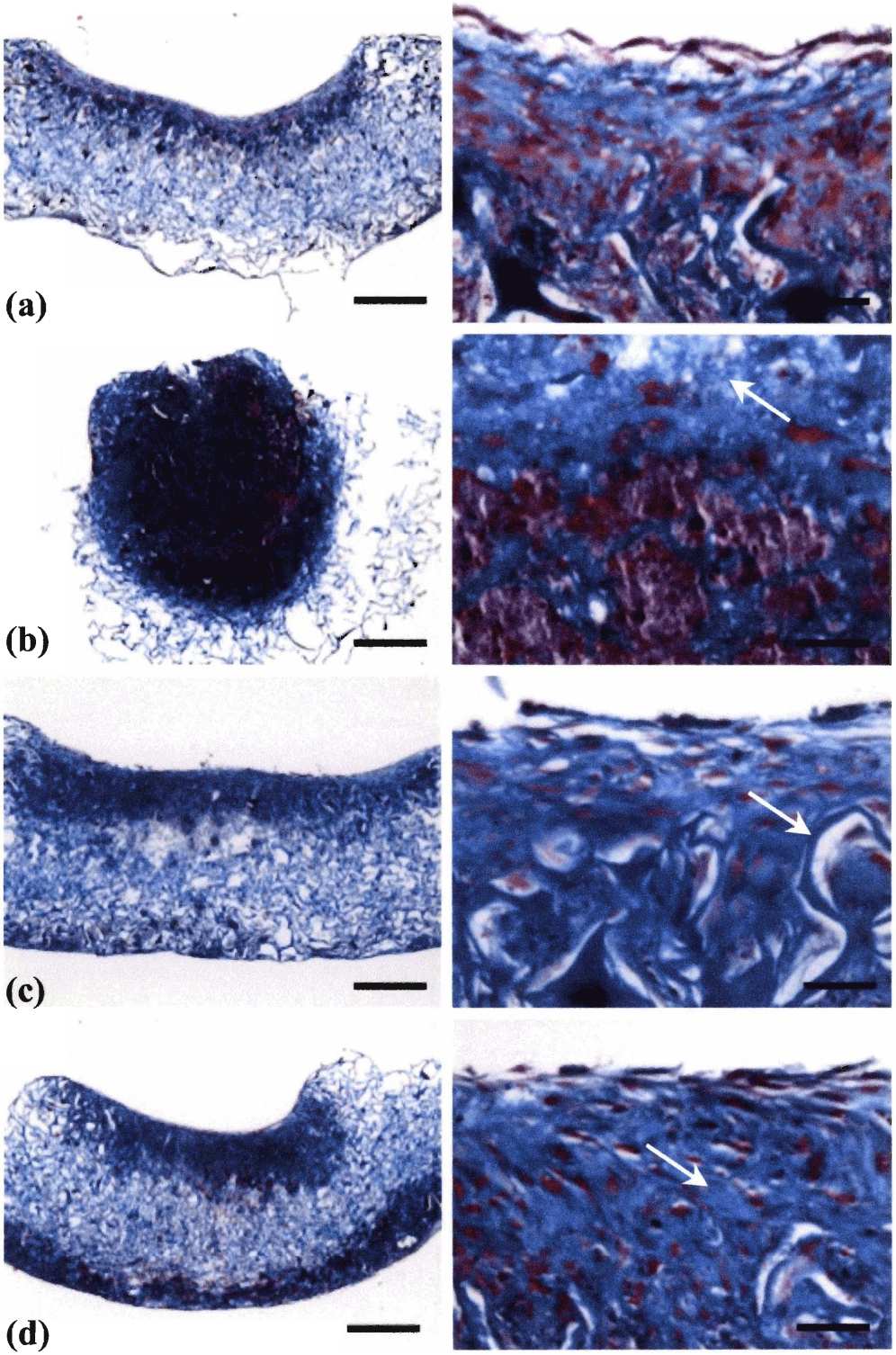


Figure 6.5 **Mason's Trichrome stain of MSC-seeded scaffolds after 2 weeks in 3D culture (a) Control group, (b) Monolayer transfected group, (c) GenePorter, x-link group, (d) GenePorter, x-link & soak group. Scale bars: 1st column is 500 μm and 2nd column is 50 μm. Blue stain is collagen and red stain is cytoplasm.**

6.4. DISCUSSION

Of significance in this study was that MSCs seeded within gene-supplemented CG scaffolds can be transfected and produce elevated levels of IGF-1 over a 2-week 3-D culture period. Control scaffolds containing MSCs seeded within CG scaffolds without gene incorporation also showed IGF-1 production that increased up to 12 days in culture. This increased production of IGF-1 from Control scaffolds may indicate that cell interaction with the CG scaffold and cultured in a defined serum-free medium may also upregulate IGF-1 expression. The method of gene incorporation within CG scaffolds significantly affected IGF-1 overexpression as reflected in the IGF-1 release kinetics for the GenePorter (x-link) group versus the GenePorter (x-link & soak) group. Cross-linking the full 10μg plasmid load to the scaffold, GenePorter (x-link) group, did not show any elevated expression levels over the Control group during the 2-week culture period. When 5μg of the GenePorter/pIGF-1 complex was cross-linked and the other half was absorbed onto the scaffold (GenePorter (x-link & soak) group) there was a significantly higher IGF-1 release in the media compared to the Control group. This may indicate that the IGF-1 overexpression produced by the GenePorter (x-link & soak) group within this 2-week culture period, is most likely the result of MSCs being transfected by the absorbed GenePorter/IGF-1 complexes that are quickly released from the scaffold at early time points. Based on the histological evaluation revealing the significant amount of undegraded scaffold left at the end of 2 weeks, it is likely that the majority of the cross-linked GenePorter/pIGF-1 complexes was not released within this time frame. Longer-term studies need to assess if IGF-1 overexpression will occur at later time points when the scaffold more fully degrades releasing the cross-linked GenePorter/pIGF-1 complexes.

Interestingly, transfecting MSCs in monolayer did not result in the highest overexpression when subsequently seeded in 3-D culture. There was only about a 2-fold higher accumulated amount of IGF-1 produced from the Monolayer Transfected group compared to the 11-fold higher level of the GenePorter (x-link & soak) group compared to the Controls. The amount of GenePorter/pIGF-1 complexes used for monolayer transfection was approximately the same amount per cell used for the MSC-seeded gene-supplemented scaffolds. This demonstrates that nonviral transfection of cells in a 3-D culture environment may be more efficient than transfection in 2-D. This was also demonstrated in the study by Xie et al. [13], in which cells were seeded onto 2-D films or 3-D scaffolds and nonvirally transfected with pCMV-bgal and pEGFP

reporter vectors. In this study, 3-D transfection was found to promote a higher gene expression level and longer expression times compared to 2-D transfection.

Unexpectedly, for all groups (including the Control group) there was a peak in IGF-1 production between days 9 and 12 and then a decrease in IGF-1 release between days 12 and 14. It is speculated that this decrease in IGF-1 release may be due to cell apoptosis due to scaffold contraction over time. This contraction may inhibit sufficient nutrient diffusion throughout the entire scaffold, eventually leading to cell death and the decreased production of IGF-1. Of note, however, is an apparent correlation between total accumulated IGF-1 released in the medium and scaffold contraction. Samples that showed elevated IGF-1 expression above the Controls (the Monolayer Transfection and Geneporter (x-link & soak) groups), also showed the most scaffold contraction over the two-week culture period. This may indicate that IGF-1 might play a role in affecting scaffold contraction by increasing enzymatic production and/or smooth muscle actin expression of seeded cells. Future studies will need to assess the possible role of IGF-1 in regulating matrix remodeling and smooth muscle actin expression.

Biochemical analysis for DNA content showed that the Monolayer Transfected and GenePorter (x-link & soak) groups had lower amounts of DNA after 2-weeks in culture. This supports the possible association between scaffold contraction and cell apoptosis since these groups were also the ones that contracted the most. Despite this decrease in DNA content, however, the GenePorter (x-link & soak) group showed the highest amount of accumulated GAG/DNA at the end of the culture period. The 2-fold increase in GAG/DNA values for the GenePorter (x-link & soak) group above all other groups may be the result of the significant elevation of IGF-1 overexpression over time.

Although GAG/DNA values were higher for the GenePorter (x-link & soak group) this increased accumulated GAG production was not clearly illustrated in the Safranin-O histologically stained sections. In all groups, there were few isolated regions of light staining for GAG. The cell distribution for the Control, GenePorter (x-link) and GenePorter (x-link & soak) groups were highly concentrated on the periphery of the scaffold with very little cell distribution in the middle cross-sections of the scaffolds. Future work will need to investigate other methods of cell-seeding MSCs into the CG scaffolds to create an even distribution of cells that results in a more ideal environment for enhancing biosynthesis throughout the entire scaffold. The higher cell concentrated regions, however, did show collagen synthesis by seeded cells (reflected in the

Mason's trichrome stain), although immunohistochemical analysis revealed an absence of type II collagen.

Of interest are the evident stages of scaffold degradation, most apparent in the outer periphery of the Monolayer Transfected group, where the degrading scaffold starts to have a "foamy" appearance together with a swelling of the scaffold struts. The Monolayer Transfected group showed the most scaffold degradation and a very high number of cells concentrated in the middle region of the scaffold. The high concentration of cells may have produced a higher concentration of secreted enzyme to more rapidly break down the scaffold. Since scaffolds from the Monolayer Transfected group deformed the most, it was difficult to obtain vertical cross-sections of samples to determine if this cell distribution was carried throughout the depth of the scaffold. Despite this high concentration of cells in the mid-region of the scaffold, however, there was very little tissue synthesis (i.e. most of this region stained red for cell cytoplasm and not collagen). Cell debris was also apparent in the high cell-populated regions, perhaps owing to insufficient nutrient diffusion to these areas. The increased scaffold degradation and lack of biosynthesis in the high cell-concentrated regions indicates that a very high cell density may not be ideal for obtaining a balanced rate of scaffold degradation and new tissue biosynthesis that is required for enhanced tissue repair.

It is important to note that the anabolic effects of IGF-1 are highly dependent on the IGF-1 tyrosine kinase receptor [14] and the accessibility of IGF-1 to its receptor is regulated by the presence of IGF-1 binding proteins [15]. It has been shown that IGF-1 itself can stimulate the production of IGF-binding proteins in ovine and bovine articular and growth-plate chondrocyte monolayers and can diminish the bioactivity of IGF-1 [16]. Therefore, it is possible that despite the significant elevation of IGF-1 expression above the Control levels for the GenePorter (x-link & soak) group, the modest response in biosynthesis reflected in the histological results may be due to the production of IGF-binding proteins that could have potentially inhibited the anabolic action of expressed IGF-1. Future work will need to assess the presence of IGF-binding proteins in these cultures to determine to what extent the goat MSCs seeded within GSCG scaffolds are producing IGF-binding proteins.

In conclusion, gene-supplemented CG scaffolds can effectively transfect seeded MSCs resulting in elevated IGF-1 expression levels and enhanced GAG biosynthesis (as reflected in biochemical analysis) after 2-weeks *in vitro*. These findings show promise in using MSCs as a

cell source for seeding within GSCG scaffolds for tissue engineering purposes, or for regenerative medicine applications, in which a GSCG scaffold can be implanted in conjunction with a microfracture procedure.

6.5. REFERENCES

1. Breinan HA, Martin SD, Hsu HP, Spector M. Healing of canine articular cartilage defects treated with microfracture, a type-II collagen matrix, or cultured autologous chondrocytes. J.Orthop.Res. 2000 Sep;18(5):781-789.

2. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp.Cell Res. 1998 Jan 10;238(1):265-272.

3. Wang WG, Lou SQ, Ju XD, Xia K, Xia JH. In vitro chondrogenesis of human bone marrow-derived mesenchymal progenitor cells in monolayer culture: activation by transfection with TGF-beta2. Tissue Cell 2003 Feb;35(1):69-77.

4. Huang D, Liang C, Wang L, Liu S, Li S. Study on chondrogenesis of rabbit bone marrow-derived mesenchymal stem cells by centrifuge-tube culture in vitro. Lin Chuang Er Bi Yan Hou Ke Za Zhi 2005 Feb;19(4):168-9, 172.

5. Solchaga LA, Penick K, Porter JD, Goldberg VM, Caplan AI, Welter JF. FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. J.Cell.Physiol. 2005 May;203(2):398-409.

6. Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumura M, Fujinaga T. Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: Influence of collagen type II extracellular matrix on MSC chondrogenesis. Biotechnol.Bioeng. 2006 Feb 9.

7. Spagnoli A, Longobardi L, O'Rear L. Cartilage disorders: potential therapeutic use of mesenchymal stem cells. Endocr Dev. 2005;9:17-30.

8. Oh CD, Chun JS. Signaling mechanisms leading to the regulation of differentiation and apoptosis of articular chondrocytes by insulin-like growth factor-1. J.Biol.Chem. 2003 Sep 19;278(38):36563-36571.

9. Worster AA, Brower-Toland BD, Fortier LA, Bent SJ, Williams J, Nixon AJ. Chondrocytic differentiation of mesenchymal stem cells sequentially exposed to transforming growth factor-beta1 in monolayer and insulin-like growth factor-I in a three-dimensional matrix. J.Orthop.Res. 2001 Jul;19(4):738-749.

10. D. Hastreiter. A collagen-GAG matrix for the growth of intervertebral disc tissue. Cambridge, MA: Massachusetts Institute of Technology; 2002.

11. Yannas IV, Lee EO,D.P., Skrabut EM, Murphy GF. Synthesis and characterization of a model extracellular matrix that induces partial regeneration of adult mammalian skin. Proc. Nat'l. Acad. Sci. USA 1989;86:933-937.

12. Lee CR, Grodzinsky AJ, Spector M. The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis. Biomat. 2001;22:3145-3154.

13. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986 Sep 4;883(2):173-7.

14. Jansen J, van Buul-Offers SC, Hoogerbrugge CM, de Poorter TL, Corvol MT, Van den Brande JL. Characterization of specific insulin-like growth factor (IGF)-I and IGF-II receptors on cultured rabbit articular chondrocyte membranes. J.Endocrinol. 1989 Feb;120(2):245-249.

15. Dore S, Pelletier JP, DiBattista JA, Tardif G, Brazeau P, Martel-Pelletier J. Human osteoarthritic chondrocytes possess an increased number of insulin-like growth factor 1 binding sites but are unresponsive to its stimulation. Possible role of IGF-1-binding proteins. Arthritis Rheum. 1994 Feb;37(2):253-263.

16. Sunic D, Belford DA, McNeil JD, Wiebkin OW. Insulin-like growth factor binding proteins (IGF-BPs) in bovine articular and ovine growth-plate chondrocyte cultures: their regulation by IGFs and modulation of proteoglycan synthesis. Biochim.Biophys.Acta 1995 Aug 17;1245(1):43-48.

CHAPTER 7: LIMITATIONS AND FUTURE WORK

The work presented in this thesis shows significant promise for the use of GSCG scaffolds in articular cartilage tissue engineering. The following discussion describes various limitations to the work presented and identifies potential areas of research for further developing GSCG scaffolds in tissue engineering applications.

One limitation associated with the use of natural biomaterials for tissue engineering scaffolds was the inconsistent properties of the type II collagen slurry and resulting freeze-dried porous scaffolds employed in the various studies conducted. Since the type II slurry or freeze-dried sheets used to make the type II slurry was provided by an outside source, the resulting slurry viscosity and subsequent freeze-dried scaffold properties (i.e. porosity, degradation properties, etc.) were difficult to predict and control from batch to batch, even when using the same protocols for creating porous constructs. This variability in inherent scaffold properties made it difficult to directly compare results from multiple experiments if different slurry batches were used. Efforts to more fully characterize the raw materials and establish standardized criteria for slurry properties (i.e. viscosity, GAG content, collagen typing, etc.) would be beneficial for the creation of scaffolds with more consistent batch-to-batch properties.

Another potential limitation was that cells were obtained and used from only one animal in experiments using adult canine articular chondrocytes. Other studies carried out in our group using multiple animals and using the same culture conditions as in the presented studies, however, demonstrated that interanimal variability using adult mongrel dogs was minimal (coefficient of variance about 10-15%). This variability is small in comparison to the difference in the outcome variables between groups that show a statistically significant difference. Hence, the overall findings presented in these experiments would most likely be confirmed if subsequent multi-animal experiments were carried out. For future developments, however, it is advantageous to use cells from multiple animals to present more robust findings so that differences between experimental groups would not be exaggerated or muted, as is the potential case when using one animal.

Although the findings of this thesis clearly demonstrates the potential of using GSCG scaffolds as gene delivery vehicles for localized and prolonged release of desired growth factors for the ultimate enhancement of articular cartilage regeneration, below is a list of potential areas of research for the further development of GSCG scaffolds:

- Evaluation of GSCG scaffolds in an *in vivo* model, assessing the ability of these scaffolds to enhance articular cartilage regeneration using either a cell-seeded GSCG construct or an unseeded GSCG scaffold used in conjunction with a microfracture procedure.
- Investigation of better seeding techniques using mesenchymal stem cells for a more even cell distribution within GSCG scaffolds.
- A more thorough understanding of cell interaction with CG scaffolds incorporating naked plasmid alone, with the lipid mediated transfection reagent, or within gelatin nanoparticles in regards to cell attachment, scaffold contraction (evaluating contributions from varying scaffold cross-link density or SMA expression of seeded cells), and transfection efficiency.
- Alternative methods for incorporation of plasmid within CG scaffolds for a better control of vector release. For example:
  - Cationization of the type II scaffold for better naked plasmid retention using a soak and freeze-dry method of incorporation (eliminates the interdependence of the amount of gene incorporation and the degree of cross-linking of the collagen scaffold associated with using the carbodiimide method of incorporation).
  - The use of the avidin-biotin system for plasmid attachment to CG scaffolds (may provide a way to have better control over the specific amount of plasmid that is incorporated).
- Incorporation of more than one gene within CG scaffolds and controlling the release of each individual gene to be expressed at varying time points in the repair process.
- A more thorough investigation of the effect of plasmid size (when incorporated within CG scaffolds) on the ability to transfect seeded cells.
- Determination of optimal conditions for synthesizing and incorporating gelatin nanoparticles within CG scaffolds.

CHAPTER 8: SUMMARY

The overall goal of this thesis was to investigate a scaffold-based gene transfer approach employing collagen (type II)-GAG scaffolds as nonviral gene delivery vehicles to provide local, elevated, and prolonged release of growth factors for enhancing articular cartilage tissue engineering *in vitro* and ultimately *in vivo*. The main variables investigated in the process of developing gene-supplemented 3-D constructs for implantation *in vivo* are: 1) media culture conditions in both monolayer expansion and 3-D culture, 2) methods to incorporated genes within type II CG scaffolds, 3) methods to enhance gene transfer using lipid-mediated transfection reagents or nanoparticles, and 4) potential cell sources. This chapter provides a summary of the significant findings from the experiments presented in the previous chapters in relation to the aforementioned hypotheses.

The first experiments in Chapter 2 involved comparing two different expansion media for growing chondrocytes in monolayer to be subsequently cultured in CG scaffolds using a serum-free culture medium. Although the initial purpose of this experiment was to assess specific expansion medium culture conditions that would result in better cartilaginous formation when using a defined serum-free medium in 3-D culture, an interesting finding was the fact that the composition of the expansion medium also had a significant impact on the ability to (nonvirally) transfect or (virally) transduce chondrocytes in monolayer. Fortunately, the expansion medium (Medium 2) that enhanced biosynthesis and chondrogenesis in 3-D culture also proved to be the expansion medium that resulted in improved gene transfer to chondrocytes in monolayer. These results commended the use of Medium 2 for expanding chondrocytes in conjunction with a defined serum-free culture medium for 3-D culture, throughout the rest of the subsequent experiments for evaluating gene-supplemented collagen-GAG scaffolds for articular cartilage tissue engineering.

The findings of this study support the first hypothesis that the make up of the monolayer expansion medium not only has a direct effect on biosynthesis and chondrogenesis in 3-D culture, but also affects gene transfer to cells.

Chapter 3 included a comparison of two different gene incorporation methods. One method involved soaking the scaffold in the plasmid solution followed by a freeze-drying process (Method 1) and the other involved chemically cross-linking the plasmid to the scaffold (Method 2). This study evaluated these two incorporation methods regarding the kinetics of IGF-1 plasmid release from the scaffolds, the ability to maintain plasmid integrity, and the resulting IGF-1 expression kinetics from cells seeded and subsequently transfected within the GSCG scaffolds. Another aspect of this study was a comparison between supplementing CG scaffolds with either naked plasmid IGF-1 alone or plasmid complexed to a (GenePorter®) lipid-mediated transfection reagent (GP/IGF), evaluating the differences in IGF-1 gene expression over time and the effects on biosynthesis and chondrogenesis. The significant findings from these studies are as follows:

- Cross-linking plasmid DNA to CG scaffolds (Method 2) results in a greater retention of plasmid DNA as opposed to the quick passive release of plasmid (majority of plasmid is released within 24 hrs in buffer) from the soak and freeze-dry method of gene incorporation (Method 1).
- Plasmid integrity is maintained for plasmid released or retained in GSCG scaffolds for both methods of incorporation.
- Plasmid release kinetics from GSCG scaffolds dictates the kinetics of IGF-1 expression from chondrocytes seeded and subsequently transfected within GSCG scaffolds; scaffolds synthesized by Method 2 result in a more steady and prolonged IGF-1 release compared to scaffolds synthesized by Method 1.
- Incorporation of a lipid-mediated transfection together with the IGF-1 plasmid results in enhanced gene transfer and higher IGF-1 release compared to using naked plasmid alone.
- A localized, elevated, and prolonged release of IGF-1 from chondrocytes seeded and subsequently transfected within GSCG scaffolds (with as little as 4-10μg of plasmid IGF-1) results in enhanced biosynthesis and chondrogenesis *in vitro.*

The results from these studies support the second hypothesis that cross-linking naked plasmid DNA to the CG scaffold can result in a more prolonged delivery of genes and expression of the encoded growth factor compared to plasmid addition without cross-linking; and the third

hypotheses that a lipid transfection reagent complexed to the plasmid can be successfully incorporated and cross-linked to CG scaffolds, and can enhance gene transfer to seeded cells.

Chapter 4 included the development and characterization of cationized gelatin plasmid IGF-1 nanoparticles (CGPIN) and evaluates 1.) the potential use of these nanoparticles as nonviral gene transfer reagents in chondrocyte monolayers and 2.) the behavior of CGPIN-transfected cells seeded within CG scaffolds in regards to the IGF-1 release kinetics and subsequent effects on GAG biosynthesis. The results from these studies showed that:

- Gelatin nanoparticles formed by a complex coacervation method using cationized gelatin and plasmid IGF-1 can effectively form uniformly-sized nanoparticles that can successfully transfect chondrocytes with the IGF-1 (and GFP) genes in monolayer culture.
- Nanoparticle morphology and zeta potential (which is affected by gelatin cationization), the weight ratio of gelatin to plasmid used to synthesize the nanoparticles, and the plasmid amount applied to cells during transfection significantly affects the resulting transfection of chondrocytes in monolayer.
- CGPIN-transfected cells subsequently seeded within CG scaffolds can maintain an elevated and prolonged expression of IGF-1 up to 2-weeks in culture.
- As demonstrated in previous studies, the effect of IGF-1 overexpression localized within CG scaffolds can result in a significant improvement in GAG biosynthesis compared to the control conditions.

This experiment corroborates the fourth hypothesis that gelatin nanoparticles can successfully enhance the transfection of chondrocytes and can be used in conjunction with a CG scaffold to produce a localized and prolonged release of encoded protein.

Based on promising results from monolayer transfection studies using CGPIN, Chapter 5 evaluated methods to synthesize GSCG scaffolds using either CGPIN or GP/IGF complexes using two different modes of incorporation: 1.) cross-linking half the total plasmid load to the scaffold and allowing the other half of the load to be absorbed into the scaffold (Method 1) or 2.) cross-linking the full amount of plasmid load (Method 2—also used in prior experiments). The significant conclusions from these studies are the following:

- CGPIN and GP/IGF complexes can be successfully incorporated within type II CG scaffolds and can result in elevated IGF-1 release into the 3-D culture medium.
- Method 1-synthesized GSCG scaffolds results in higher IGF-1 release levels compared to Method 2 over the 2-weak period for scaffolds containing either CGPIN or GP/IGF complexes.
- Scaffolds incorporating GP/IGF complexes demonstrate higher IGF-1 expression levels compared to scaffolds containing CGPIN. The type of gene supplementation, however, also significantly affects the cross-link density as well as the resulting cell-mediated contraction of GSCG scaffolds.
- IGF-1 overexpression resulting from successful transfection of chondroctyes seeded within GSCG scaffolds can result in enhanced biosynthesis and chondrogenesis in 3-D culture.

These findings also support the third and fourth hypothesis that a lipid-mediated transfection reagent or gelatin nanoparticles can successfully enhance the transfection of chondrocytes and can be successfully incorporated within a CG scaffold to produce a localized and prolonged release of encoded protein.

Chapter 6 evaluated mesenchymal stem cells (MSCs) as an alternative cell source to be used in conjunction with GSCG scaffolds for articular cartilage tissue engineering, by assessing the the ability of MSCs to be transfected within GSCG scaffolds and to maintain prolonged overexpression of the encoded protein. The results from this study showed that:

- MSCs can be successfully transfected by incorporated gene vectors when seeded within the CG scaffolds and can maintain prolonged IGF-1 overexpression up to 2 weeks in culture.

These results accept the fifth hypothesis that MSCs can be transfected using GSCG scaffolds and can maintain overexpression of desired proteins over prolonged times.

Based on these investigations, the optimal GSCG scaffold commended for subsequent *in vivo* studies for articular cartilage repair (particularly with a microfracture procedure) would consist of a type II CG scaffold supplemented with 10-20 μg of total plasmid IGF-1 complexed with a lipid-mediated transfection reagent (GenePorter®), incorporating half of the total amount

of the GenePorter/pIGF-1 complexes using a carbodiimide cross-link method and incorporating the other half by simple absorption. This type of construct will allow immediate transfection of cells through passive release of the absorbed plasmid complexes, and prolonged transfection and protein overexpression as the cross-linked GenePorter/pIGF-1 complexes are released upon scaffold degradation over time.

CHAPTER 9: CONCLUSIONS

Below are the main conclusions that this thesis supports:

1. The composition of the monolayer expansion medium not only has a direct effect on biosynthesis and chondrogenesis in 3-D culture, but also on gene transfer to cells in monolayer.
2. Cross-linking plasmid DNA to the CG scaffold achieves a more steady delivery of genes, resulting in a more stable and prolonged overexpression of the encoded growth factor from seeded chondrocytes compared to plasmid incorporation without cross-linking.
3. A lipid mediated transfection reagent complexed to plasmid DNA can be successfully incorporated and cross-linked to CG scaffolds and results in enhanced gene transfer to seeded cells.
4. Cationized gelatin nanoparticles can successfully enhance gene transfer to chondrocytes, and when used in combination with a CG scaffold, can produce a localized and prolonged release of encoded protein.
5. IGF-1 overexpression resulting from successful IGF-1 gene transfer to chondrocytes seeded within GSCG scaffolds enhances the resulting synthesis of cartilage matrix molecules and chondrogenesis.
6. MSCs can be transfected when seeded into GSCG scaffolds and can maintain overexpression of desired proteins over prolonged times.

APPENDICES

The following appendices include additional studies that supplement the main text of the thesis and current protocols used in this thesis work.

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APPENDIX A: IMPORTANCE OF TRANSFORMING GROWTH FACTOR (TGF)-β1 AND/OR OSTEOGENIC PROTEIN (OP)-1 IN THE 3-D SERUM-FREE CHONDROGENIC CULTURE MEDIUM

A.1. INTRODUCTION

Several studies have demonstrated the favorable effects of human recombinant transforming growth factor (TGF)-β1[1-7] and human recombinant osteogenic protein (OP)-1 (or bone morphogenetic protein-7, BMP-7) [8-14] on chondrogenesis *in vitro* and on cartilage repair *in vivo*. The current study investigated the effects of supplementing the 3-D serum-free differentiation medium w/ TGF-β1, OP-1, or TGF-β1 & OP-1 together, on chondroctyes grown in collagen (type II)-GAG (CG) scaffolds. This investigation was performed to assess the necessity of adding TGF-β1 in the 3-D culture medium for evaluating CG scaffolds incorporating the gene encoding for OP-1 and to also verify that OP-1 has beneficial effects on tissue regeneration in our 3-D culture system.

A.2. MATERIALS AND METHODS

A.2.1. Type II Collagen Scaffold Fabrication

Porous sheets of type II collagen were fabricated by freeze-drying a porcine cartilage-derived slurry (Geistlich Biomaterials, Wolhusen, Switzerland). The collagen sheets were sterilized and cross-linked dehydrothermally by placing the samples in a vacuum oven at 105 °C for 24 hours. Eight-mm diameter disks (~2 mm thick) were punched out with a dermal punch (Moore Medical, New Britain, CA) and additionally cross-linked by a ten minute carbodiimide treatment containing an aqueous solution of 14 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 5.5 mM N-hydroxysuccinimide (EDAC; Sigma Chemical Co., St. Louis , MO). Excess EDAC was removed by rinsing in PBS.

A.2.2. Chondrocyte Isolation and Expansion

Chondrocytes were isolated from the trochleae of both knees (stifle joints) from one adult mongrel dog (approximate age 2-4 years). The cells were isolated using a sequential digestion of pronase (20 U/ml, 1hr) and collagenase (200 U/ml, overnight). The cells were then expanded in monolayer culture using a medium consisting of high glucose Dulbecco's modified Eagle's medium, DMEM (4.5 g/L D-glucose, without L-glutamine and with 1 mM sodium pyruvate), containing 10% (v/v) fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 10 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic (HEPES) buffer, 100 U/mL penicillin, and 100 μg/mL streptomycin glutamate. The medium was supplemented with the following growth factors (all from R&D Systems, Minneapolis, MN): 5 ng/mL of fibroblast growth factor-2 (FGF-2), 10 ng/mL of platelet-derived growth factor-bb (PDGF-bb), 1 ng/mL of transforming growth factor- β1 (TGF-β1). The cells were incubated at 37°C and 5% CO_2. Once cells reached confluence, they were trypsinized, re-suspended, and re-plated to obtain passage (P)1 cells for seeding into the scaffolds.

A.2.3. Cell Seeding and Chondrocyte Culture in Collagen (type II)-GAG Scaffolds

For seeding collagen type II scaffolds, hydrated scaffolds (previously cross-linked) were briefly dried on filter paper and placed on pre-warmed agarose-coated wells. Four million cells were added to each scaffold by pipetting a suspension of 2 million cells (in 20 μl medium) onto each side with a 10 minute incubation period in between. Cell-seeded scaffolds were cultured in a defined serum-free medium (SFM) which consisted of high glucose DMEM (4.5 g/L D-glucose, without L-Glutamine and with 1mM Sodium Pyruvate), 0.1 mM nonessential amino acids, 10 mM HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin glutamate, ITS^{+1} (100x, by Sigma Chemical, St. Louis, MO), 0.1 mM ascorbic 2-phosphate, 1.25 mg/ml bovine serum albumin, and 100 nM dexamethasone. To examine the effects of adding human recombinant TGF-β1 and/or OP-1 to the SFM, the following conditions were investigated: 1.) no growth factor addition (NoGF group), 2.) addition of 10 ng/ml TGF-β1 (TGF group), 3.) addition of 10 ng/ml OP-1 (OP(10) group), 4.) addition of 10 ng/ml TGF-β1 + 10 ng/ml OP-1 (TGF/OP(10) group), or 4.) addition of 10 ng/ml TGF-β1 + 100 ng/ml OP-1

(TGF/OP(100) group). Media was changed every 2-3 days (1.5 ml per scaffold). At each medium change, the diameters of the scaffolds were monitored to assess scaffold contraction over time. Cultures were terminated after 2 weeks for histological evaluation and biochemical analysis. For DNA and GAG analysis, scaffolds were lyophilized and enzymatically digested using proteinase K (Roche Diagnostics, Indianapolis, IN).

A.2.4. DNA Analysis

The DNA content of cell-seeded scaffolds was measured using the Picogreen Dye assay kit (Molecular Probes, Inc, Eugene, OR) (n=4). The Picogreen dye was used with the reagents and standard provided according to the manufacturer instructions.

A.2.5. GAG Analysis

The sulfated GAG content of cell-seeded scaffolds after the 2-week culture period was determined by the dimethylmethylene blue (DMMB) dye assay [15] (n = 4). An aliquot of the proteinase K digest was mixed with the DMMB dye and the absorbance at 525 nm was measured with a spectrophotometer. The results were obtained by extrapolating from a standard curve using shark chondroitin-6-sulfate. Newly accumulated GAG was determined by subtracting the unseeded values from the sample values.

A.2.6. Histology of Cell-Seeded Scaffolds

Cell-seeded scaffolds (n = 2-3) were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. Six-micron thin sections were stained with Safranin-O to assess the presence of sulfated GAG.

A.2.7. Statistical Analysis

Data were analyzed by one- or two-factor analysis of variance (ANOVA), and the Fisher's protected least squares differences (PLSD) post-hoc test using StatView (SAS Institute Inc, Cary, NC). Data are presented as mean ± standard error of the mean.

A.3. RESULTS

A.3.1. Macroscopic Appearance and Contraction of Cell-Seeded CG Scaffolds

There was an obvious difference in appearance and contraction comparing scaffolds grown with or without the addition of TGF-β1 at the end of the 2-week culture period (Fig. A.1). Scaffolds grown with TGF-β1 (Figs. A.1d-f) seemed to have more tissue formation and possessed a more homogeneous opaque appearance throughout the entire scaffold. In contrast, scaffolds grown without TGF-β1 (Figs. A.1a-c) were much smaller in size, had greater deformation, and showed an opaque area mostly in the center of the scaffold (around the periphery, the scaffold appeared more transparent). The color of the resulting medium was also more pink for cultures grown without TGF-β1. There was no obvious macroscopic difference in scaffold appearance after 2 weeks resulting from the addition of OP-1 at either concentration (10 ng/ml or 100 ng/ml) with or without TGF-β1.

Measurements of scaffold diameter at the end of 2 weeks showed that scaffolds grown in medium without TGF-β1 contracted to 3.5-4 mm in diameter, whereas, the scaffolds cultured with TGF-β1 supplementation had diameters in the range of 5-6 mm (Fig. A.2). Two-factor ANOVA revealed a significant effect of time ($P < 0.0001$, power = 1) and type of growth factor supplementation ($P < 0.0001$, power = 1) on cell-mediated contraction. Additional post hoc analysis showed significant differences between all scaffolds grown with TGF-β1 versus all scaffolds grown without TGF-β1 ($P < 0.0001$, power = 1). Of the scaffolds grown without TGF-β1, the NoGF group was significantly different from the OP(10) group ($P < 0.0005$, power = 1). For the scaffolds grown with TGF-β1 supplementation, there was a significant difference between the TGF and TGF/OP(10) groups ($P < 0.0001$, power = 1).

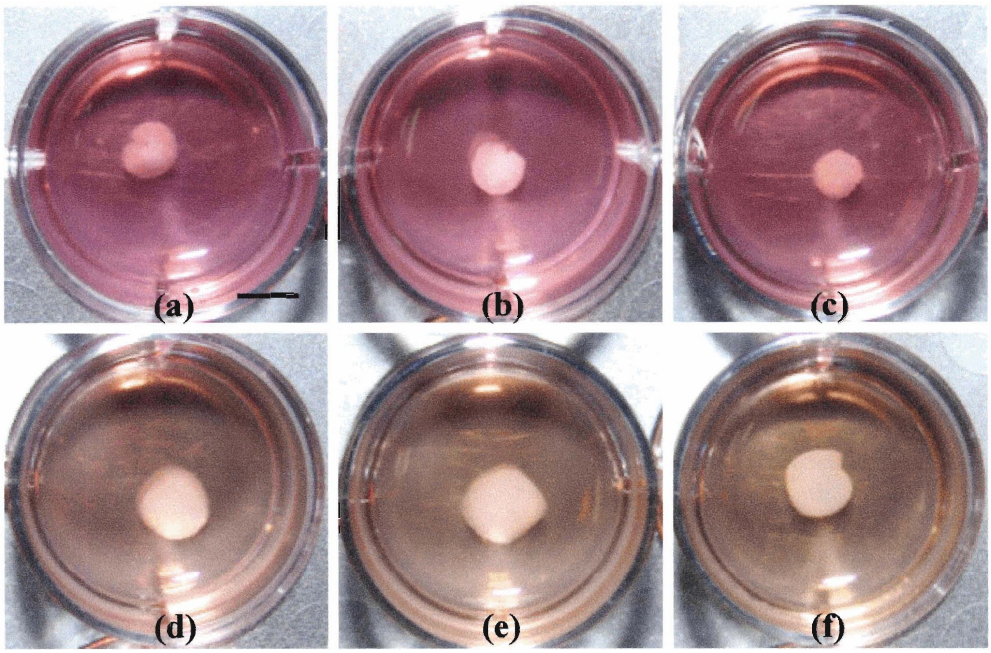


Figure A. 1 Macroscopic view of cell-seeded 3-D scaffolds grown in serum-free medium supplemented with no growth factors (a), 10ng/ml OP-1 (b), 100ng/ml OP-1 (c), 10ng/ml TGF-β1 (d), 10ng/ml TGF-β1+10ng/ml OP-1 (e), 10ng/ml TGF-β1+100ng/ml OP-1. Scale bar is 400μm.

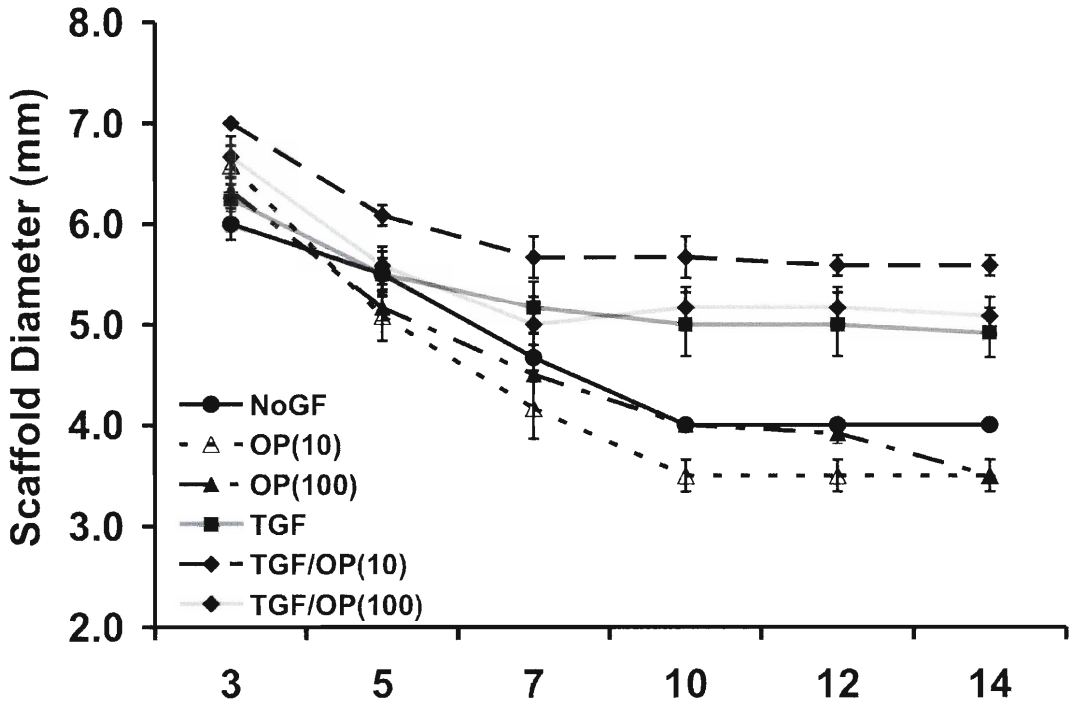


Figure A. 2 Contraction of cell-seeded CG scaffolds over the 2-week culture period. n = 6; mean ± SEM.

A.3.2. Biochemical Analysis of Cell-Seeded CG Scaffolds

At the end of the 2-week culture, DNA (Fig. A.3a) and accumulated GAG/DNA content (Fig. A.3b) were noticeably higher for scaffolds grown in TGF-β1-supplemented medium. ANOVA showed a significant effect of type of growth factor supplementation on DNA content and on accumulated GAG/DNA content ($P < 0.0001$, power = 1) with significant difference between all scaffolds grown with TGF-β1 versus those grown without TGF-β1 ($P < 0.0001$, power = 1). Post hoc analysis revealed that for scaffolds grown without TGF-β1 supplemented in the medium, the addition of OP-1 at 100 ng/ml did result in a significantly higher DNA content (~20% higher value) compared to the NoGF and OP(10) groups ($P < 0.002$, power = 1). Additionally, there was a slight significant difference (~10%) in DNA content between the TGF/OP(10) and the TGF/OP(100) groups ($P < 0.04$, power = 1). There was no significant difference in GAG/DNA content among the cultures grown without TGF-β1. For scaffolds grown with TGF-β1, however, there was a significant effect of adding OP-1 at the 100 ng/ml concentration on GAG/DNA at the end of the 2-week period, with a 22% and 31% higher GAG/DNA value compared to the TGF group and the TGF/OP(10) group, respectively ($P < 0.004$, power = 1).

A.3.3. Histological Analysis of Cell-Seeded CG Scaffolds

Safranin-O staining for GAG revealed an obvious difference in tissue formation and staining between scaffolds grown without TGF-β1 (Figure A.4a and b) verses scaffolds cultured with TGF-β1 (Figure A.4c). There were no obvious differences in histological appearance when OP-1 was added at either concentration (with or without TGF-β1). Scaffolds that were cultured without TGF-β1 showed a very dense population of cells in the middle of the scaffold with very little tissue formation and Safranin-O staining (Figure A.4a and b). In contrast, scaffolds grown in medium with TGF-β1 demonstrated significantly more tissue formation that stained intensely and uniformly for GAG. Some cells within these scaffolds were also rounded and located in lacunae (Figure A.4c).

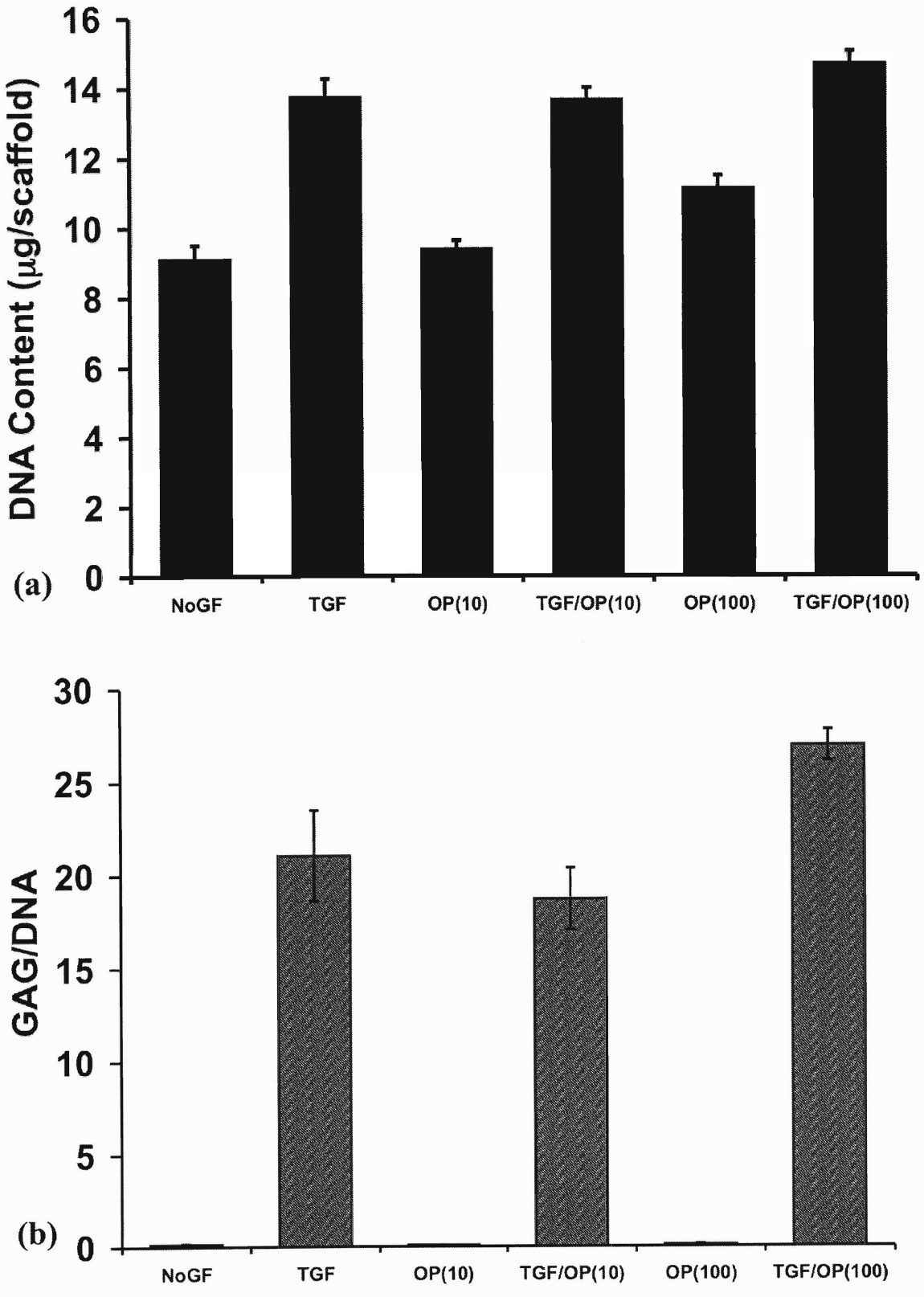


Figure A. 3 DNA content (a) and GAG/DNA content (b) at the end of the 2-week 3-D culture period. n = 4; mean ± SEM.

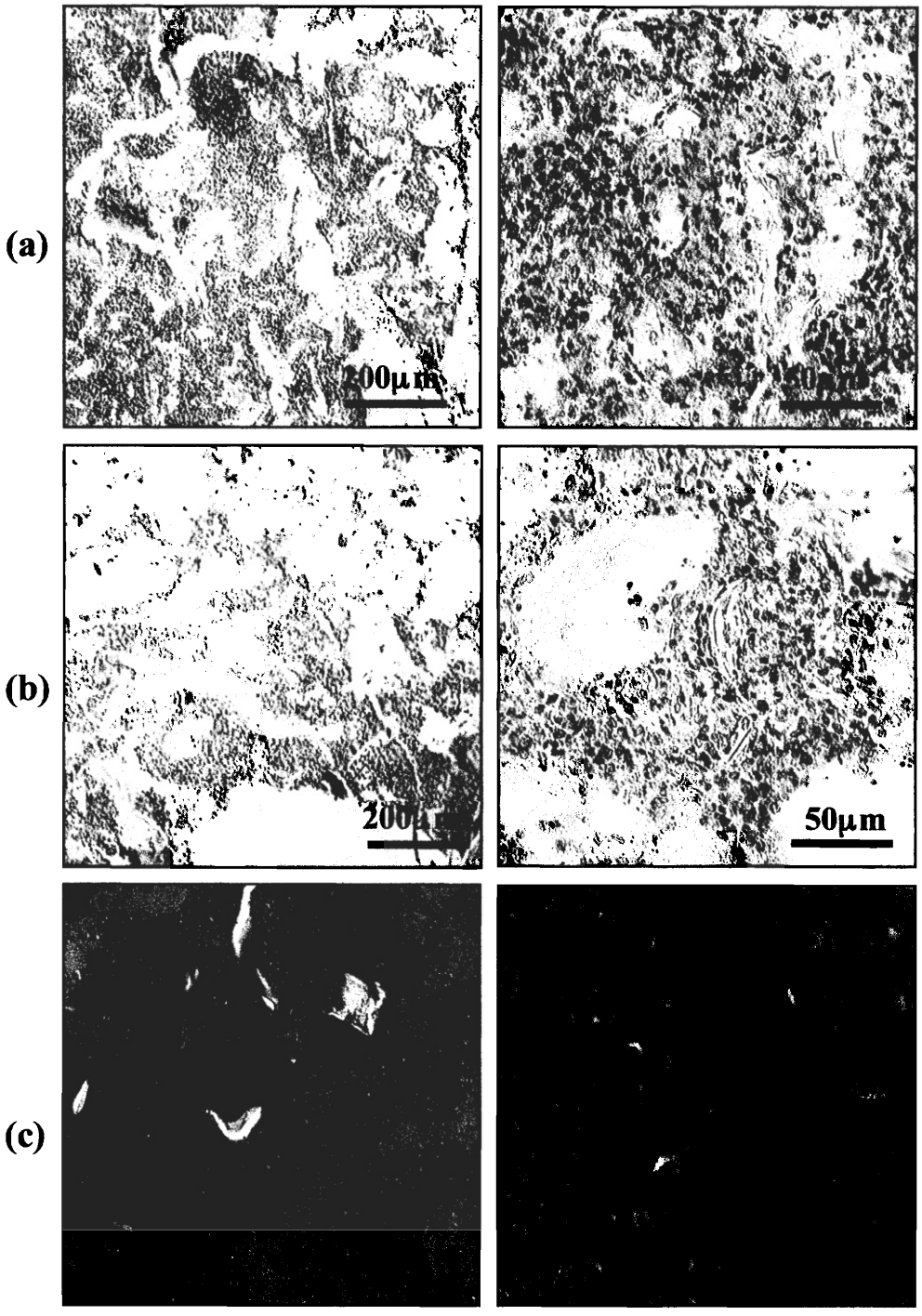


Figure A. 4 Representative sections stained with Saffranin-O (red is positive stain for GAG) of scaffolds cultured without growth factor supplementation (a), with OP-1 (no TGF-β1) (b), or with TGF-β1.

A.4. DISCUSSION

The results of this study verified the importance of adding TGF-β1 in the 3-D serum-free culture medium. The addition of TGF-β1 significantly increased tissue formation, GAG biosynthesis, and chondrogenesis in chondrocyte-seeded CG scaffolds. The increased tissue formation in scaffolds cultured with TGF-β1 resulted in a larger construct size compared to scaffolds grown without TGF-β1. Without TGF-β1 supplementation, the cells contracted the scaffold (resulting in high cell densities in the scaffold center), but hardly any tissue or GAG was produced. The effects of OP-1 were not as obvious in the macroscopic or histological appearance of scaffolds cultured with OP-1, however, there was a significant beneficial effect on accumulated GAG/DNA at the end of the 2-week culture when using 100 ng/ml of OP-1 (with 10 ng/ml TGF-β1) supplemented in the medium compared to using TGF-β1 alone. This demonstrates the potential synergistic effects of TGF-β1 and OP-1 on cartilage formation. Future work should determine the concentrations of OP-1 (when used with TGF-β1) required for optimal biosynthesis and chondrogenesis in CG scaffolds.

These findings commend the continued use of TGF-β1 as a supplement in the 3-D culture medium for evaluating gene-supplemented collagen-GAG (GSCG) scaffolds, and justify the development of GSCG scaffolds incorporating the gene encoding for OP-1.

A.5. REFERENCES

1. van der Kraan PM, Buma P, van Kuppevelt T, van den Berg WB. Interaction of chondrocytes, extracellular matrix and growth factors: relevance for articular cartilage tissue engineering. Osteoarthritis Cartilage 2002 Aug;10(8):631-637.

2. Mauck RL, Nicoll SB, Seyhan SL, Ateshian GA, Hung CT. Synergistic action of growth factors and dynamic loading for articular cartilage tissue engineering. Tissue Eng. 2003 Aug;9(4):597-611.

3. Kim SE, Park JH, Cho YW, Chung H, Jeong SY, Lee EB, et al. Porous chitosan scaffold containing microspheres loaded with transforming growth factor-beta1: implications for cartilage tissue engineering. J.Control.Release 2003 Sep 4;91(3):365-374.

4. Lee JE, Kim KE, Kwon IC, Ahn HJ, Lee SH, Cho H, et al. Effects of the controlled-released TGF-beta 1 from chitosan microspheres on chondrocytes cultured in a collagen/chitosan/glycosaminoglycan scaffold. Biomaterials 2004 Aug;25(18):4163-4173.

5. Holland TA, Bodde EW, Baggett LS, Tabata Y, Mikos AG, Jansen JA. Osteochondral repair in the rabbit model utilizing bilayered, degradable oligo(poly(ethylene glycol) fumarate) hydrogel scaffolds. J.Biomed.Mater.Res.A. 2005 Oct 1;75(1):156-167.

6. DeFail AJ, Chu CR, Izzo N, Marra KG. Controlled release of bioactive TGF-beta 1 from microspheres embedded within biodegradable hydrogels. Biomaterials 2006 Mar;27(8):1579-1585.

7. Chou CH, Cheng WT, Lin CC, Chang CH, Tsai CC, Lin FH. TGF-beta1 immobilized tri-co-polymer for articular cartilage tissue engineering. J.Biomed.Mater.Res.B.Appl.Biomater. 2006 May;77(2):338-348.

8. Klein-Nulend J, Louwerse RT, Heyligers IC, Wuisman PI, Semeins CM, Goei SW, et al. Osteogenic protein (OP-1, BMP-7) stimulates cartilage differentiation of human and goat perichondrium tissue in vitro. J.Biomed.Mater.Res. 1998 Jun 15;40(4):614-620.

9. Koepp HE, Sampath KT, Kuettner KE, Homandberg GA. Osteogenic protein-1 (OP-1) blocks cartilage damage caused by fibronectin fragments and promotes repair by enhancing proteoglycan synthesis. Inflamm.Res. 1999 Apr;48(4):199-204.

10. Louwerse RT, Heyligers IC, Klein-Nulend J, Sugihara S, van Kampen GP, Semeins CM, et al. Use of recombinant human osteogenic protein-1 for the repair of subchondral defects in articular cartilage in goats. J.Biomed.Mater.Res. 2000 Mar 15;49(4):506-516.

11. Nishida Y, Knudson CB, Eger W, Kuettner KE, Knudson W. Osteogenic protein 1 stimulates cells-associated matrix assembly by normal human articular chondrocytes: up-

regulation of hyaluronan synthase, CD44, and aggrecan. Arthritis Rheum. 2000 Jan;43(1):206-214.

12. Jelic M, Pecina M, Haspl M, Kos J, Taylor K, Maticic D, et al. Regeneration of articular cartilage chondral defects by osteogenic protein-1 (bone morphogenetic protein-7) in sheep. Growth Factors 2001;19(2):101-113.

13. Huch K. Long-term effects of osteogenic protein-1 on biosynthesis and proliferation of human articular chondrocytes. Clin.Exp.Rheumatol. 2001 Sep-Oct;19(5):525-531.

14. Loeser RF, Pacione CA, Chubinskaya S. The combination of insulin-like growth factor 1 and osteogenic protein 1 promotes increased survival of and matrix synthesis by normal and osteoarthritic human articular chondrocytes. Arthritis Rheum. 2003 Aug;48(8):2188-2196.

15. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986 Sep 4;883(2):173-7.

APPENDIX B: GENE-SUPPLEMENTED COLLAGEN-GAG SCAFFOLDS FOR NONVIRAL GENE DELIVERY OF OSTEOGENIC PROTEIN (OP)-1

B.1. INTRODUCTION

The recombinant protein for osteogenic protein (OP)-1 or bone morphogenetic protein (BMP)-7 has been shown to have favorable affects on chondrogenesis *in vitro* [1-4] and to stimulate cartilage formation and aggrecan synthesis *in vivo* [5-7]. A local and prolonged administration of OP-1 may be beneficial for articular cartilage formation. Therefore, a plasmid encoding for (OP)-1 was another plasmid of interest to use in conjunction with the collagen (type II) scaffold for providing a prolonged, elevated, and local release of OP-1 via a nonviral scaffold-based approach. This preliminary study investigates the potential use of CG scaffolds for the delivery of a plasmid containing the OP-1 gene (supplied by Stryker Biotech). Plasmid OP-1 (pOP-1) was cross-linked to the CG scaffold with or without a GenePorter® transfection reagent. The OP-1 released in the 3-D serum-free medium from seeded chondroctyes was assessed over a 2-week culture period. At the end of the 2-weeks, scaffolds were allocated for biochemical and histological analysis.

B.2. MATERIALS AND METHODS

B.2.1. Type II Collagen-GAG Scaffold Fabrication

Porous sheets of type II collagen were fabricated by freeze-drying a porcine cartilage-derived slurry (Geistlich Biomaterials, Wolhusen, Switzerland). The collagen sheets were sterilized and cross-linked dehydrothermally by placing the samples in a vacuum oven at 105 °C for 24 hours. Eight-mm diameter disks (~2 mm thick) were punched out with a dermal punch (Moore Medical, New Britain, CA).

B.2.2. OP-1 Plasmid Propagation and Isolation

Multiplication of plasmids encoding for OP-1 (pOP-1) was accomplished by heat shock transformation into *Escherichia coli* DH5α competent cells grown overnight in Luria-Bertani (LB) medium containing ampicillin. Plasmid was isolated and purified using a Mega QIAfilter™ Plasmid kit (Qiagen, Valencia, CA). The absorption ratio at 260 nm and 280 nm was used to determine plasmid concentration and purity while plasmid integrity was demonstrated by polyacrylamide gel electrophoresis. The size of pOP-1 was 11-12kb.

B.2.3. Plasmid Incorporation into CG Scaffolds

Plasmid OP-1 was cross-linked to the CG scaffold with or without a GenePorter® (GP) transfection reagent. An aliquot of diluted pOP-1 solution containing 10 or 50 μg of pOP-1 was placed onto the DHT-treated scaffolds followed by incubation for an hour at room temperature. For scaffolds incorporating the GP/pOP-1 complexes, a 10 μg plasmid load per scaffold was employed and a 5:1 (μl/μg) ratio of GP:plasmid was used to synthesize the GP/pOP-1 complexes. An aliquot of an aqueous carbodiimide cross-linking solution consisting of 14 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 5.5 mM N-hydroxysuccinimide (EDAC; Sigma Chemical Co., St. Louis , MO) was then added to the scaffold and incubated for about 30 minutes to allow cross-links to form among the collagen molecules and between the plasmid and collagen. Excess EDAC was removed by rinsing the scaffolds in PBS. The resulting OP-1 plasmid load was assessed by enzymatically digesting unseeded GSCG scaffolds using proteinase K (Roche Diagnostics, Indianapolis, IN) and assessing the amount of DNA using the Picogreen Dye assay kit (Molecular Probes, Inc, Eugene, OR).

B.2.4. Chondrocyte Isolation and Expansion

Chondrocytes were isolated from the trochleae of both knees (stifle joints) from one adult mongrel dog (approximate age 2-4 years). The cells were isolated using a sequential digestion of pronase (20 U/ml, 1hr) and collagenase (200 U/ml, overnight). were expanded in monolayer culture using a medium consisting of high glucose

Dulbecco's modified Eagle's medium, DMEM (4.5 g/L D-glucose, without L-glutamine and with 1mM sodium pyruvate), containing 10% (v/v) fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 10 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic (HEPES) buffer, 100 U/mL penicillin, and 100 μg/mL streptomycin glutamate. The medium was supplemented with the following growth factors (all from R&D Systems, Minneapolis, MN): 5 ng/mL of fibroblast growth factor-2 (FGF-2), 10 ng/mL of platelet-derived growth factor-bb (PDGF-bb), 1 ng/mL of transforming growth factor- β1 (TGF-β1). The cells were incubated at 37°C and 5% CO_2. Once cells reached confluence, they were trypsinized, re-suspended, and re-plated to obtain passage (P)1 cells for seeding into the scaffolds.

B.2.5. Nonviral Gene Transfer in GSCG Scaffolds and Scaffold Contraction

For seeding GSCG scaffolds, hydrated scaffolds were briefly dried on filter paper and placed on pre-warmed agarose-coated wells. Four million cells were added to each scaffold by pipetting a suspension of 2 million cells (in 20 μl medium) onto each side with a 10-minute incubation period in between. Cell-seeded scaffolds were cultured in a defined serum-free medium (SFM) which consisted of high glucose DMEM (4.5 g/L D-glucose, without L-Glutamine and with 1 mM Sodium Pyruvate), 0.1 mM nonessential amino acids, 10 mM HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin glutamate, ITS^{+1} (100x, by Sigma Chemical, St. Louis, MO), 0.1 mM ascorbic 2-phosphate, 1.25 mg/ml bovine serum albumin, 10 ng/mL of TGF-β1, and 100 nM dexamethasone. Medium was collected and changed at various time points over a two-week culture period, and the amount of OP-1 in the collected medium (n = 6) was detected by an OP-1 DuoSet ELISA kit (R&D Systems). During each medium change, the diameters of the scaffolds were monitored to assess scaffold contraction. Cultures were terminated after 2 weeks for histological evaluation and biochemical analysis of the resulting DNA and GAG content. For DNA and GAG analysis, scaffolds were lyophilized and enzymatically digested using proteinase K (Roche Diagnostics, Indianapolis, IN).

B.2.6. DNA Analysis

The DNA content of cell-seeded scaffolds was measured using the Picogreen Dye assay kit (Molecular Probes, Inc, Eugene, OR) (n = 4). The Picogreen dye was used with the reagents and standard provided according to the manufacturer instructions.

B.2.7. GAG Analysis

The sulfated GAG content of cell-seeded scaffolds after the 2-week culture period was determined by the dimethylmethylene blue (DMMB) dye assay [8] (n = 4). An aliquot of the proteinase K digest was mixed with the DMMB dye and the absorbance at 525 nm was measured with a spectrophotometer. The results were obtained by extrapolating from a standard curve using shark chondroitin-6-sulfate. Newly accumulated GAG was determined by subtracting the unseeded values from the sample values.

B.2.8. Histology of Cell-Seeded GSCG Scaffolds

Cell-seeded scaffolds (n = 2-3) were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. Six-micron thin sections were stained with Safranin-O to assess the presence of sulfated GAG.

Statistical Analysis

Data were analyzed by one- or two-factor analysis of variance (ANOVA), and the Fisher's protected least squares differences (PLSD) post-hoc test using StatView (SAS Institute Inc, Cary, NC). Data are presented as mean ± standard error of the mean.

B.3. RESULTS

B.3.1. OP-1 Plasmid Load in Unseeded GSCG Scaffolds

OP-1 plasmid detected in unseeded scaffolds after plasmid incorporation using the carbodiimide cross-linking method revealed ~85% incorporation of the 10 μg load within the scaffold for the OP(10) group (without the GP reagent) and 24% incorporation of the 50 μg load for the OP(50) group (Fig. B.1). It appeared that the GenePorter/pOP-1 complexes incorporated within the CG scaffold for the GP/OP(10) group could not be detected by the Picogreen assay.

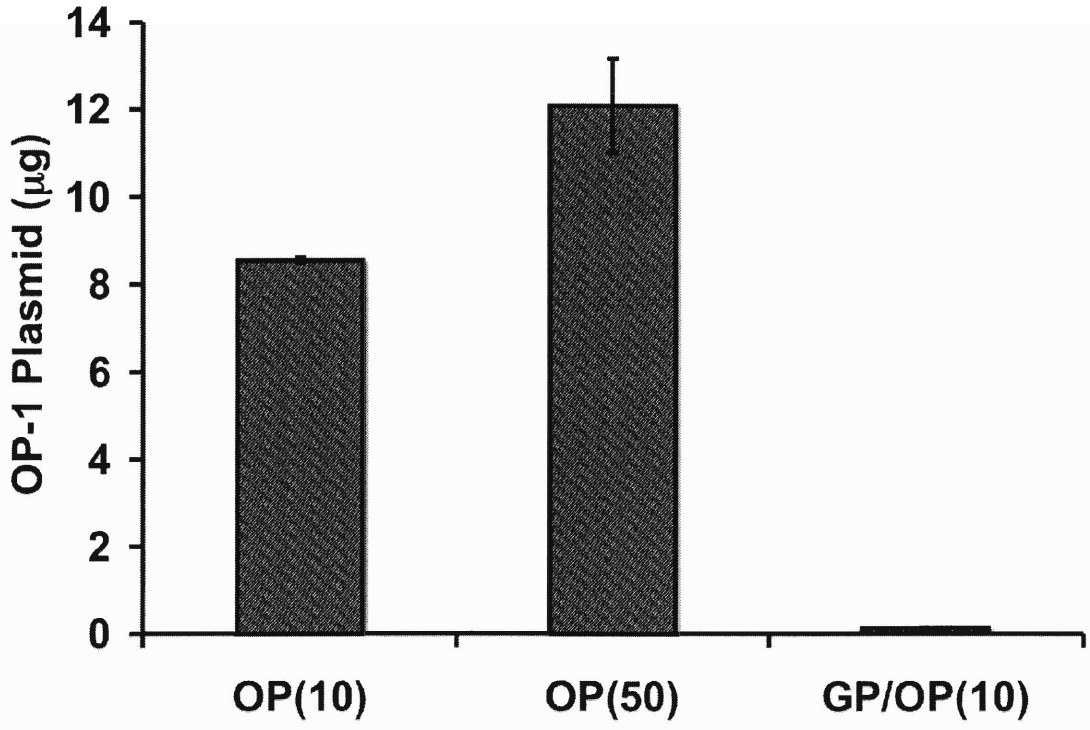


Figure B. 1 Incorporated pOP-1 alone or with the GP transfection reagent. n = 2; mean ± SEM.

B.3.2. Cell-Mediated Contraction of Chondrocyte-Seeded GSCG Scaffolds

Over the 2-week culture period, the GP/OP(10) group displayed the most cell-mediated contraction with a 28% decrease in scaffold diameter. The OP(10) and OP(50) groups both showed about a 20% size reduction and the Control group had a 10% decrease in scaffold diameter at the end of the culture period. Two-factor ANOVA revealed a significant effect of type of gene supplementation ($P < 0.0001$, power = 1) and

time ($P < 0.0001$, power = 1) on cell-mediated contraction. Post hoc analysis showed a significant difference between the Control group and all the other groups ($P < 0.0001$, power = 1) and between the GP/OP(10) group verses the rest of the groups ($P < 0.0001$, power = 1).

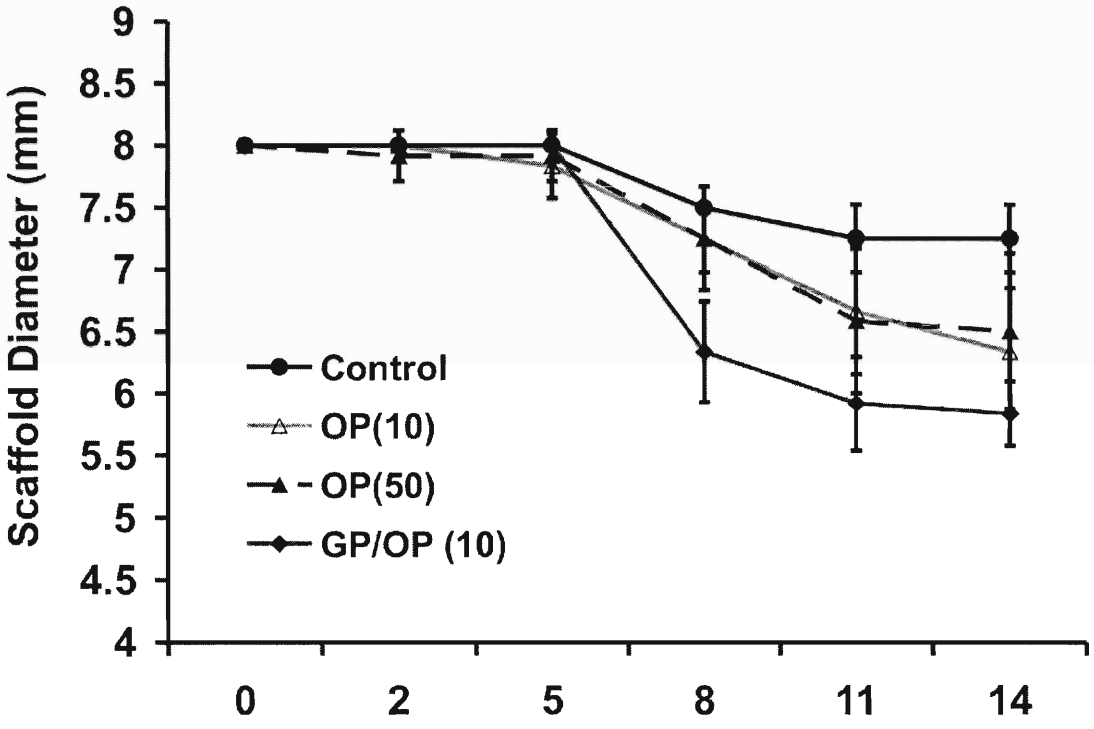


Figure B. 2 Contraction of cell-seeded GSCG scaffolds over the 2-week culture period. n = 6; mean ± SEM.

B.3.3. OP-1 Release in the 3-D Culture Medium

Detectable OP-1 in the medium (Fig. B.3a) was not apparent for any of the groups until about 8 days in culture (at the Day 11 and Day 14 collections). At these time points, OP-1 release in the medium from the Control group was about 27 pg/ml. The GP/OP(10) group showed a noticeably higher elevation in OP-1 compared to the rest of the groups. Two-factor ANOVA revealed a significant effect of type of gene supplementation ($P < 0.01$, power = 0.8) on resulting OP-1 expression, but no significant effect of time. Post hoc analysis showed a significantly higher elevation in OP-1 release for the GP/OP(10) group above all other groups ($P < 0.03$, power = 0.8). There was no significant difference in OP-1 expression between the Control group and scaffolds supplemented

with pOP-1 alone. Total accumulated OP-1 at the end of the 2-week period showed a 30% higher level for the GP/OP(10) group compared to the Control group (Fig. B.3b).

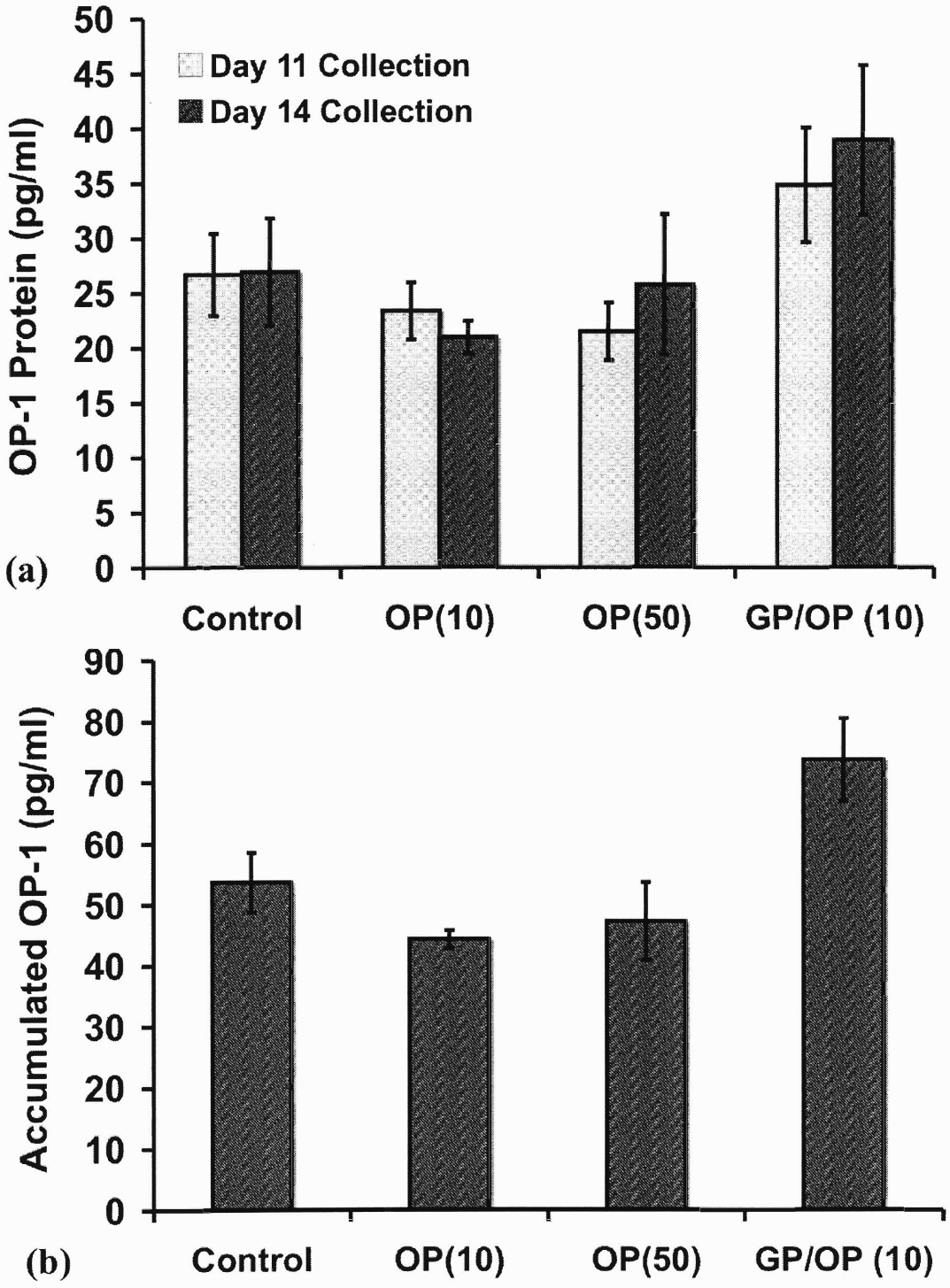


Figure B. 3 OP-1 detected in the media (a) at Day 11 and Day 14 and total accumulated OP-1 (b) produced from chondrocytes seeded in control and GSCG scaffolds over the 2-week culture period. n = 6; mean ± SEM.

B.3.4. Biochemical Analysis of GSCG Scaffolds Incorporating pOP-1

The GP/OP(10) group had a slight, but significantly higher (11%) DNA content (Fig. B.4) compared to the other groups at the end of the 2-week culture ($P < 0.03$, power = 0.8). Since DNA content detected by the Picogreen assay includes both nuclear material and pOP-1 remaining in the scaffold after the 2-week period, GAG contents were not normalized to DNA values. The GP/OP(10) group showed a 16%, 34%, and 47% higher accumulated GAG after 2-weeks compared to the Control, OP(10), and OP(50) groups, respectively (Fig. B.5). ANOVA and post hoc analysis revealed a significant effect of the type of gene supplemenation on accumulated GAG with significant differences between the Control group versus the other groups ($P < 0.05$, power = 1) and the GP/OP(10) group compared to the rest of the groups ($P < 0.05$, power = 1).

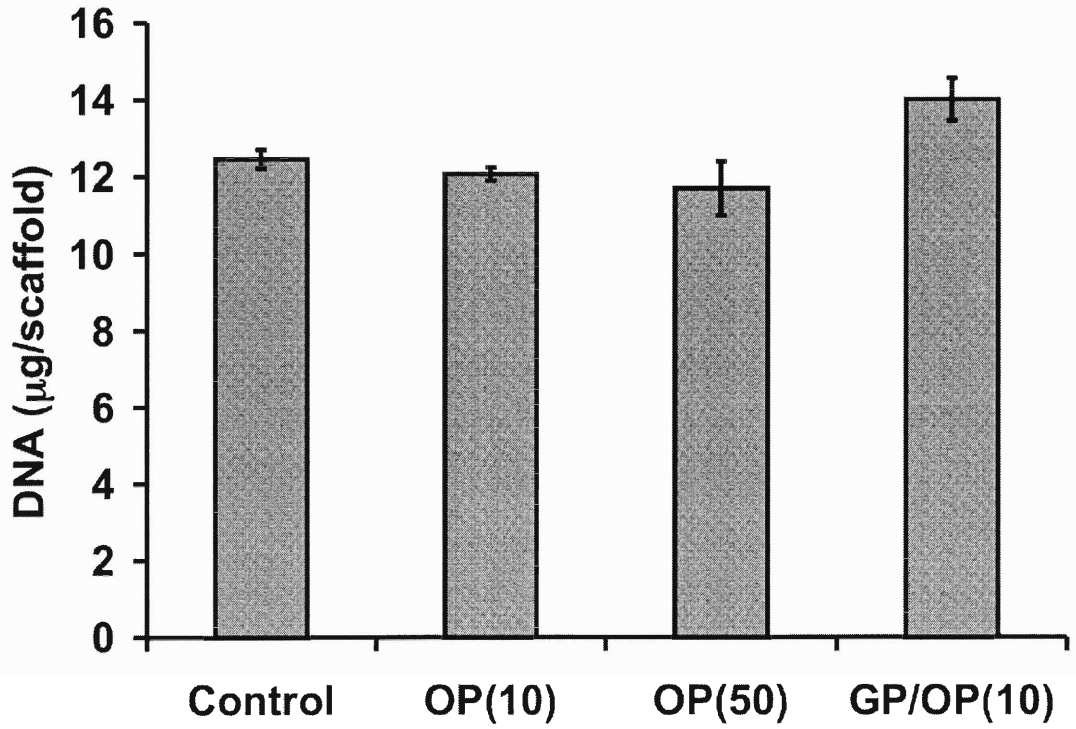


Figure B. 4 **DNA content at the end of the 2-week 3-D culture period. n = 4; mean ± SEM.**

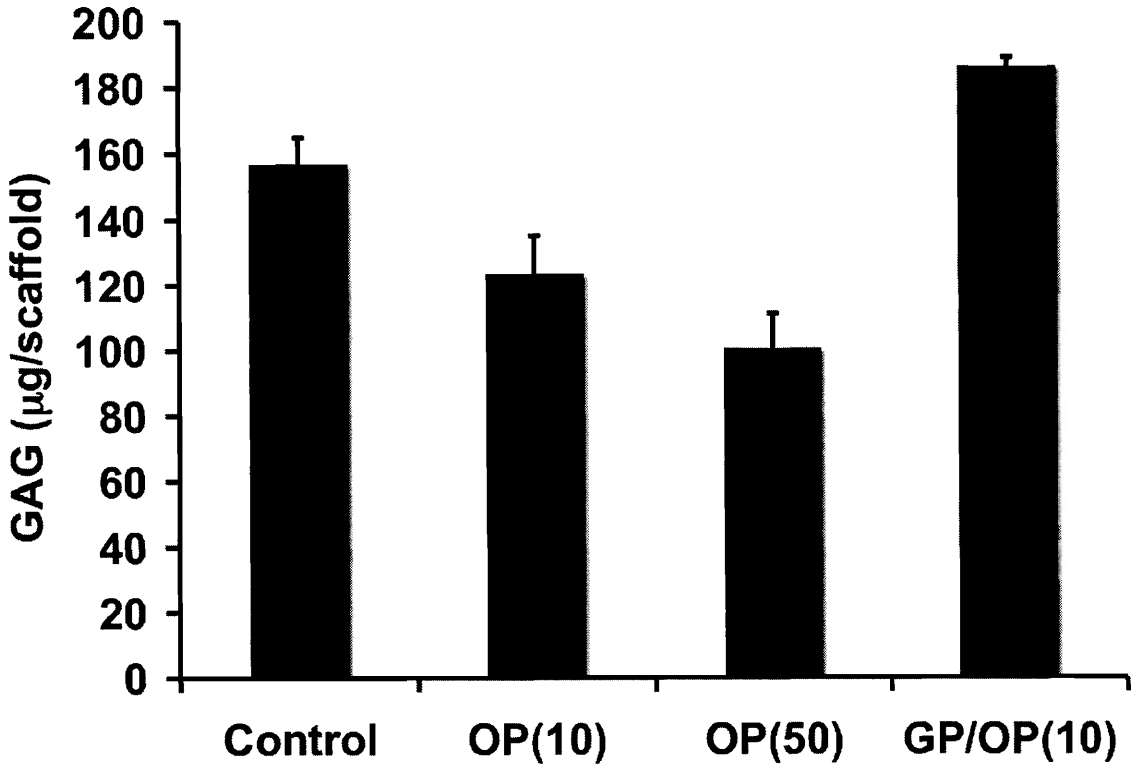


Figure B. 5 **Accumulated GAG content at the end of the 2-week 3-D culture period. n = 4; mean ± SEM.**

B.3.5. Histological Analysis of GSCG Scaffolds Incorporating pOP-1

Cell-seeded scaffolds from the Control group displayed a high concentration of tissue formation and GAG staining in the center of the scaffold with a significant amount of scaffold still remaining after 2 weeks in culture (Fig. B.6a). The morphology of the cells in the control scaffolds appeared more elongated and fibroblast-like compared to the other groups. Scaffolds incorporating pOP-1 alone (Fig. B.6b) demonstrated tissue synthesis throughout most of the scaffold, although GAG staining was more diffuse with a lighter pink appearance. Interestingly, there was a high number of cells in this group, that seemed to display a chondrocytic phenotype, with a rounded morphology and located in lacunae (see arrows in Fig. B.6b). The GP/OP(10) group displayed the most tissue formation and GAG staining, but also with a higher concentration in the center of the scaffold. Some cells in this group were also rounded and located in lacunae. In most of the scaffolds, a significant amount of residual scaffold still remained (undegraded

scaffold struts stained green) mostly in the outer periphery of the scaffolds where the least amount of cells and tissue was present.

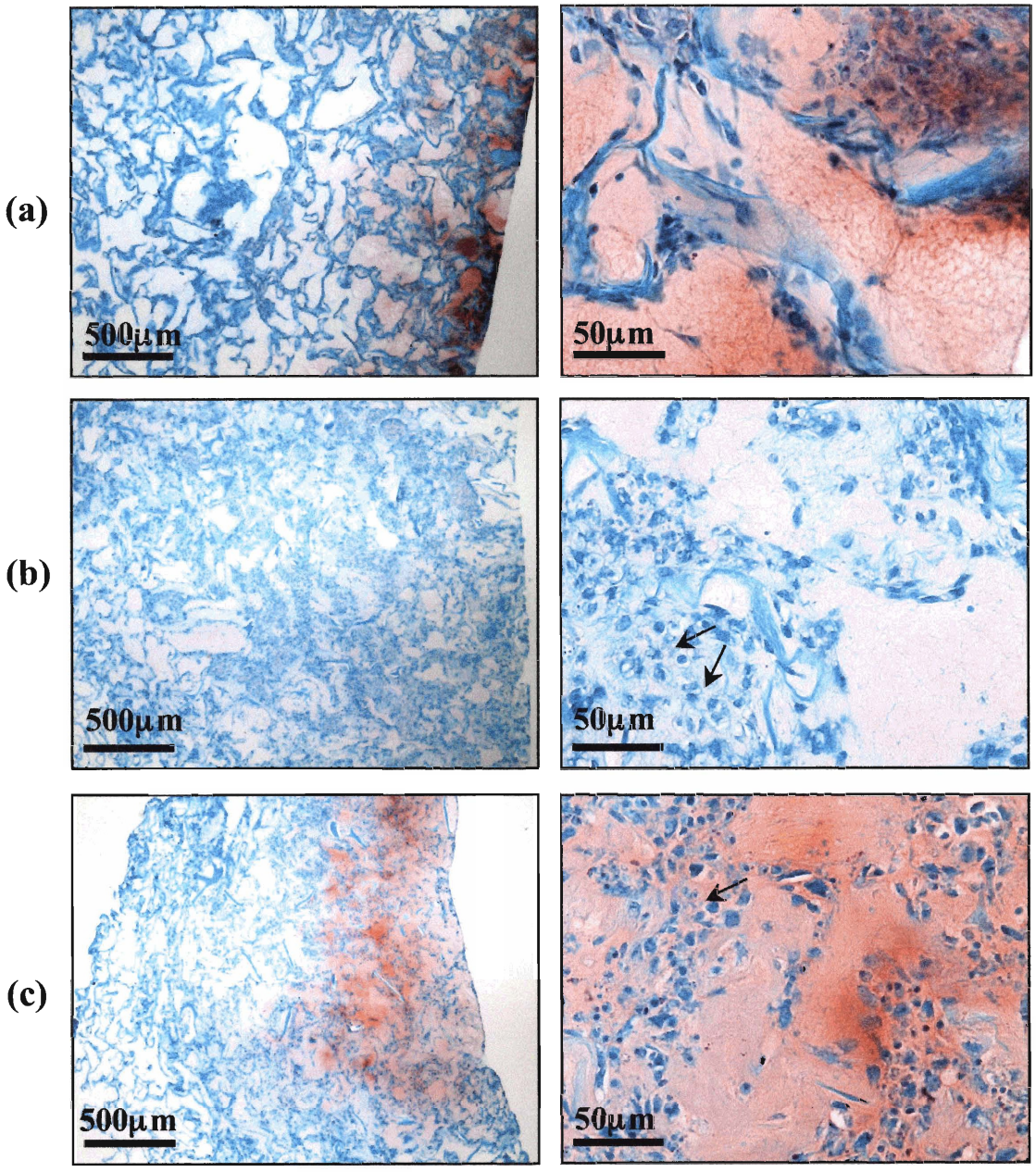


Figure B. 6 Saffranin-O stained sections (red is positive stain for GAG) of scaffolds from the Control group (a), OP(10) group (b), and GP/OP(10) group (c) after 2 weeks in 3-D culture.

B.4. DISCUSSION

Of significance in this study is that the plasmid containing the gene for OP-1 could be successfully incorporated within the CG scaffold by a carbodiimide cross-linking treatment. Addition of the GP transfection reagent resulted in successful gene transfer to and subsequent OP-1 expression by seeded chondrocytes. Interestingly, at the lower plasmid load (10 μg), a high percentage (85%) of the plasmid was incorporated within the scaffold, whereas, at a higher plasmid load (50 μg) only 24% of the plasmid was successfully incorporated. This indicates that when incorporating naked pOP-1 alone by this cross-linking method, there seems to be a limit as to how much plasmid can be incorporated. The large size of the OP-1 plasmid (between 11-12 Kb) may also be a limiting factor in the amount of plasmid that can be incorporated within the scaffold. Unfortunately, the quantitative amount of incorporated pOP-1 that was complexed with the GP transfection reagent could not be detected with the Picogreen assay. It is speculated that the complexation of the plasmid to the GP reagent interferes with the binding of the Picogreen dye to the plasmid DNA and, therefore, detection was not possible. The presence of the GP/OP complexes within the scaffold, however, was apparent due to the resulting OP-1 overexpression detected from cells seeded within scaffolds incorporating the GP/OP complexes above the control levels. Future work should investigate other methods of tracking the plasmid amounts incorporated within the CG scaffold when employing the GP transfection reagent (e.g. tagging the plasmid with small fluorescent molecules prior to mixing with the GP reagent).

Of interest is the effect of gene incorporation on resulting cell-mediated contraction during 3-D culture. In this study, control scaffolds contracted the least, scaffolds incorporating pOP-1 alone contracted a little more than the controls, and scaffolds incorporating the GP/OP complexes contracted the most. Cell-mediated contraction can be affected by the scaffold cross-linking properties (stiffness and degradation), smooth muscle actin (SMA) expression of cells, or cell remodeling of the extracellular matrix (ECM). Histological analysis verified that there was a greater amount of residual scaffold and the least amount of scaffold degradation in the control scaffolds compared to the other groups. This may be an indication that these scaffolds

may have been more cross-linked than the other groups. The difference in contraction between scaffolds incorporating pOP-1 alone and scaffolds incorporating GP/OP complexes also indicates that these vectors may significantly affect the resulting scaffold mechanical and degradation properties, induce cell expression of SMA, or stimulate remodeling of the ECM. Future work needs to more fully understand the mechanism(s) by which vector incorporation within CG scaffolds affects cell-mediated contraction.

Interestingly, measurements detecting OP-1 in the culture medium revealed that OP-1 was produced by cells seeded in control scaffolds after about a week in culture. This delayed response in OP-1 expression from controls seems to indicate that interaction between the cell and the type II collagen scaffold with time, may induce the expression of OP-1. At this collection point, there was also an indication of OP-1 overexpression from chondrocytes seeded in scaffolds incorporating the GP/OP complexes above control levels. Compared to prior work employing the plasmid containing the gene encoding for insulin-like growth factor (IGF)-1 within GSCG scaffolds, there seems to be a slower gene transfer rate and lower expression levels when using plasmid OP-1. Past studies have shown that IGF-1 overexpression can be detected as early as 3 days after seeding cells within the GSCG scaffolds using this scaffold-based approach. This difference in gene transfer kinetics and expression levels may be due to the significantly larger plasmid size of the pOP-1 compared to the IGF-1 plasmid (with pOP-1 being about 2 times larger in size than pIGF-1). The lack of overexpression from scaffolds incorporating the naked pOP-1 alone (i.e. without the GP transfection reagent) may also be due to the size of the OP-1 plasmid. The vector size may have been too large for sufficient cellular uptake. The addition of the GP transfection reagent, however, may have helped condense the plasmid, increasing the probable entry into the cell and leading to the resulting OP-1 overexpression above the control levels. Future work needs to further investigate the effect of plasmid size on the ability to use this scaffold-based nonviral gene transfer approach for efficient gene transfer to seeded cells.

Of significance is that the 30% higher accumulated OP-1 level for the GP/OP group compared to controls resulted in a slight but significantly higher DNA content and ~20% higher accumulated GAG content found in the scaffolds after 2 weeks in culture. Since the Picogreen assay could not detect plasmid complexed to the GP transfection

reagent, the higher DNA content of the GP/OP group is most likely a reflection of cell proliferation and not due to the residual plasmid left in the scaffold. DNA content detected for the OP(10) and OP(50) groups, however, could have contained both nuclear material and pOP-1 still remaining in the scaffold. Since histological analysis revealed a considerable amount of residual scaffold left after 2 weeks for these groups, it is likely that pOP-1 was still present within the constructs and contributed to the DNA amount detected by the Picogreen assay for scaffolds incorporating pOP-1 alone. Accumulated GAG content was therefore reported per scaffold and not normalized to the DNA values.

The quantitative accumulated GAG values determined biochemically seemed to coincide with the histological findings. Safranin-O staining for GAG revealed the most tissue formation and GAG staining for the GP/OP group and the least amount of GAG staining in scaffolds incorporating pOP-1 alone. Interestingly, the scaffolds incorporating 10 μg of pOP-1 without the GP reagent still contained a high percentage of cells that were rounded and located in lacunae despite the lack of GAG biosynthesis. In contrast, the control scaffolds showed a high concentration of GAG present in the center of the scaffold, but cells displayed a more elongated and fibroblast-like appearance. It is important to note that differences in biosynthesis and chondrogenesis between groups could have also been affected by the resulting cell-mediated contraction of the scaffold. Future work needs to further understand the contributions of cell-mediated contraction, the presence of plasmid vectors within the scaffold, and localized OP-1 overexpression on the resulting biosynthesis and chondrogenesis within GSCG scaffolds.

In conclusion, plasmid vectors containing the OP-1 gene are able to be incorporated within CG scaffolds and can result in successful gene transfer to seeded chondrocytes when incorporated with the GP transfection reagent. The large size of the plasmid OP-1 may be a significant factor inhibiting gene transfer to chondroctyes when incorporated alone. Supplementation of these vectors within the CG scaffold also has a significant effect on the resulting cell-mediated contraction. These findings warrant further research in the development of GSCG scaffolds for OP-1 gene delivery in articular cartilage tissue engineering.

B.5. REFERENCES

1. Masuda K, Pfister BE, Sah RL, Thonar EJ. Osteogenic protein-1 promotes the formation of tissue-engineered cartilage using the alginate-recovered-chondrocyte method. Osteoarthritis Cartilage 2005 Nov 29.

2. Loeser RF, Pacione CA, Chubinskaya S. The combination of insulin-like growth factor 1 and osteogenic protein 1 promotes increased survival of and matrix synthesis by normal and osteoarthritic human articular chondrocytes. Arthritis Rheum. 2003 Aug;48(8):2188-2196.

3. Nishida Y, Knudson CB, Eger W, Kuettner KE, Knudson W. Osteogenic protein 1 stimulates cells-associated matrix assembly by normal human articular chondrocytes: up-regulation of hyaluronan synthase, CD44, and aggrecan. Arthritis Rheum. 2000 Jan;43(1):206-214.

4. Klein-Nulend J, Louwerse RT, Heyligers IC, Wuisman PI, Semeins CM, Goei SW, et al. Osteogenic protein (OP-1, BMP-7) stimulates cartilage differentiation of human and goat perichondrium tissue in vitro. J.Biomed.Mater.Res. 1998 Jun 15;40(4):614-620.

5. Flechtenmacher J, Huch K, Thonar EJ, Mollenhauer JA, Davies SR, Schmid TM, et al. Recombinant human osteogenic protein 1 is a potent stimulator of the synthesis of cartilage proteoglycans and collagens by human articular chondrocytes. Arthritis Rheum. 1996 Nov;39(11):1896-1904.

6. Louwerse RT, Heyligers IC, Klein-Nulend J, Sugihara S, van Kampen GP, Semeins CM, et al. Use of recombinant human osteogenic protein-1 for the repair of subchondral defects in articular cartilage in goats. J.Biomed.Mater.Res. 2000 Mar 15;49(4):506-516.

7. Grgic M, Jelic M, Basic V, Basic N, Pecina M, Vukicevic S. Regeneration of articular cartilage defects in rabbits by osteogenic protein-1 (bone morphogenetic protein-7). Acta Med.Croatica 1997;51(1):23-27.

8. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986 Sep 4;883(2):173-7.

APPENDIX C: FABRICATION OF COLLAGEN (TYPE II)-GAG SCAFFOLDS

C.1. TYPE II COLLAGEN SLURRY PREPARATION

For 100ml type II collagen slurry:

1. Prepare 0.001N HCl (pH~3): add 25ul of 12N HCl to 3.25ml dH2O to make 0.1N HCl (pH~1.1); add 1ml of 0.1N HCl to 99ml dH20 to make 0.001N HCl (pH~3)
2. Add 1g of type II Chondrocell sponge—cut collagen sheet into small pieces with clean scissors
3. Blend at 1500 rpm (slightly past setting 3) for 5 minutes—use a small glass container (~150 ml) and place on ice to keep cool during blending
4. Degas (vacuum or centrifuge on low setting) and freeze-dry (50ml per ~5in. x 5in. metal pan) with normal ramping protocol

C.2. VITREOUS FREEZE-DRYING "RAMP" PROTOCOL

1. The ramp program used for freeze-drying a collagen slurry consists of a hold at 20°C for 5 minutes, ramp down to -40°C over 65 minutes (fastest cooling time when actual time is set for 15min), and held at -40°C for a minimum of 60min (set time for this step is longer than 60 minutes)

Auto set program reads:	Step 1	T = 20	t = 5	Hold
	Step 2	T = -40	t = 15	Ramp
	Step 3	T = -40	t = 165	Hold

2. Press auto switch off, turn on heater and freezer switches, and set temperature to -40°C
3. Make sure chamber release button is off and turn on the vacuum. Press door shut while vacuum is being pulled until a sufficient seal is produced
4. Once vacuum is below 200 mtorr (~30min), set the temp. to 0°C. Leave overnight or at least 12 hrs for sublimation
5. Set temp to 20°C and turn off freeze button

6. When freezer is at 20°C, turn off the heat, vacuum, and condenser buttons. Release the chamber and drain the condenser chamber. After defrosting, chamber and condenser should be wiped dry.

APPENDIX D: SWELLING RATIO

D.1. SWELLING RATIO PROTOCOL TO DETERMINE DEGREE OF CROSS-LINKING

D.1.1. Materials

Distilled water

Hot plate
100 ml beaker
Tweezers
Thermometer
Filter paper sheets (Whatman #1)
1.0 kg weight

D.1.2. Procedure

1. Fill beaker w/ distilled water and heat to 90°C on hot plate. Place thermometer inside beaker to adjust the hot plate over time.
2. Place matrix in the hot water bath for 2 min. in order to denature the collagen and allow it to swell w/ water--samples will shrink in size
3. Expel water from pores by placing hydrated matrix between sheets of filter paper w/ the 1.0 kg weight placed on top for 20 seconds. Need sufficient number of filter paper so that there is no water visible on the outer layers of the filter paper when the weight is taken off. Usually 7 pieces of filter paper on the bottom and 4 pieces on top of the matrix is sufficient
4. Immediately weigh sample after being pressed and record mass as the wet mass (WM)
5. Dry samples in the DHT oven overnight at 110°C
6. Weigh samples after taken out of the oven and record mass as dry mass (DM)
7. Calculate the swelling ratio (which is the inverse of the volume fraction of dry collagen, Vf) using this equation:

$$\mathbf{r^* = 1/Vf = [(DM/rc) + ((WM-DM)/rwater)]^*rc/DM}$$

where rc = 1.32 g/cm^3 (density of collagen) and rwater = 1.00g/cm^3 (density of water)

APPENDIX E: SYNTHESIS OF GENE-SUPPLMENTED COLLAGEN-GAG SCAFFOLDS

E.1. BACTERIAL TRANSFORMATION AND PLASMID ISOLATION

E.1.1. Materials Needed

- LB Broth Base (LENNOX L BROTH BASE, Invitrogen, Cat# 12780-052): follow instructions
- LB Agar (LENNOX L AGAR, Invitrogen, Cat# 22700-025): follow instructions on bottle; plate in petri dishes overnight (store in cold room)
- Antibiotic (see chart below)
- Plasmid
- Mega QIAfilter^{TM} Plasmid kit (Qiagen, Cat #12281)

TE buffer for plasmid storage:

- 1ml 1M Tris (pH=8) (UltraPure Tris, Invitrogen, Cat# 15504-020, FW=121.1; or use 1M Tris (pH=8.0), Ambion, Cat# 9855G)
- 0.2 ml 0.5M EDTA (pH=8) (Disodium Ethenediamine Tetraacetate, Fisher, Cat#S311, FW=372.24; or use 0.5M EDTA (pH=8.0), Invitrogen, Cat# 15575-038)
- 98.8 ml dH_2O

E.1.2. Bacterial Transformation Protocol Using Heat Shock

(Place LB agar plates in warm room)

1. Take competent *E.coli* cells (DH5α, Invitrogen, Cat# 18258-012) from –80C freezer and thaw them on ice (~20min).
2. Turn on water bath to 42C.
3. Put 50ul competent cells in 1.5 ml tube (Eppendorf or similar) for transforming a DNA construct and keep tubes on ice.
4. Add 1-10 ng of circular DNA into *E.coli* cells. Never exceed (1/10) of the total volume. Ideally add 5ul of construct to 50 ul of competent cells. Mix gently by swirling pipette tip in solution (DO NOT pipette up and down).
5. Incubate on ice for 30 min.

6. Put tube(s) with DNA and *E.coli* into water bath at 42C for 45 seconds.
7. Put tubes back on ice for 2 minutes to reduce damage to the *E.coli* cells.
8. Add 500 ul of pre-warmed LB (with no antibiotic added).
9. Incubate tubes for 1 hour at 37C and shake at 225 rpm.
10. Spread about 100 ul (can include also a different spread volume on another plate—20 to 200ul) of the resulting culture on LB plates (with appropriate antibiotic added – usually Ampicillin or Kanamycin.)—wait until plates are dry before putting in the cold room. Grow overnight.
11. Pick and grow colonies about 12-16 hours later.

E.1.3. Bacterial Growth and Isolation

1. Place 5 ml of LB medium w/ Ampicillin (100ug/ml) in culture tube.
2. Pick isolated colony w/ a sterile stick and place it in the tube.
3. Spin at max revolutions in the warm room for 6-8hrs (medium should look cloudy when ready).
4. For mega prep, transfer whole contents of tube into a sterilized 3000ml flask containing 1000ml of LB medium w/ antibiotic.
5. Put on shaker (~260rpm—max to prevent severe shaking of bench) overnight.
6. Isolate plasmid w/ Qiagen Plasmid Purification kit.
7. Check amount of plasmid isolated w/ spectrophotometer and integrity w/ gel electrophoresis (cutting w/ appropriate restriction enzymes).

E.1.4. Antibiotics Used for GSCG Scaffolds

Antibiotic	Concentration	Storage	Working concentration (dilution)
Ampicillin (sodium salt) (Sigma Cat# A-8351)	100 mg/ml in water	–20°C	100 µg/ml (1/1000)
Kanamycin Sulfate (Invitrogen Cat# 11815-024)	50 mg/ml in water	–20°C	50 µg/ml (1/1000)

- ***Stored in 1ml aliquots at -20C and used 1 tube per 1L LB medium***
- Ampicillin used for pIGF-1 prep; Kanamycin used for pEGFP prep

E.2. FABRICATION OF GELATIN/PLASMID NANOPARTICLES

E.2.1. Materials Needed

- Gelatin from porcine skin, Type A, ~175 bloom (Sigma, Cat #G2625-100G)
- Ethylenediamine (Sigma, Cat# 107-15-3)
- N-(3-Dimethylaminopropyl-N'-ethyl) carbodiimide hydrochloride, EDAC—commercial grace (Sigma, Cat # E7750)
- HCL (Fisher Scientific, Cat # A508-212)
- Plasmid DNA
- 50mM Sodium Sulfate solution (Fisher, Cat # 421-1)
- PBS solution (0.1M, pH = 5.0)—Mix equal volumes of the following solutions & pH w/ HCl:
  - 0.1M Sodium phosphate dibasic (Mw = 141.96g/mol, Sigma, Cat # S0876)
  - 0.1M Sodium phosphate monobasic (Mw = 137.99g/mol, Sigma, Cat # S9638)

E.2.2. Gelatin Cationization

1. Add 2.5g gelatin in 125ml PBS solution (0.1 M, pH 5.0)
2. Stirr and put in waterbath (37C) or on a hotplate in a container w/ water, until gelatin is completely dissolved
3. Under hood, add 7.9ml ethylendiamine and 1.34g EDAC
4. Adjust pH to 5 with 5-6N HCl
5. Stirr (medium-fast setting) for 16-18h, overnight (in a hood)
6. Soak dialysis tubing overnight in dH_2O
7. Next day, open dialysis tubing completely, add the gelatin solution, and clamp securely
8. Soak for dialysis 48hrs, changing dH_2O preferably every 8hrs
9. Pour gelatin solution in containers for freezedrying and put in freezer until solution is frozen (~2hrs)
10. Lyophilize until gelatin is completely dry (1-2 days depending on amount)

E.2.3. Preparation of Cationized Gelatin-Plasmid Nanoparticles (Complex Coacervation Method)

1. 200ng/ul plasmid DNA in 50mM filtered Na_2SO_4, keep at 55^0C
2. Mix 5-8% (w/v) lyophilized cationized gelatin (in dH_2O), filter, keep at 55C
3. mix equal volume of 1) and 2) 1min, usually 100-150ul

E.3. GENE INCORPORATION OF CG SCAFFOLDS

** Scaffolds previously DHT crosslinked, 105C-24hrs in vacuum oven & 8mm diameter discs cut out with dermal biopsy punch (Moore Medical, Cat# 52443)*

E.3.1. EDAC Calculations—1:1:5 (EDAC:NHS:COOH)

(Change calculations accordingly depending on number of scaffolds)

100 discs x 0.0023g collagen/disc x 0.0012mol COOH/g collagen x 5mol EDAC/5mol COOH x 191.7g EDAC/mol EDAC = **0.0529g EDAC** (small bottle)

100 discs x 0.0023g collagen/disc x 0.0012mol COOH/g collagen x 2mol NHS/5mol COOH x 116.0g NHS/mol NHS = **0.0128g NHS**

E.3.2. Gene supplementation by soak and freeze-dry

E.3.2.1. EDAC Cross-linking of CG scaffolds

1. Place scaffolds previously DHT cross-linked in sterile petri dishes.
2. Hydrate scaffolds w/ sterile dH_2O (0.5ml/scaffold).
3. Weigh out necessary EDAC and NHS and dissolve in same volume amount of dH_2O used to hydrate scaffolds and sterile filter.
4. Add EDAC/NHS solution to hydrated scaffolds (pipette over scaffolds several times to ensure good mixing) and cross-link at room temperature for ~30minutes.
5. Remove EDAC/NHS solution and rinse in PBS by swirling petri dish.
6. Transfer scaffolds in falcon tubes containing fresh PBS and rinse for at least 1hr on rocker.
7. Transfer scaffolds back to petri dishes and remove PBS.

E.3.2.2. Plasmid Incorporation

1. Place 60-100ul diluted plasmid solution onto scaffolds (briefly dry on filter paper before adding plasmid aliquot if supplementing right after PBS rinse).
2. Incubate for at least and hour.
3. Freeze-dry scaffolds using similar freeze-drying protocol used to make scaffolds from slurry (See Appendix C).

E.3.3. Gene supplementation of Scaffolds by EDAC Cross-linking

E.3.3.1. Plasmid Supplementation by EDAC Cross-linking

1. Hydrate scaffolds w/ aliquots of plasmid solution or TE buffer (~60ul/scaffold).
2. Dissolve the EDAC and NHS in 50ml dH_2O and sterile filter (0.45 mm).
3. Pipette 0.5ml solution/scaffold over scaffolds to ensure good mixture.
4. Allow chemical cross-link at room temperature for about 15 minutes.
5. Pipette 0.5ml sterile dH2O/scaffold over scaffolds to ensure good mixture.
6. Allow chemical cross-link at room temperature another 15 minutes.
7. Remove EDAC/NHS solution w/ pipette, rinse w/ PBS and transfer scaffolds to 15ml falcon tubes containing sterile PBS and rock for ~1hr. (prepare cells & SF medium during this time).
8. Transfer back to petri dish, remove PBS w/ pipette—ready to seed.

APPENDIX F: MEDIA PREPARATIONS FOR CHONDROCYTE AND MESENCHYMAL STEM CELL CULTURES

(modified protocol from Jakob et. al, J Cell Biochem 2001;81(2):368-77)

F.1. MEDIUM FOR EXPANDING CHONDROCTYES

F.1.1. J-Base Medium

- 500 ml **hg-DMEM** (high glucose: 4.5%) without L-Glutamin with Sodium Pyruvate (by GIBCO®)
- 5 ml **MEM Nonessential Amino Acids** (NEAA) (solution 10mM, by GIBCO® cat. No. 11140 050)
- 5 ml **Hepes** Buffer (solution 1M, by GIBCO® cat. No. 15630 056)—can also make 1M solution w/ Hepes powder (238.3g/mol) in distilled water (ie. 0.2383g Hepes powder/ml dH_2O—need to sterile filter)
- 5 ml **PSG** consisting in 10000U/ml penicillin, 10000mg/ml streptomycin glutamate (by GIBCO®, cat. No. 10378 016)

**** J-Base is the base medium used for both making both expansion and serum-free medium***

F.1.2. J-FBS

- 450ml J-Base medium
- 50 ml **FBS** (by GIBCO® , cat. No. 10270 106 or equivalent)

F.1.3. J-Expansion Medium

- J-FBS medium

ADD growth factors just before use:

- human **TGFβ1**—1 ng/ml of media (R&D Systems, cat. No. 240-B-002)
  →Reconstitute in sterile 4mM HCl containing at least 1mg/ml human serum albumin or bovine serum albumin in the vial to prepare a stock solution of no less than 1ug/ml of TGFβ1
    - Obtain sterile eppendorf tubes (preferably 0.5ml tubes)

- 166.5ul (7.5% g/ml) **BSA solution** + 500ul **0.1N HCl** + 11.5ml **H_2O** in 5ml centrifuge tube and vortex (we have HCl *plus* that is 12.1N HCl so add 0.5ml of 12.1N HCl + 60.5ml H_2O to get 0.1N HCl)
- Sterile filter 10ml of the buffer and add it to 10ug of TGF-β (concentration = 1ug of TGF-β1/ml)

-- Per ml of media, add 1ul of the TGFβ1 stock to have 1ng TGFβ1 per ml of media

- Aliquot in sterile microtubes and store @ -70°C for three months w/out detectable loss of activity
- Avoid repeated freeze-thaw cycles for all growth factors

➢ human FGF basic (**FGF-2**)—5 ng/ml (R&D Systems, cat. No. 233-FB-025)

→Reconstitute in sterile PBS containing at least 0.1% (1mg/ml) human serum albumin or bovine serum albumin and 1mM DTT (154.3g/mol) in the vial to prepare a stock solution of no less than 10ug/ml of cytokine:

- Obtain sterile eppendorf tubes (preferably 0.5ml tubes)
- Add 0.0025g **BSA** OR 33.3ul (7.5% g/ml) **BSA solution** + 0.0004g **DTT** + 2.5 ml **PBS**
- Sterile filter w/ syringe, and add to the 25ug of FGF-2 in vial

--Per ml of media, add 0.5ml of the FGF-2 stock to have 5ng FGF-2 per ml of media

- Aliquot in sterile microtubes and store @ -70°C for three months w/out detectable loss of activity
- Avoid repeated freeze-thaw cycles for all growth factors

➢ human **PDGFββ**—10 ng/ml (R&D Systems, cat. No. 220-BB-010)

→Reconstitute in sterile 4mM HCl containing at least 0.1% human serum albumin or bovine serum albumin in the vial to prepare a stock solution of no less than 10ug/ml of cytokine

- Obtain sterile eppendorf tubes (preferably 0.5ml tubes)
- Add 0.0025g **bovine serum albumin** + 100ul **0.1N HCl** + 2.4ml **H_2O** OR 33.3ul (7.5% g/ml) **BSA solution** + 50ul **0.2N HCl** + 2.4ml **H_2O** in 5ml

centrifuge tube and vortex (we have HCl *plus* that is 12.1N HCl so add 0.5ml of 12.1N HCl + 60.5ml H_2O to get 0.1N HCl)

- Sterile filter 1ml of the buffer and add it to the 10ug of PDGFββ (concentration = 10ug of PDGFββ/ml)

-- Per ml of media, add 1ul of the PDGFββ stock to have 10ng PDGFββ per ml of media

- Aliquot in sterile microtubes and store @ -70°C for three months w/out detectable loss of activity
- Avoid repeated freeze-thaw cycles for all growth factors

F.2. MEDIUM FOR EXPANDING MESENCHYMAL STEM CELLS

- 500mL low glucose DMEM (Invitrogen # 11885-092)
- 50mL FBS (Invitrogen #16000-044)
- 5mL penicillin/streptomycin

F.3. 3-D CULTURE DIFFERENTIATION MEDIUM

F.3.1. SF Base Medium

- 500ml J-Base medium
- 5 ml **ITS+1** (100X) (SIGMA—Cat. No. I2521)
- 9 ml (7.5% g/ml) **BSA solution** (1.25mg **BSA**/ml of media OR 17ul (7.5% g/ml) **BSA solution**/ml of media)

F.3.2. SF Medium

ADD supplements at the last minute before use:

- TGFβ1 (10ng/ml)—Add 10ml of TGFβ1 stock solution per ml of media
- **Dexamethasone** (100 nM) non-water soluble (MW=392.5g/mol—Sigma D-4902) *or* water soluble (*~65mg dexamethasone/gram of powder—MW of dexamethasone = 392.5g/mol*—by Sigma 2915)

  → 10ul of a 1/100 dilution of 10^{-3} M dexamethasone stock solution/ml of media

- Make 10^{-3} M dexamethasone (DM) stock in 100% ethanol (stable for 1 yr, stored @ -20°C) by adding 3.92mg of **dexamethasone** (non-water soluble) per 10ml of **100% ethanol** *or* 6.03mg of dexamethasone (water soluble) per 1ml of 100% ethanol
  (Calc: 1ml 10^{-3} M DM x 0.001mol DM/1000ml x 392.5g DM/1mol DM x 1g powder/ 0.065g DM = 0.00603g powder -- in 1ml of ethanol)
- Make 10^{-5} M dexamethasone in **low glucose DMEM** (LG) by adding 20ul of 10^{-3} M dexamethasone stock + 1.98 ml LG-DMEM
- Store as sterile aliquots @ -20°C

➢ **L-Ascorbic acid 2-Phosphate** (0.1 mM)

→ 10ul of a 1/100 dilution of ascorbate 2-phosphate (MW = 289.54g/mol)

- Add 37.5 mg **ascorbate 2-phosphate** (Wako Chemical) + 10ml **Tyrodes** solution (Sigma)
- Sterile filter the ascorbate 2-phosphate solution and store frozen (-20°C) in 2ml aliquots

APPENDIX G: CELL ISOLATION METHODS

G.1. CHONDROCYTE HARVEST PROTOCOL

(modified from H. A. Breinan, BWH Orthopaedics Research Lab and Kuettner et al., 1982 and C. Lee Thesis, 2001)

G.1.1. Materials

G.1.1.1. OR Supplies

Scalpel handle (#3)

Scalpel blades (2 #10)

Sterile gloves

If removing whole joint:

Surgical saw

Sterile specimen bags (plastic bags in histo room)

Sterile towels/wrap

Sterile PBS to moisten towels

If removing shavings (ie: for subsequent autologous seeding):

Extra scalpel blades

50 ml tubes w/ complete PBS: 40 ml D-PBS (Gibco #14190-144) +

0.4 ml Pen/Strep/Fungizone cocktail (100X; Gibco #15240-062)

G.1.1.2. Lab Supplies

Sterile petri dish (100 mm diameter)

Sterile spatula

Sterile forceps (2)

Sterile razor blades (flat edges are easiest to use)

Sterile centrifuge tube/bottle with sterile stir bar

Clean stir plate in incubator (one that doesn't heat up too much during prolonged operation)

10-20 ml/joint complete PBS (see above)

T-75 and/or T-25 tissue culture flasks

G.1.1.3. Solutions

1. *Pronase solution* (20 U/ml) – ~ 40ml/2 joints
   a. Dissolve appropriate amount of pronase (Sigma protease type XIV #P 5147) in 10ml HG-DMEM (high glucose: 4.5%, without L-Glutamin with Sodium Pyruvate by GIBCO®)

$$\boxed{pronase(mg) = \frac{V \times 20U/ml}{\#Units/mg}}$$

   where V=volume of solution
   b. Sterile filter (0.2 mm) solution (use syringe filter; it will take a while for pronase to dissolve and it will be difficult to pass solution through filter)
   c. Add remaining volume of DMEM/F12 (ex: 30 ml if making 40 ml/2 joints)
   d. Add 1% v/v of 100X pen/strep/fungizone cocktail (antibiotic) (ex: 0.4 ml)

2. *Collagenase solution* (200 U/ml) – approx 40ml/2 joints
   a. Dissolve appropriate amount of collagenase (Worthington Biochemical CLS2) in 10 ml HG-DMEM (this dissolves quickly)

$$\boxed{collagenase(mg) = \frac{V \times 200U/ml}{\#Units/mg}}$$

   where V = volume of solution
   b. Sterile filter solution using a 0.2 mm syringe filter
   c. Add remaining volume of HG-DMEM
   d. Add 1% v/v 100X pen/strep/fungizone cocktail
3. *Jakob Base or Expansion Media (see Appendix H)*

G.1.2. Procedure

1. Using scalpel blades, remove slices of articular cartilage from joint surfaces and place in complete PBS (or in tubes and store on ice if harvesting in OR)

- Don't dig into the hard calcified cartilage or subchondral bone, cartilage should offer little resistance to the blade; full thickness slices should be 0.5-1.5 mm thick depending on joint location
- If harvesting whole joint, open joint in sterile hood, cutting ligaments and removing menisci; take cartilage from tibial, femoral and patellar surfaces
- If slices are large, use razor/scalpel blade to cut into pieces no larger than 3x3x1mm

2. Using forceps and spatula, transfer cartilage pieces into tube/bottle with stir bar and pronase solution, cap loosely
3. Incubate 1hr (37°C, 5% CO_2) on spinner plate (or shake every 15 minutes or so if can't use spinner plate in incubator)
4. Centrifuge the pronase and tissue solution, remove the pronase, and resuspend pellet in collagenase solution
5. Incubate overnight (maybe as short as 4-6 hours until all tissue is digested) on spinner plate; make sure cap is loose!
6. Strain through 40-70 mm pore strainer into new 50ml centrifuge tube
7. Centrifuge (10 min @ 1500 rpm)
8. Remove supernatent and resuspend in ~30 ml complete media; centrifuge again and resuspend in known amount of complete media to do cell count (usually 20ml)
9. Count cells and assess viability (should have at least ~7-8x10^6 cells/joint with >90% viable if cap was loose during digestion)

G.2. MESENCHYMAL STEM CELL HARVEST AND EXPANSION

1. Aspirate cells from the iliac crest into tube containing heparin
2. To a 15mL tube, add 2mL of anticoagulant treated blood to equal volume of PBS
3. Mix well with pipette
4. Mix Ficoll-Paque by inverting bottle several times
5. Using 20mL syringe and 18 gauge needle – add 5mL air and add 3mL Ficoll to 15mL tubes (**Falcon 352059 tubes**)
6. Layer 4mL diluted blood onto 3mL Ficoll – carefully so as not to mix
7. Spin at 3000 for 30 minutes
8. Transfer lymphocyte layer to clean 15mL tube with 5mL PBS

   This means:

   -- Carefully aspirate the Ficoll above the lymphocyte layer leaving ~15mm above. With p1000, carefully remove the lymphocyte band **PLUS** the material above and below. You can take the mixture almost to the bottom pellet. This should be about 2 ml total volume per tube.
9. Put all bands from the same animal in one 15 ml conical tube
10. Mix with pipette
11. Centrifuge 1500 rpm for 10 minutes
12. Aspirate supernatant (note - leave a little bit above pellet)
13. Resuspend lymphocytes and MSCs in DMEM – 1mL
14. Add 29mL DMEM in 50 mL Falcon tube and mix gently
15. Put in T-150 (count)
16. Check cells after 2-3 days for attachment.
17. After 3 days - Aspirate cell that are not adhered to the plate
18. And feed remaining MSCs with 30mL of warm medium.
19. Subsequently change medium twice a week.
20. Passage cells or freeze when they have reached 90% confluence
21. Reseed cells in T-150 flasks at 1000 cells/cm sq in T-150 flasks.
22. Freeze cells P0 at 0.5 ml at 5E5
23. Thaw cell at 5E5 and seed new flask (P1)

APPENDIX H: CHONDROCYTE MONOLAYER CELL CULTURE

H.1. THAWING CELLS

H.1.1. Materials

J-FBS Medium

75 cm^2 TC flasks

Sterile pipettes and pipetteman

Flame

Sterile glass pasteur pipettes

Vacuum flask and tubing to hook up to vacuum

15 ml tube(s)

H.1.2. Procedure

1. Place cells directly into a 37°C water bath. Agitate gingerly while cells thaw for 40-60 seconds.
2. When defrosted minimally (liquid appears around outer edges) add a drop of complete medium.
3. Wait a minute and add another drop of medium. Repeat until tube is full—ensures that the cells thaw into the medium.
4. Transfer cells suspension in a 15 ml centrifuge tube and spin for 10 minutes at 1500 rpm.
5. Resuspend the pellet and count the number of cells.
6. Add appropriate amount of medium to obtain the desired concentration.
7. Should culture the cells at least 3-4 days before being used for experimentation (or before changing medium).

H.2. FREEZING CELLS

H.2.1. Materials

J-FBS Medium

Dimethyl Sulfoxide (DMSO)

0.45 μm sterile filter

Sterile pipettes and pipetteman

Sterile cryogenic tubes

H.2.2. Procedure

1. Add 10% v/v DMSO to J-FBS medium.
2. Filter solution through the 0.45 um sterile filter.
3. Adjust cell concentration to $1x10^6$ cells/ml of J-FBS/DMSO solution.
4. Aliquot cell suspension in sterile cryogenic tubes (be sure to account for expansion during freezing—1-1.5ml in 2 ml cryogenic tubes is a safe amount)
5. Freeze in the -20°C freezer for 2-4 hrs (longer the better) and then transfer to the -80C freezer **or** use isopropanol freezing containers and place straight into -80C freezer. For long-term storage, place cells in liquid nitrogen tank.

H.3. PASSAGING CELLS

H.3.1. Materials

J-FBS Medium

J-Expansion Medium

Trypsin

PBS

Collagenase type II (in fridge, Rm 108)

50ml centrifuge tubes

Centrifuge tube holders

Sterile pipettes and pipetteman

Vacuum flask and tubing to hook up to vacuum

150 cm^2 culture flasks (5)

H.3.2. Procedure:

1. Warm the medium, trypsin, and PBS in 37°C water bath.
2. Make necessary amount of J-FBS (See Appendix H)—8ml/150cm^2 flask; 5ml/75cm^2

3. Remove the medium in flasks w/ the vacuum pipettes.
4. Rinse once w/ solution of 0.15% collagenase II in PBS (g/ml)—for 1, 75cm^2 flask need ~5ml (ie. 0.0015 g Collagenase + 5 ml PBS, sterile filter w/ syringe filter)—incubate 3-5 minutes
5. Remove CollII/PBS solution from flask **w/ a pipette and put this solution in a 50 ml centrifuge tube—DO NOT ASPIRATE w/ vaccum** (cells do get detached during this rinse so we need to keep this).
6. Add trypsin (3-5 ml for 75 cm^2 flask)
7. Incubate for 3-5 minutes. Tap on the sides of the flask to loosen the cells and check under the microscope to ensure cells are no longer attached. If some are still attached, incubate longer checking every minute until all are unattached.
8. Once the cells are floating, collect trypsin and add it to the collected collagenase type II solution.
9. Add **J-FBS** medium to rinse the flask, collect, and add to the collagenase/trypsin solution to inactivate the trypsin (~5 ml for 75 cm^2 flask)
10. Balance the tubes and centrifuge for 10 minutes at 1500 rpm.
11. Once the pellet is on the bottom, draw off the medium w/ the vacuum pipette.
12. Resuspend in **J-Expansion** medium (20ml) and count number of cells (see following protocol for cell counting).
13. Once the cells are counted and recorded, split the cell suspension into 5 flasks by adding 4ml of the cell suspension in each 150cm^2 flask.
14. Bring volume of medium in each flask to 30ml by adding 26ml more of J-Expansion medium to each flask.

H.4. PLATING DENSITIES MONOLAYER CULTURES

TC container	Plating Density (x 10^6)	Volume of Media (ml)
150cm^2 flask	4	30
75cm^2 flask	2	15
25cm^2 flask	0.65	5
6-well	0.25	2
12-well (2.2cm diam)	0.125	1
24-well (1.5cm diam)	0.05	0.5

H.5. CELL COUNTING PROTOCOL

H.5.1. Materials

Jakob-FBS Medium + 3GFs (J-FBS-GF)

Trypan Blue

Hemacytometer & glass slide cover

Micropipetter

Sterile pipette tips and pipette extender

70% alcohol

Kimwipes

Cell counter

Calculator

H.5.2. Procedure

1. Resuspend pellet by adding 20 ml of **J-FBS-GF** media to cell pellet and mix well w/ pipette to ensure equal spacing of cells.
2. Clean the hemacytometer and cover slide w/ alcohol and a kimwipe.
3. Place the cover slide on top of the hemacytometer so that it covers the tip of the grove near the edge of the hemacytometer.
4. For a dilution factor of 2, mix a 1:1 ratio of a 15μl aliquot of trypan blue w/ a 15μl aliquot of the cell suspension into a microcentrifuge tube (use the sterile pipette extender to obtain the aliquot of cell suspension if needed)—mix well by pipetting mixture up and down.
5. Obtain a 15ml aliquot of the trypan blue/ cell suspension mixture and release it into the groove at the edge of the hemacytometer so that the mixture reaches the edges of the silvered surface—try not to overfill as this may make the count inaccurate.
6. Place the hemacytometer on the microscope stage, remove yellow glass filter, and view with standard 10x objective.
7. Count cells in each of the four corner squares and the central square (clear "glowing" cells are viable, blue stained cells are dead). To prevent overcounting,

count cells that lie on the top and left lines but not those on the bottom or right lines of each square.

8. Calculate the viability w/ the following equation:

$$\textbf{Viability} = (1 - \#\text{dead cells}/\#\text{viable cells}) * 100$$

9. Calculate and record the total cell number w/ the following equation:

$$T = N_C/N_S \times 10^4 \times D \times V$$

Where T = Total number of cells in suspension

N_C = Number of viable cells counted

N_S = Number of squares counted (ie. 5 boxes)

D = Dilution factor (ie. 2)

V = Volume of media used to suspend the cell pellet (ie. 20ml)

--For this case, I would like to split the PO cells (in the 75cm^2 flask) into 5 flasks (150cm^2 flasks) with at least 4 million cells per flask using 30 ml of media in each 150cm^2 flask (I'm estimating we'd get 20-30 million P0 cells from first plating). However, regardless of the number of cells counted (if less than 20 million or more than 30 million), still split the total number into 5 flasks and note what the exact plating number is.

H.6. STAINING CELLS WITH CELLTRACKER DYE FOR CONFOCAL MICROSCOPY ANALYSIS

H.6.1. Materials Needed

- CellTracker™ Red CMTPX Fluorescent Stain (Molecular Probes)
- Sterile PBS (Gibco)
- Trypsin-EDTA (Invitrogen)
- Culture medium (Expansion & Serum-free)
- Polystyrene cell culture 12-well plate (Falcon, VWR)
- Ultra-low Attachment 6-well plate (Corning, Fisher Scientific)
- Sterile filter paper
- 8 mm dermal biopsy punch (Moore Medical)

H.6.2. Procedure

1. Cut scaffolds to desired dimension with 8 mm dermal biopsy punch.
2. After EDAC cross-linking scaffolds, scaffolds should be rinsed in sterile PBS for ~1 hour.
3. While rinsing scaffolds, start the fluorescent cell staining process in parallel. Thaw CMTPX in hand. Mix 1ul CMTPX stain per 1ml medium (1:1000). (use expansion medium w/ FBS)

   Stock CMTPX (10mM in DMSO):

   --Add 250ul sterile DMSO to 1mg CMTPX powder provided.

   --Aliquot into 10ul stocks (to be mixed w/ 10ml media—1:1000 dilution)
4. Aspirate medium from expansion flask & add Medium + CMTPX solution to flask (30ml/150cm^2 flask). Place flask back into incubator for 20 minutes.
5. Aspirate medium. Add sterile PBS to wash. Aspirate PBS and continue basic procedures for trypsinizing monolayer cultures.

H.7. MONOLAYER TRANSFECTION OF CHONDROCYTES WITH GENEPORTER TRANSFECTION REAGENT

1. Expand P0 chondrocytes in 24-well plates, plated at 50,000 cells per well.
2. At confluence, prepare dilute GenePorter solution and plasmid solutions in SF medium (For 24-well plate, according to manufacturer's instructions, per well):
   - 2ug plasmid in 125ul SF medium (if stock is at 1mg/ml, add 2ul)
   - 10ul GP reagent in 125ul SF medium (5:1, GP:plasmid)
   - Add diluted DNA to the diluted GP reagent, vortex, and incubate at room temperature for 30minutes
3. Aspirate medium from well, add an aliquot of the GP/plasmid solution (250ul/well) and incubate at 37C for 4 hrs.
4. Four hours later, remove GP/plasmid solution and replace with 0.5ml of SF medium.
5. Collect and change every 2-3 days after infection (Day 2, 5, 7, 9. 12, and 14)
6. Assess amount of expressed protein released in the medium w/ the sandwich ELISA kit for the human IGF-1 protein (R&D Systems).

H.8. TRANSFECTING MONOLAYERS WITH GELATIN NANOPARTICLES

1. Seed chondrocyte at a density of 20,000 cells per well for a 24-well tissue culture plate in expansion medium. Change media every 2-3 days until cells become confluent.
2. At 80-90% confluence, remove media and wash with PBS (500ul).
3. Add 250ul NP/plasmid solution (~10ug plasmid per well)—diluted in serum-free medium.
4. Incubate 4 hrs.
5. Remove NP/plasmid solution and add 500ul of fresh serum-free medium to each well.

APPENDIX I: 3-D CHONDROCYTE CULTURES

I.1. MAKING CHONDROCYTE CELL PELLETS

1. After expanding cells to P1, collect cells in monolayer and resuspend in SF medium at 1 x 10^6 cells/ml.
2. Aliquot 500ul of cell suspension in sterile tubes (15ml falcon tubes or microcentrifuge tubes).
3. Centrifuge at 4500rpm for 30 seconds.
4. Vent caps and place in incubator.
5. Change medium every 2-3 days.

I.2. MAKING MSC CELL PELLETS

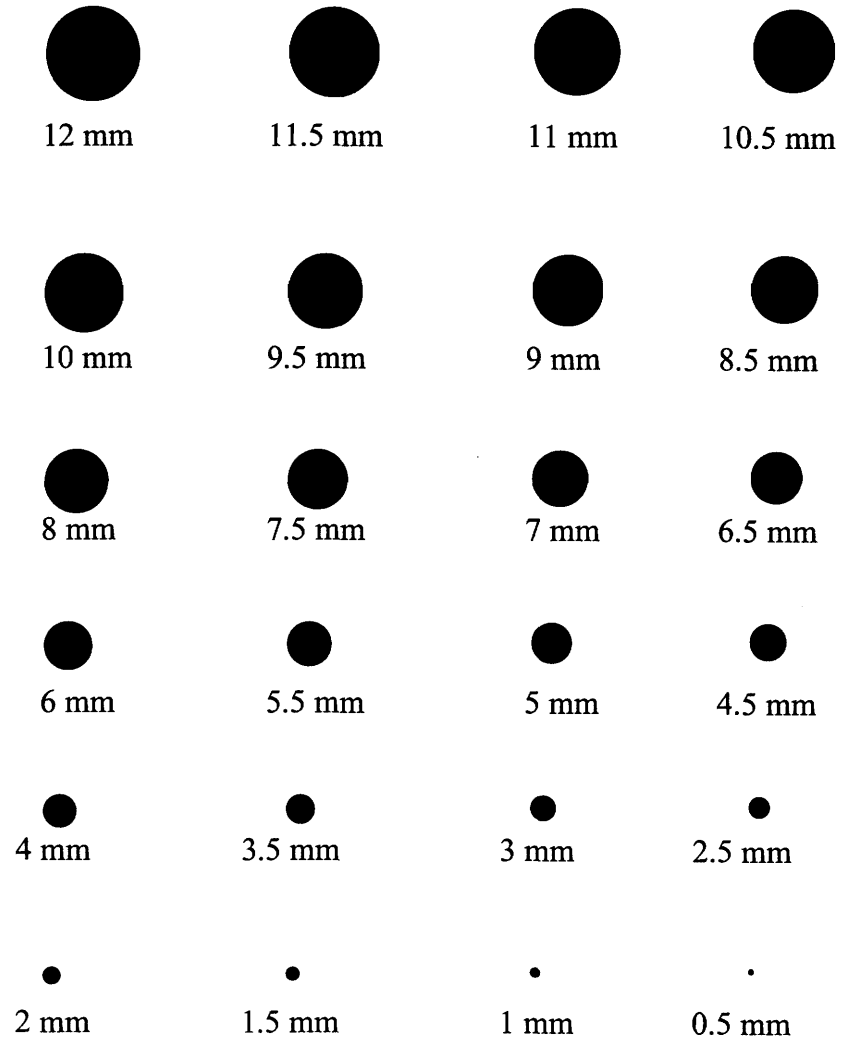
1. After expanding MSCs to P2, collect cells in monolayer and resuspend in SF medium at 400,000 cells/ml.
2. Aliquot 500ul of cell suspension in sterile tubes (15ml falcon tubes or microcentrifuge tubes).
3. Centrifuge at 1500rpm for 10 minutes.
4. Vent caps and place in incubator.
5. Change medium every 2-3 days.

I.3. CELL SEEDING SCAFFOLDS

Day of seeding (for seeding density of 4million cells/scaffold):

1. EDAC cross-link scaffolds.
2. Trypsinize confluent cells and count.
3. Resuspend in serum-free medium @ 100,000 cells/ul.
4. Prewet scaffolds (in PBS after EDAC), dry on sterile filter paper (~6-8 scaffolds at a time) then transfer to warm agarose-coated 12-well TC plates.
5. Seed 2million per side (20ul/side) with ~10min incubation in between.
6. Incubate for 2hrs and then add 0.5ml SF medium—dispense slowly against well wall.
7. Next day, add 0.5 ml medium.
8. Change media every 2-3 days.

I.4. SCAFFOLD MEASUREMENT TEMPLATE



APPENDIX J: BIOCHEMICAL ASSAYS

J.1. PROTEINASE K SAMPLE DIGESTION

(Lyophilize samples prior to digestion)

1. Digest samples overnight (12-24 hours) at **60° C** in the following solutions:

   --If digesting ***8mm*** *scaffolds,* use ***500ug/ml*** proteinase K solution:

   For 100 ml solution: 100 ml Tris-HCl buffer (see below)

   50 mg proteinase K powder (Sigma, Cat# P-6556, -20C)

   --If digesting ***cell pellets*** (or 4mm scaffolds), use ***100ug/ml*** proteinase K solution:

   For 100 ml solution: 100 ml Tris-HCl buffer (see below)

   10 mg proteinase K powder
2. Sterile filter proteinase K solution & keep left over solution at 4C
3. Add 1ml of Proteinase K solution per sample
4. Vortex before putting in water bath & once again before leaving it overnight
5. Keep digested samples at -20C until used for biochemical analysis

Tris-HCl buffer (1L)—keep at room temperature

- 0.05M UltraPure Tris—6.1g/L (Mw=121.14g/mol, Invitrogen, Cat #15504-020)
- 1mM $CaCl_2$ Dihydrate—0.147g/L (Mw=147.02g/mol, Sigma (Fluka), Cat #21097)
- Dissolve above salts in 900 ml distilled water
- Adjust pH to 8.0 with 1N NaOH
- Bring to IL with distilled water

J.2. DETERMINING DNA CONTENT USING THE PICOGREEN ASSAY

J.2.1. Materials Needed

Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes, Cat# P7589) contains:

- PicoGreen dye reagent (1mL solution in DMSO)—light sensitive (keep covered)
- 20X TE (25mL of 200mM Tris-HCl, 20mM EDTA, pH 7.5)
- Lambda DNA standard (1mL of 100 mg/mL in TE)

TE buffer for diluting samples:

- 1ml 1M Tris (pH=8) (UltraPure Tris, Invitrogen, Cat# 15504-020, FW=121.1; or use 1M Tris (pH=8.0), Ambion, Cat# 9855G)
- 0.2 ml 0.5M EDTA (pH=8) (Disodium Ethenediamine Tetraacetate, Fisher, Cat#S311, FW=372.24; or use 0.5M EDTA (pH=8.0), Invitrogen, Cat# 15575-038)
- 98.8 ml dH_2O

96-well plate—Black Isoplate (Clear bottom plates, Wallac, Cat# 1450-571)

J.2.2. Procedure

1. Assay Buffer Preparation: Add 25ml (20X TE) + 500 ml sterile, dH_2O
2. DNA std working solution (2mg/ml): Add ***294ml of TE buffer*** + ***6ml DNA stock*** (100 mg/mL)
3. Dilute all digested samples ***1:10*** with TE buffer (not from kit): ***180ul TE + 20ul digest*** (can be diluted in microcentrifuge tubes or 96-well plate)
4. Prepare the DNA standards as follows in the first 8 wells of Picogreen 96-well plate:

Standard No.	Standards	TE buffer (ul)	DNA std-2mg/ml
Blank	0	100	0
1	10	99	1
2	50	95	5
3	100	90	10
4	250	75	25
5	500	50	50
6	750	25	75
7	1000	0	100

5. Add 20ul of diluted digested sample to each well—***vortex samples before adding***
6. Add 80ul TE buffer (from kit) to each well (for total of 100ul solution per well)
7. Dilute PicoGreen dye stock with TE buffer (from kit), ***200X***—*Prepare just before use*

   --100 ul of working dye solution needs to be added to well used

   --Make more working dye then needed (i.e. add solution for 5 additional wells)

   For example, if there are ***96 wells*** to fill, make enough dye for 110 wells:

   110 x 100ul = 11000ul (or 11ml); 11000/200 = ***55ul of PicoGreen stock + 11ml TE***
8. Dispense 100 ul PicoGreen working dye solution to each well being used
9. Take fluorescence reading on microplate reader (WALLAC VICTOR^2 1420 Multilabel Counter, Perkin Elmer Life Sciences)): Protocol assigned in computer program under DNA Assay—"Fluorescein (485nm/535nm, 1.0s)" (includes a 5 min incubation period & shake)

**** If samples end up having a reading greater than the highest standard, samples need to be diluted more and re-run***

J.3. GAG ASSAY USING DIMETHYLMETHYLENE BLUE (DMMB) DYE

J.3.1. Solutions Needed:

Color Reagent

For 500ml of DMMB dye solution, mix the following:

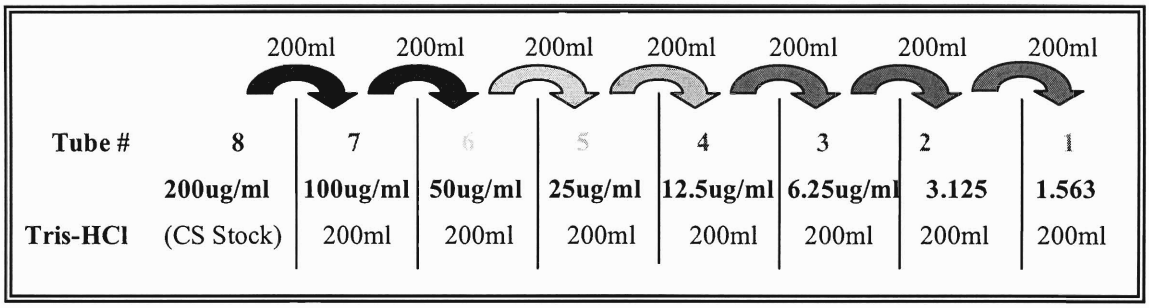
- 425 ml dH_2O
- 1.52 g glycine (Sigma, Cat# G-8898)
- 1.19 g NaCl (Fisher, Cat# S642)
  a. Adjust pH to 3.0 with concentrated HCl and NaOH
  b. Add more dH_2O to bring volume up to 500ml
- Add 8mg of DMMB dye
- Solution good for 3 months and should be kept in a light-protected bottle (stir 30 min prior to use)

TE buffer for diluting samples

- 1ml 1M Tris (pH=8) (UltraPure Tris, Invitrogen, Cat# 15504-020, FW=121.1
  or use 1M Tris (pH=8.0), Ambion, Cat# 9855G)
- 0.2 ml 0.5M EDTA (pH=8) (Disodium Ethenediamine Tetraacetate, Fisher, Cat#S311, FW=372.24)
  or use 0.5M EDTA (pH=8.0) (Invitrogen, Cat# 15575-038)
- 98.8 ml dH_2O

J.3.2. Procedure

1. Stir DMMB dye working solution at least 30min before use
2. Prepare the Chondroitin Sulfate (CS) stock solution at 2 mg/mL (keep in –20C freezer)
3. CS working solution (200mg/ml): Add 100 ml of CS stock + 900 ml dH_2O
4. Prepare the CS standards as follows: (Start w/ 7 labeled tubes filled w/ 200ul TE buffer)



5. Dilute all digested samples ***1:10*** with TE buffer: ***180ul TE + 20ul digest*** (can be diluted in microcentrifuge tubes or 96-well plate)
6. Add 20ul of each standard and sample to the 96-well plate (Clear plates, Packard Bioscience Spectraplate-96, Cat# P12-106-043)—good to do this in duplicate, at least for the standard (Use 20ul aliquot of TE buffer as blank)
7. Dispense 200 mL of DMMB dye solution to each well used
8. Take reading at 530 nm on microplate reader (WALLAC VICTOR^2 1420 Multilabel Counter, Perkin Elmer Life Sciences): Protocol assigned on computer program for the microplate reader is under GAG Assay—"GAG-DMMB Assay protocol (530nm-1s)"

**** If sample reading is outside the standard curve range (or at the extremes of the standard curve), it is recommended that another dilution and reading of the sample be performed—the ideal is to have the reading land on the mid-range of the standard curve (usually a 2nd order polynomial is the best fit curve for this standard)***

J.4. IGF-1 DUOSET SANDWICH ELISA ASSAY (R&D SYSTEMS CAT. #DY291)

J.4.1. Solutions Needed

PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2-7.4

--Used 1packet powdered PBS + 1L DI water, 0.2μm filtered

Wash buffer: 0.05% Tween 20 in PBS, pH 7.2-7.4

1L PBS + 0.5g Tween 20 (per liter) ***or*** 0.5L PBS + 0.25g Tween 20 (per liter)

(Weigh out Tween 20 in 15 ml falcon tube and dissolve in PBS before adding to rest of PBS solution—Tween 20 very viscous)

Block buffer: 5% Tween 20 + 0.05% NaN3 in PBS

For 100ml: 100 ml PBS + 5g Tween 20 + 0.05g NaN3

Reagent Diluent (RD): 5% Tween 20 in PBS—*0.2μm filtered*

For 50ml: 50ml PBS + 2.5g Tween 20

Substrate Solution: 1:1 mixture of Color Reagent A (H2O2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems Cat. # DY999)

Stop Solution: 2 N H2SO4

J.4.2. Day 1: Plate Preparation

Coat plates: Capture Antibody (R&D, Part 840264—stock concentration 720 μg/ml, reconstituted w/ 1.0 ml PBS, working concentration 4μg/ml in PBS, 100 μl/well

Ex (96 wells): 58 μl Capture Ab stock + 10.4 ml PBS

(adjusted for weak signal: 70ul CapAb stock + 10.5 ml PBS)

Tap plate to cover bottom of wells, check for bubbles

Cover plate with plate sealer, incubate overnight at room temperature

J.4.3. Day 2: IGF-1 ELISA

1. Wash plate 3x with wash buffer, 400ul/well—after last wash, invert plate and blot against clean paper towels
2. Block plates w/ Block Buffer, 300 µl/well
3. Cover plate, RT, 1 hr minimum
4. Wash plate 3x
5. Prepare IGF-1 standard dilutions:

--Standard (R&D, Part 840266, 1 vial)—stock concentration 70 ng/ml, reconstituted w/ 0.5 ml Reagent Diluent. Store reconstituted standard at 2-8°C for up to 60 days

10-point standard curve using 2-fold serial dilutions in Reagent Diluent w/ high standard at 2ng/ml:

STOCK	**8**	**7**	6	**5**	4	3	**2**	**1**	**Tube #**
	(70ng/ml)								
	582.9ul	300µl	300µl	300µl	300µl	300µl	300µl	300µl	**Reagent**
Diluent									
(ng/ml)	2	1	0.5	2.5	0.125	0.0625	0.0313	0.0156	**Std. Conc.**

6. Wash plate 3x
7. Add standards or samples: run standard in duplicate, 100ul/well—Include blank well w/ Reagent Diluent only
8. Cover plate, RT, 2 hrs
9. Wash plate 3x
10. Add Detection Antibody (Part 840265)—stock concentration 14.4 µg/ml, reconstituted w/ 1.0 ml Reagent Diluent (store at 2-8C up to 6 months), working concentration 80ng/ml in Reagent Diluent, 100 µl/well

    Example (96 wells): 56ul Det. Ab stock, 10 ml Reagent Diluent

    (adjusted for weak signal: 80ul Det. Ab stock + 10.5 ml RD)

    Cover plate, RT, 2 hrs
11. Wash plate 3X

12. Add Streptavidin HRP (Part 890803, store at 2-8C up to 6 months)—working concentration: dilute stock solution 1:200 in Reagent Diluent, 100 µl/well

    Example (96 wells): 50ul Streptavidin HRP stock, 10 ml Reagent Diluent

    (adjusted for weak signal: 80ul CapAb stock + 10.5 ml RD)

    Cover plate, RT, 20 minutes—**Avoid direct light**

13. Wash plate 3X
14. Substrate Solution: mix 1:1 Color Reagent A & Color Reagent B, 100ul/well

    Example (96 wells): 5.2 ml A, 5.2 ml B

    Wipe pipette tips with paper towel in between rows

    Cover plate, RT, 20 minutes—**Avoid direct light**

15. Add Stop Solution—50ul per well, gently tap plate to ensure thorough mixing
16. Determine optical density immediately using microplate reader set to 450 nm. Subtract readings at 540nm or 570 nm from 450nm readings for wavelength correction (corrects for optical imperfections in the plate).

APPENDIX K: HISTOCHEMICAL STAINS

K.1. HEMATOXYLIN AND EOSIN STAIN

Formalin fixed, paraffin embedded specimens

K.1.1. Solutions

- Harris Hematoxylin Solution (Sigma Cat# HHS-128)—filter 200ml of stock solution into staining dish
- Eosin Y Solution Aqueous (Sigma Cat# HT110-2-128)
- Acid Alcohol 0.5 % in 80% alcohol (99.5ml of 80% alcohol + 0.5 ml HCl)

K.1.2. Other Materials

- Cytoseal 60 (Cat# 18006, Electron Microscopy Sciences)

K.1.3. Methods

1. Deparaffinize and Rehydrate

Xylene (or substitute)	2 x 5 min.
100% alcohol	2 x 3 min.
95% alcohol	2 x 2min.
80% alcohol	1 min.
Wash in tap water	5 min.

2. Hematoxylin, 3 min. Note: be sure to filter hematoxylin prior to use!
3. Wash in tap water for 5 min.
4. One quick dip in acid alcohol.
5. Wash in tap water for 5 min.
6. Eosin, 3 quick dips.
7. Dehydrate

100% alcohol	2 x 3 min.
Xylene (or substitute)	2 x 3 min.

8. Coverslip with Cytoseal

K.2. SAFRANIN-O STAIN

K.2.1. Solutions

- Safranin-O: 0.2% w/v. Add 1 ml acetic acid per 100 ml dH_2O.

  Example: 0.2 g Saf-O + 1ml acetic acid + 100 ml dH_2O.
- Fast Green Stock Solution: 0.2g Fast green + 1 ml acetic acid + 100 ml dH_2O.
- Fast Green Working Solution: 1:500 dilution of stock solution in dH_2O.
- 0.5% Acetic Acid: 1 ml acetic acid in 200 ml dH_2O.

K.2.2. Other Materials

- Cytoseal 60 (Cat# 18006, Electron Microscopy Sciences)
- Gill's Hematoxylin

K.2.3. Methods

1. Deparaffinize and Rehydrate:

Xylene (or substitute)	2 x 5 min.
100% EtOH	2 x 3 min.
% EtOH	2 x 2min.
80% EtOH	1 min.
Wash in tap water	5 min.

2. Hematoxylin, 3 min. Note: be sure to filter hematoxylin!
3. Wash in tap water for 5 min.
4. Fast green – 3 quick dips.
5. 0.5% acetic acid – 3 quick dips.
6. Safranin-O – 30 minutes
7. Dehydrate

95% EtOH	a few quick dips
100% EtOH	2 x 3 min.
Xylene (or substitute)	2 x 3 min.

8. Coverslip with Cytoseal

K.3. TYPE II COLLAGEN IMMUNOHISTOCHEMISTRY

1. Put slides on hot plate (setting 6 for 2 hours or overnight at setting 4 or 5)
2. Prepared TBS solution as necessary (from Dako packets – box on shelf)
3. Set up protocol on autostainer & fill all necessary reagents (see steps below)
   - Open "Scott" file
   - 150ul on bottom and middle position of each slide
4. Prior to loading in autostainer, de-paraffin and rehydrate
   - Xylene 2 x 5min
   - 100% alcohol 2 x 3min
   - 95% alcohol 2 x 2min
   - 80% alcohol 1min
   - Water 5min
5. Keep slides wet with TBS while loading in autostainer.
6. Prime the pumps (make sure fluids freely moving) and start the program
7. The autostainer steps:
   * Rinse
   * Proteolytic digestion to unmask antigenic sites (40 min.)
     --Prepared 0.1% (w/v) Protease XIV (0.015g in 15ml TBS)
   * Rinse
   * Endogenous peroxidase quench (10 min.)
     --Use from Dako kit
   * Rinse
   * Block non-specific binding (30 min.)
     --Prepared 5% horse serum. (0.75ml serum in 14.25ml TBS)
   * Rinse
   * Primary Antibody or negative control (30 min.)
     --Prepare 1:20 dilution of CIICI collagen 2 antibody in Dako diluent
     (Add 0.375ml antibody to 7.125ml diluent)
     --If using diluent with BSA as a protein block, incubation with horse serum may not be necessary

* Rinse

* Secondary Antibody (10 min.)

--Biotinylated link: biotin labeled goat anti-rabbit and goat anti-mouse IgG.

--Yellow liquid in Dako kit. (Prepared 15ml)

* Rinse

* Tertiary reagent (10 min.)

--Streptavidin HRP: streptavidin conjugated to horseradish peroxidase

--Red liquid in Dako kit (prepared 15ml)

* Rinse

* "switch"

* Subtrate-Chromogen solution (10 min.)

--Diaminobenzidine (DAB)

--Add 1 drop (20ul) of DAB Chromogen per 1ml of buffered substrate (from DAKO kit).

--Mix in supplied graduated test tube and transfer pipette. (prepared 15ml)

8. Remove slides from autostainer
9. Wash in dH_2O (3 min.)
10. Stain with hematoxylin (3min. Be sure to filter hematoxylin prior to using)
11. Wash in dH_2O (3 min.)
12. Acid Alcohol - a few quick dips

--Prepare 1% acid alcohol (1ml HCl in 99ml of 80% ethanol)

13. Wash in dH_2O (3 min.)
14. Dehydrate through xylenes (times rather arbitrary)

--80% ethanol, a few dips

--95% ethanol, a few dips

--100% ethanol, 1 minute

--Xylene

15. Coverslip

K.4. MASON'S TRICHROME STAIN

1. Dehydrate (as described in previous protocols)
2. Hematoxylin (Gill or Harris-do not use the Weigert hematoxylin)-5min
3. Rinse-5min
4. Rinse in deionized water-a few dips
5. Biebrich-Scarlet Acid Fuchsin (deep red)-for 10 min
6. Phosphotungstic/Phosphomolybid Acid (Prepare by mixing equal volumes of each)-1min
7. Aniline Blue-5 min
8. 1% Acetic Acid-2 quick dips
9. Rehydrate rapidly and coverslip

APPENDIX L: TRASMISSION ELECTRON MICROSCOPY OF UNSEEDED GSCG SCAFFOLDS

L.1. FIXATION

L.1.1. Solutions

- 0.2M cacodylate stock buffer: Sodium Cacodylate 4.28 g + 100ml distilled water
  Note: addition of acid produces Arsenic gas, work in the hood if possible
  Store in refrigerator

  For pH 7.2 add 8.4 ml of 0.2N Hydrochloric Acid to 100 ml of the Sodium cacodylate buffer.

  For pH 7.4 add 5.6 ml of 0.2N Hydrochloric Acid to 100 ml of the Sodium cacodylate buffer.

L.1.2. Procedure

L.1.2.1. Primary Fix

10 ml 8% glutaraldehyde
6 ml 0.2M buffer
6 ml distilled water

- Fix specimens in 2% glutaraldehyde in 0.1M cacodylate buffer, on ice, 20 minutes rinse with 0.1 M cacodylate working buffer 5 minutes, x3

L.1.2.2. Post Fixation

- 2% osmium tetraoxide in 0.1 M cacodylate buffer for 1 hr on ice in dark
- Rinse with 0.1 M cacodylate buffer 5 minutes, x 3

L.2. ENBLOC STAINING

- 2% Uranyl Acetate in 50% EtOH for 20 min.

L.3. DEHYDRATION

- 70% ethanol, 5 minutes
- 80%, 90%, 10 minutes each
- 100% ethanol x 3, 10 minutes each
- 50% ethanol, 50% propylene oxide (PO) for 5 min
- Pure PO for 5min x 2

L.4. INFILTRATION

Spurr's embedding resin: medium hard mixture; can use 50ml tri-pour beakers to measure this out

- Mix Spurr's embedding resin *gently* for 5 min with tongue depressor
- 1:1 Spurr:PO o/n on rotator
- 100% Spurr's mixture (not more than 24 hours old) for 4-6 hours on rotator
- Embed 70C for 24 hours

L.5. MICROTOMY

- Trim specimens to 1mm square block face
- Thick sections can be cut 0.5mm, stained with toludine blue to check morphology
- Thin sections approx 60nm thick (silver to gold)
- Pick up sections on 400 mesh copper grids

L.6. POST STAIN

- In Evaporator, clean grids by glow discharging
- On bench set up two petri dishes and 3 50-ml beakers of distilled wate
- Place drop of 2%-8% uranyl acetate in petri dish on parafilm
- On each drop place grid sample side down, 15 min in darkness
- Rinse 3x in series of 50 ml beakers containing H2O
- Blot dry on lens paper
- While the samples are staining in UA, place one drop of Reynold's lead citrate (with 4 or 5 pellets of NaOH). *Do not breathe on lead!*
- Stain grids 7-10 min, covered petri dish
- Rinse 3x in series of beakers containing H2O and dry on lens paper
- Store in grid box

* Protocol provided by Patricia Reilly (Assistant Director, DuPont MIT Alliance)