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ORIGINAL ARTICLE

Collagen scaffolds for nonviral IGF-1 gene delivery in articular cartilage tissue engineering

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This study investigated the use of a type II collagen-glycosaminoglycan (CG) scaffold as a nonviral gene delivery vehicle for facilitating gene transfer to seeded adult articular chondrocytes to produce an elevated, prolonged and local expression of insulin-like growth factor (IGF)-1 for enhancing cartilage regeneration. Gene-supplemented CG (GSCG) scaffolds were synthesized by two methods: (1) soaking a pre-cross-linked CG scaffold in a plasmid solution followed by a freeze-drying process, and (2) chemically cross-linking the plasmid DNA to the scaffold. Two different plasmid solutions were also compared: (1) naked plasmid IGF-1 alone, and (2) plasmid IGF-1 with a lipid transfection reagent. Plasmid release studies revealed that cross-linking the plasmid to the CG scaffold prevented passive bolus release of plasmid and resulted in vector release controlled by scaffold degradation. In chondrocyte-seeded GSCG scaffolds, prolonged and elevated IGF-1 expression was enhanced by using the cross-linking method of plasmid incorporation along with the addition of the transfection reagent. The sustained level of IGF-1 overexpression resulted in significantly higher amounts of tissue formation, chondrocyte-like cells, GAG accumulation, and type II collagen production, compared to control scaffolds. These findings demonstrate that CG scaffolds can serve as nonviral gene delivery vehicles of microgram amounts of IGF-1 plasmid (< 10 μg per scaffold) to provide a locally sustained therapeutic level of overexpressed IGF-1, resulting in enhanced cartilage formation.

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Introduction

Three-dimensional scaffolds and recombinant growth factors have been proven to be effective stimulants for chondrogenesis in articular cartilage tissue engineering. One challenge in implementing exogenous recombinant growth factors *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 restrict the protein to a localized area. One promising method for the prolonged, localized release of the therapeutic protein over the time course of regeneration, combines tissue engineering and gene therapy strategies by incorporating growth factor gene-transfected/transduced cells into tissue engineering scaffolds.

Ex vivo gene transfer to cells in two-dimensional (2D) monolayer culture for subsequent transplantation, with or without a three-dimensional (3D) scaffold, has been used to provide a 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* owing to the migration of these cells from the defect site and apoptosis.[5] An alternative approach for incorporating transfected cells for prolonged protein release is a 'substrate-mediated'[6] gene transfer method, in which tissue-engineering scaffolds are used as gene delivery vehicles to seeded cells and/or endogenous cells *in vivo*. This method provides for the continual transfection of cells and subsequent protein expression which can be achieved over extended periods of time (weeks to months).

Both viral[1,3,7] and nonviral[4,8–13] 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 efficiency of gene transfer, 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

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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),[11,12,14] poly(D,L-lactide)–poly(ethyleneglycol) (PLA–PEG),[12] and collagen.[8–10,13,15]

In the present study, gene-supplemented type II collagen-glycosaminoglycan (GSCG) scaffolds were investigated using naked plasmid DNA encoding for the insulin-like growth factor (IGF)-1 protein, alone and with a lipid transfection reagent, GenePorter (GP). These type II collagen scaffolds were commended for this application because of their favorable performance as non-cell-seeded[16] and cell-seeded[17] implants in cartilage defects. Moreover, earlier work[15] has demonstrated the versatility of collagen scaffolds to be physically and chemically modified in ways that could allow for a wide range of plasmid DNA release kinetics. Two different methods of gene incorporation were investigated: (1) soak of the scaffold in the plasmid solution followed by freeze-drying (method 1), and (2) covalent linkage of the plasmid to the scaffold using a carbodiimide cross-linking agent (method 2). We hypothesized that the first method would provide a rapid release of the plasmid and the second a prolonged release, thus offering control of the timing of transfection. The plasmid release kinetics, encoded IGF-1 protein overexpression, and the resulting biochemical and histochemical outcomes from GSCG scaffolds fabricated using the two methods of incorporation along with the addition of a lipid transfection reagent were evaluated in this study.

A local and prolonged administration of 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.[18–23] Although studies have shown effective IGF-1 gene transfer to cells using an *ex vivo* approach for enhancing articular cartilage tissue engineering,[1,4,24] there has not yet been an investigation demonstrating IGF-1 gene transfer to cells using a substrate-mediated nonviral gene transfer approach using collagen scaffolds.

Results

Transmission electron microscopy of unseeded GSCG scaffolds

Transmission electron micrographs of GSCG scaffolds (fabricated using method 2 – cross-linking method) revealed distinct differences in the ultrastructure of scaffolds supplemented with or without plasmid. CG scaffolds incorporating plasmid encoding for IGF-1 (*pIGF-1*) alone displayed a thin electron-dense layer (less than 100 nm) lining the wall of the scaffold struts (Figure 1b). This layer was not present in control samples (Figure 1a). Scaffolds incorporating GP/IGF complexes showed clusters of electron-dense particles (from about 50 to 100 nm in diameter) along the walls of the scaffold struts (Figure 1c). These electron-dense particles were greater in number and appeared with substantially greater regularity on the surfaces of the struts of scaffolds incorporating GP/IGF complexes (Figure 1c) than in the control scaffolds with no plasmid (Figure 1a).

IGF-1 plasmid release from GSCG scaffolds – release kinetics and plasmid integrity

The release kinetics of naked plasmid *IGF-1* from CG scaffolds comparing both types of plasmid incorporation methods were assessed by soaking the GSCG scaffolds in buffer over various time points. There were dramatic differences in the amounts of *pIGF-1* initially incorporated into the GSCG scaffolds and the plasmid release profiles comparing the two different incorporation methods (Figure 2). Approximately 60% of the 20 μg plasmid load was incorporated into scaffolds using method 1 (soak and freeze-dried), whereas $\sim$40% was incorporated using method 2 (cross-linking plasmid to scaffold, Figure 2). GSCG scaffolds synthesized by method 1, however, released most of the incorporated plasmid ($\sim$83%) within the first 2 days of soaking in the buffer solution (Figure 2). Thereafter, there was little release of plasmid, leaving $\sim$12% of the incorporated plasmid in the method 1 scaffolds at the end of the 2-week plasmid release study (Figure 2). In contrast, GSCG scaffolds with plasmid cross-linked to the scaffold (method 2) released only minute amounts of plasmid during the leaching period, resulting in $\sim$99% plasmid retention after 2 weeks in buffer (Figure 2).

Since most of the plasmid from method 1–produced GSCG scaffolds produced with method 1 was released in the buffer, and most of the plasmid was retained in the method 2–produced 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 (Figure 3). The resulting gel analysis (Figure 3) revealed that 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 (Figure 3). This demonstrates, that the structural integrity of plasmid released

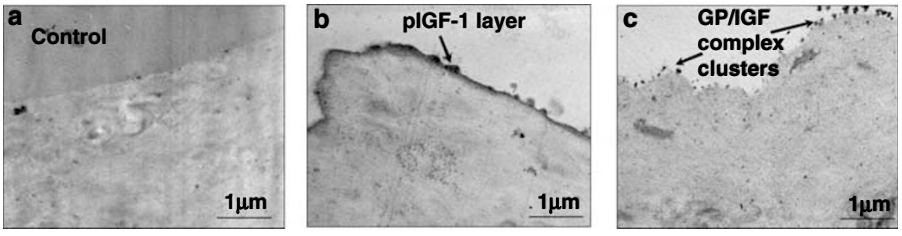

Figure 1 Transmission electron micrographs of control (**a**), GSCG scaffolds incorporating *pIGF-1* alone (**b**) and GP/IGF complexes (**c**).

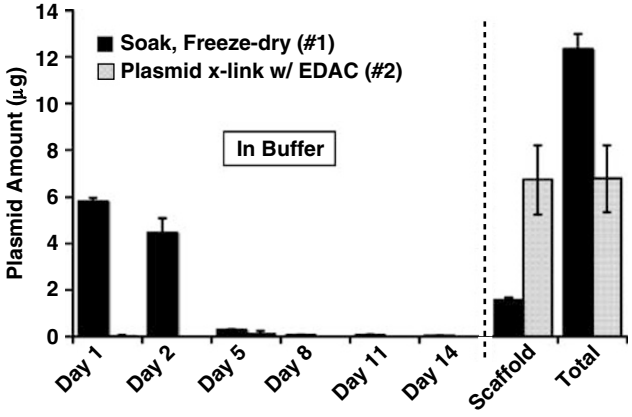

Figure 2 Plasmid amounts detected in the leaching buffer and remaining in the GSCG scaffolds after the 2-week leaching study (scaffolds loaded with 20 μg plasmid *IGF-1*). $n=3$; mean $\pm$ s.e.m.

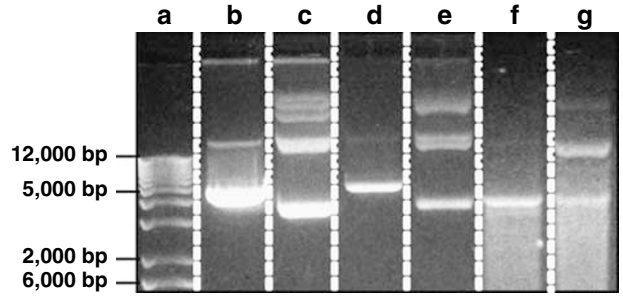

Figure 3 Gel electrophoresis (on a 1% agarose gel) of 1 kb DNA ladder (**a**); *IGF-1* plasmid stock solution (**b**) cut and (**c**) uncut; plasmid released from method 1-synthesized GSCG scaffolds (**d**) cut and (**e**) uncut; and plasmid retained within method 2-synthesized GSCG scaffolds after the 2-week leaching study (**f**) cut and (**g**) uncut. Restriction enzyme *Xba*I was used to cleave the various *IGF-1* plasmid samples. The size of the linearized *IGF-1* plasmid was about 5 kb.

from and retained in the GSCG scaffolds was maintained.

Nonviral gene transfer to chondrocytes seeded in GSCG scaffolds

For GSCG scaffolds synthesized using method 1, there was no difference in IGF-1 released in the medium for scaffolds incorporating naked *pIGF-1* alone (IGF group) over the control scaffolds (Figure 4a). Incorporation of the GP transfection reagent (GP/IGF group) into the GSCG 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 (Figure 4a). The GP/IGF group showed a peak in IGF-1 release rate after about 1 week in culture ($\sim$20 pg/h), followed by a rapid decline from the day 11 to day 14 collection. There was no difference in the amount of IGF-1 detected in the day 14 collection for the GP/IGF group compared to the control and IGF groups. After the 2-week culture period, the GP/IGF group showed a sevenfold higher accumulated IGF-1 level at around 5 ng/ml versus the control and IGF groups at about 0.7 ng/ml (Figure 4b). Two-factor analysis of variance (ANOVA) showed significant effects of time ($P<0.01$, power $=0.88$) and gene supplementation ($P<0.0001$, power $=1$) on IGF-1 release.

GSCG scaffolds created by cross-linking plasmid *IGF-1* to the collagen scaffolds (method 2) both with (x-GP/IGF) and without (x-IGF) the transfection reagent, resulted in noticeably elevated IGF-1 expression levels compared to controls (Figure 4c). 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 scaffold containing plasmid *IGF-1* alone (Figure 4c). Scaffolds containing GP and prepared using method 2 (x-GP/IGF group) showed a significant increase in IGF-1 release rate between the first two

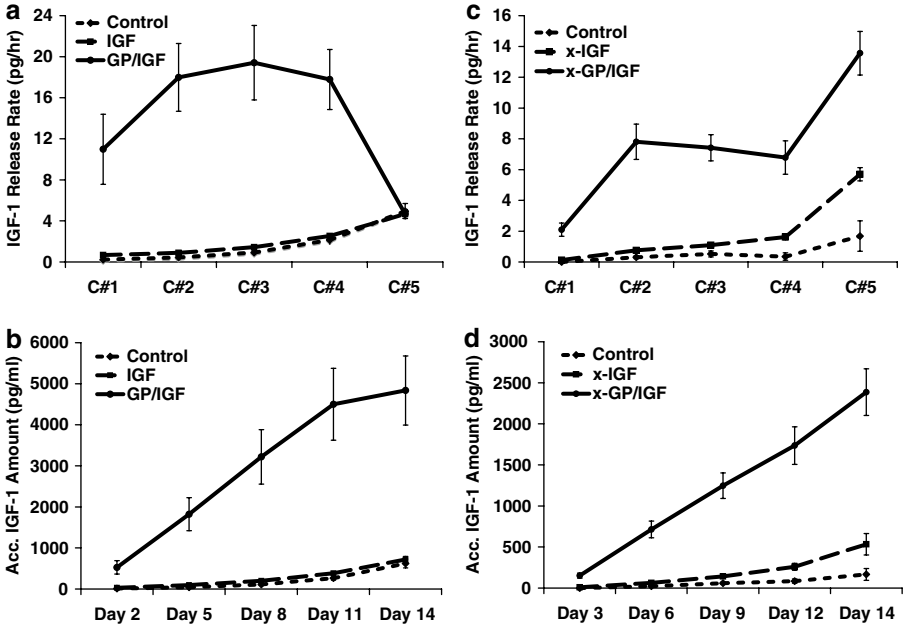

Figure 4 IGF-1 release rates (**a**, **c**) and accumulated IGF-1 (**b**, **d**) detected in the serum-free medium over the 2-week 3D culture period from GSCG scaffolds synthesized using method 1 (**a**, **b**), $n=3$ and method 2 (**c**, **d**), $n=6$. mean $\pm$ s.e.m.

collection time points (after 3 and 6 days), followed by a steady release between collections 2 and 4 (days 2 and 12), and finally a rise in the IGF-1 release rate to 14 pg/h at the end of the 2-week culture period (Figure 4c). There were 4.5-fold and 14.5-fold differences in accumulated IGF-1 levels for the x-GP/IGF group above the x-IGF and control groups, respectively (Figure 4d). Two-factor ANOVA revealed significant effects of time ($P<0.0001$, power $=1$) and gene supplementation on the IGF-1 release ($P<0.0001$, power $=1$) for GSCG scaffolds made using method 2.

GFP expression in chondrocytes seeded onto scaffolds supplemented with plasmid encoding for green fluorescent protein (*pEGFP*) with or without the transfection reagent using method 2 was observed with fluorescence (Figure 5a–c) and confocal microscopy (Figure 5d). After 5 days in culture, chondrocytes seeded onto control scaffolds (no plasmid) did not show any evidence of GFP expression (Figure 5a). Chondrocytes seeded in scaffolds supplemented with *pEGFP* alone (Figure 5b) or with the GP transfection reagent (Figure 5c) displayed evidence of transfection, with a significantly higher number of cells fluorescing in scaffolds incorporating the plasmid with the transfection reagent (Figure 5c versus Figure 5b). Of note was that transfection was still apparent up to 2 months in the scaffolds. Confocal microscopy of GSCG scaffolds containing the GP/plasmid complex (7 days after seeding) revealed that a small percentage of cells within the scaffold were transfected at that given time point (Figure 5d).

Light micrographs taken in the brightfield mode were not included because the cells were not clearly defined in the collagen scaffold (i.e., the cell membrane did not display sufficient contrast relative to the scaffold background), especially when the cells were located deeper within the scaffold. The presence and relative number of cells were better assessed through analysis of the histological sections.

Biochemical analysis of cell-seeded GSCG scaffolds

Control scaffolds and scaffolds incorporating *IGF-1* plasmid alone made using method 1 were similar in DNA content after 2 weeks in culture with $\sim 10\ \mu$g DNA/scaffold (Figure 6a). The DNA content for the GP/IGF group, however, was significantly lower, at about half the amount found in the IGF and control groups (Figure 6a). In contrast, GSCG scaffolds incorporating plasmid *IGF-1* with the transfection reagent using method 2 (x-GP/IGF) showed a 25% higher DNA content ($\sim 21\ \mu$g/scaffold) compared to the x-IGF and control scaffolds (Figure 6b). 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 μg/mm^3 for all groups (Figure 6c). For method 2-synthesized scaffolds, there was a significant elevation ($\sim$two-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 (Figure 6d).

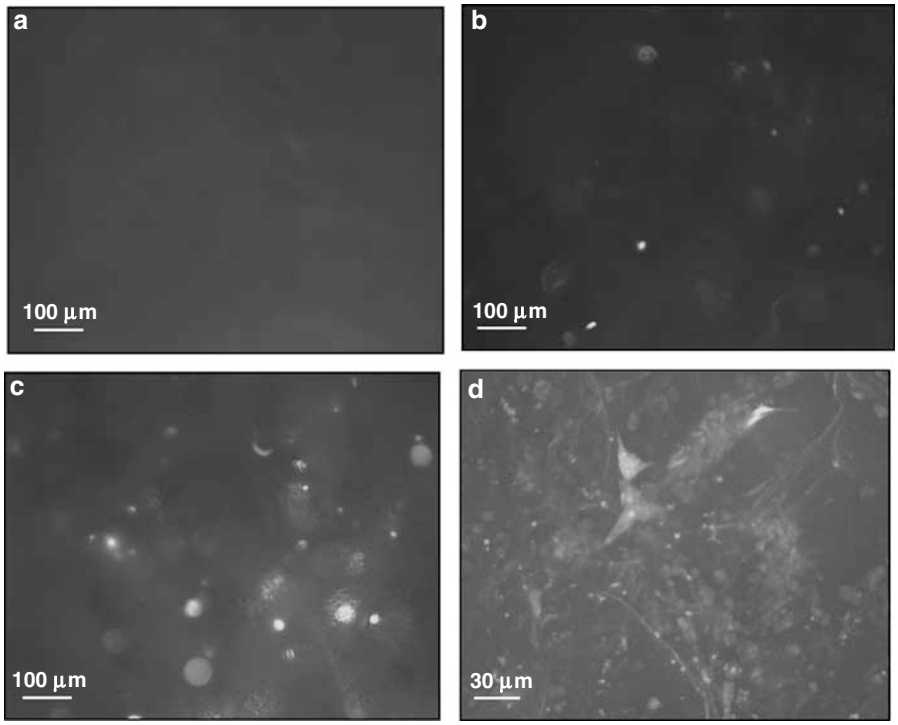

Figure 5 Fluorescent microscopy images of GSCG scaffolds synthesized using method 2 (**a**) without any supplementation (control), (**b**) with incorporation of *pEGFP* alone, and (**c**) with incorporation of *pEGFP* and the GP transfection reagent after 5 days in 3D culture. Confocal image of GSCG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

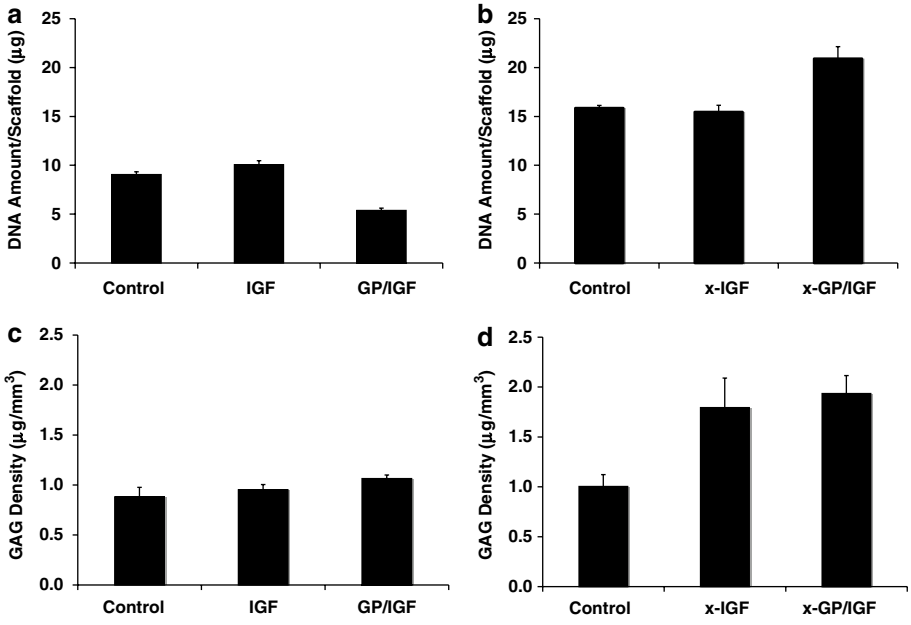

Figure 6 DNA contents (**a**, **b**) and accumulated GAG densities (**c**, **d**) measured at the end of the 2-week 3D culture period in GSCG scaffolds synthesized using method 1 (**a**, **c**) and method 2 (**b**, **d**). $n = 3$–4; mean $\pm$ s.e.m.

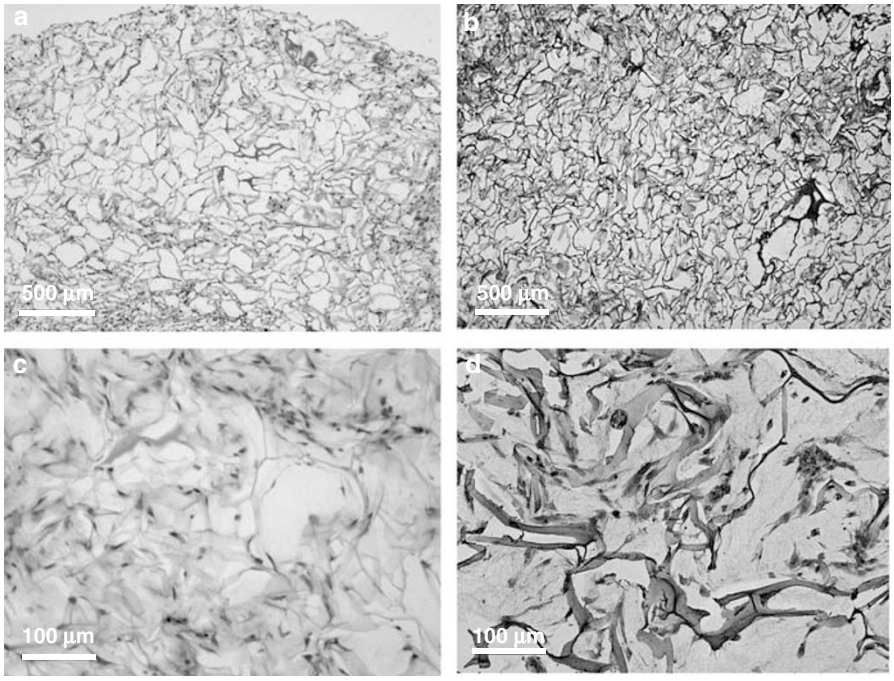

Figure 7 Representative safranin-O stain (red is positive stain) for GAG (**a**, **c**) and immunohistochemical stain (brown is positive stain) for type II collagen (**b**, **d**) from chondrocyte-seeded control and GSCG scaffolds synthesized using method 1 after the 2-week culture period. Both control and GSCG scaffold conditions showed similar histological characteristics. Two million cells per scaffold were seeded onto the scaffolds.

Histology and immunohistochemistry of 3D cultures

Histochemical results for method 1-synthesized GSCG scaffolds and the controls were similar with regard to the amount of tissue in the pores, safranin-O staining, and type II collagen staining (Figure 7). 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 or collagen type II in the synthesized tissue and a significant amount of the scaffold was still present after the 2-week culture period (Figure 7).

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 (Figures 8 and 9). For all groups in this experiment, there were some cells present in the

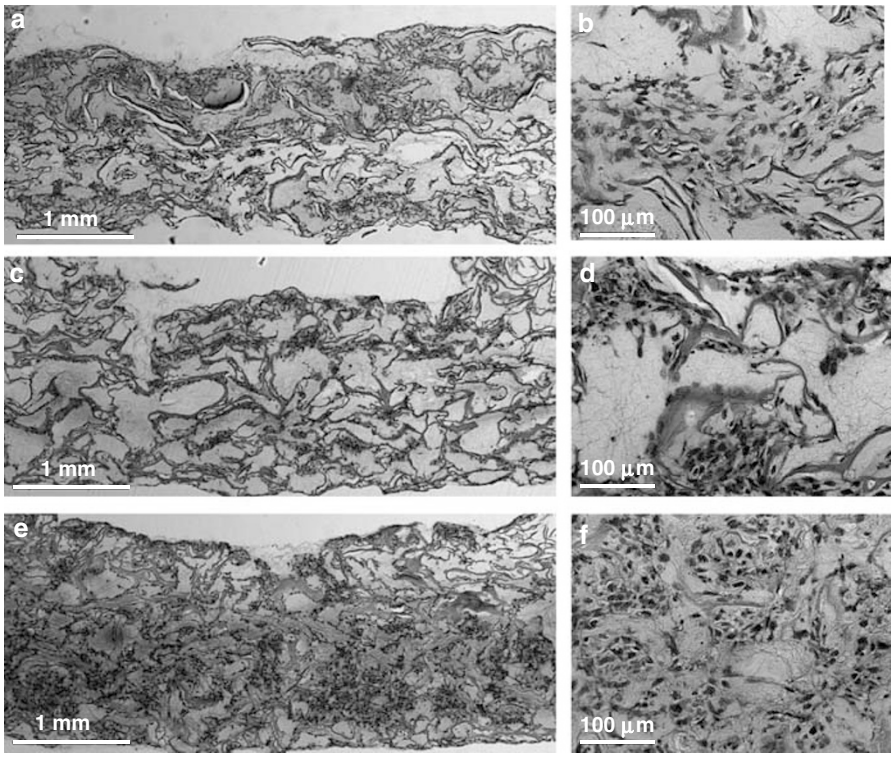

Figure 8 Safranin-O stain for GAG (red is positive stain) of scaffolds synthesized using method 2 for controls (**a**, **b**) and GSCG scaffolds incorporating *IGF-1* plasmid alone (**c**, **d**) or the GP/IGF complexes (**e**, **f**) after 2-weeks in 3D culture. A total of 4 million cells were seeded onto the scaffolds.

constructs that displayed a rounded chondrocytic morphology and were located in lacunae. The x-IGF group showed more tissue, GAG staining, and collagen type II staining compared to the control group (Figure 8c and d; Figure 9c and d). For the control and x-IGF groups, more tissue was present at the periphery of the constructs. The x-GP/IGF group, however, showed the most tissue formation (penetrating through the middle of the construct), number of cells with chondrocyte-like features, safranin-O staining, and presence of type II collagen (Figures 8e and f; 9e and f) compared to the other groups. There was still evidence of the collagen scaffold still present for all groups (stained green in Figure 8) after 2-weeks in 3D culture.

Areas of histological sections that stained for GAG generally seemed to correlate with the areas that stained for type II collagen (Figures 8 and 9), with more intense staining for GAG and type II collagen where more tissue formation was evident. Areas with more tissue present also demonstrated a smaller amount of residual scaffold (indicating a greater degree of scaffold degradation), a higher cell density, and more cells displaying a chondrogenic phenotype (Figures 8 and 9).

Discussion

The current investigation demonstrated the efficacy of a type II collagen-GAG scaffold as a gene delivery vehicle for tissue engineering applications. 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 a growth factor, thus allowing (nonviral) gene transfer to be safely joined to tissue engineering. Previous work using naked plasmid DNA alone incorporated within scaffolds has also demonstrated elevated levels of encoded protein, but using plasmid loads that were significantly higher (on the order of milligram levels per scaffold)[8,14,25,26] compared to the current study. The significantly lower amounts of plasmid needed in the present study to produce elevated and prolonged IGF-1 expression levels may be explained by lipid-mediated transfection along with the controlled delivery of plasmid, enhancing the conditions for gene uptake.

The kinetics of *IGF-1* plasmid released from these scaffolds can be modified using different incorporation methods. Submerging scaffolds in a plasmid solution followed by a freeze-drying step resulted in about 82% of incorporated plasmid released within the first 2 days in buffer. It is likely that this passive release was due to the weak mechanical entrapment of the plasmid among 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 that would only be released upon degradation of the collagen scaffold. Chemically cross-linking the plasmid to the scaffold, on the other

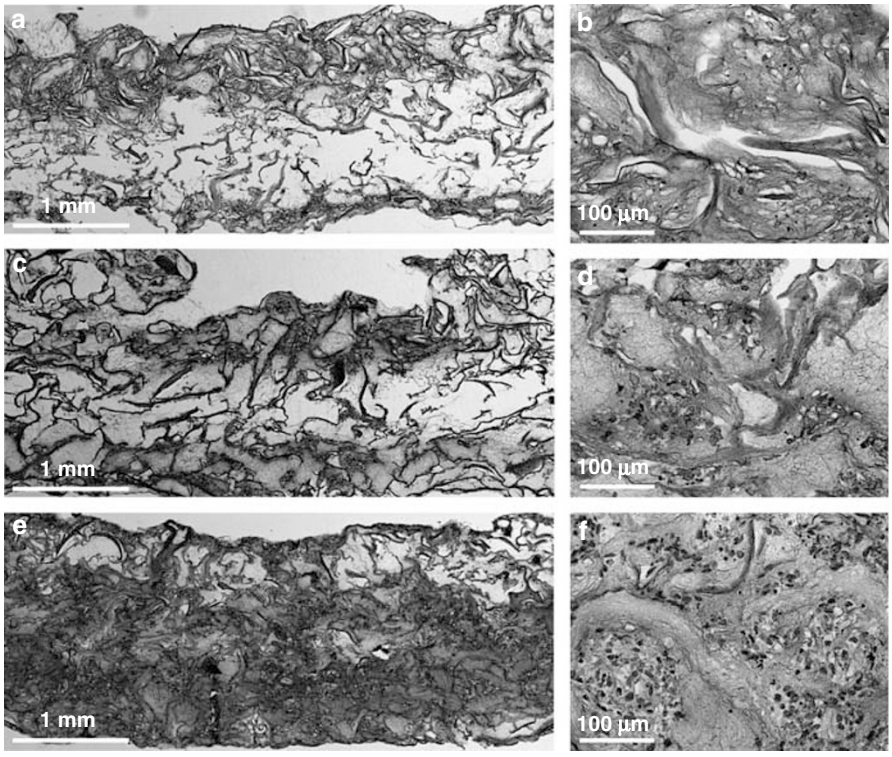

Figure 9 Immunohistochemical stain for collagen type II (brown is positive stain) of scaffolds synthesized using method 2 for controls (**a**, **b**) and GSCG scaffolds incorporating plasmid *IGF-1* alone (**c**, **d**) or the GP/IGF complexes (**e**, **f**) after 2-weeks in 3D culture. A total of 4 million cells were seeded onto the scaffolds.

hand, resulted in 99% retention of incorporated plasmid in the scaffold after the 2-week release 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 commends this method of supplementation for prolonged delivery. In this case, incorporated plasmid would only be released as the scaffold is degraded, and therefore transfection and subsequent gene expression could occur over the duration of scaffold degradation and time course of tissue repair. Of importance was the finding that the *pIGF-1* released in buffer and retained in GSCG constructs was similar to the original plasmid by gel electrophoresis, indicating that plasmid integrity and functionality was not altered by 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 and released by the adult articular chondrocytes. Interestingly, chondrocytes placed in a scaffold without any plasmid supplementation also showed production of IGF-1 which was released in the medium after about 1 week in 3D 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 CG scaffold. For method 1-synthesized scaffolds that released most of the incorporated plasmid 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, the peak and subsequent decline in IGF-1 release to control levels 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 was evidence that the plasmid/transfection reagent complex most likely was no longer present in the scaffold at the end of the culture period.

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. Cross-linking naked plasmid to the collagen scaffold (x-IGF group) allowed for a continued presence and concentration of plasmid as the scaffold was degraded by the seeded cells, which may have increased the probability of cellular plasmid uptake and subsequent gene expression. The addition of the transfection reagent using the cross-linking method of plasmid incorporation further elevated IGF-1 expression levels. The spike in the IGF-1 release rate within the last 2 days of culture and the continued presence of fluorescent cells that were seeded in *GFP*-GSCG scaffolds for up to 2 months in culture, indicated prolonged cell transfection as the scaffold was degraded over time. Although confocal imaging showed only a small percentage of cells that were fluorescent at any one given time point, a high transfection efficiency may not be required to produce therapeutic results locally when using the gene encoding for a functional protein (i.e., IGF-1). 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. It will be of interest in future work to carry out experiments to longer time points to determine the entire chronological profile

of overexpression to the complete degradation of the scaffolds, and to quantify the IGF-1 protein that is retained within the scaffold (in addition to that which is released to the medium) to determine the minimum *local* therapeutic concentration required for enhanced biosynthesis.

Of note is the effect of the plasmid and the lipid transfection reagent on the number of cells (reflected in the DNA measurements) in the scaffolds. Interestingly, there was a lower DNA content for the GP/IGF group in the method-1-prepared scaffolds and an elevated DNA content for the x-GP/IGF group in the method-2-fabricated samples. This result could be attributed to differences in cell attachment and/or differences in cell proliferation and death rates over the course of the 2-week culture. Initial cell attachment may have been affected by the interaction of the cells with the GP/IGF complexes on the scaffold walls. It is speculated that for method 1-fabricated scaffolds, 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, 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. The continued presence of the GP/IGF complexes on the scaffold walls that resulted in increased transfection and IGF-1 release may have also stimulated cell proliferation, since IGF-1 has been shown to have a mitogenic effect on both chondrocytes[27] and human mesenchymal stem cells.[28] Future work (viz, cell attachment and proliferation assays) may be needed to further understand the cellular interactions with these GSCG scaffolds.

It is important to note that the DNA content in digested 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 without cells (data not presented), however, 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 plasmid/transfection reagent complex within the scaffolds was indirectly determined through functional assays (i.e., gene expression of IGF-1 or GFP proteins), which proved that plasmid was present within the cell-seeded 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 DNA detection. Therefore, the majority of the DNA reported for the GSCG groups that incorporated the *IGF-1* plasmid/transfection reagent complex was most likely a reflection of the number of cells present at the end of the 2-week culture period.

The difference in accumulated GAG amounts and histogenesis between method 1 and method 2 synthesized scaffolds may have been due to an insufficient cell-seeding density used in the method 1 experiment (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. Previous studies[29,30] have demonstrated the importance of cell density on biosynthesis and chondrogenesis in 3D culture. Future work needs to investigate if increasing the cell seeding number in GSCG scaffolds supplemented using method 1 will show a more pronounced effect of IGF-1 overexpression on differentiation and the quality of tissue that forms.

Unlike 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. Both of the groups with plasmid supplementation showed an almost twofold increase in accumulated GAG density over controls. Histochemical analysis further revealed the superior outcome in scaffolds that produced an elevated and prolonged IGF-1 overexpression during the full 2-week culture period. Of note in the histological results is the positive association between cell density, degree of scaffold degradation, amount of tissue formation and chondrogenesis. These findings demonstrate that localized IGF-1 overexpression resulting from the transfection of cells seeded within GSCG scaffolds was sufficient enough to significantly enhance chondrogenesis and the production of cartilage matrix molecules *in vitro*.

In conclusion, plasmid release profiles from type II CG scaffolds depend on the method of preparation, with carbodiimide cross-linking of the plasmid to the scaffold resulting in prolonged release. The kinetics of IGF-1 plasmid release has a direct effect on gene expression and IGF-1 release rates from adult canine articular chondrocytes growing in the GSCG scaffolds. 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 cells that are transfected while growing in the GSCG scaffolds (with or without a transfection reagent) results in enhanced biosynthesis and chondrogenesis.

Materials and methods

The first experiments in the present investigation compared the plasmid release kinetics from GSCG scaffolds fabricated using the two methods of incorporation. Owing to the resulting dramatic difference in the plasmid release kinetics, we selected different conditions (viz, plasmid loading and cell seeding density) for tissue culture studies. 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 evaluated GSCG scaffolds incorporating pIGF-1 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 in GSCG scaffolds made using method 2

(cross-linking the plasmid to scaffolds). For the second experiment, the number of cells seeded on to the scaffolds was doubled per scaffold in order to increase the rate of scaffold degradation and facilitate the release of the plasmid which was cross-linked to the collagen fibers.

The goal of the present work was to determine the nonviral transfection that results from selected cases of cell seeding density and plasmid loading for the soaked/freeze-dried and cross-linked scaffolds, and to compare the effects of incorporating a lipid transfection reagent into the scaffolds. The current study was necessary before a comprehensive parametric analysis of the effects of cell seeding density and plasmid loading on the transfection of cells seeded in the two types of scaffolds. The experimental conditions and the evaluation techniques used in this study are outlined in Table 1.

Type II collagen-GAG scaffolds

Porous sheets ($\sim$2 mm thick) of a type II CG scaffold were fabricated by freeze-drying a porcine type II collagen-GAG slurry (Geistlich Biomaterials, Wolhusen, Switzerland). The collagen slurry (1 weight percent collagen) was poured into a stainless steel tray, inserted into the freeze-dryer (VirTis, Gardiner, NY, USA), and frozen using a temperature ramping protocol[31] that reduced the temperature to -40°C over about 65 min. A vacuum of 200 mTorr was then applied while the temperature remained at -40°C. Once the vacuum was achieved the temperature was raised to 0°C. The samples were sublimated for at least 17 h, after which the temperature was raised to 20°C. The collagen sheets were sterilized and cross-linked by dehydrothermal treatment[32] at 105°C and 50 mTorr for 24 h. Disks (8 mm in diameter) were prepared using a dermal biopsy punch. Similar scaffolds have been reported in prior studies to have a porosity of $89 \pm 2\%$ (mean $\pm$ s.d.) and a pore diameter of 125 ± 42 μm.[33]

Plasmid propagation and isolation

Multiplication of *pIGF-1* and enhanced green fluorescent protein (*pEGFP*) was performed 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, USA). The absorption ratio at 260 and 280 nm was used to determine plasmid concentration and purity, whereas plasmid integrity was demonstrated by polyacrylamide gel electrophoresis. The size of *pEGFP* was 4.7 kb, and the size of *pIGF-1* was 5 kb.

Plasmid incorporation into 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 10-min carbodiimide treatment[34] 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, USA) at room temperature. Excess EDAC was removed by rinsing in phosphate-buffered saline (PBS). An aliquot of a diluted *pIGF-1* solution (at pH 8) was then added to each scaffold followed by incubation for a minimum of 1 h 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 physical entrapment of the plasmid.

The second method (method 2) investigated to synthesize GSCG scaffolds involved placing an aliquot of the diluted *pIGF-1* solution onto the DHT-treated scaffolds followed by incubation for an hour at room temperature. An aliquot of the EDAC cross-linking solution described above was then added to the scaffold and incubated for about 30 min to allow chemical cross-links to form among the collagen molecules and between the plasmid and collagen. Excess EDAC was removed by rinsing the scaffolds in PBS. Additional GSCG scaffolds incorporating *pEGFP* were prepared using method 2 to visualize chondrocyte transfection with time.

For cell culture studies, a lipid transfection reagent was included along with the *pIGF-1* for incorporation into additional groups of CG scaffolds using methods 1 and 2, described above. For these groups, the *pIGF-1* solution contained a GP lipid-mediated transfection reagent (Gene Therapy Systems, Inc., San Diego, CA, USA) complexed to the plasmid using a 5:1 (v/w) ratio of GP:plasmid.

The *pIGF-1* load was 20 μg per scaffold for plasmid release studies and 4 or 10 μg for cell culture experiments.

Transmission electron microscopy

Nonseeded samples of GSCG scaffolds prepared using method 2 were allocated for transmission electron

Table 1 Outline of experimental conditions for GSCG scaffold evaluation

Experiment	*Method of preparing GSCG scaffolds*	*Plasmid*	*Loading (μg)*	*Number of cells seeded ($\times 10^6$)*	*GenePorter*	n
Release kinetics/integrity	1 and 2	*IGF-1*	20	–	–	3
Chondrocyte cultures	1	*IGF-1*	4	2	$\pm$	ELISA (3) Biochemical (4) Histochemical (2)
Chondrocyte cultures	2	*IGF-1*	10	4	$\pm$	ELISA (6) Biochemical (4) Histochemical (2-3)
Chondrocyte cultures	2	*EGFP*	10	4	$\pm$	Flourescence (2) Confocal (2)

microscopy (TEM). Samples were primary-fixed in glutaraldehyde, post-fixed in osmium tetroxide, uranyl acetate stained and embedded in Epon. Specimens were then sectioned (60 nm thick) and post stained with uranyl acetate before imaging. TEM images of the ultra-thin sections were obtained on a JEOL 1200EX microscope.

Plasmid release from CG scaffolds and analysis of structural integrity

GSCG scaffolds containing naked *pIGF-1* alone were submerged in Tris ethylenediamine tetraacetic acid buffer (pH 8) to investigate the plasmid release kinetics, comparing the two methods of gene incorporation. At selected time points, buffer was collected from the samples and the wells were replenished with equal volumes of fresh buffer. After 2 weeks, GSCG scaffolds were digested overnight using Proteinase K (Roche Diagnostics, Indianapolis, IN, USA) at 60°C to release the plasmid remaining within the scaffolds. Plasmid released in the buffer and retained in the scaffolds were quantified by the Picogreen assay (Molecular Probes, Inc., Eugene, OR, USA). Aliquots of the released and residual plasmid were analyzed by gel electrophoresis using a 1% agarose gel in Tris-borate-ethylenediamine tetraacetic acid (TBE) buffer with ethidium bromide for comparison with the stock *pIGF-1* used to synthesize the GSCG scaffolds. Samples run on the gel were digested with the restriction enzyme, *Xba*I (New England BioLabs, Ipswich, MA, USA) to assess plasmid integrity. A 1 kb ladder was run in parallel to assess plasmid size.

Chondrocyte isolation and expansion

Chondrocytes were isolated from the trochleae of both knees (stifle joints) from one adult mongrel dog ($\sim$2-year 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, 1 h) and collagenase (200 U/ml, overnight) as previously described.[35] 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, USA): 5 ng/ml of fibroblast growth factor-2 (FGF-2), 10 ng/ml of platelet-derived growth factor-bb (PDGF-bb) and 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.

Nonviral gene transfer in GSCG scaffolds

After GSCG scaffolds were prepared and pre-wet in culture medium, scaffolds were placed briefly onto sterile filter paper, and then placed on agarose-coated 12-well tissue culture plates. 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-min 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 a defined serum-free medium (SFM), found in previous work[36] to enhance differentiation. The SFM 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, insulin–transferrin–selenium (ITS)$^{+1}$ ($\times$100, Sigma Chemical, St Louis, MO, USA), 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 every 2–3 days over a 2-week culture period.

The amount of IGF-1 in the collected medium ($n = 3$–6) from the cultures of the cell-seeded scaffolds was detected by a sandwich ELISA kit for the human IGF-1 protein (R&D Systems). The IGF-1 values were reported as rates of release by dividing by the time period since the last medium 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 freeze-dried and enzymatically digested using proteinase K (Roche Diagnostics, Indianapolis, IN, USA).

Confocal and fluorescence microscopy of cell-seeded scaffolds

Chondrocytes cultured within scaffolds incorporating *pEGFP* were observed under fluorescence and confocal microscopy to visualize chondrocyte transfection over time. At various time points, constructs were placed on a sterile glass bottom Petri dish for observation under the fluorescence or confocal microscope. Samples were then returned to tissue culture wells containing medium for further culture.

DNA analysis

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

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[37] ($n = 4$). 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. Although newly accumulated GAG was determined by subtracting the unseeded scaffold values from the seeded sample values, the GAG amount for the unseeded, non-degraded scaffolds barely showed a reading and did not significantly change the relative amounts of accumulated GAG for the experimental conditions.

The GAG content was normalized to the estimated volume of the cell-seeded scaffolds; reported as the GAG density. The accumulated GAG was normalized to the volume of the scaffold to reflect the differences in scaffold size at the end of the culture period, and this coincided well with the histological results (i.e., the higher the GAG density, the more intense the safranin-O stain for GAGs). The volume was assessed by measuring the scaffold diameter using a template, whereas the thickness for all scaffolds was about 2 mm (the largest dimensional change throughout the culture period was the scaffold diameter). The data were not normalized to DNA content because any plasmid DNA left in the GSCG scaffolds incorporating naked plasmid DNA alone, might have contributed to the amount of DNA in the scaffolds. Therefore, using DNA content for normalization might not give an accurate measure of GAG produced relative to cell number (or genomic DNA). The weight of the scaffolds was very difficult to measure accurately and therefore was not used for normalization.

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 sectioned (6-μm 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 min and stained with a standard avidin–biotin complex peroxidase-based antibody staining technique (Vectastain, Vector Laboratories, Burlingame, CA, USA). Mouse anti-chick monoclonal antibody for type II collagen was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA).

Statistical analysis

Data were analyzed by one- or two-factor ANOVA, and the Fisher's protected least-squares differences (PLSD) *post hoc* test using StatView (SAS Institute Inc., Cary, NC, USA). Data are presented as mean $\pm$ s.e.m.

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