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Factors influencing the long-term behavior of extracellular matrix-derived scaffolds for musculoskeletal soft tissue repair

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Abstract

Musculoskeletal connective tissues such as tendon, ligament, and cartilage possess a limited ability for self-repair. Tissue engineering seeks to use combinations of cells, bioactive molecules, and biomaterials to develop new treatment options for the repair or replacement of damaged tissues. The use of native extracellular matrix as scaffold material for tissue engineering has become increasingly attractive as such tissues can not only provide structural support, but also regulate cell behavior. While demineralized bone matrix has long been recognized for its osteoinductive abilities, recent studies have identified the ability of cartilage and tendon extracellular matrices to stimulate the differentiation of mesenchymal or adipose-derived adult stem cells toward chondrogenic or tenogenic lineages respectively. This review discusses the motivation for fabricating scaffolds from musculoskeletal tissues, the *in vitro* and *in vivo* efficacy of these tissue-derived scaffolds, and various processing techniques such as decellularization or crosslinking that can mitigate immunogenic responses, moderate degradation profile, and enhance the mechanical properties of these constructs following long-term implantation *in vivo*.

Keywords

articular cartilage; tendon; ligament; decellularized tissue; decellularization; tissue engineering; mesenchymal stem cell; adipose stem cell; MSC; ASC

Introduction

Tissue engineering combines cells, growth factors, and biomaterials to generate new tissue *in vitro* for implantation or to stimulate *in vivo* regeneration.^{1, 2} Biomaterials play a prominent role in musculoskeletal tissue engineering because they can provide a template for defining the geometric shape of the tissue-engineered construct, offer a substrate for cell adhesion, organize and orient new matrix deposition, and support physiological loads until cells synthesize a mechanically functional tissue.^{1–7} Further, scaffolds can provide three-dimensional, environmental cues that can dictate cell attachment, morphology, migration, proliferation, differentiation, and the orientation of matrix assembly.^{7–13} Scaffold composition governs its mechanical properties, degradation rate and byproducts, ability to influence cell behavior, and biocompatibility.^{14–17} Synthetic scaffolds are attractive because they are generated from cheap, plentiful raw materials with well-characterized physical and chemical properties.^{18, 19} These materials are amenable to a variety of processing techniques, which allows precise control over their mechanical²⁰ and degradation²¹

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properties. Despite these advantages, cells do not necessarily express receptors for these materials; hence they possess a limited ability to modulate cell behavior.¹⁹

In this regard, the extracellular matrix (ECM) of native tissues present an attractive material option for tissue engineering, due to their ability to support cell viability and metabolism, as well as perform physiological function.^{22–25} The exceptional performance of ECMs in their native role led to their investigation as scaffold material and the discovery of their potent bioinductive properties.^{22–25} For example, the field of bone repair was revolutionized by the discovery by Marshall Urist that demineralized bone matrix (DBM) contains osteoinductive and osteogenic factors, termed “bone morphogenetic proteins”, that can greatly enhance bone repair and regeneration.²⁶ More recently, it has been shown that porcine small intestinal submucosa (SIS) similarly contains growth factors and morphogens within its extracellular matrix (ECM), and these factors can induce a variety of cellular responses that enhance tissue repair.²²

In this paper, we review recent advances on the use of native ECM from two other tissues – cartilage and tendon – as potential scaffold materials for tissue engineering. These studies have shown that such ECM-based materials can have a significant influence on cell responses, including proliferation, differentiation, gene and protein expression, and matrix accumulation. Furthermore, modification of ECM-based scaffolds using composite materials, chemical crosslinking, or other methods can enhance the functional properties while increasing the potential for long-term success.

Extracellular matrix-derived scaffolds for cartilage repair

Articular cartilage is the connective tissue that lines the surfaces of diarthrodial joints. Under normal physiological conditions, it is able to withstand decades of cyclic loading; however, once damaged it has a very limited ability to self-repair.²⁷ A variety of surgical approaches including arthroscopic debridement, microfracture, and autologous grafting are used clinically to enhance cartilage repair, but have shown little long-term success.^{28, 29} Cell-based approaches, such as autologous chondrocyte implantation, hold significant promise for promoting tissue regeneration, but still have not shown improved efficacy over standard surgical methods such as microfracture.³⁰

In this regard, several approaches to cartilage tissue engineering have examined the potential for scaffolds based on cartilage ECM components to provide the functional requirements of chondrogenic induction and the physiologic mechanical properties of native cartilage. For example, several studies have examined the use of purified ECM components such as type II collagen, chondroitin sulfate, and hyaluronan on chondrogenesis.^{31–34} Chondrocytes embedded in tri-copolymers of these components have demonstrated repair using an *in vivo* porcine osteochondral defect models.³¹ Further, exogenous delivery of these matrix constituents *in vitro* generates dose-dependent upregulation of cartilage specific gene and protein expression.³² Lyophilized, collagen-glycosaminoglycan (GAG) scaffolds provide two major components of the cartilage ECM; however, they do not recapitulate the complexity of the native composition. In addition to these matrix constituents, cartilage ECM contains collagens III, VI, IX, X, XI, XII, and XIV,³⁵ small leucine rich-proteins including decorin, fibromodulin, lumican, and biglycan,^{36, 37} cartilage oligomeric matrix protein,³⁸ anchorin,³⁸ fibronectin,³⁸ matrilins 1 and 3,³⁹ as well as growth factors such as transforming growth factor β (TGF- β), insulin-like growth factor-1, and bone morphogenic protein-2.⁴⁰ While these elements comprise only a small fraction of cartilage ECM, they play a crucial role in organizing type II collagen fibrils and mediating cell-matrix interactions due to the multiple adhesive domains they possess for cells and matrix constituents.^{35, 36, 38, 39} In addition to pericellular matrix components such as type VI

collagen, perlecan, laminin, and nidogen,⁴¹ cartilage also contains “matrikines”, peptides that arise from the proteolytic degradation of extracellular matrix macromolecules, which have been shown to influence cellular proliferation, migration, apoptosis, matrix synthesis and degradation.^{42, 43} Thus, many structural components of cartilage have the ability directly modify chondrocyte metabolism and response to extracellular stimuli.

Encapsulating the intricate complexity of cartilage composition has been shown to be a critical factor in promoting stem cell differentiation down a chondrogenic lineage.⁴⁴ Studies comparing the chondrogenic potency of collagen alone to more complex ECM formulations found that only compositions containing multiple ECM constituents stimulated GAG synthesis in embryonic stem cells.⁴⁴ Furthermore, functionalizing culture surfaces with cartilage ECM extract prevented dedifferentiation of the chondrocyte phenotype better than coating with type I collagen.⁴⁵ Artificial surfaces functionalized with cartilage ECM extract exhibited increased type II collagen and GAG production and decreased levels of type I and X collagen gene expression.⁴⁵ These findings coupled with the success of other tissue-derived constructs have prompted the investigation of cartilage ECM as a scaffold material.^{46, 47}

Regardless of material, scaffolds for cartilage tissue engineering must facilitate cellular infiltration, stimulate chondrogenic differentiation, minimize host-inflammatory responses *in vivo*, and support physiologic loads.^{1, 14} However, native cartilage has a very dense ECM characterized by an effective pore size of only a few nanometers, which presents a major challenge for the removal of native chondrocytes and infiltration with new cells.^{48–51} Homogenization and pulverization processing techniques physically break cartilage into fine particles. These cartilage particles can be suspended in water, frozen, and lyophilized to form scaffolds with a high degree of porosity and interconnectivity, which expedite cellular infiltration and provide a large surface area for cell attachment.⁴³ Cryosectioning has also been used to enable cellular infiltration into the dense cartilage ECM.^{40, 52} Cryosectioned cartilage slices have been seeded with cells and stacked to form a 3-D construct with uniform cell distribution.^{40, 52} Additionally, homogenized cartilage fragments have been solubilized in hexafluoro-2-propanol and poly(ϵ -caprolactone) (PCL), which were then electrospun to form porous, nano-fiber scaffolds containing cartilage ECM within each fiber.⁵³ These electrospun scaffolds possess a pore size that permits cellular infiltration and chondrogenic differentiation. Fabricating scaffolds from dense cartilage ECM requires processing techniques that increase the effective pore size in order to facilitate uniform cell infiltration. Constructs synthesized from cartilage ECM processed in any manner will be referred to as cartilage-derived matrix (CDM) scaffolds.

The most salient quality of CDM is its ability to stimulate chondrogenic differentiation of mesenchymal stem cells and foster the native phenotype of chondrocytes *in vitro*, as well as promote cartilage repair *in vivo*. Several *in vitro* studies have utilized gene expression, biochemical assays, immunohistochemistry, and mechanical testing to demonstrate the chondrogenic potential of CDM.^{43, 54, 55} These studies revealed that CDM scaffolds support cell proliferation, upregulate cartilage-specific gene and protein expression, stimulate cartilaginous matrix synthesis, and increase in stiffness throughout *in vitro* culture.^{43, 54, 55} CDM constructs did not require exogenous growth factor supplementation in order to elicit chondrogenic differentiation of adipose-derived stem cells (ASCs, Figure 1).⁴³ Additionally, CDM scaffolds prompted porcine and human chondrocytes to synthesize cartilaginous proteins without exogenous growth factors.⁵⁴ While CDM alone promotes cartilage tissue formation, perhaps more interesting is the synergistic effect that occurs in the presence of chondrogenic growth factors. Across various chondrogenic growth factor cocktails, CDM scaffolds yielded enhanced matrix production from ASCs or bone marrow-derived stem cells (MSCs) compared to alginate beads.⁵⁵ Furthermore, CDM prevented differentiating MSCs from progressing towards a hypertrophic phenotype, which is typically seen with

TGF- β 3 supplementation.⁵⁵ In conjunction with these promising *in vitro* results, a variety of studies have evinced the chondrogenic capacity of CDM constructs *in vivo*^{40, 50, 52, 56} as well as their ability to promote cartilage repair in osteochondral defect models.⁵¹ Decellularized CDM porous scaffolds⁵⁰, acellular cartilage sheets^{40, 52}, and osteoarthritic cartilage fragments⁵⁶ have been seeded with MSCs^{40, 50, 56} and chondrocytes⁵² and implanted subcutaneously in nude mice. *In vivo*, these CDM constructs induced chondrogenesis and stimulated the production of cartilaginous matrix staining positive for cartilage-specific proteins. In rabbit⁵¹ and canine⁵⁷ osteochondral defect models, MSC-seeded, decellularized CDM scaffolds improved cartilage repair as evidenced by mechanical, biochemical, histological, and immunohistochemical evaluation. While the mechanisms by which CDM promotes chondrogenesis remain to be elucidated, cell-matrix interactions,⁴³ the release of entrapped growth factors,⁴⁰ and the presence of matrikines⁴³ are likely to play a prominent role.

CDM derived from an allogeneic or xenogeneic source faces concerns of acute and chronic immune-mediated rejection; decellularization may be necessary for alleviating potential inflammatory reactions upon implantation.^{49, 58} Complete cell removal is paramount to the long-term efficacy of CDM scaffolds because exogenous cellular fragments have been implicated as the primary stimulus of immunogenic responses.^{50–52} In order to mitigate potential immunogenic reactions towards CDM, various studies have analyzed a variety of decellularization strategies.^{49–52, 58, 59} Decellularization begins with physical processing techniques to break up the dense cartilage ECM and provide access to the embedded chondrocytes. Pulverization can reduce cartilage ECM into a fine powder, which facilitates decellularization approaches by minimizing the diffusion distance of chemical reagents.^{50, 51, 57} Cryosectioning cartilage into 10 μ m slices has also been used to facilitate the removal of cellular components.^{40, 52} Additionally, freeze-thaw cycles have utilized ice crystal formation to loosen the dense ECM.⁴⁹ Hypo-osmotic solutions and non-ionic detergents (TritonX-100) result in cell lysis and solubilization of cell membranes, respectively.^{49–51} Protease inhibitors such as phenylmethylsulfonyl fluoride and ethylenediaminetetraacetic acid (EDTA) prevent autolysis, and nucleases remove DNA and RNA.^{49, 50} Other approaches have limited reagent use to sodium dodecyl sulfate, which removes cellular content by solubilizing membranes.^{40, 52, 59} The overarching goal of decellularization protocols is to deplete cellular debris that is responsible for immunogenicity, while retaining the native composition of the ECM, which is crucial for maintaining the chondroinductive properties of CDM.^{50, 59} Decellularizing cartilage ECM with harsh reagents for prolonged periods of time results in the loss of GAGs, denaturation of proteins, and attenuation of mechanical properties.^{49, 58}

However, removal of cellular contents does not necessarily eliminate immunological concern over the use of CDM harvested from bovine and porcine tissues, which possess an inflammatory galactosyl- α (1,3)galactose (α -gal) epitope.^{60–62} The presence of anti-Gal antibodies in human serum mediates acute and chronic rejection of xenografts from bovine and porcine sources.⁶³ Further, human serum contains anti-non gal antibodies against multiple xenogeneic antigens, which may result in rejection of even those xenografts lacking the α -gal epitope.⁶⁴ Efforts have sought to eliminate the α -gal epitope in porcine-derived cartilage via treatment with α -galactosidase⁶² or sodium hydroxide, guanidine hydrochloride, and sodium acetate.⁵⁸ These treatment conditions successfully eliminated detection of the α -gal epitope via enzyme-linked immunosorbent assay. Unfortunately, these treatment conditions completely removed non-collagenous ECM components, such as GAGs, and significantly increased the fraction of denatured collagen, which substantially decreased the linear modulus of the treated cartilage.⁵⁸ Thus strategies to remove the α -gal epitope from cartilage must be optimized, such that the native, chondroinductive composition is not detrimentally changed. Decellularization of CDM has been shown to

prevent inflammatory reactions in a variety of animal models.^{7, 50–52, 57} A subcutaneous mouse model using $\alpha 1,3$ -galactosyltransferase knock out mice has been used to simulate human immunological responses towards implanted CDM constructs.⁴⁹ Reactions towards untreated CDM scaffolds exhibited typical foreign body responses while decellularized constructs underwent infiltration by macrophages and progenitor cells characteristic of tissue remodeling.⁴⁹ Interestingly, other studies have forgone cell-removal processing and did not report any immune responses towards implanted CDM.^{56, 65}

Degradation rate is another critical factor influencing the long-term efficacy of implanted CDM constructs. Scaffold clearance is desired because the inability of cells to dynamically remodel an implant leads to fibrotic encapsulation of the construct, which inhibits integration with host tissue and attenuates mechanical properties.²⁵ The natural composition of CDM makes it amenable to enzymatic degradation via matrix metalloproteinases. Clearance through native pathways enhances construct biocompatibility and obviates the generation of cytotoxic by products. However, enzymatic activity may be patient-specific, which makes *in vivo* degradation rates difficult to predict.⁶⁶ Some studies have found complete resorption of CDM scaffolds as early as 28 days after implantation,⁶⁵ while other groups observed remnants of CDM as late as 12 weeks post-surgery.⁵² The disparity of these results arises from differences in fabrication techniques, crosslinking treatments, implant location, and species of animal model. Hence, a variety of factors contribute to the degradation rate of CDM scaffolds, and must be tailored to balance the deterioration of the scaffold with new tissue formation. Matching the degradation profile to the rate of matrix synthesis is crucial for maintaining the mechanical integrity of the tissue-engineered construct throughout the regeneration process.

Since cartilage is exposed to large compressive loads under physiologic conditions, replacement constructs must have sufficient mechanical properties to sustain loading upon implantation. While processing techniques to increase porosity and surface area enhance cellular infiltration, they concomitantly diminish the mechanical properties of the cartilage ECM.^{43, 51} CDM scaffolds have a relatively low stiffness as compared to native cartilage, and thus may exhibit changes in shape and porosity after cell seeding as a result of cell-mediated contractions.^{43, 54, 55} Researchers have implemented a variety of approaches to enhance scaffold structural integrity. For example, increased concentrations of CDM result in a denser matrix.⁵⁴ Additionally, collagen crosslinking treatments such as dehydrothermal treatment, UV irradiation, and chemical reagents have also been shown to mitigate cell-mediated contraction.^{7, 50, 51, 57} While these treatments do increase mechanical stiffness, they also modulate cell-matrix interactions, which can negatively affect the chondroinductive properties of CDM.⁶⁷ Perhaps the most promising method of recapitulating the physiologic mechanical properties of native cartilage is through the combination of CDM with synthetic scaffolds to generate multifunctional hybrid implants.⁶⁸ At the macroscale, these hybrid scaffolds mimic the anisotropic, nonlinear, and viscoelastic properties of cartilage through the use of a woven polymer fiber scaffold.²⁰ and provide bioinductive signals through the incorporation of CDM around the synthetic fibers.⁶⁸ At the nanoscale, CDM can be incorporated into electrospun fibers to increase the chondrogenic potential of electrospun scaffolds compared to nanofibrous scaffolds composed of synthetic polymer alone.⁵³

In summary, a growing body of research has shown that cartilage ECM induces stem cell chondrogenesis without exogenous growth factors, and accentuates matrix production and construct mechanical properties. *In vivo* studies demonstrate that removal of cellular components via established protocols is sufficient to prevent inflammatory reaction towards the implantation of CDM constructs. The low initial modulus of CDM represents one potential shortcoming for loading-bearing applications; however, formation of composite

scaffolds using combinations of CDM and synthetic materials such as textiles could circumvent this limitation. Overall, these preliminary studies demonstrate the biomimetic properties of CDM and suggest that it would not produce detrimental long-term effects *in vivo*, although this requires further evaluation.

Extracellular matrix-derived scaffolds for tendon or ligament repair

Tendon and ligament autografts, allografts and xenografts have been widely used for reconstruction or augmentation of tendon or ligament repair and frequently result in good clinical and functional results. However each of these materials has distinct disadvantages; autograft harvest causes problems associated with donor site morbidity.^{69, 70} Allograft tendons have been associated with a 3% contamination rate,⁷¹ slower revascularization compared to autografts,^{72–76} and may require in excess of three years for complete recellularization and remodeling.⁷⁷ Xenografts may induce production of both anti-galactosidase (gal) and anti-non-gal antibodies, which are responsible for both acute and chronic immune-mediated implant rejection although the relative contribution of each of these antibodies, and the ultimate functional or clinical outcome may depend on implantation site.^{64, 78} Xenografts and allografts elicit different host responses based on species and tissue, post-harvest treatments, *in vivo* degradation rate, and presence of residual cellular components.^{25, 78–83} Furthermore, material and suture retention properties may be limited by initial architecture, tissue type, method of application, degree of tissue regeneration and degradation rate of the graft *in vivo*.⁷⁸ Many of the currently available graft materials have at least one of these limitations but nonetheless these extracellular matrix based grafts have many beneficial biomimetic properties, similar to those described earlier for CDM. Decellularization of xeno- or allograft tendon or ligament has been used as a strategy to reduce immunogenicity, while maintaining biomimetic properties, but decellularization may reduce mechanical properties of the graft and impair subsequent cellular repopulation and maintenance of cell viability deep within the graft.^{84–87} These factors have been the major driving force for the development of porous tendon or ligament-derived matrices within the field of tendon tissue engineering.

As an alternative approach for improving cellular repopulation compared to use of intact decellularized tendon xenografts, tendons have been harvested, decellularized, sliced into 50µm-thick slices, re-seeded with bone marrow-derived mesenchymal stem cells then bundled together as a single construct.^{88, 89} Interestingly, bone marrow-derived mesenchymal stem cells seeded in these constructs exhibited a tendon phenotype and aligned along collagen fibrils within the decellularized slices.^{88, 89} Tendon slices less than 30 µm thick had reduced ultimate tensile strength and Young's modulus compared to thicker slices, probably due to disruption of tendon fascicles. However, while bone marrow-derived mesenchymal stem cells remained viable for two weeks *in vitro* sandwiched between two 300 µm thick slices, cellular repopulation within the slices themselves appeared to be limited.⁹⁰ It has been suggested that tendon or ligament extracellular matrix is a logical choice for cell-mediated tendon repair.²⁴, and others have investigated the effect of powdered, film or gel tendon or ligament derived extracellular matrix on multipotent cell differentiation.^{91, 92} Human ASCs demonstrated enhanced novel type I and III collagen synthesis and proliferation when cultured with powdered porcine ligament-derived matrix in a rat type I collagen gel compared to rat type I collagen gel alone.⁹¹ (Figure 2). Interestingly, in this study treatment of the ligament-derived matrix with 0.1% peracetic acid (PAA) to disinfect and decellularize the xenograft material did not reduce DNA content compared to treatment with phosphate-buffered saline (PBS) and pulverizing under liquid nitrogen alone, although DNA-release into the media was enhanced in unseeded scaffolds treated with PAA compared to PBS treated scaffolds, emphasizing the need for continued study in this area. Compared to tissue culture plastic, tendon stem cells cultured on a processed tendon matrix

film demonstrated up-regulation of several tendon-related genes, and demonstrated increased ability to differentiate along adipogenic, chondrogenic and osteogenic pathways.⁹² Further, after subcutaneous implantation into nude rats, only tendon stem cells injected with a tendon matrix gel demonstrated type I collagen synthesis; tendon stem cells injected alone did not produce type I collagen. When similar treatments were applied to patellar tendon, tendon matrix gel supported improved repair of the tendon compared to tendon stem cells alone.⁹² Powdered, gel and film formulations of tendon matrix have limited *in vivo* application despite their ability to induce tendon-like tissue formation and support cellular proliferation because they have no clinically relevant mechanical properties under physiological tensile loading. In order to address this problem, we have recently produced electrospun nanofibrous scaffolds composed entirely of tendon-derived matrix, or a tendon-derived matrix – polymer hybrid on which human ASCs demonstrated rapid proliferation and infiltration, matrix synthesis and up-regulation of tendon-related gene expression (unpublished data).

The predominant potential long-term beneficial effects of porous tendon or ligament matrices are improved cellular repopulation, proliferation and extracellular matrix synthesis compared to currently available tendon grafts. However, powdered, gel or film formulations of these porous scaffolds have poor tensile properties that may limit their clinical application. These materials are able to retain their beneficial, biomimetic properties despite extensive processing, supporting approaches that incorporate ECM components into synthetic scaffolds for tendon augmentation or interposition. Composite scaffolds can be tailored to mimic the mechanical properties of the target tissues more closely than many currently available grafts. Future studies will reveal whether these porous tendon matrices can regenerate new tissue that recapitulates the biological and mechanical properties of native tendons and ligaments, particularly at the interface between the tissue-engineered construct and the host tissue. Slicing or pulverizing donor tissue dramatically increases the surface area of these tendon- or ligament-derived matrices. This increased surface area is expected to enhance cellular attachment and infiltration. However there is also potential for increased exposure of infiltrating cells to both anti-gal and anti-non-gal epitopes for xenograft materials, and to allogeneic DNA, RNA and infectious agents for allograft materials. Processing of these porous matrices should be evaluated at an early design stage to reduce these possibilities. The increased surface area of the porous matrix is also likely to accelerate the proteolytic degradation of these porous matrices compared to intact tendon or ligament grafts. The host immune response to these porous matrices is likely to be dependent on particle size and overall scaffold stability *in vivo*, particularly in synovial environments. Porous tendon- or ligament- matrices release substantial amounts of glycosaminoglycans and collagen into cell culture media,⁹¹ and the potential effect of any similar *in vivo* release on the host response to these novel processed epitopes, particularly within the synovium is unknown and should be thoroughly investigated during scaffold development. In summary porous tendon- or ligament-derived matrices may bestow many beneficial properties on the tissue engineered tendon or ligament, but thorough evaluation of their potential interactions with the host synovial environment is critical during the design phase.

Conclusions

ECM-derived scaffolds show significant promise for enhancing the repair of soft connective tissues such as cartilage, tendon, or ligament by providing biocompatible and biodegradable materials that are capable of influencing cell proliferation and differentiation. Reconstitution of native ECMs as a slurry or powder allows a variety scaffold shapes to be created, including porous materials, gels, or fiber-reinforced composites that have macroscopic mechanical properties similar to those of native tissues.

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Figure 1.

Immunohistochemistry and histology of CDM constructs. At day 0, unseeded CDM scaffolds (blank) stained positive for type II collagen and chondroitin-4-sulfate, but did not contain type I and X collagens. At day 28 and 42 time points, D28 and D42 respectively, ASC-seeded CDM constructs synthesized cartilaginous matrix staining positive for type II collagen and GAGs, with minimal type I collagen content and no type X collagen. Positive controls are porcine osteochondral sections for immunohistochemistry and porcine cartilage for histology. Scale bars = 200 μ m. Reproduced with permission from Cheng et al.⁴³

Figure 2.

Positive (+) and Negative (-) control tendon, unseeded ligament-derived matrix (LDM) treated with peracetic acid (PAA) or phosphate buffered saline (PBS) constructs, or constructs prepared with PAA, PBS or type I rat tail collagen gel alone (COL) and seeded with 1x10⁶ human ASCs per ml and stained for human Type I collagen (green) (A) or human Type III collagen (green) (B). Cell-seeded constructs showed significant labeling for Type I collagen throughout the construct, whereas Type III collagen was located peripherally (5 μm sections, 100x magnification, scale bar = 100 μm). Reproduced with permission from Little et al.⁹¹