Many regenerative medicine studies have successfully engineered tissues *in vitro* with combinations of progenitor cells and biomaterials. However, these approaches typically require elaborate, laborious, and expensive procedures for cell isolation, expansion, and *in vitro* cell conditioning for proper cell differentiation and tissue formation. A primary challenge has been the translation of these promising preclinical studies into methods that are not only straightforward to practice but also effectively direct cell differentiation and tissue formation that recapitulates the complexity of natural tissues. Technologies that capitalize on advances in cell engineering but minimize *in vitro* cell manipulation can greatly enhance the promise of regenerative medicine.

Our ***long-term goal*** is to develop strategies for the *in situ* regeneration of complex musculoskeletal tissues that lead to normal physiological structure, function, remodeling, and integration with surrounding native tissue. Towards this goal, we have demonstrated the directed differentiation of adult mesenchymal stem cells (MSCs) into osteoblastic and chondrocytic phenotypes by genetic engineering with a variety of morphogenetic factors. We have also engineered anisotropic 3D composite scaffolds by microscale weaving of multi-filament yarns that recapitulate the complex mechanical properties of native cartilage. Finally, by combining these two approaches, we have developed a novel method for highly efficient and spatially-defined gene delivery by viral gene carriers immobilized to a highly structured 3D biomaterial scaffold. The ***rationale*** for the proposed work is that biomaterial-directed differentiation of progenitor cells will minimize the need for extensive cell conditioning and *ex vivo* manipulation. Additionally, spatially-defined functionalization of 3D-woven scaffolds with gene delivery vehicles encoding inducible morphogenetic factors can provide regenerative cues to progenitor cells in a spatiotemporally controlled manner. This strategy facilitates the engineering of complex tissues with precise control over the 3D organization of progenitor cell differentiation and tissue development without the need for culturing multiple cell types or using expensive growth factors. Functionalization of scaffolds with gene carriers also provides a novel method to direct the activity of endogenous progenitor cells that infiltrate the construct in microfracture-based cartilage regeneration. The ***overall objective*** of this research proposal is to use spatiotemporally regulated gene expression of differentiation factors from a woven polymer scaffold to engineer inhomogeneous and anisotropic osteochondral tissues *in vivo* and enhance the quality of tissue regeneration following microfracture surgery. The ***central hypothesis*** is that preferential gene delivery of differentiation factors from specific regions of the woven scaffold will induce controlled tissue development *in vitro* and *in vivo* that replicates the transition zones of natural tissues. We will pursue the following aims:

Aim 1: Engineer osteochondral tissues via spatially controlled gene delivery of differentiation factors

Specific fibers of the 3D-woven PCL scaffolds will be coated with poly-L-lysine (PLL) that binds lentiviral particles. Following weaving, doxycycline (dox)-inducible lentiviral vectors encoding osteogenic and chondrogenic factors will be loaded onto the scaffolds containing PLL-modified yarns in specified layers. These constructs will be seeded with human MSCs and cultured in optimized media conditions that support both chondrogenesis and osteogenesis. Our preliminary results show that this approach leads to localized MSC differentiation that is highly spatially-defined. Organized cartilaginous and mineralized tissue development determined by the gene immobilization geometry and dynamics of dox induction will be evaluated by microCT, histology, and immunohistochemistry. Autocrine transcription factors, including Runx2 and Sox9, and autocrine/paracrine factors, including BMP-2 and TGF-β3, will be tested to evaluate the effects of secretion and diffusion of signaling molecules on spatially organized tissue formation.

Aim 2: Enhance the mechanical and biochemical quality of cartilage regeneration* in vivo *by spatially defined material-mediated gene delivery and temporally controlled gene expression

Lentivirus-functionalized PCL scaffolds will be implanted into a rabbit full-thickness osteochondral defect model of microfracture surgery. It is expected that the differentiation of endogenous progenitor cells that populate the scaffold will be guided by morphogenetic factors encoded by the lentiviral vectors. We will test the histologic quality of tissue regeneration guided by empty scaffolds, scaffolds functionalized uniformly with lentivirus encoding chondrogenic factors, and scaffolds preferentially functionalized with chondrogenic factors at the joint surface or osteogenic factors at the bone-cartilage interface. Dox-inducible vectors will be used to determine the role of duration of transgene expression on the quality of tissue regeneration *in vivo*.

This approach is ***innovative*** because it incorporates unique scaffold fabrication methods and biomaterial-mediated gene delivery to engineer organized tissues and direct regeneration with a degree of spatiotemporal precision that is not achievable by current methods. This work is ***significant*** in that it provides a means of biologically directing osteochondral repair without extensive cell manipulation *in vitro*, leading to the regeneration of complex tissue substitutes that mimic their native counterparts and integrate with surrounding tissues. Ultimately, we envision this approach leading to a more feasible strategy for the engineering of osteochondral tissues *in situ*.

A. SIGNIFICANCE

A1. Limitations of standard of care for cartilage degeneration. The treatment of injuries or diseases affecting articular cartilage poses important unmet challenges to the medical community. Damage to articular cartilage is a significant clinical problem with over 50% of orthopaedic injuries involving cartilage in synovial joints. Furthermore, osteoarthritis (OA) is a prevalent degenerative joint disease characterized by dysfunction of articular chondrocytes and articular cartilage degradation that occurs frequently secondary to trauma and other factors.[1] The economic burden associated with this disease costs the U.S. public health system more than 40 billion dollars annually.[2] Surgeons have few available options for the repair of acute cartilage injury. Current treatment options include joint lavage, tissue debridement, abrasion arthroplasty, the transplantation of autologous or allogeneic osteochondral grafts, or commonly the microfracture of the subchondral bone.[3-14] Microfracture entails penetration of the subchondral bone to induce flow of the bone marrow, and presumably stem cells, into the tissue defect to form a clot which will hopefully remodel into cartilage. Although this procedure yields initial clinical success with improvement noted up to 24 months after surgery, the outcome deteriorates after the initial postoperative period.[15-20] In particular, microfracture results in the formation of fibrocartilage, rather than hyaline cartilage which is necessary to maintain long-term cartilage function.

Autologous chondrocyte implantation has also experienced early success, though nearly 40% of all patients undergo revision operations due to graft hypertrophy and graft delamination, among other complications.[21] Additionally, the harvest of autologous cartilage for the cell implantation procedure carries significant iatrogenic risk for the patient. The harvest of even non-load-bearing cartilage is associated with the onset of articular cartilage degeneration and osteoarthritic changes in the joint.[22] Therefore current procedures have yielded promising initial clinical results, but have long-term limitations due to the formation of fibrous tissue, apoptosis, and further cartilage degeneration.[23-25] Many of these limitations can be attributed to the lack of two specific components for tissue regeneration: 1) the *proper biologic signals* for guiding cell differentiation, hyaline cartilage formation, and integration with the underlying bone, and 2) the *proper scaffolding biomaterial* to support physiologic loading, retain the cells at the site of implantation, guide and constrain the growth of the tissue, and integrate with the host cartilage. Therefore our goal is to develop an approach for *in situ* tissue engineering that combines our novel *3D-woven polymer scaffolds*[26-30] with *spatially-defined, inducible gene delivery of morphogenetic factors*[31-38] to specifically address these two limitations in a single "off-the-shelf" device that possesses biomimetic cartilage properties as well as controlled cell-instructive signals.[39]

A2. In situ tissue engineering. Microfracture is popular among surgeons because it is fast and simple. In contrast, most proposed tissue engineering strategies are complex, lengthy, and require multiple surgeries. Additionally, engineering of cartilage tissues *in vitro* requires the expensive and laborious expansion of cells in culture under delicate proliferation and differentiation conditions. The field of tissue engineering has made remarkable progress in the areas of biomaterials and bioreactors that can direct the differentiation of progenitor cells *ex vivo*,[41, 42] but new approaches are necessary to translate these advances into commercializable therapies that can benefit patients in the clinic. We propose to use a mechanically and structurally appropriate biomaterial scaffold loaded with gene carriers of morphogenetic factors to spatially guide the differentiation and tissue formation by endogenous progenitor cells following microfracture surgery. If successful, our proposed approach will require only a single surgery with an off-the-shelf product that can provide spatially- and temporally-regulated cues via patterning of chemically-inducible gene vectors in the scaffold. Success in this microfracture repair model will provide a template for *in situ* tissue engineering to repair other types of defects.

B. INNOVATION

B1. 3D Woven Scaffolds. As described above, biomaterial scaffolds that support tissue growth and maturation while providing necessary mechanical properties for tissue function and integrity are necessary to improve upon current methods for osteochondral regeneration. To address this need, we have developed 3D-woven scaffolds with biomimetic mechanical properties similar to those of native articular cartilage as the basis for composite tissue-engineered constructs (**Fig. 1**).[26-28] With this technique, pore size and geometry may be controlled by manipulating machine variables during the weaving process. Directional mechanical properties may be controlled by independently varying constituent fibers in any of the three directions. Anisotropy can be controlled by changing diameter, material, or spacing of each individual fiber. The placement of individual fibers and pores throughout the depth of the scaffold permits inhomogeneous construct architectures that mimic the osteochondral tissue structure. Because there is no crimping or bending of fibers during fabrication, there is reduced fiber buckling relative to typical 2D weaving processes. This results in increased strength and stiffness of 3D-woven fabrics in both compression and tension, and prevents crimping when the 3D-woven fabrics are molded into curved geometries that can replicate the native geometry of the joint.

B2. Next Generation Gene Delivery Vehicles. Although gene therapy encountered multiple serious adverse events during its early development, several notable advances in recent years have revitalized the field. This is highlighted by the first approved gene therapy in Europe earlier this year, which consists of *in vivo* viral gene delivery to skeletal muscle,[40] and several recent successful business deals by gene therapy-focused startups in the U.S.[41-43] Over the past decade, there have been roughly 100 new gene therapy clinical trials each year worldwide.[44] More than 60% of these trials were in the U.S. and greater than 66% of all trials have used viral vectors.[44] A particular concern has been the integration of viral vectors into the genome that has the potential to disrupt endogenous genes, such as critical oncogenes and tumor suppressors.[45] However, over a decade of research since earlier adverse events has led to enhanced gene delivery vehicles that address this issue. In particular, self-inactivating (SIN) vectors have been developed in which the strong viral promoters and viral splice sites have been disabled and therefore the virus is unlikely to alter the expression of nearby genes.[46] Additionally, lentiviral vectors are now preferred over the retroviral vectors used in the previous clinical trials that were associated with adverse events, as retroviral transduction is dependent on cell division whereas lentiviral transduction is not,[47] and lentiviral vectors are less likely to integrate into regions of the genome that disrupt gene expression.[48] Our proposal incorporates all of these innovations into our gene delivery system. Many other clinical successes for viral gene therapy in recent years, particularly with next generation lentiviral vectors,[49-59] are anticipated to pave the way for multiple new approved gene and cell therapies.[42] As a result of these *enabling technological innovations*[46-48] and the precedent of *efficacy and safety of lentiviral gene therapy* in several other recent clinical trials for rare diseases,[49-53, 57-59] we are proposing a combination of gene therapy and tissue engineering to translate these positive results to orthopaedic regenerative medicine.

B3. Spatiotemporally-Controlled Biomaterial-Mediated Gene Delivery. Conventional methods for gene therapy involve *in vitro* gene delivery to cultured cells or *in vivo* delivery by direct injection of gene carriers. However, both of these approaches are often limited by inefficient transgene delivery and/or poor specificity. Recent attempts to overcome these limitations include biomaterial-mediated gene delivery, wherein the gene carrier is immobilized to, or encapsulated within, a biomaterial scaffold.[37, 60, 61] The hybrid gene-activated biomaterial is then seeded with cells *in vitro* or implanted. By co-localizing the gene delivery vehicle and cell adhesion, this method enhances gene transfer and specifically targets cells at the biomaterial interface, thereby reducing the risks associated with direct injection of the gene carrier. Therefore, this approach provides several advantages over conventional gene delivery modalities, including reduced cytotoxicity and immunogenicity of freely diffusible gene carriers, eliminated or reduced risk of ectopic transgene expression in neighboring tissues, improved stability of the gene carrier, and controlled levels of gene transfer and expression. Biomaterial-mediated gene delivery has been used recently in a variety of successful model studies and in generating musculoskeletal tissues.[62-74] Our innovative approach will build on these successes by adding spatial control over the development of a biphasic tissue in a single scaffold (**Fig. 2**). Importantly, our preliminary results suggest that growth factors secreted using this method act in an autocrine manner, as evidenced by site-specific differentiation of MSCs into osteogenic or chondrogenic lineages.

In addition to spatial control, temporal control of the expression of morphogenetic factors is critical to proper tissue development and function and the prevention of off-target effects on surrounding tissues. We are achieving temporal control of gene expression from the lentiviral vectors by using a third-generation single-vector doxycycline (dox)-inducible gene expression system[75-77] engineered for low levels of leaky expression and high levels of induction.[77-80] We use the dox-on system as a model gene regulatory system, but other inducible gene expression systems with similar properties controlled by synthetic inducers that have no detectable effect on human physiology are currently being assessed in gene therapy clinical trials (NCT01397708, NCT01703754) with early signs of safety and efficacy.[81, 82] This represents a potentially promising clinical path forward for our spatiotemporally-regulated *in situ* tissue engineering strategy.

B4. In Situ Tissue Engineering. Despite nearly two decades of successful preclinical models of tissue engineering strategies,[83] there are only a few approved tissue engineering products and these focus on simple tissues consisting of a single cell type.[84] The lack of products for treating complex tissue defects is due to the challenges and obstacles associated with commercialization and regulatory approval for biologic combination products. In order to address these hurdles but also capitalize on the advances in tissue engineering research, we propose *in situ* tissue engineering, in which the implanted construct provides all the mechanical and biological cues necessary to guide functional tissue development by endogenous progenitor cells. By removing cell isolation and expansion procedures, as well as *in vitro* construct maturation, we expect that this approach will have specific advantages in translation to a clinical therapy for patients.

C. APPROACH

C1. Preliminary Results

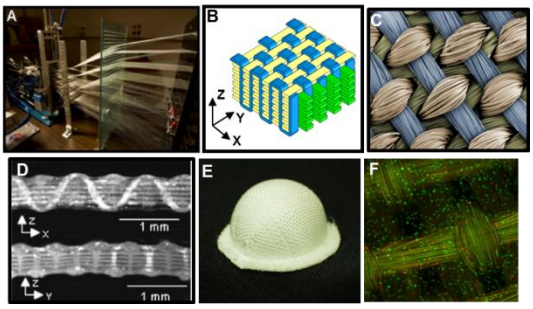

Fig. 1. 3D-woven scaffolds. (A) Custom-built weaving machine. (B) Scaffold structure. (C) SEM micrographs of the scaffold. (D) Photomicrographs of scaffold cross-sections. (E) 3D woven fabric molded in the shape of a hemispherical hip surface; (F) Calcein AM staining of cells in 2% agarose seeded onto woven scaffold.

C1.1. Woven Polymer Scaffolds. We have developed 3D-woven scaffolds as the basis for composite tissue-engineered constructs with similar mechanical properties to native articular cartilage.[26-28] The 3D-woven scaffolds were made by weaving yarns in three orthogonal directions (**Fig. 1**). PCL was selected because of its structural and mechanical properties. Specifically, PCL is a biocompatible, FDA-approved material[85, 86] that supports chondrogenesis[87] and degrades very slowly (i.e., less than 5% degradation at 2 years, as measured by mass loss) into byproducts that are entirely cleared from the body.[88, 89] The 3D-woven PCL scaffolds were constructed from ~600 multi-filament yarns that were woven in three orthogonal directions using 11 total fiber layers (5 warp, 6 weft).[28] By varying the weaving parameters, PCL scaffolds can be made with pore sizes ranging from 150 to 1000 μm, porosities ranging from 50 to 75%, and cartilage-mimetic compressive mechanical properties that can be maintained for more than 6 weeks in standard *in vitro* culture conditions.[26, 27]

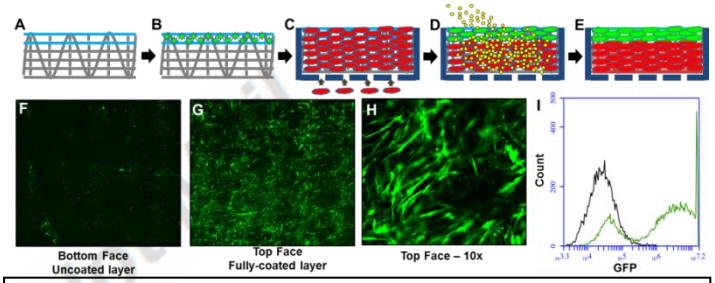

Fig. 2. Spatial control of gene expression for *in situ* tissue engineering. (A) Specific PCL fibers (grey) are coated with PLL (blue) prior to weaving. (B) After incubation with lentiviral supernatant, viral particles (green) bind to only the PLL-coated fibers. (C) The acellular virus-scaffold composite is then implanted into the osteochondral defect, where the microfracture procedure leads to infiltration of bone marrow progenitor cells (red) into the scaffold. (D) The infiltrating cells are transduced in the pattern dictated by the immobilization geometry, but gene expression is only activated following oral or local delivery of a small molecule inducer (yellow). (E) Gene expression is silenced following removal of the inducer, but permanent tissue development occurs in a structure dictated by the scaffold-mediated gene delivery. (F-H) Gene delivery is localized to one face of the scaffold when only one plane of fibers is coated with PLL, as in (A-B). (I) Highly efficient transduction (>70%) of hMSCs seeded onto a scaffold uniformly coated with PLL and GFP-encoding lentivirus.

C1.2 Spatially Controlled Biomaterial-Mediated Gene Delivery. In order to engineer structurally complex tissues that replicate the organization of natural tissues, we have developed methods for spatially-controlled gene transfer within the 3D-woven scaffolds. We previously showed that enveloped retroviral and lentiviral vectors interact electrostatically with biomaterial surfaces coated with the cationic polymer poly-L-lysine (PLL)[35] and this approach was subsequently used to engineer mineralized tissues on fibrous collagen scaffolds.[62] In our recent publication,[39] we have extended this approach to our 3D-woven PCL scaffolds to engineer cartilaginous tissue. In more recent unpublished work, we have taken advantage of the ability to independently modify each of the 600 fibers of the 3D woven scaffold to coat specific fibers in one direction with PLL via passive adsorption prior to weaving (**Fig. 2A**). After weaving, the scaffold was incubated in viral supernatant, leading to site-specific virus binding to the PLL-coated fibers (**Fig. 2B**). These scaffolds were then washed and seeded with human MSCs, leading to efficient lentiviral transduction only of cells in the plane of PLL-coated fibers (**Fig. 2F-H**). Notably, >70% of the cells were transduced when the complete scaffold was coated with PLL and incubated with GFP-encoding virus before cell seeding, demonstrating the high efficiency of gene transfer with this system (**Fig. 2I**).

C1.3 Temporally Controlled Cell Differentiation and Tissue Formation. Gene delivery of morphogenetic factors is essential for guiding proper cell differentiation and tissue development, but prolonged and uncontrolled production of these factors is undesirable and can lead to hypertrophy or osteophyte formation.[90, 91] In order to control the dynamics of tissue development, we have previously used dox-inducible vectors to control morphogenetic factors driving bone formation *in vitro* and *in vivo*.[32] These studies used a two-vector tet-off system, but more recently we have developed a single tet-on lentiviral vector engineered for low levels of leaky expression and high levels of induction[77-80] that will be used for this proposal.

C2. Research Design

C2.1 Aim 1: Engineer osteochondral tissues via spatially controlled gene delivery of differentiation factors. The ***objective*** of this aim is to engineer constructs consisting of multiple tissue types by spatially controlling the expression of morphogenetic factors that direct stem cell differentiation and tissue formation. This aim will build on our preliminary studies (**Fig. 2**) by delivering lentiviral vectors encoding differentiation factors rather than reporter genes. Our ***working hypothesis*** is that spatial regulation of morphogenetic gene

expression and signaling in a 3D construct of hMSCs will lead to the formation of distinct tissue types in different regions of the scaffold. We will also test these methods in rabbit MSCs as well to verify their efficacy before performing Aim 2 *in vivo*.

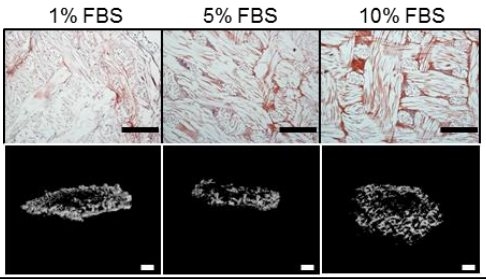

Fig. 3. Concomitant GAG (top) and mineral deposition (bottom) on 3D woven scaffolds after biomaterial-mediated transduction of hMSCs with a BMP-2-encoding lentivirus. Samples cultured for 28 (top) or 14 (bottom) days in chondrogenic medium supplemented with 10mM β-glycerophosphate and indicated FBS (scale bar 500μm). Constructs with untransduced cells did not mineralize (not shown).

C2.1a Differentiation Factors. We have thoroughly characterized the osteogenic and chondrogenic effects of Runx2, BMP-2, Sox9, TGF-β3 and several other chondrogenic factors in adult progenitor cells.[31-38] The cDNA sequences for Runx2, Sox9, BMP-2, and TGF-β3 will be transferred from existing constitutive lentiviral vectors into the pTet vector, such that dsRed2 will be co-expressed from an internal ribosomal entry site (IRES), allowing for fluorescent and immunohistochemical tracking of cells expressing the various factors. The choice of these particular differentiation factors is two-fold: First, we and others have extensively characterized the directed differentiation of several different types of adult progenitor cells by these transcription factors (Runx2 and Sox9) and growth factors (BMP-2 and TGF-β3). Second, we have shown that Runx2 acts predominantly through an autocrine mechanism in genetically engineered cells,[31] whereas it is well known that BMP-2 acts as a potent paracrine signal in addition to autocrine function. We hypothesize there may be a similar relationship with Sox9 and TGF-β3. Since we are attempting to precisely control gene expression and cell differentiation over small distances (<1 mm), the diffusion of potent morphogenetic growth factors may dramatically influence spatially-regulated tissue formation. Nonetheless, our preliminary data suggest that even with soluble growth factors such as TGF-β3 or BMP-2, we are able to induce MSC differentiation in a spatially-defined manner. However, since the relative benefits or limitations of these approaches are currently unclear, we will pursue both in parallel.

C2.1b Media Conditions. The goal of this aim is to generate multiple tissue types from a single cell population on a single construct in the presence of a uniform culture media. We have previously tested a variety of media conditions in combination with overexpression of these differentiation factors[31-34, 38, 92, 93], and our preliminary studies show proof-of-concept that concomitant production of cartilage and mineralized tissue is possible (**Fig. 3**). Nonetheless, additional media conditions will be tested in order to optimize the influence of chondrogenic and osteogenic supplements. We hypothesize that using chondrogenic media conditions for the scaffolds containing a subpopulation of cells overexpressing a potent osteogenic factor will lead to cartilage formation by the unmodified cells and matrix mineralization by the genetically engineered cells. Similarly, osteogenic media conditions on constructs modified with chondrogenic differentiation factors may lead to the formation of both tissue types. We will test these hypotheses first by culturing constructs that have been uniformly functionalized by each of the four gene carriers, or a control eGFP virus, in various media conditions before moving on to scaffolds with spatially controlled gene transfer.

C2.1c Construct Analysis. Scaffolds will be uniformly coated with PLL or only coated with PLL on the yarns of specific x-y planes, as described above (**Fig. 2**). These scaffolds will then be incubated with one of the four lentiviral vectors encoding a differentiation factor, or the negative control vector encoding eGFP only. The constructs will then be cultured in the various media conditions for 3, 7, or 21 days before harvesting and analysis. Live/dead staining will be used to assay cell viability throughout the scaffold. For controls that have been uniformly seeded with virus throughout the scaffold, mRNA samples will be collected for analysis of osteogenic (Runx2, osteocalcin, alkaline phosphatase, bone sialoprotein, type I collagen, type X collagen) and chondrogenic (Sox9, type II collagen, aggrecan) gene expression, indicating differentiation of the hMSCs. ELISA of the conditioned media will be used to validate overexpression of BMP-2 or TGF-β3. RNA in situ hybridization (ISH), immunohistochemistry, and histology of cross-sections of the scaffold will be used to evaluate spatial control of gene expression, protein expression, and tissue formation, respectively, in the z-direction of the scaffold. ISH will be performed against the viral transgenes, whereas immunohistochemistry will be performed for the transgene products as well as other markers of directed cell differentiation, including type II collagen. Histology stains will include hematoxylin and eosin for cellular distribution, von Kossa for mineralized tissue, Masson's trichrome stain for collagen and osteoid, and safranin-O for glycosaminoglycans (GAGs). Importantly, the images of each of these stains will be segmented and evaluated by image analysis software for a quantitative relationship of the intensity of each marker or tissue stain and distance in the z-direction. This will facilitate a rigorous analysis of the degree of success in obtaining spatially regulated tissue formation. Furthermore, microcomputed tomography (Skyscan 1176 microCT, Bruker) will be performed on scaffolds using a high resolution (8 μm) system to determine the density and spatial distribution of mineral

within the scaffolds. All of these assays have all been performed successfully by our groups.[26, 27, 31-34, 38, 93]

C2.1d Alternative Strategies. Our preliminary analysis of scaffold-mediated gene delivery of TGF-β3 to hMSCs in our recent publication[39] shows efficient and robust TGF-β3 secretion and induction of chondrogenesis, indicating a high probability of success for these experiments. In addition to Runx2, Sox9, BMP-2, and TGF-β3 there are many other factors that can be assessed in this system, including BMP-7, BMP-6, IGF-1, FGFs, caALK2, and LMP-1, which have been used in gene therapies for producing bone or cartilage.[37] Finally, lyophilized collagen or hydrogels can be incorporated to enhance biological properties.[94]

C2.2 Aim 2: Enhance the mechanical and biochemical quality of cartilage regeneration *in vivo* by spatially defined material-mediated gene delivery and temporally controlled gene expression

Lentivirus-functionalized PCL scaffolds will be implanted into a rabbit full-thickness osteochondral defect model of microfracture surgery. It is expected that the differentiation of endogenous progenitor cells that populate the scaffold will be guided by morphogenetic factors encoded by the lentiviral vectors. We will test the relative histologic quality of tissue regeneration guided by empty polymer scaffolds, scaffolds functionalized uniformly with lentivirus encoding chondrogenic factors, and scaffolds preferentially functionalized with chondrogenic factors at the joint surface or osteogenic factors at the bone-cartilage interface (**Fig. 2A-E**). Tetracycline-inducible lentiviral vectors will be used to evaluate the effects of various time courses of transgene expression on the quality of tissue regeneration *in vivo*.

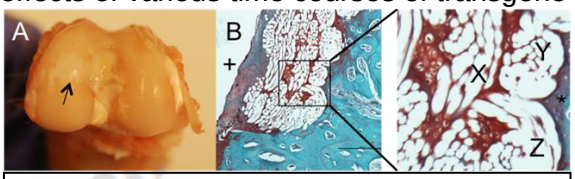

Fig. 4. (A) Gross picture of explanted rabbit femur after 6 weeks demonstrating complete filling of the defect (arrow). (B) SafO/FastGreen histological cross-section of the cartilage surface covering the 3D woven scaffold and interface with the underlying bone. Bone incorporation is noted by FastGreen staining of collagenous tissues growing directly into the pore structure of the PCL scaffold (denoted by *). Magnified section shows X, Y, and Z fiber bundles. Note that all white space in defect (B) is PCL fibers.

C2.2a Assess the efficacy of the 3D-woven scaffold in a rabbit articular cartilage defect model. We propose use of a well-accepted model[95-100] of articular cartilage repair using New Zealand White rabbits to assess the ability of the 3D-woven scaffold to repair the joint defect. Dr. Guilak's laboratory has extensive experience with animal models of cartilage injury and repair, including this rabbit model of defect repair. His laboratory includes a full-time veterinary surgeon, a full-time veterinary technician who will perform the surgical procedure, and numerous orthopaedic surgery residents and fellows who are available to assist with these surgeries. The Department of Orthopaedic Surgery sponsors 3 fellows per year for specialized training in sports medicine surgery. As part of their research training, fellows are supported for up to 6 weeks.

Scaffolds will be prepared as described in the previous aim. Using a surgical drill, a full thickness defect 1 mm deep by 4 mm in diameter will be created through the articular cartilage and into the subchondral bone of the weight-bearing surface of the medial femoral condyle of the knee. Any diameter greater than 3 mm is considered a “critical size” defect in the lapine model which does not heal spontaneously (as reviewed in[101]). With penetration of the subchondral bone, the defect will fill with blood and marrow, as occurs with the clinical microfracture procedure. 4 mm scaffolds matching the curvature of the femoral condyle will be press fit into the osteochondral defect, where they will be infiltrated with cells and marrow from the defect (**Fig. 2C**). Our initial pilot surgeries showed all scaffold remained in place and no additional fixation methods are necessary (**Fig. 4**). An unfilled defect will serve as a control. To induce gene expression, animals will receive dox through their drinking water beginning at 2 days prior to the surgery. Animals (n=8/time point) will be sacrificed at 12 weeks and evaluated histologically and biomechanically. First, a micro-indentation test will be used to obtain the biphasic, compressive material properties of the repair tissue, the surrounding articular cartilage, and the control cartilage from the contralateral condyle.[102] Second, joints will be fixed and assessed using high-resolution digital radiographs and microCT analysis to ascertain bone density and subchondral thickness in the femoral condyle in response to the injury and reparative process. Additionally, the degree of calcification in the implanted scaffold will be assessed. Finally, to evaluate overall cartilage pathology, the joints will be decalcified and assessed histologically by modified Mankin and ICRS scoring systems[103] by experienced, blinded graders, specifically noting regenerative changes at the defect site, as well as degenerative changes in cartilage structure throughout the joint. Proteoglycan staining, hematoxylin and eosin, the presence of fibrocartilage, the presence of hypertrophic chondrocytes, and subchondral bone thickness will all be assessed. In addition, any indication of synovitis, inflammation, and/or an immune response of the knee will be assessed using histopathologic techniques at sacrifice, as described previously.[104] Additionally, the synovium will be analyzed for gross signs of inflammation and the presence of macrophage infiltration.[104] Completion of this aim will demonstrate the construct is able to regenerate cartilaginous tissue and also yield valuable data on the ability of tissue-engineered repair to prevent joint degeneration as a long-term sequela of cartilage damage.[105]

We will evaluate the following groups with a sample size of 8 rabbits/group/time point, and as discussed above each sample will be sequentially examined by mechanical testing, microCT, and histology.

Group 1: Unfilled defect. While rabbits have some ability for osteochondral repair, defects of 4mm diameter or greater do not exhibit extensive repair and consist of fibrocartilaginous tissue, providing a baseline against which new repair methods can be tested. ***Group 2: Uniform immobilization of GFP-encoding virus.*** This group will be included as a control to show successful lentiviral transduction *in vivo*. In our *in vitro* studies, we have observed no effect of PLL coating by itself. PLL is not expected to elicit an *in vivo* response[106] and lentiviral vectors are not immunogenic.[107] ***Group 3: Uniform immobilization of TGF-β-encoding virus.*** A limitation of the use of MSCs in cartilage defect repair has been the conversion to a hypertrophic phenotype after long-term implantation *in vivo*, resulting on endochondral ossification of the implant. We propose that the continued expression of TGF-β in the scaffold will accelerate repair and maintain the chondrogenic phenotype of MSCs as compared to scaffold with GFP virus. ***Group 4: Preferential localization of TGF-β-encoding virus.*** TGF-β overexpression at the bone-cartilage interface may inhibit osteogenic differentiation of infiltrating MSCs and successful osseointegration with mineralized tissue. We hypothesize that by preferentially localizing TGF-β expression to the top half (1 mm) of the scaffold, the regenerating tissues will more closely recapitulate the natural osteochondral interface. ***Group 5: Preferential localization of Runx2-encoding virus.*** The integration of an osteochondral construct to the native bone is critical for rapid repair and the potential for returning to physiologic load-bearing. We propose that the integration of the construct into the defect site will be significantly improved by the spatially-controlled expression of Runx2 in the bottom half of the scaffold, as compared to scaffold without virus. ***Group 6: Preferential localization of TGFβ and Runx2.*** We hypothesize that a construct with spatially defined control of chondrogenesis and osteogenesis will show the best repair scores and lowest osteoarthritis scores in the joint, as compared to other control groups or those with only one localized transgene. For these experiments, each viral supernatant will be separately applied to each side of the scaffold. ***Success criteria:*** Success will be defined as generating mechanical properties and histological quality most similar to natural control tissues. We hypothesize that Group 6 will most successfully recapitulate the properties of these tissues. ***Surgery schedule:*** This study includes 48 rabbit surgeries, which will be completed in four surgeries per day, for a total of 12 days of surgery. Samples from each group will be randomly assigned to each day of surgery.

C2.2b Determine the necessary duration of transgene expression for enhanced osteochondral regeneration. It is possible that long-term constitutive expression of these differentiation-inducing factors may be detrimental to long-term tissue formation and health of the joint. Consequently, it may be desirable to have temporal control over the level of transgene expression. In this sub-aim, we will evaluate the minimal duration of transgene expression necessary to achieve tissue regeneration without adverse effects on the joint using the best condition from the *in vivo* experiment described above. Based on the findings from C2.2a, we will examine whether short-term delivery of transgenes improves osteochondral repair. We will choose the best performing condition from the experiment above (Groups 3-6, described above). The treated scaffold will be implanted as described above, and dox will be provided in the drinking water for 0, 4, 8, or 12 weeks, and all rabbits will be sacrificed at the 12 week time point (n=8 rabbits/time point). This study includes 32 rabbit surgeries, which will be completed in four surgeries per day, for a total of 8 days of surgery. Samples from each group will be randomly assigned to each day.

C2.2c Alternative Strategies. Based on our initial pilot work in rabbits, we do not foresee any issues with this model. Based on previous studies, we do not expect the PLL or lentivirus to be toxic or immunogenic.[106-108] A highly appealing, but risky, aspect of the proposed technique is that we do not propose to seed the scaffold with exogenous MSCs, but we instead hypothesize that the endogenous progenitor cells from the marrow that repopulate the scaffold (as shown in our preliminary data, **Fig. 4**) will respond to the transgenes in a similar manner as cultured MSCs have in our preliminary *in vitro* work.[39] If successful, this approach represents a major advance toward the development of a one-step procedure for enhancing microfracture-based repair through implantation of a multifunctional, cell-instructive scaffold. However, if we find that the progenitor cells within the microfracture clot do not exhibit osteogenesis and chondrogenesis in response to the delivered transgenes *in vivo*, we will perform repair studies using 3D woven scaffolds that have been exogenously seeded with allogeneic bone-marrow-derived rabbit MSCs.

C3. Future Studies. As this is a R21 submission to establish proof-of-principle, the short-duration animal study is primarily to evaluate the first line response to the scaffold in an *in vivo* model. With promising results of the current study, an R01 application is envisioned for full evaluation of this approach. Positive results in this model will also justify moving forward with larger animal models. Finally, this principle of *in vivo* gene delivery to endogenous progenitor cells for *in situ* tissue engineering can be applied to other tissue interfaces.