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Quantitative MRI assessment of matrix development in cell-seeded natural urinary bladder smooth muscle tissue-engineered constructs

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ABSTRACT

The approach of cell-seeded natural scaffolds holds great promise for tissue-engineering complicated soft-tissue organs such as the urinary bladder and heart. However, relatively little is known about cell-natural scaffold interactions or their influence on magnetic resonance image (MRI) characterization, which is valuable for non-invasive monitoring. Ideally, MRI should provide information on tissue biochemistry in addition to structure and function. In this study, quantitative MRI was performed on control and smooth muscle cell-seeded natural bladder matrices at different time-points up to 7 days post-seeding. Measurements of MR relaxation times (T1, T2) and diffusion coefficient (D) showed an overall change that was incompatible with cell presence. Multicomponent T2 provided greater specificity, revealing time-course changes in the short T2 fraction that were consistent with biochemically determined matrix degradation from collagenase released from seeded cells. These matrix alterations are noted for the first time, and their relatively early occurrence may be unique to soft tissue matrices compared with synthetic materials. More importantly, they are not evident on histology but are revealed on quantitative MRI. We conclude that quantitative MRI may provide specific information on cell-matrix interaction and is a promising non-invasive approach to understand and monitor cell-seeded natural scaffold-based regeneration.
INTRODUCTION

The engineering of whole soft tissue organs such as the urinary bladder and heart is considerably more complex than hard tissue regeneration\(^1\). Regenerating tissue over a large area generally requires a scaffold for three-dimensional (3D) guidance and cell-seeding to improve continued regeneration \textit{in vivo} post-implantation\(^2\). Recent suggestions of using acellular matrices (ACMs) derived from biologically similar natural tissue have been particularly promising. Unlike conventional synthetic or tissue-mimicking materials, ACMs have the advantages of biological recognition\(^3\) and built-in structural and biochemical similarities with the target tissue to be engineered that are not easily reproduced using synthetic methods. A recent study shows that the ACM composition provides a culture microenvironment that dramatically improves cell growth and differentiation\(^4\). The approach of combining cell-seeding with natural scaffolds, thus, holds great promise to create 3D organs with proper mechanics, structure, and function. In fact, ACMs derived from the urinary bladder have shown capable of regenerating not only bladder tissue but also esophageal, skeletal muscle, heart valve, and myocardial tissue\(^5,6,7\). Therefore, there is broad interest in further investigation of ACM-based tissue-engineering.

Experience with cell-seeded natural scaffolds is young compared with other tissue-engineering paradigms, and advancing this approach requires an understanding of the chosen cells, scaffold, and their interaction\(^8,9,10\). These questions are ideally probed non-invasively. Amongst imaging techniques, magnetic resonance imaging (MRI) has emerged as the most useful \textit{in-vitro} and \textit{in-vivo} method for 3D assessment of tissue structure, composition, and function in a variety of tissue-engineering applications (mainly cartilage\(^11,12,13,14\) and bone\(^15,16,17\); some heart valve\(^18\), liver\(^19\), pancreas\(^20\), and bladder\(^21,22\)). The size of the implant\(^23\) or the location of locally injected cells\(^24\) or newly formed tissue\(^12,25\) can be reliably determined. Beyond these
qualitative metrics, however, is a need for absolute measurements that correspond to underlying biology. Quantitative MRI has the unique potential to provide sensitive and specific information on tissue biophysics and biochemistry, and there have been recent efforts in its application to monitoring cartilage \textsuperscript{13,14,26} and bone development\textsuperscript{15}. Advances in tissue-engineering methods will rely on developing robust quantitative techniques to monitor and optimize all stages of regeneration (cell growth, implantation, and integration with host tissue) that, to date, remain limited.

This study explores quantitative MRI at 1.5 Tesla for monitoring matrix changes in smooth muscle cell-seeded urinary bladder ACMs intended for bladder replacement. The goals are to: 1) understand the relatively unexplored interaction between cells and natural ACMs, and 2) develop quantitative MRI methods at clinical field strengths for sensitive and specific monitoring of matrix progression. This matrix characterization on MRI is an essential step towards interpreting and distinguishing MRI characterization of multiple concurrent processes (e.g. ACM composition and degradation, cell growth and penetration) that occur during regeneration. Single quantitative MRI measurements (T1, T2, diffusion coefficient) that are useful for assessing cell-seeding and expansion\textsuperscript{11,15} and biophysical and biochemical properties\textsuperscript{13} will be investigated. T2 relaxation data will also be acquired and analysed for multicomponents, an approach that distinguishes different water compartments (e.g. water tightly bound to macromolecules such as collagen) and can potentially provide more specific information on tissue composition\textsuperscript{16,23,27}. We hypothesize that matrix changes occur in natural ACMs due to cell-seeding, that on MRI these changes counter those from cell growth and need to be accurately quantified for potential distinction of matrix- and cell-related information.
MATERIALS AND METHODS

Acellular matrix (ACM) preparation

Acellular tissue matrices were prepared according to a published protocol\(^2^8\). Briefly, fresh urinary bladders were harvested from pigs weighing 20-50 kg. Bladders were immediately washed in sterile phosphate buffer saline (PBS; Sigma), longitudinally sectioned, and placed in a hypotonic solution [5 mM EDTA, 10 mM Tris HCl, pH 8.0, 1% Triton X-100, 0.1 mg/mL Pefabloc Plus\(^\text{TM}\) (Alexis) and Penicillin/Streptomycin] (Sigma) for 72 hours, stirring at 4°C, to lyse all cell structures and inhibit proteases. Bladder tissues were then immersed in hypertonic solution [5 mM EDTA, 10 mM Tris HCl, pH 8.0, 1% Triton X-100 and 1.5 M KCl] (Sigma), stirring for an additional 72 hours at 4°C to denature residual proteins. Subsequently, bladders were washed in Hank’s Balanced Salt Solution (HBBS), followed by overnight incubation at 37°C with Benzonase (2U/mL) diluted in HBSS to degrade DNA and RNA components. Final extraction was performed by transferring bladders to 0.25% CHAPS (non-denaturing detergent) based solution [50 mM Tris HCl, pH 8.0, 1% Triton X-100 and Penicillin/Streptomycin]. The resulting ACM was washed with sterile dH\(_2\)O and stored in 70% ethanol. Hematoxyline and eosin (H&E) sections were taken to confirm acellularity.

All protocols were approved by the institutional Animal Care Committee and compliant with national policies on the humane use of laboratory animals (CCAC guidelines).

Cell-seeding

Cell cultures were obtained from passage 2 smooth muscle cells (SMCs) isolated from porcine bladder\(^2^9\) and expanded in SMC medium (high glucose DMEM (Wisent Inc), 10% FBS). Cells were seeded on 2×2 cm\(^2\) pieces of ACM in a 6 well plate at 1×10\(^6\) cells/cm\(^2\) in 100 μL of
SMC medium. The cells were allowed to attach on the ACM for 3 hrs (37°C in a humidified atmosphere of 5% CO₂) before immersing in 2 mL of SMC medium. Unseeded ACMS were also prepared as controls and incubated in SMC medium. The medium was changed every 72 hours.

**Experimental design**

Cell-seeded \( N_s=24 \) and unseeded \( N_u=15 \) ACMS were prepared at three time-points of 1, 3, and 7 days prior to MRI. The first week was chosen, as cell number remained relatively stable over this time interval and, hence, time-course changes in the matrix could be assessed without confounding influence from cellularity differences. The samples were prepared for MRI by transferring each ACM to a 5 mL round Falcon tube containing SMC medium, and placing the tubes in a water-filled container. Histology was performed on all ACMS, and samples were taken prior to imaging to verify no changes occurred during MRI. Additional samples were prepared for evaluation on immunofluorescence microscopy and biochemical assay.

**Histology**

ACM samples were fixed in 10% formalin for 24 hours, embedded in paraffin, and sectioned into 5 µm thick slices. Sections were stained with H&E and Masson’s Trichrome to provide qualitative assessment of cellularity and collagen fibers in the extracellular matrix, respectively, and examined under a light microscope (Nikon Eclipse E 400).

**Quantitative MRI**

MRI was performed on a 1.5 Tesla clinical scanner (Signa EXCITE TwinSpeed, GE Healthcare, Milwaukee, WI) using a single 3-inch surface coil positioned under the water-filled container in which ACM-containing tubes were placed. Pilot scans were taken to determine
Quantitative MR of cell-seeded natural constructs

placement of imaging slices perpendicular to the tubes and encompassing the upper and lower ends of the ACMs.

Quantitative T1, T2, and diffusion imaging was performed. The longitudinal relaxation time T1 was measured using a rapid 3D technique\textsuperscript{30} based on a fast spoiled gradient echo sequence with radio-frequency field correction [flip angle=2,3,10,20°, number of averages (NEX)=4]. The transverse relaxation time T2 was measured using a spin echo sequence [nine echoes with echo times (TE)=9-300 ms, repetition time (TR)=3000 ms, NEX=1]. Multi-component T2 data was also acquired using a 96-echo CPMG sequence\textsuperscript{31} [TR=2500 ms, TE=11.4-1094 ms, NEX=2]. Diffusion coefficients (D) were measured using a spin echo diffusion-weighted imaging sequence up to high b-values [b=0-3000 s/mm\textsuperscript{2} in steps of 200 s/mm\textsuperscript{2}, TR=4000 ms, NEX=16]. Slice thickness was 3 mm for all sequences and 5 mm for CPMG; in-plane resolutions were 0.4 mm (T1, T2) and 0.8 mm (D).

For single T1 and T2 analysis, pixelwise maps were computed. T1 analysis was conducted as previously described\textsuperscript{30}. T2 was analysed by fitting a mono-exponential decay model to signal intensities obtained at various echo times. To obtain an average T1 or T2 value for each ACM, the imaging slices through which each ACM traversed were identified. The mean parameter value was then computed by averaging within the ACM on each slice and through all slices. All analysis was performed using Levenberg-Marquardt algorithm in Matlab (v.7.0). For multicomponent T2 or diffusion data, a region encompassing the ACM was drawn and the average signal intensity at each echo time or b-value, respectively, was taken for analysis. T2 components were identified using non-negative least-squares analysis, while high b-value diffusion data was analysed by fitting a biexponential decay model to estimate diffusion coefficients and their fractions. All data are expressed as mean value ± standard deviation.
Immunofluorescence

Immunofluorescence staining for matrix metallopeptidase 1 (MMP1), or interstitial collagenase, was performed as follows: SMCs were cultured on sterilized plastic cover slips in 48-well plates at a density of $5 \times 10^5$ cells/cover slip in complete DMEM medium (10% FBS and Penicillin/Streptomycin). At confluence cells on the cover slip were washed 3 times with PBS, fixed in 4% paraformaldehyde (Sigma) in PBS for 10-15 minutes at room temperature, washed again 3 times with PBS, and blocked with 4% BSA (Bovine Serum Albumin, Ph 7.5, MULTICELL #800-095-EG) for 60 minutes at constant shaking, followed by incubation for one hour with the addition of primary antibody rabbit polyclonal MMP1 (catalog no. ab38929, dilution 1:100) and FITC-conjugated secondary antibody chicken anti-rabbit immunoglobulin G (Invitrogen, cat no. A21441, dilution 1:500). Cells were washed in PBST (Tween 20) 3 times for 5-10 minutes, mounted on glass slides using DAPI and Vectashield (Vector Laboratories), and examined on fluorescence microscopy (Olympus BX60).

Biochemical assay

Degradation of type I collagen in seeded ACMs, which was previously identified as a major component of the extracellular matrix\textsuperscript{32}, was assessed quantitatively. The total activity of porcine type I collagen from tissue was determined using a commercial Porcine Type I Collagen Detection Kit according to the manufacturer protocol (Chondrex Inc, catalog no: 6015, Richmond, WA, 98052, USA). Briefly, unseeded and seeded ACMs were prepared at the same time-points prior to MRI ($n=3$ per time-point). The ACMs were lyophilized overnight followed by immersion in pepsin solution (Sigma, P-7012, dissolved in 0.1 mg/mL in 0.05M acetic acid) and porcine pancreatic elastase enzyme (Worthington Biochemical Corporation, USA, 0.1mg/mL, dissolved in 0.01M Tris-base 0.15M NaCl and 5mM CaCl$_2$, PH 7.8) digestion for 96
hours. Afterwards, samples were centrifuged at 10,000 RPM; the supernatant was kept in 0.2mL buffered normal goat serum (Invitrogen, 0.1M Tris-base and 0.15M NaCl, PH 7.5). Capture antibody (100μL) was added to each well and incubated overnight at 4°C and washed 3 times with 1X wash buffer. A range of samples/standards were prepared (5μg/mL-0.16μg/mL). Both standards and samples (100 μL) were added to the wells as duplicates, incubated for 2 hours at room temperature, and washed 3 times with 1X wash buffer. Detection antibody was added followed by a 2-hour incubation. Streptavidin Peroxidase (100μL) was then added to each well followed by 1-hour incubation and 3-times washing with 1X wash buffer. OPD solution (100μL) was added to each well, and the reaction was stopped by adding 50μL 2N sulphuric acid after 30 minutes of incubation. Plates were read on an ELISA plate reader at 490 nm. The activities of collagen I in each sample were determined by interpolation of the standard curve ($R^2 = 0.997$).

Statistical analysis

Differences between groups were assessed using a two-tailed Student’s $t$-test. Significance was declared at $P < 0.05$.

RESULTS

Histological results

H&E sections (Fig. 1) showed slight variations in cellularity from sample to sample, but, overall, cellularity was maintained during the first 7 days post-cell seeding. Cells were aggregated mainly at the surface of the ACM, with minimal penetration inside the matrix. No difference in cellularity was observed in samples taken before and after MRI from the same ACM, indicating that the time spent for MRI did not affect cell viability.
Masson’s Trichome sections (Fig. 2) illustrate the main constituents of the ACM: smooth muscle bundles (SMB) within a collagen extracellular matrix. No noticeable difference in collagen content was detected between unseeded and cell-seeded ACMs or with longer times during the first 7 days post-cell seeding.

Quantitative MRI measurements

Quantitative MRI measurements of single T1, single T2, and diffusion coefficient (D) in the unseeded ACMs were relatively constant over the 7-day duration of the experiment (Fig. 3). Cell-seeded ACMs also exhibited stable T1 and T2 values that were not significantly different from those in unseeded ACMs. Only variations in diffusion were observed, where seeded ACMs showed increasing D values that were significantly different from those in unseeded ACMs at 3 and 7 days post-cell seeding. An important but unexpected observation was the direction of change in MRI parameters between the unseeded and seeded ACMs. Fig. 3 shows a lower T1 in seeded ACMs at each time-point, which is consistent with the presence of cells. However, both T2 and D are higher in seeded ACMs, which counters the notion that cellularization in seeded ACMs was the sole distinguishing feature from unseeded ACMs. This contradictory result suggests changes in the underlying matrix that are present soon after cell-seeding and exerting a competing influence on MRI parameters.

More specific information on tissue composition is provided by multicomponent T2 results (Fig. 4). Two components, representing tightly bound (short T2) and loosely bound (long T2) water protons, were consistently detected in all ACMs. In unseeded ACMs, the short T2 relaxation time and its population fraction did not vary significantly throughout the 7 days, although a slight decrease in the long T2 relaxation time was noted on day 7. In contrast, significant changes were noted in seeded ACMs: the short T2 increased (23 ms to 36 ms) and its
population fraction decreased (68% to 48%) from day 1 to 7. There was also a trend of lowered long T2 with time, similar to unseeded ACMs, although the decrease was not significant.

**Immunofluorescence and biochemical assay results**

Evidence of collagenase secretion from SMCs is confirmed on immunofluorescence microscopy at 1, 3, and 7 days (Fig. 5). Collagenase images (green) are overlaid on DAPI for SMCs (blue) to show the presence of collagenase in both intra- and extra-cellular space.

Quantitative assay for type I collagen showed lower collagen content with time in cell-seeded ACMs (Fig. 6). In contrast, no changes with time were observed in unseeded ACMs, indicating that time and culture medium had no effects on collagen content. Therefore, a single average across all samples is shown to represent the “control” unseeded matrix. The lowered collagen content observed in seeded ACMs, which can now be attributed to the presence of cells, confirms matrix degradation (due most likely to collagenase released from SMCs) and is responsible for higher T2 and improved water diffusion observed on MRI.

**DISCUSSION**

The present work provides the first characterization by quantitative MRI of cell-seeded acellular matrices in an in-vitro system and offers new insight into cell-matrix interactions possibly unique to natural matrices. Most imaging studies of tissue-engineered systems to date have focused on seeding synthetic or tissue-mimicking materials, primarily in bone and cartilage regeneration. Biologically derived materials, such as smooth muscle matrices investigated in this study, offer the advantages of biological recognition, required structural and mechanical properties, and improved cell growth and function. However, experience with natural tissue matrices remains in its infancy, and there is scarce imaging data to guide further
development. The purpose of this study was to investigate the feasibility of quantitative MRI at clinical field strengths for characterizing cell-seeded acellular tissue matrices and to explore potentially concurrent biological processes and their effects on MRI signatures.

In-vitro quantitative MRI measurements of single T1, T2, and diffusion (D) were taken to capture overall changes in cell-seeded ACMs, while more specific association with tissue characteristics was attempted with multicomponent T2 measurements. During the first 7 days post-cell seeding, cell number remained stable, which was consistent with constant T1 and T2 values at all time-points. However, T2 in seeded ACMs changed in a direction (an increase) incompatible with cell presence. Diffusion coefficient D was also inconsistent, being higher in seeded ACMs and increasing with time. These inconsistencies captured on MRI can only reflect alterations in matrix composition and structure in response to cell-seeding, changes that histology failed to reveal throughout the first week. Multicomponent T2 data provided more specific clues, suggesting degradation of structures (e.g. collagen in the extracellular matrix) with which tightly bound water is associated. Immunofluorescence confirmed collagenase secretion from the SMCs used for seeding, and collagen I assay demonstrated decreased collagen content with time in the seeded matrices. This phenomenon of collagen degradation in the matrix due to the release of enzymes from seeded cells is unique as it has not been seen in studies where other scaffold materials are used\textsuperscript{15,17}, which suggests soft tissues may be desirable for its easier and earlier degradation to accommodate cell growth. Our results show not only that MRI may provide specific information on tissue composition and can uncover subtle tissue changes before they appear on histology, but also that soft tissue-based regeneration is likely unique and will require appropriate imaging methods to study concurrent biological processes.
The quantitative MRI changes observed need to be interpreted within the framework of other studies that attempt to elucidate underlying biological mechanisms. Although there exist scarce MRI data from similar regeneration methods for comparison, it is well recognized that in general, tissue formation characterized by cell growth is related to a decrease in T1, T2, and diffusion. This relationship has been verified by controlled cell suspension experiments in cancer applications, and is explained by a higher macromolecular content (T1, T2) and restricted water diffusion (D) through the extracellular space with increasing cell density. The same trend has also been observed in tissue-engineering applications. Since the expected lowering of T2 and D from cell-seeding was not obtained in this study, we hypothesized confounding influences from matrix changes, which were confirmed to be matrix collagen degradation due to collagenase release from seeded cells.

Matrix degradation is an important process in tissue regeneration, and although its characterization on imaging has been much less studied compared to cell growth, an increase in T1, T2, and D can be expected due to reduced matrix integrity and possibly higher matrix hydration. With the exception of T1, all parameters were higher in seeded matrices, and all changes were all consistent with the presence of collagen, which is hydrophilic and slows down water. Therefore, collagen degradation would have the largest impact on T2 and a lesser effect on D but negligible effect on T1; similar results were also observed in a study of matrix-depleted cartilage. However, these single parameters do not distinguish potential competing tissue changes, and only multicomponent T2 data yielded significant time-course description of matrix component evolution. Our multicomponent T2 results can be interpreted based on other studies that examine water compartments in a variety of tissues. Assignment of T2 compartments to intra- and extra-cellular water can be ignored, since cells comprised a relatively small
fraction of the ACM. A more likely interpretation is the association of the short T2 component with water tightly bound to macromolecules (e.g. collagen) and the long T2 component with more mobile water in the matrix. This interpretation is supported by studies of cartilage and muscle in which collagen, proteoglycans, and “free” extracellular matrix water were assigned to progressively longer T2\textsuperscript{27,38,39}. Our short T2 component (23-36 ms), which we ascribe to collagen, is higher than that reported in cartilage (2.3 ms)\textsuperscript{27} but falls within the T2 range for varying collagen concentrations in tissue\textsuperscript{38}. The increase in short T2 relaxation time is consistent with the action of collagen breakdown\textsuperscript{40} and reflects increased mobility of water formerly attached to intact collagen that are now attached to collagen fragments. A shift (decrease) from the short to the long T2 fraction is consistent with a loss of water bound to collagen accompanied by a relative increase of loosely bound water. The long T2, however, did not increase as predicted from cartilage degradation studies\textsuperscript{27,40} but showed a downward albeit insignificant trend. One explanation for this trend is a slight reduction in free water mobility due to its interaction with collagen fragments that are not depleted from the matrix. However, the trend was observed also in unseeded matrices, suggesting that other mechanisms must be involved. Perhaps slight changes in hydration were present, but if so, they were not considerable to be evident on single T1 or T2. The underlying mechanisms for long T2 changes remain unclear, and further investigation is warranted.

This study has provided a basis for evaluating cell-seeded tissue-engineered soft tissue matrices using quantitative MRI. Although the focus was on bladder tissue, the value of this MRI technology extends to other engineered systems using different soft tissue matrices and cell types. It is important to note that our goal was to demonstrate the value of MRI quantification, which is distinct from ongoing efforts to optimize our regeneration paradigm. A number of
biological questions regarding this regeneration system remain, and they are only beginning to be answered. For instance, cell penetration into the matrix was suboptimal but could possibly be improved using cell types different from SMCs, such as bone marrow stem cells, or altering matrix properties through the addition of biochemicals. Also, significant cell growth was not observed over the 7-day period. Further investigation on appropriate growth factors, different cell types and seeding densities, and matrix manipulation is warranted. Independent of these biological challenges, this study demonstrates that MRI can provide information on biochemical properties and is a useful non-invasive probe for tissue-engineering.

In conclusion, this is the first characterization of bladder smooth muscle cell-seeded tissue-engineered natural tissue matrices using quantitative MRI. Our results indicate collagen degradation of the matrix due to collagenase released from seeded cells. These matrix alterations are noted for the first time, and their relatively early occurrence may be unique to soft tissue matrices compared with synthetic materials. More importantly, they are not evident on histology but are revealed on quantitative MRI, with the greatest specificity provided by multicomponent T2. Our results underscore the potential difficulty of distinguishing cell- and matrix-related events. As an initial step towards addressing this challenge, we provide the necessary baseline MRI characterization for matrix evolution. Our results provide a framework for further development of quantitative MRI for correlative biological imaging and are valuable in guiding ACM-based organ regeneration.
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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.
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FIGURE LEGENDS

FIG. 1. H&E staining for cellularity at different time-points post cell-seeding reveals relatively stable cell number throughout the first 7 days. Arrows indicated seeded cells. Scale bar represents 40 μm (magnification, 10×).

FIG. 2. Masson’s Trichome staining for collagen content in the extracellular matrix reveals no observable differences between unseeded (Control) and cell-seeded matrices during the first 7 days post cell-seeding. Remaining smooth muscle bundles (SMB) are also present. Scale bar represents 40 μm (magnification, 4×).

FIG. 3. Quantitative MRI parameters single T1, single T2, and diffusion coefficient (D) at different times post cell-seeding. Parameter values in unseeded (Control) matrices were relatively stable. In cell-seeded matrices, only parameter D exhibited a significant increase at both 3 days and 7 days. A lowering of all parameters expected from cell presence was not seen for T2 and D, which suggests concurrent matrix changes dominating effects from cellularization. Values are reported as mean ± standard deviation. (* P < 0.05)

FIG. 4. Quantitative MRI multicomponent T2 data at different times post cell-seeding. The seeded matrix short T2 component undergoes a significant increase in relaxation time and decrease in its population fraction on day 7, relative to day 1 and also to unseeded matrices. A downward trend in the long T2 was observed in both matrices. Values are reported as mean ± standard deviation. (* P < 0.05)

FIG. 5. (A) Immunofluorescence staining for collagenase (green) confirms its release from SMCs (blue, DAPI) at all time-points (magnification, 10×). (B) Images are shown with
and without overlay with DAPI to illustrate the presence of collagenase both inside and outside cells (magnification, 20×).

**FIG. 6.** Collagen I content (μg/mL) progressively declines in cell-seeded matrices (grey) and is lower than collagen content in unseeded matrices (Control), which showed no changes with time and thus represented as a single average across all time-points. Values are reported as mean ± standard error.
FIG. 1

1 day

3 days

7 days
FIG. 2

Control                  Cell-seeded

1 day

3 days

7 days

SMB

FIG. 2
FIG. 3.
**FIG. 4.**
FIG. 5.
FIG. 6.