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Effects of extracellular matrix analogues on primary human fibroblast behavior

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Abstract

In vitro cell culture is a vital research tool for cell biology, pharmacology, toxicology, protein production, systems biology and drug discovery. Traditional culturing methods on plastic surfaces do not accurately represent the in vivo environment, and a paradigm shift from two-dimensional to three-dimensional (3-D) experimental techniques is underway. To enable this change, a variety of natural, synthetic and semi-synthetic extracellular matrix (ECM) equivalents have been developed to provide an appropriate cellular microenvironment. We describe herein an investigation of the properties of four commercially available ECM equivalents on the growth and proliferation of primary human tracheal scar fibroblast behavior, both in 3-D and pseudo-3-D conditions. We also compare subcutaneous tissue growth of 3-D encapsulated fibroblasts in vivo in two of these materials, Matrigel™ and Extracel™. The latter shows increased cell proliferation and remodeling of the ECM equivalent. The results provide researchers with a rational basis for selection of a given ECM equivalent based on its biological performance in vitro and in vivo, as well as the practicality of the experimental protocols. Biomaterials that use a customizable glycosaminoglycan-based hydrogel appear to offer the most convenient and flexible system for conducting in vitro research that accurately translates to in vivo physiology needed for tissue engineering.

Keywords

3-D culture; Cell proliferation; Bioresorbable hydrogel; Biomimetic substrate; Hyaluronan; Gelatin; *In situ* cross-linking; ECM remodeling; Cell morphology

1. Introduction

The practice of culturing cells in three dimensions has increased exponentially in the past decade, following numerous reports that classical two-dimensional (2-D) culturing conditions lead to aberrant cell behavior that may have limited relevance to in vivo conditions [1–4]. In organisms, a complex network of proteins and proteoglycans constitute the extracellular matrix (ECM) that surrounds every cell. Cell proliferation, migration, differentiation, angiogenesis and invasion are orchestrated by the ECM components and the signaling cascades in this three-dimensional (3-D) cellular microenvironment [5–7]. The ECM dictates the morphology and overall behavior of cells and, in turn, is constantly remodeled by matrix-specific enzymes produced by cells [8].

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ECM scaffolds derived from natural sources meet many key requirements, such as biological recognition, presentation of receptor-binding ligands, cell-induced proteolytic degradation and remodeling [7]. One such material, PureCol™ (Vitrogen®), consisting of 99.9% pure type I collagen, is widely used in cell culture and tissue engineering, and as a coating material for medical devices [9–13]. A more complete suite of matricellular proteins and growth factors is provided by Matrigel™, an ECM preparation extracted from Engelbreth–Holm–Swarm (EHS) mouse sarcoma [14]. Matrigel™ has been successfully utilized for a variety of applications, such as cell growth and differentiation, angiogenesis and invasion assays, and promotes a natural cell morphology and behavior [14–18]. However, limited availability, batch-to-batch variability, pathogen transmission, immunogenicity, technical challenges in handling, and the inability to experimentally vary composition and compliance suggested the need for a more versatile ECM equivalent. Synthetic analogs of the natural ECMs were developed as 3-D scaffolds in an effort to accomplish in vivo-like environments in culture dishes, ex vivo tissue growth and engineering, and other scientific applications without posing health risks. One such material, PuraMatrix™, is a synthetic, self-assembling, peptide-based material that forms fibrous scaffolds and can be used for 3-D cell embedding or surface plating [19–23]. This non-animal-derived material is non-immunogenic and can be used for in vivo studies. Another ECM equivalent, Extracel™, consists of a chemically modified hyaluronan (CMHA-S, also known as Carbylan™-S) and gelatin (Gtn-DTPH), which are co-cross-linked with polyethylene glycol diacrylate (PEGDA) [24,25]. This synthetic ECM (sECM) has been used successfully in numerous tissue engineering applications, has broad applicability in drug discovery [26], and is suitable for both 3-D and pseudo-3-D plating [27–34].

To date, no direct comparison of these four ECM equivalents with a primary human cell type has been described. Herein, we evaluate each of these materials for their efficiency as artificial scaffolds and convenience of use. Cell proliferation assays were designed to test the pseudo-3-D properties of these materials, while 3-D growth and cytotoxicity experiments were included to analyze the effect of the matrices on embedded cells. The results can be used to rank these sECM from a researcher's perspective, taking into consideration the biological performances of the matrices, as well as their preparation protocols and “user-friendliness”. In an in vivo study, we also demonstrate that cells injected in vivo in Extracel™ show improved cell proliferation and hydrogel degradation relative to cells injected in Matrigel™.

2. Materials and methods

2.1. Preparation of gels and sponges

Matrigel™ (BD, Biosciences Discovery Labware, Bedford, MA) gels were made according to the manufacturer's protocol. Briefly, the material was thawed overnight on ice at 4°C. Subsequently, the product was kept on ice and handled with cold pipettes. After casting, the material was allowed to gel for 10 min at room temperature in the hood before adding medium.

PuraMatrix™ (BD, Biosciences Discovery Labware) was prepared according to manufacturer's instructions. The peptide mix was bath-sonicated for 30 min prior to use. Sterile sucrose solution (10% w/v) was used to dilute the material twofold. The protocol instructions were followed to obtain gels. Briefly, at 5 min after casting, culture medium was gently added on the material to increase the pH of the matrix and promote gelation. The medium was changed three more times during the next 30 min.

PureCol™ (Inamed, Fremont, CA) was mixed with 10× phosphate-buffered saline (PBS) as recommended by the supplier and the pH of the solution was adjusted to 7.4 with 1 M NaOH. The prepared collagen solution was then filtered through a 0.45 µm syringe driven filter unit prior to casting, to ensure sterility.

ExtracelTM hydrogels (Glycosan BioSystems, Inc., Salt Lake City, UT) were obtained by mixing 1% w/v CMHA-S solution (Glycosil) with 1% w/v Gtn-DTPH solution (Gelin-S) in a 1:1 volume ratio and cross-linking this mixture with 2% w/v PEGDA (mol. wt 3400) in a 4:1 volume ratio. All components were dissolved in DMEM/F12 + 10% newborn calf serum + 2 mM L-glutamine + penicillin/streptomycin (T31 fibroblast growth medium), and the pH of the CMHA-S and Gtn-DTPH solutions were adjusted to 7.5 with 0.1 M NaOH. All solutions were then filtered through a 0.45 μ m syringe driven filter unit prior mixing to ensure sterility. The hydrogels were cast and allowed to gel in the hood at room temperature before adding medium.

2.2. Pseudo-3-D cell proliferation assay

T31 human tracheal scar fibroblasts were a generous gift from Dr. S.L. Thibeault, Department of Surgery, University of Wisconsin, Madison, WI). T31 fibroblasts [29,35] are sensitive, non-immortalized cells derived from primary culture and were selected as being representative primary cells. The T31 cells were evaluated under pseudo-3-D plating conditions in 96-well plates. Each material tested (50 μ l well⁻¹) was used to coat one row per plate (a total of seven plates were used, one for each day of the assay). The hydrogels were allowed to gel and were seeded with 3.5×10^4 cells ml⁻¹. On the third day of culture, the medium was refreshed. Cell numbers were monitored each day by using the Cell-Titer 96 Aqueous One Solution Cell Proliferation assay (MTS assay) (Promega, Madison, WI). The A_{490} values, which are directly proportional to the number of viable cells, were plotted against the time course of the assay to yield the growth profile of the cells seeded on various sECMs.

2.3. 3-D cell culture

To determine the growth of T31 fibroblasts encapsulated in various gels, cells were entrapped in different materials at a final concentration of 10^5 cells ml⁻¹ and 100 μ l of cell + sECM mix was cast per well of a 24-well plate containing Corning Transwell permeable supports (inserts) with 8.0 μ m membrane pore size (Corning Inc., Corning, NY). After gelation, cells were incubated with DMEM/F12 + 10% newborn calf serum + 2 mM L-glutamine + penicillin/streptomycin for 72 h and the MTS assay was used to estimate the number of viable cells in each material.

2.4. Live/dead cell assay

The cytocompatibility of sECMs with T31 fibroblasts was evaluated by analyzing cell viability by a double staining procedure that uses calcein AM and ethidium homodimer-1 (EthD-1) (LIVE/DEAD[®] Viability/Cytotoxicity Kit, Invitrogen, Carlsbad, CA). Calcein AM is a nonfluorescent, cell-permeant molecule that is cleaved inside the cell by intracellular esterases to yield its fluorescent counterpart (green fluorescence). EthD-1 is a nucleic acid stain impermeant to viable cells but can diffuse through the membrane of dead cells where it binds to the DNA and gives a red fluorescence. Cells (3×10^5 cells ml⁻¹) were encapsulated in various sECMs and cultured for 6 days (the medium was replaced with fresh one on the third day of culturing). The staining solution used was obtained by diluting a stock solution of calcein AM to a final concentration of 2 μ M in a 4 μ M EthD-1 solution in 1 $\times$ PBS, pH 7.4. The inserts with gels were washed three times in 1 $\times$ PBS, pH 7.4, then incubated for 45 min at room temperature, on a rocker, with the staining solution. After 45 min, the samples were washed again three times in 1 $\times$ PBS, pH 7.4, then covered with one drop of VectaShield mounting medium for fluorescence (Vector Laboratories, Inc., Burlingame, CA) to prevent photo-bleaching. Cells were analyzed with an Olympus IX70 microscope equipped with Microfire/QCAM CCD (Olympus America Inc., Melville, NY) at $\times 100$ magnification using DAPI and GFP filters.

2.5. Subcutaneous tissue growth *in vivo*

Twelve 6-week-old female athymic nude mice (Charles River, Wilmington, MA) were anesthetized by intraperitoneal injections of ketamine (80 mg kg⁻¹) and xylazine (10 mg kg⁻¹) according to the protocol approved by the University of Utah Institutional Animal Care and Use Committee (IACUC). The dorsal skin was then sterilized with iodine and alcohol swabs.

T31 fibroblasts were trypsinized and resuspended in 2.5% CMHA-S and 3% Gtn-DTPH in a 1:1 volume ratio. One volume of 4% PEGDA solution was then added to four volumes of cell suspension, and the resulting suspension was mixed gently by vortexing. The final cell concentration was 5×10⁷ cells ml⁻¹. All solutions for the *in vivo* injections were prepared in serum-free growth medium. When the cell suspension was becoming viscous (~3–5 min at 20° C), 100 µl of gel was injected subcutaneously bilaterally into the back of each mouse by means of an 18-gauge needle for a total of six mice. Another six mice received the bilateral injection of same number of cells in MatrigelTM according to the manufacturer's protocol. The concentrations of CMHA-S and Gtn-DTPH were increased compared with the *in vitro* protocols to support a higher cell seeding density.

Animals were euthanized at 4 weeks post-injection in a CO₂ chamber, and the implants with the surrounding tissues were removed from the mice, fixed in 10% buffered formalin solution (Sigma, Milwaukee, WI) for 24 h, embedded in paraffin, cut into 5 µm sections, mounted onto slides, and stained with Hematoxylin and Eosin B (H&E; Fisher Scientific, Hanover Park, IL) following standard protocols. Cellular densities in histological slides from both sECM-derived tissues were microscopically counted at identical magnifications.

2.6. H&E staining

Same culturing conditions and experimental duration were used as described under Section 2.4. The hydrogels were removed from the inserts and fixed for 5 min in methanol. Samples were then further processed via H&E staining at the LDS Hospital Electron Microscopy Laboratory (Salt Lake City, UT). The slides were then analyzed with an Olympus IX70 microscope equipped with Microfire/QCAM CCD (Olympus America Inc., Melville NY) at ×400 magnification.

2.7. Statistical analysis

Values, represented as mean ± standard deviation (SD) were compared using Student's *t*-test (2-tailed) with *p* < 0.05 considered statistically significant and *p* < 0.005 or *p* < 0.001 considered highly significant.

3. Results

3.1. Pseudo-3-D proliferation and morphology

First, the proliferation rates and morphology of fibroblasts cultured on thin layers of different biomaterials or tissue culture plate (TCP) were analyzed. We refer to biomaterial-coated culturing conditions as “pseudo-3-D” because the thin layer of biomaterial provides a metabolizable substratum of variable compliance and composition that cells can penetrate and degrade, while the tissue culture plates used for conventional 2-D cell culture present cells with a stiff, non-metabolizable, impenetrable surface.

Two major morphological patterns were observed: stretched, spindle-like shaped fibroblasts and clustered rounded cells. Cells grown on MatrigelTM adopted a rounded, clustered morphology. In contrast, cells grown on surfaces such as TCP (control), PureColTM, PuraMatrixTM or ExtracelTM had a more spindle-shaped appearance (Fig. 1). This experiment

was repeated three times with consistent results, showing that the observed differences in cellular morphology were clearly attributable to variations in biomaterial nature and composition.

Consistent with the aforementioned results, cell proliferation rates of fibroblasts cultured on various hydrogels were also different (Fig. 2). All statistical results are reported relative to the TCP control. Matrigel™ and PureCol™ sustained a slower cell division rate when compared with cells grown on TCP (control) ($p < 0.001$). Cells grown on Extracel™ elicited similar proliferation rates with those on TCP, up to day 6. On day 7, cell numbers appeared higher on Extracel™ than on TCP ($p < 0.001$), most probably due to 2-D growth surface limitation of the TCP vs. the pseudo-3-D surface of Extracel™. Cells grown on PuraMatrix™ behaved similarly to the control group grown on TCP ($p > 0.05$). This was caused primarily by the inconvenient preparation protocol, which causes an incomplete surface coating with PuraMatrix™; as a result, many cells actually attached to the TCP instead of to the biomaterial.

We did not further characterize T31 fibroblast behavior in pseudo-3-D environments, as the aim of this experimental section was to underscore the importance of transitioning from 2-D to 3-D culture systems for accurate in vivo mimicry. Therefore, we cannot speculate on the different spreading trends on sECMs, since the mechanistic data needed to support any such assumptions were not the object of this study.

3.2. 3-D cell culture

In order to assess cell proliferation rates under 3-D conditions, T31 fibroblasts were encapsulated in hydrogels, as shown in Fig. 3. Three days after seeding, the number of cells in each biomaterial was determined by colorimetric assays. The variance for Matrigel™ was high due to its rapid gelation at room temperature, with the increasing viscosity of the material causing some pipetting inconsistencies. Therefore, when compared with Matrigel™, the composition of hydrogels appears to have a minimal, statistically non-significant influence on cellular properties ($p > 0.05$ relative to Matrigel™; Fig. 4). A small difference in cellular proliferation rates was observed between PuraMatrix™ and Extracel™ ($p < 0.05$). However, during encapsulation of cells in PuraMatrix™, problems similar to those in the pseudo-3-D assays were encountered. Cells cultured in PureCol™ settled to the bottom of the inserts because of the prolonged gelation time of the material (45–60 min at 37°C). Thus, this condition was further excluded from the assay because it failed to mimic a true 3-D growth environment.

3.3. Cytological analysis

The viability and morphology of encapsulated T31 fibroblasts was determined by a double staining procedure that stains live cells green and dead cells red. Three-dimensionally cultured cells were examined 6 days after seeding. This experimental end time-point was arbitrarily chosen based on the assumption that during this time the embedded cells have completely adapted to their new microenvironment. Consistent with the results described above, the composition of biomaterials influenced cell morphology and behavior (Fig. 5). Matrigel™-encapsulated cells appeared to be spread, with fibroblasts eliciting spindle-shaped morphology (Fig. 5A). As already mentioned, PuraMatrix™ and PureCol™ allowed the gravitational settling of cells onto the insert membrane. Cells embedded in both of these biomaterials, grown under pseudo-3-D conditions, had a spindle-shaped morphology (Fig. 5B and C). In Extracel™, cells were three-dimensionally distributed, but were mostly round-shaped (Fig. 5D). Cell viability in all hydrogels was estimated to be 85–90% based on the double staining microscopic evaluation.

The classical approach of quantifying cell viability in 3-D was not feasible here, since there are multiple vertical and horizontal planes in the 3-D microenvironment that could have led to

erroneous interpretations. Thus, Figs. 4 and 5 offer complementary information. Fig. 4 provides a quantitative interpretation of the viability of cells embedded in hydrogels, while Fig. 5 presents additional information on the 3-D embedded cell distribution and morphology.

Next, embedded cells were stained with H&E for a more detailed cytological analysis (Fig. 6). Fibroblasts embedded in Matrigel™ or Extracel™ are organized in clusters that correspond to the structures observed by fluorescent staining. PuraMatrix™ and PureCol™ encapsulated cells appear isolated and concentrated in just a few areas of the hydrogel cross-sections. These observations are consistent with the fact that most of the cells embedded in these materials settled on the bottom of the inserts, resulting in attachment and growth on the insert membrane.

3.4. *In vivo* subcutaneous injections

Based on our preliminary *in vitro* evaluations, Matrigel™ and Extracel™ were the only two biomaterials suitable for injections into immunocompromised mice. To this end, T31 fibroblasts mixed with either Matrigel™ or Extracel™ were injected subcutaneously into nude mice and the quality of the newly formed tissue was assessed. This experiment was modeled on the successful growth of healthy fibrous tissue *in vivo* from T31 cells cultured on an earlier formulation of the sECM [29]. Both matrices were enclosed in a thin layer of fibrous tissue that delimited them from the surrounding native tissues. We could thus confirm that the injected fibroblasts were able to survive and proliferate, and that endogenous cell migration did not occur, or occurred to an insignificant extent.

Differences in cell densities between the two matrices are clearly visible in the histological sections (Fig. 7). Fig. 7A shows the histological analysis after the injection of T31 fibroblasts in Matrigel™ after 4 weeks *in vivo*. Very little degradation of the ECM equivalent has occurred, and some newly formed immature fibrous tissue is evident but with low cell density. In contrast, 4 weeks after subcutaneous injection of T31 fibroblasts in Extracel™ (Fig. 7B), the hydrogel has been almost completely degraded and remodeled into newly formed fibrous tissue, with cell density and ECM deposition resembling that of native fibrous tissue.

4. Discussion

Two-dimensional proliferation assays are useful research tools in providing valuable information regarding cell behavior as a result of genetic manipulation or to evaluate side-by-side various cell lines or types. Analysis of growth factor activity, drug screening and serum batch testing are just a few of the applications that rely on cell proliferation assays [36–39]. Commonly, cells are plated on poly-lysine-coated tissue culture plates, and cell proliferation is assessed for a predetermined period of time. Our experimental results show clear differences in cell morphology and proliferation rates that are dependent on the nature of the culturing surface. Cells could attach and either spread out or stay clustered, all depending on the composition and nature of the material used for culture plate coating. These radically different patterns should definitely be taken into account when designing or selecting assays, especially for experiments such as drug screening, which will be further extrapolated for *in vivo* testing. Moreover, it is important to acknowledge that *in vivo* cells are presented with 3-D microenvironments that provide environmental signals impossible to attain in 2-D substrata.

A more physiological alternative would be the use of 3-D cultures for all *in vitro* cellular testing. These culturing conditions most adequately mimic *in vivo* biology, and experimental results would thus be most pertinent to physiologic situations [1,25,40]. Cellular growth and morphology is heavily influenced by mechanical stimulation and cues coming from the ECM, as well as soluble autocrine and paracrine regulatory signals [7,41]. Matrix composition, stiffness and topography play crucial roles in cellular morphology, behavior and tissue development [42,43].

As evident from the in vitro data, Matrigel™ represents a good choice for 3-D culturing, but its use is exclusively limited to in vitro applications. Since it is a murine sarcoma-derived product, it would be immunogenic in non-murine or immunocompetent hosts. Moreover, Matrigel™ gels rapidly at room temperature, limiting its use to small-scale experiments.

PuraMatrix™ is a synthetic, peptide-based biomaterial that overcomes the issue of the immunogenicity. However, when compared with the other materials tested in this study, it was the most cumbersome to use and gave the least consistent and least satisfactory results. The pH of this material is 3.0, which strictly limits the time of cell exposure to this environment. Furthermore, the gelation procedure for this material requires extensive handling; for example, the medium needs to be changed three times in 30 min. Increased handling increases the risk of cell culture contamination, and thus limits use to small-scale experimental protocols.

PureCol™ is collagen based and has long gelation times (45–60 min at 37°C) that make this material unsuitable as a vehicle for cell retention in vivo or for 3-D applications. However, this material is easy to use, has a very long history in cell culture and is suitable for pseudo-3-D plate coating.

Extracel™ is based on cytocompatible thiol-modified hyaluronan and gelatin components that can be chemically cross-linked in situ. This biomaterial sustains cell growth and proliferation, while eliminating many of the issues posed by other biomaterials. Its preparation protocol is very “user-friendly” and suitable for large-scale experimental protocols, its gelation time can be adjusted by varying pH or temperature, and its compliance can be altered by adjusting the degree of cross-linking [44]. In addition, its nature overcomes the issue of immunogenicity in in vivo applications [27–33]. In this study, the cellular morphology in 3-D encapsulated Extracel™ was mostly rounded rather than the stretched, spindle-shape appearance seen in other scaffolds. Nevertheless, in a separate study we observed that, by simply increasing the ratio of Gtn-DTPH to CMHA-S, the fibroblastic phenotype seen in Matrigel™ could be completely restored in an Extracel™-based sECM with increased gelatin content (M.A. Serban and G.D. Prestwich, unpublished results). In addition, we found that T31 fibroblast proliferation rates were higher in this high-gelatin version of Extracel™ in comparison to Matrigel™.

Finally, injection of T31 fibroblast embedded in Extracel™ into nude mice resulted in the formation of better quality fibrous tissue than when the cells were delivered at the same cell density in Matrigel™. Extracel™ appears to function as a cellular scaffold for the first week or two after the injection, and then is biodegraded and resorbed synchronously with the need of the cells to proliferate and deposit the newly secreted ECM. In contrast, Matrigel™ appears relatively dense, thus potentially limiting cell proliferation at early times post-injection. Moreover, the slower degradation rate of Matrigel™ also limits the deposition of newly formed ECM, resulting in the formation of immature fibrous tissue with low cell density and less cell-secreted ECM.

Taken together, the results of this study underline the diversity of the synthetic scaffolds available and their unique properties, and may serve as a guide to selection of the appropriate application-specific ECM equivalent for both in vitro and in vivo applications. Compositions should be specifically formulated for individual cell types and applications by incorporation of additional ECM components and growth factors specific for each cell type.

5. Conclusion

Classical 2-D culturing has long been the benchmark for cell-based assays. Although the limitations of this technique are becoming widely recognized, at present there is no accepted “TCP equivalent” for the 3-D marketplace [25]. In this study, we evaluated four ECM-

equivalent biomaterials that are presently commercially available for 3-D cultures. While these materials provide researchers with a certain flexibility in selecting the proper substratum for a given cell type and experimental design, it may be that no one 3-D cell culture product can meet all needs. Nonetheless, to accommodate the complexity and diversity [45] of naturally occurring cellular microenvironments, one is best served by an ECM equivalent that offers the greatest experimental flexibility in composition, compliance, methods of use and utility in translating from in vitro formats to in vivo protocols. Cell types and the experimental goals will ultimately dictate the composition and compliance requirements for the ECM equivalent. The data herein offer a rational basis for selecting and optimizing an ECM equivalent for specific 3-D culturing experiments that have the potential to enable a seamless transition from in vitro and in vivo models to clinical applications in regenerative medicine.

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Fig. 1. Morphology of T31 human tracheal scar fibroblasts on thin layers of various sECMs after 5 days in culture. (A) Tissue culture plate (TCP); (B) Matrigel™; (C) PuraMatrix™; (D) PureCol™; (E) Extracel™.

Fig. 2. Pseudo-3-D proliferation of T31 human tracheal scar fibroblasts on wells coated with a thin layer of biomaterials. Values represented are mean $\pm$ SD, $n = 6$. Cell proliferation rates on TCP (control), PuraMatrixTM ($p > 0.05$), PureColTM ($p < 0.001$), MatrigelTM ($p < 0.001$) and ExtracelTM ($p < 0.001$). All reported statistical data are relative to TCP on day 7 data points.

Fig. 3.
Schematic representation of 3-D cell encapsulation into hydrogels (original drawing by Xiaoyu Chen).

Fig. 4. Proliferation rates of fibroblasts encapsulated within various ECM analogues. Columns represent mean $\pm$ SD, $n = 4$. $p > 0.05$ relative to MatrigelTM.

Fig. 5. Live/dead staining of 3-D encapsulated cells. (A) Matrigel™; (B) PuraMatrix™; (C) PureCol™; (D) Extracel™. Green = live cells, red = dead cells.

Fig. 6. H&E staining of hydrogel-embedded cells. Nuclei = purple to dark purple; cytoplasm = pink to light purple. Cells or cell clusters are indicated with arrows. (A) Matrigel™; (B) PuraMatrix™; (C) PureCol™; (D) Extracel™.

Fig. 7. H&E staining of newly formed fibrous tissues after the injection of T31 fibroblasts in either Matrigel™ (A) or in Extracel™ (B). The arrowheads indicate selected regions of undegraded hydrogel.