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Controlled release of TGF-β3 from cartilage extra cellular matrix derived scaffolds to promote chondrogenesis of human joint tissue derived stem cells

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Abstract

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1. Introduction

Articular cartilage has a limited capacity for self-repair, leading to increased interest in tissue engineering and regenerative medicine therapies targeting this tissue. Mesenchymal stem cells or multipotent stromal cells (MSCs) isolated from various tissues such as bone marrow [1-7], subcutaneous fat [8-11], infrapatellar fat pad (IFP) [6, 12-21] and synovium [9, 22-26], combined with various scaffolds and hydrogels, have been explored for tissue regeneration. The IFP is a particularly attractive source of MSCs for this type of application as it is easy to access and provides a large number of cells with the potential to generate cartilaginous tissue [12, 13, 20]. Identifying the optimal combination of material and soluble cues that leads to robust chondrogenesis will be central to the success of any new MSC based therapy for articular cartilage regeneration.

Scaffolds fabricated using devitalized extracellular matrix (ECM) have shown great promise for the regeneration of damaged tissues [27]. This approach has been used to develop different tissue-specific (e.g. heart valves, blood vessels, skin and cartilage [8, 28-44]) scaffolds. In the case of articular cartilage, numerous studies have demonstrated that scaffolds derived from devitalized cartilage are chondroinductive and show great promise for regenerating damaged joints [8, 29-47]. For example, it has been demonstrated that adipose tissue derived stem cells secrete a matrix rich in glycosaminoglycans (GAGs) and type II collagen when seeded onto cartilage ECM derived scaffolds, resulting in the development of a cartilaginous tissue with mechanical properties approaching that of the native tissue after 42 days in culture [43]. One of the factors that may limit ECM derived scaffolds for cartilage tissue engineering applications is cell-mediated contraction of the construct either during *in vitro* culture or following implantation into a defect [44]. To overcome such problems different crosslinking techniques can be used to minimise or prevent contraction such as dehydrothermal (DHT) [28, 42, 48-55], UV light [42, 50] and chemical crosslinking [42, 54, 56, 57]. Chemical methods include the use of glutaraldehyde [54, 58-60], 1-Ethyl-3-3dimethyl aminopropyl carbodiimide (EDAC) [42, 49, 54, 61, 62] and genipin [57]. DHT treatment consists of the removal of water present in the scaffold polymeric chains under vacuum and with temperature. By removing the water from the collagen molecules, a condensation reaction occurs, leading to intermolecular crosslinking [54]. However it is unclear how these different crosslinking methods will influence the chondroinductive properties of cartilage ECM derived scaffolds, with recent studies demonstrating that high levels of crosslinking do not support new cartilaginous ECM accumulation [57].

While cartilage ECM derived scaffolds most likely retain endogenous growth factors that contribute to their chondroinductive properties, it may be that the combination of such a scaffold with additional exogenous growth factors will lead to more robust chondrogenesis of cells that are seeded onto or migrate into the construct. It has been shown that ECM acts as a reservoir for growth factors [27, 31, 47, 63, 64], opening up the possibility of using such materials as growth factor delivery systems. Members of the transforming growth factor-β (TGF-β) family of growth factors, which play a key role in driving chondrogenesis of MSCs [7, 8, 14, 47, 65-67], have a natural affinity for ECM components such as proteoglycans [63, 68]. This suggests that cartilage ECM derived scaffolds could be used as natural growth factor delivery systems, overcoming the need to introduce additional components into a scaffold such as microspheres to control the release of such soluble cues [7, 14, 68-71].

Building on previous work in this field [42, 57], the global aim of this study was to develop a cartilage ECM derived scaffold capable of promoting robust chondrogenesis of human IFP derived stem cells (FPSCs). To this end, we first sought to compare chondrogenesis of FPSCs within a cartilage ECM derived scaffold in the presence or absence of exogenously supplied TGF-β3. Next, we sought to determine whether EDAC crosslinking could be used to prevent scaffold contraction without impacting the chondroinductive properties of the construct. Having fabricated a suitable cartilage ECM derived scaffold, we then compared the chondro-permissiveness of this scaffold to a biomimetic scaffold produced from two key components of cartilage tissue, specifically collagen and hyaluronic acid (HA) [55]. Finally, having demonstrated that a cartilage ECM derived scaffold promotes robust chondrogenesis of human FPSCs in the presence of exogenously supplied TGF-β3, we sought to determine whether the scaffold itself could be used as a delivery system to control the release of this growth factor and hence facilitate chondrogenesis of progenitor cells that are seeded into such a construct.

2. Material and methods

2.1 Scaffold preparation

Cartilage used in the fabrication of ECM derived scaffolds was harvested, in sterile conditions, from the femoral condyles and tibial plateaus of female pigs (3 months old) shortly after sacrifice. The cartilage was first broken up into small pieces (approximately $1mm^3$) using a scalpel. These small

pieces of cartilage where then minced in distilled water (dH_2O) using a homogenizer (IKAT10, IKA Works Inc, NC, USA) to create a cartilage slurry. The homogenized tissue was centrifuged and the supernatant was removed. The remaining material was re-suspended in dH_2O at a concentration of either 500 or 1000 mg/ml. The slurry was transferred to custom made moulds (containing wells 5 mm in diameter and 3 mm in height) and freeze-dried (FreeZone Triad, Labconco, KC, USA) to produce porous scaffolds. Briefly, the slurry was frozen to -30˚C (1˚C/min) and kept at that temperature for one hour. The temperature was then increased to -10˚C (1˚C/min), followed by a hold of 24 hours and then finally increased to room temperature (0.5˚C/min). Next, two different crosslinking techniques were applied to the scaffolds. The scaffolds underwent either DHT crosslinking or both DHT and EDAC crosslinking as previously described in literature [54]. The DHT process was performed in a vacuum oven (VD23, Binder, Germany), at 115˚C, in 2 mbar for 24 hours. The EDAC (Sigma-Aldrich, Germany) crosslinking consisted of chemical exposure for 2 hours at a concentration of 6 mM in the presence of N-Hydroxysuccinimide (NHS) (Sigma-Aldrich, Germany), a catalyst that is commonly used with EDAC. A molar ratio of 2.5 M EDAC/M N-Hydroxysuccinimide was used [54, 61]. After EDAC crosslinking the scaffolds were washed twice in sterile PBS (Sigma-Aldrich, Germany).

Collagen-HA scaffolds were fabricated using a freeze-drying method and were DHT crosslinked, as previously described [55]. Briefly, collagen-HA scaffolds were composed of collagen type I derived from bovine Achilles tendon (Collagen Matrix, USA) and hyaluronic acid sodium salt derived from *streptococcus equi* (Sigma-Aldrich, Arklow, Ireland). The final concentrations of the suspensions were composed of 0.5% (w/v) collagen and 0.05% (w/v) hyaluronic acid (HA) [55].

2.2 Helium ion microscopy (HIM)

Freeze-dried ECM derived scaffolds were imaged using Helium ion microscopy (HIM) (Zeiss Orion Plus, Germany). Image resolution of the microscope is manufacture specified at 0.35 nm, working distance was 10 mm and a 10 µm aperture was used. The beam current was 0.8 pA with a tilt angle of 15 degrees. Charge compensation was enabled using an electron beam flood gun and no additional conductive coating of the specimens was employed.

2.3 Area, porosity and pore size determination

After 28 days in culture constructs were removed from culture and imaged next to a ruler. Pictures were analyzed with Image J to quantify area. Using a previously described approach [72],

mean pore size was determined using Image J. The diameter of 15 pores present in cross-section of each scaffold was measured and averaged (n = 3). Porosity was determined using liquid displacement method (n=10), as previously described in literature [73]. Hexane was used as the displacement liquid [73].

2.4 Cell culture

Ethical approval for the isolation of human FPSCs was obtained from the institutional review board of the Mater Misericordiae University Hospital Dublin. Cells were isolated from the IFP of patients undergoing total joint arthroplasty. The IFPs were harvested from the knee joint capsule, weighed and washed thoroughly in phosphate buffered saline (PBS) (Sigma-Aldrich, Germany). Subsequently, the IFPs were diced in sterile conditions and followed by incubation under constant rotation at 37˚C in high-glucose Dulbecco's Modified Eagle Medium (hgDMEM, GlutaMAX^{TM})(GIBCO, Biosciences, Ireland) containing collagenase type II (750 U/ml, Worthington Biochemical, LaganBach Services, Ireland) and 1% penicillin (100 U/ml)-streptomycin (100 µg/ml) for 4 hours. A ratio of 4 ml of collagenase (750 U/ml) per gram of tissue was found to be optimal based on previous work [15-18]. After tissue digestion, cells were washed, filtered (40 µm nylon cell strainer) and centrifuged at 650 g for 5min. The supernatant (which include the majority of adipocytes) was then removed. The remaining cells were re-suspended, counted and finally plated ($5x10^3$ cells/cm^2) in T-175 flasks (Sarsted, Wexford, Ireland). Cells were cultured in a standard media formulation, which consisted of hgDMEM containing 10% foetal bovine serum and 1% penicillin (100 U/ml)-streptomycin (100 mg/ml) (GIBCO, Biosciences, Ireland) with the addition of fibroblast-growth factor-2 (FGF-2, 5 ng/ml; ProSpec-Tany TechnoGene Ltd, Israel). Cells were expanded to passage 2 (P2), with an initial seeding density of $5x10^3$ cells/cm^2 at each passage. Media changes were performed twice a week.

Each scaffold was seeded with $0.5x10^6$ human FPSCs. Each experiment used FPSCs from different donors. Scaffolds were maintained in chemically defined chondrogenic medium (CDM), as previously described, for 28 days (at 5% O_2 and 37˚C) [15]. CDM consisted of DMEM GlutaMAX^{TM} supplemented with penicillin (100 U/ml)-streptomycin (100 µg/ml) (both GIBCO, Biosciences, Ireland), 100 µg/ml sodium pyruvate, 40 µg/ml L-proline, 50 µg/ml L-ascorbic acid-2-phosphate, 1.5 mg/ml BSA, 1x insulin-transferrin-selenium, 100 nM dexamethasone (all from Sigma-Aldrich, Ireland) and 10 ng/ml recombinant human growth factor-β3 (TGF-β3; ProSpec-Tany TechnoGene Ltd, Israel). In

certain experiments, TGF-β3 (3.2 ng in 40 µl of media) was soak loaded into the scaffold. In such cases, TGF-β3 was not directly added to the culture media. The scaffolds were kept in 12 well plates and each scaffold was placed within cylindrical agarose moulds (slightly bigger than the scaffold) to prevent cell migration into the culture wells. After seeding, the scaffolds with the cells plus 40 µL of CDM were left in the incubator for two hours. After two hours, 2.5 ml of supplemented CDM were added to each well. Media changes were performed twice a week. The media was stored at -85˚C for further analysis.

2.5 Biochemical analysis

Constructs were biochemically analyzed at day 0 (cell free), day 1 and day 28, for sulphated glycosaminoglycan (sGAG) and collagen content. Four scaffolds were analysed for each time point. The scaffolds were enzymatically digested by incubating the constructs in papain (125 µg/ml) in 0.1 M sodium acetate, 5 mM cysteine HCl, 0.05 M EDTA, pH 6.0 (all from Sigma-Aldrich, Ireland) at 60˚C under rotation (10 rpm) for 18 h. The proteoglycan content was estimated by quantifying the sGAG in constructs using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), using bovine chondroitin sulphate as a standard. Collagen content was determined by measuring hydroxyproline content, after acidic hydrolysis of the samples at 110°C for 18 h in concentrated HCL (38%). Samples were assayed using a chloramine-T assay assuming a hydroxyproline/collagen ratio of 1:7.69 [74]. sGAG release from the scaffolds into the media was also determined. To this end, the culture medium was sampled for each construct at each media exchange.

2.6 Histology and immunohistochemistry

The cartilage derived constructs were fixed overnight at 4˚C in a 4% solution of paraformaldehyde (Sigma-Aldrich, Ireland). After being washed in PBS (Sigma-Aldrich, Ireland), samples were cut in half longitudinally, dehydrated and wax embedded. Wax embedded constructs were sectioned in 6 µm thick slices and mounted in microscope slides. Sections were stained with 1% alcian blue 8GX (Sigma-Aldrich) in 0.1 M HCl for sGAG and with picro-sirius red for collagen.

As previously described [15], immunohistochemical analysis was performed on 6 µm sections using monoclonal antibodies to type II collagen (Abcam, UK). Samples were washed in PBS and subjected to peroxidase activity (20 min). Slides were then incubated (1 hour, 37˚C in a moist

environment) with chondroitinase ABC (Sigma, 0.25 U/ml) with the aim of enhancing the permeability of the ECM by removing the chondroitin sulphate. Slides were rinsed with PBS and blocked with 10% goat serum (30 minutes) and incubated with mouse monoclonal anti-collagen type II diluted 1:100 (Abcam, UK) (concentration 1 mg/ml) (1 hour at RT). A secondary antibody for type II collagen (Anti-Mouse IgG Biotin antibody produced in goat) (concentration 1 g/L) binding was then applied (1 hour). By using Vectastain ABC reagent (Vectastain ABC kit, Vector Laboratories, UK) for 5 min in peroxidase DAB substrate kit (Vector laboratories, UK), it was possible to observe a colour alteration. Samples were dehydrated with graded ethanol and xylene and mounted with Vectamount medium (Vector Laboratories, UK).

2.7 ELISA - TGF-B3 release to the media

The amount of TGF-β3 release from the growth factor loaded cartilage ECM derived scaffold was determined via ELISA. 96 well plates were coated with capture antibody, with the concentration of 360 µg/ml of mouse anti-human TGF-β3 (R&D Systems, UK). The samples (8 time points) and TGF-β3 standards (ProSpec-Tany TechnoGene Ltd, Israel) were incubated for 2 hours. After washing and drying, detection antibody (18 µg/ml of biotinylated goat anti-human TGF-β3) was added to the plate and incubated (2 hours). The next step was to wash, dry and incubate the plate in streptavidin-HRP (horseradish-peroxidase) (R&D Systems, UK) for 20 minutes in the dark. Substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine; R&D Systems, UK) was added to each well, followed by incubation (20 min) avoiding direct light. Stop solution (2 N H_2SO_4; Sigma-Aldrich, Germany) was added and the optical density was determined immediately with a plate reader set to 450 nm.

2.8 Statistical analysis

Results are presented as mean ± standard deviation. Statistical analysis was performed with MINITAB 15.1 software package (Minitab Ltd., Coventry, UK). Experimental groups were analyzed for significant differences using a general linear model for analysis of variance with factors including crosslinking technique, material used in scaffold (ECM or collagen-HA) and growth factor supplementation. Tukey's test for multiple comparisons was used to compare conditions. Significance was accepted at a level of $p<0.05$.

3. Results

3.1 Cartilage ECM derived scaffolds promote more robust chondrogenesis of human infrapatellar fat pad derived stem cell in the presence of exogenously supplied TGF-β3

The architecture of scaffolds derived from freeze-dried slurries of cartilaginous ECM was found to depend on the initial concentration (either 500 mg/ml or 1000 mg/ml) of cartilage matrix within the slurry. More homogenous and spherical pores were observed in the 1000 mg/ml scaffolds, whilst less spherical pores and a wider distribution of pore sizes was observed in the 500 mg/ml scaffolds (Figure 1; Table 1).

We next sought to explore if the ECM derived scaffolds, in the absence of exogenously supplied chondrogenic growth factors, could promote chondrogenesis of human FPSCs. The 1000 mg/ml scaffolds were chosen for these experiments based on the results of preliminary studies which demonstrated better handleability and less cell mediated contraction when using the higher concentration scaffolds. Tissues engineered using these ECM derived scaffolds, in the absence of exogenously supplied TGF-β3, stained less intensely for sGAG and collagen deposition after 28 days in culture (Figure 2 A) compared with constructs stimulated with this growth factor (Figure 2 B). Immunohistochemistry revealed that only fragments of articular cartilage used to produce the scaffolds stained strongly for type II collagen when constructs were not additionally stimulated with TGF-β3 (Figure 2). Conversely, more diffuse deposition of type II collagen was observed in constructs where the media was additionally supplemented with TGF-β3. sGAG deposition was also significantly greater within scaffolds additionally stimulated with TGF-β3 (Figure 2 C). In all cases, a certain amount of cell mediated scaffold contraction was observed, with the diameter of the construct reducing by 16.0±4.0% over 28 days in culture (Table 1).

3.2 EDAC crosslinking of ECM derived scaffolds limits cell mediated contraction without suppressing chondrogenesis

In an attempt to minimise cell mediated contraction, scaffolds were additionally crosslinked chemically with EDAC as well as undergoing physical crosslinking with DHT. Collagen and sGAG accumulation within FPSC seeded constructs was unaffected by EDAC crosslinking (Figure 3). Significantly less contraction over 28 days in culture was observed in scaffolds that underwent both EDAC and DHT crosslinking (Figure 4).

3.3 Cartilage ECM derived scaffolds promote at least comparable chondrogenesis to biomimetic collagen-HA scaffolds

We next sought to compare the chondro-permissiveness of the cartilage ECM derived scaffold (DHT crosslinking only) to a biomimetic scaffold produced from two key components of cartilage tissue, specifically collagen and hyaluronic acid [55]. Greater cell mediated contraction was observed in the collagen-HA scaffold compared with the ECM derived scaffold, where the construct diameter reduced by 54.0±6.0% over 28 days in culture (Table 2). Both scaffolds stained intensely for alcian blue, indicating significant sGAGs deposition, while certain regions of the collagen-HA appeared to stain more intensely for picro-sirius red, suggesting greater levels of collagen accumulation in these scaffolds (Figure 5 A & B). Quantitative biochemical assays were undertaken to quantify the composition of the scaffolds at the onset of the experiment (Table 2), the levels of sGAG released into the media over the course of the 28 day culture period and the composition of the scaffold after 28 days of culture (Figure 5). The total level of sGAG synthesised was assumed to be equal to the sum of the scaffold composition at day 28 and that released to the media, less the scaffold sGAG content at day 0. Greater levels of sGAG accumulation were observed within the cartilage ECM derived scaffolds, but no significant difference in total collagen accumulation was observed between the two scaffold types (Figure 5 C, D & E). Interestingly, this was not due to FPSCs synthesising less sGAGs within the collagen-HA scaffold, but rather due to superior retention of proteoglycans within the ECM derived scaffold (Figure 5 C). The overall levels sGAG synthesized during the 28 days of culture were similar for both scaffolds (Figure 5 C).

3.4 An ECM derived scaffold can be used as a delivery system for TGF-β3 to induce chondrogenesis of diseased human infrapatellar fat pad derived stem cells

Having previously demonstrated that superior chondrogenesis was observed in the presence of exogenously supplied TGF-β3, the final phase of the study explored whether an ECM derived scaffold could be used as a growth factor delivery system to promote robust chondrogenesis of FPSCs. It was found that the majority of TGF-β3 that was loaded into the construct was released in a relatively controlled manner over the first 8-10 days of culture, with a burst release (of approximately 25% of the total released) occurring within the first day of culture (Figure 6). Chondrogenesis, as measured histologically (Figure 7 A & B) and using biochemical assays for total sGAG and collagen

accumulation (Figure 7 C & D), appeared comparable in TGF-β3 loaded scaffolds and in scaffolds where the growth factor was added to the media.

4. Discussion

ECM derived scaffolds have shown great promise for cartilage tissue engineering and regenerative medicine applications. This has been attributed to a number of factors, including providing appropriate structural and mechanical cues to resident cells, as well as the retention of bioactive molecules present in the native ECM. We hypothesised that an ECM derived scaffold, and specifically a scaffold derived from a cartilaginous ECM, could additionally be used as a growth factor delivery system to improve chondrogenesis of stem cells that are either seeded onto, or potentially recruited into, such a scaffold. We observed that a cartilage ECM derived scaffold could indeed retain and slowly release TGF-β3, and in doing so drive chondrogenesis of human FPSCs. These findings open up the possibility of using such a construct as an 'off-the-shelf' scaffold for articular cartilage repair, where stem or progenitor cells are either chemotactically recruited into the TGF-β loaded construct [75, 76], or where freshly isolated autologous stromal cells [13, 21, 77] or allogeneic MSCs from a stem cell bank are seeded into the scaffold prior to implantation.

Unlike previous studies exploring the chondroinductive nature of cartilage ECM derived scaffolds for adipose derived stem cells [43, 57], we found that additional supplementation with TGF-β3 was required to induce robust chondrogenesis of IFP derived stem cells. There are a number of reasons that may explain this discrepancy in findings. Firstly, we used MSCs isolated from diseased (osteoarthritic) human IFP tissue as opposed to adipose derived stem cells (ADSCs) from presumably non-osteoarthritic donors. It is still unclear how diseases like osteoarthritis will influence resident MSCs within the joint space. While we have found that FPSCs isolated from osteoarthritic joints possess a comparable chondrogenic capacity to those derived from healthy donors in a pellet culture system (unpublished data), there may still be changes with disease that leave such stem cells less responsible to the cues provided by an ECM derived scaffold. In addition, the scaffold crosslinking methods used in previous studies were different to that utilized in this study [43, 57]. The use of different crosslinking methods and concentrations can influence the degradation kinetics of the scaffold and may alter release rates of growth factors [41], which in turn could explain the limited chondrogenesis we observed in ECM derived scaffolds not additionally supplemented with TGF-β3.

Furthermore, the use of DHT treatment could have degraded/denatured some of the cytokines present in the ECM matrix. It should be noted, however, that a recent comparison of different crosslinking techniques demonstrated that although they all influenced the composition of newly synthesized matrix within the scaffold, that DHT treatment lead to the development of constructs that best matched the composition of native cartilage [42]. Finally, crosslinking will also stiffen the scaffold. Stiffer scaffolds have been shown to suppress chondrogenesis of stem cells [55].

In an attempt to prevent cell-mediated contraction of the ECM derived scaffold, we incorporated an additional EDAC crosslinking step into the fabrication procedure. It has been reported previously that different crosslinking methods (e.g. EDAC) can prevent cell-mediated contraction [42]. EDAC crosslinking had the effect of minimizing contraction, without suppressing cartilaginous ECM accumulation within the cell seeded construct. Such chemical crosslinking may be necessary to prevent scaffold contraction *in vitro* and *in vivo* and to ensure mechanical stability of the construct [42]. Reduction of contraction associated with matrix formation may be necessary in for the regeneration of cartilage defects as it should support integration of the ECM derived scaffold with the surrounding tissue. Further *in vivo* studies are also necessary to examine other untested impacts of EDAC crosslinking, including immunological response [78].

An alternative to the use of decellularized ECM as a scaffold for cartilage repair are biomimetic scaffolds fabricated using specific ECM components. Several natural biomaterials derived from components of ECM have been used as scaffolds for tissue regeneration including collagen, chondroitin sulphate and hyaluronic acid [55, 79]. Collagen-based scaffolds incorporating chondroitin sulphate and hyaluronic acid, key sGAGs in cartilage ECM, have previously been shown to promote the proliferation and chondrogenic differentiation of MSCs [55]. We found that sGAG accumulation was higher in FPSC seeded cartilage ECM derived scaffolds compared to collagen-HA acid scaffold, primarily due to greater retention rather than superior synthesis of sGAGs. Structural differences between the two scaffold types, such as porosity, or the greater levels of cell mediated scaffold contraction in the collagen-HA scaffolds, may explain these differences.

The final phase of the study sought to manipulate a key property of ECM, namely that certain matrix components such as collagen and proteoglycans can act as growth factor reservoirs [63, 68], to deliver TGF-β3 from such scaffolds in a controlled manner. Proteoglycans present in the

pericellular matrix (PCM) and interstitial ECM have been shown to bind and modulate TGF-β3 supply and consequently control their availability [63]. The majority of the growth factor was released from the scaffolds within the first 10 days of culture. A certain amount of scaffold degradation may have occurred in that timeframe, although it is unlikely that this is the only mechanism by which TGF-β3 is released. Rather it is suggested that the majority of TGF-β3 is not permanently bound to the ECM, allowing the growth factor to be released within what appears to be an optimal dosing window [80]. Previous work has demonstrated that two weeks of *in vitro* culture in the presence of TGF-β3 is sufficient to promote robust expression of collagen type II, aggrecan and sox 9 in MSCs [81]. Furthermore, proteoglycan deposition in poly(ethylene glycol) diacrylate hydrogels seeded with human MSCs was shown to be enhanced by the temporal withdrawal of TGF- β3 from the media [82]. Therefore the timeframe over which this growth factor is released by the ECM derived scaffold may be near optimal for promoting robust chondrogenesis of MSCs. Further experiments are required to verify this hypothesis.

5. Conclusion

In conclusion, we found that the combination of a porcine cartilage ECM derived scaffold and stimulation with TGF-β3 can induce robust chondrogenesis of human diseased infrapatellar fat pat derived stem cells. When compared with a well-established chondro-inductive collagen-HA scaffold, we found that this ECM derived scaffold was at least as effective in promoting chondrogenesis of FPSCs. The finding that such an ECM derived scaffold can be used as delivery system for TGF-β3 to induce chondrogenesis of MSCs opens the possibility of using such a construct as an “off-the-shelf” product for cartilage tissue regeneration. For clinical translation it will be necessary to ensure that minimal porcine DNA remains within the cartilage ECM-derived scaffold to prevent eliciting an inappropriate immune response upon implantation [83]. Future work will focus on appropriate decellularization of this cartilage ECM-derived scaffold.

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Figure and table captions

Figure 1 - Porous scaffolds at day 0. Alcian blue staining of freeze-dried extracellular matrix (ECM) derived scaffolds (**A**) 500 mg/ml and (**B**) 1000 mg/ml at day 0. Helium ion microscopy (HIM) micrographs of porous freeze-dried ECM derived scaffold (**C**) 500 mg/ml and (**D**) 1000 mg/ml at day 0 (scale bar: 100µm).

Figure 2 - Robust chondrogenesis with exogenously supplied TGF-β3. Alcian blue (AB), picro-sirius red (PR) and collagen type II (Coll II) staining of ECM derived scaffold histological sections, after 28 days of culture. (**A**) No TGF-β3 supplementation; (**B**) With TGF-β3 supplementation. Higher

sulphated glycosaminoglycan (sGAG) and collagen accumulation in the supplemented group (scale bar: 100µm). Biochemical assays results for ECM derived scaffold with no TGF-β3 supplementation and with TGF-β3 supplementation seeded with human infrapatellar fat pad derived stem cells (FPSC). (**C**) sGAG and (**D**) Collagen content (n=4, *p□0.05).

Figure 3 - Chondrogenesis was not affected by EDAC crosslinking. Alcian blue (AB), picro-sirius red (PR) and collagen type II (Coll II) staining of ECM derived scaffolds after 28 days of culture. (**A**) Dehydrothermal (DHT) crosslinking; (**B**) DHT + 1-Ethyl-3-3dimethyl aminopropyl carbodiimide (EDAC) crosslinking. Similar sGAG and collagen accumulation in both groups (scale bar: 100µm). Biochemical assays results for ECM derived scaffold DHT and DHT + EDAC seeded with human FPSC. (**C**) sGAG content and (**D**) Collagen content (n=4, *p□0.05). Day 0 values for EDAC group of sGAG is 82.7±15.0 µg and 1116.8±139.8 µg for collagen.

Figure 4 - EDAC crosslinking limits contraction. Area for ECM derived scaffolds with DHT crosslinking: with and without EDAC after 28 days in culture. (**A**) DHT only; (**B**) DHT + EDAC crosslinking; (**C**) Scaffolds area in mm^2 (n=6, *p□0.05).

Figure 5 - Comparable chondrogenesis with a collagen-hyaluronic acid (coll-HA) scaffold. Alcian blue (AB), picro-sirius red (PR) and collagen type II (Coll II) staining of ECM derived scaffold and coll-HA histological sections, after 28 days of culture. (**A**) ECM derived scaffold; (**B**) coll-HA scaffold (scale bar: 100µm). Biochemical assays results for ECM derived scaffold and coll–HA scaffold seeded with human infrapatellar fat pad derived stem cells (FPSC). (**C**) sGAG for ECM derived scaffold and coll-HA scaffold. sGAG values for day 28 in both constructs, in the media and total sGAG synthesized, by subtracting day 0 values (n=6). Coll-HA scaffold lost to the media the majority of the sGAG synthesized sGAG synthesized was calculated by subtracting day 0 value to total. (**D**) sGAG content per wet weight; (**E**) Collagen content per wet weight. Significantly higher sGAG accumulation for the ECM derived scaffold and similar collagen content when compared with coll-HA scaffold (n=4, *p□0.05).

Figure 6 - TGF-β3 release profile. ELISA results for TGF-β3 release into the media from the TGF-β3 loaded ECM derived scaffold (n=3). Cumulative release values are presented as a percentage of the initial amount of TGF- β3 loaded into the scaffold.

Figure 7 - ECM derived scaffold loaded with TGF-β3 can induce robust chondrogenesis. Alcian blue (AB), picro-sirius red (PR) and collagen type II (Coll II) staining of ECM derived scaffold loaded with TGF-β3 and TGF-β3 in media, after 28 days of culture (scale bar: 100µm). (**A**) TGF-β3 loaded; (**B**) TGF-β3 in media. Similar sGAG and collagen accumulation for both groups (n=4).

Table 1 - ECM derived scaffold 500 and 1000 mg/ml (DHT crosslinked) parameters before culture. Note that there is batch-to-batch variability in these parameters. Values presented are mean ± standard deviation.

Table 2 - ECM derived scaffold 1000 mg/ml and collagen-hyaluronic acid (DHT crosslinked) parameters before culture. Note that there is batch-to-batch variability in these parameters. Values presented are mean ± standard deviation. FPSCs from different donors were used in each experiment.

Figure 1

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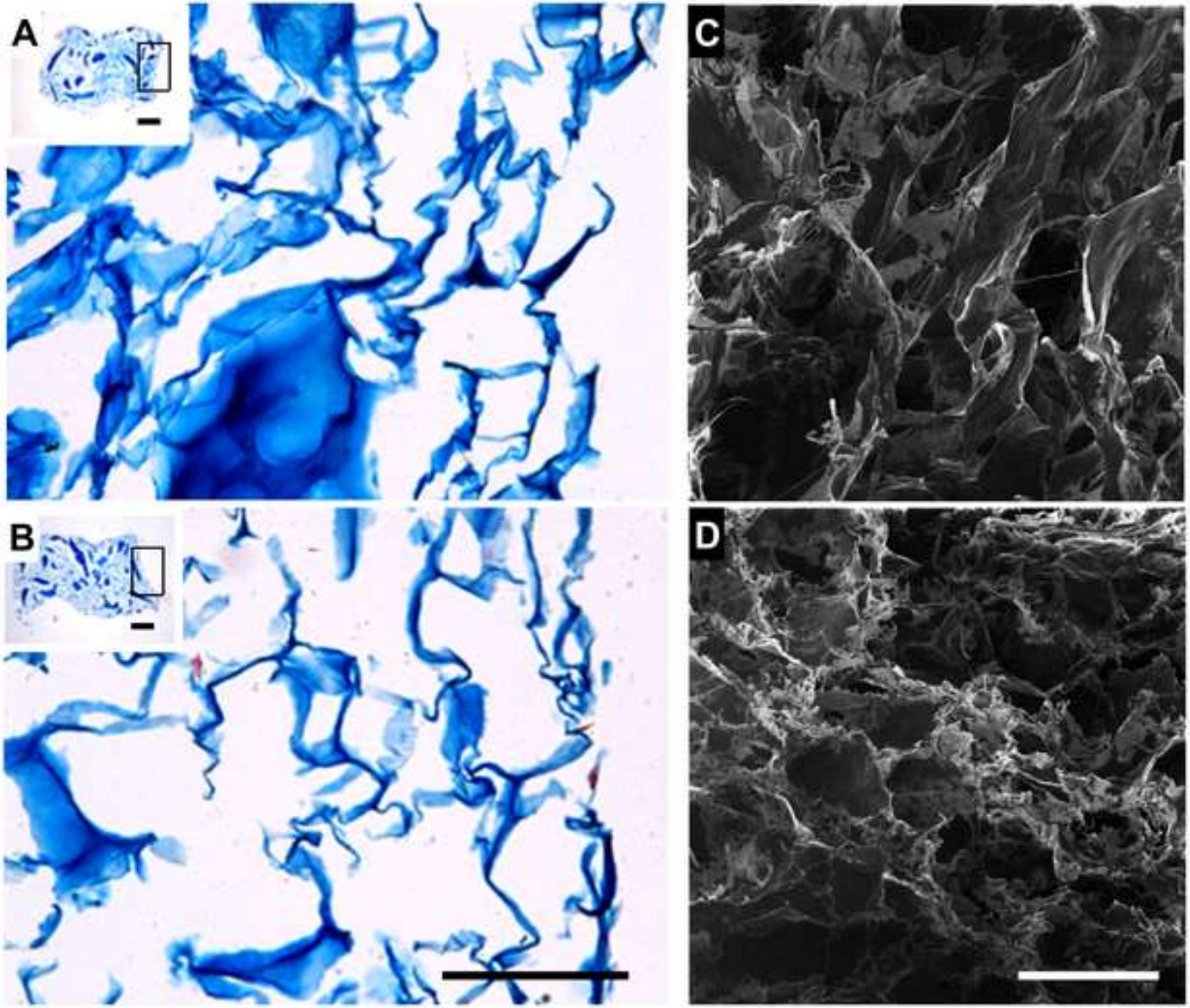

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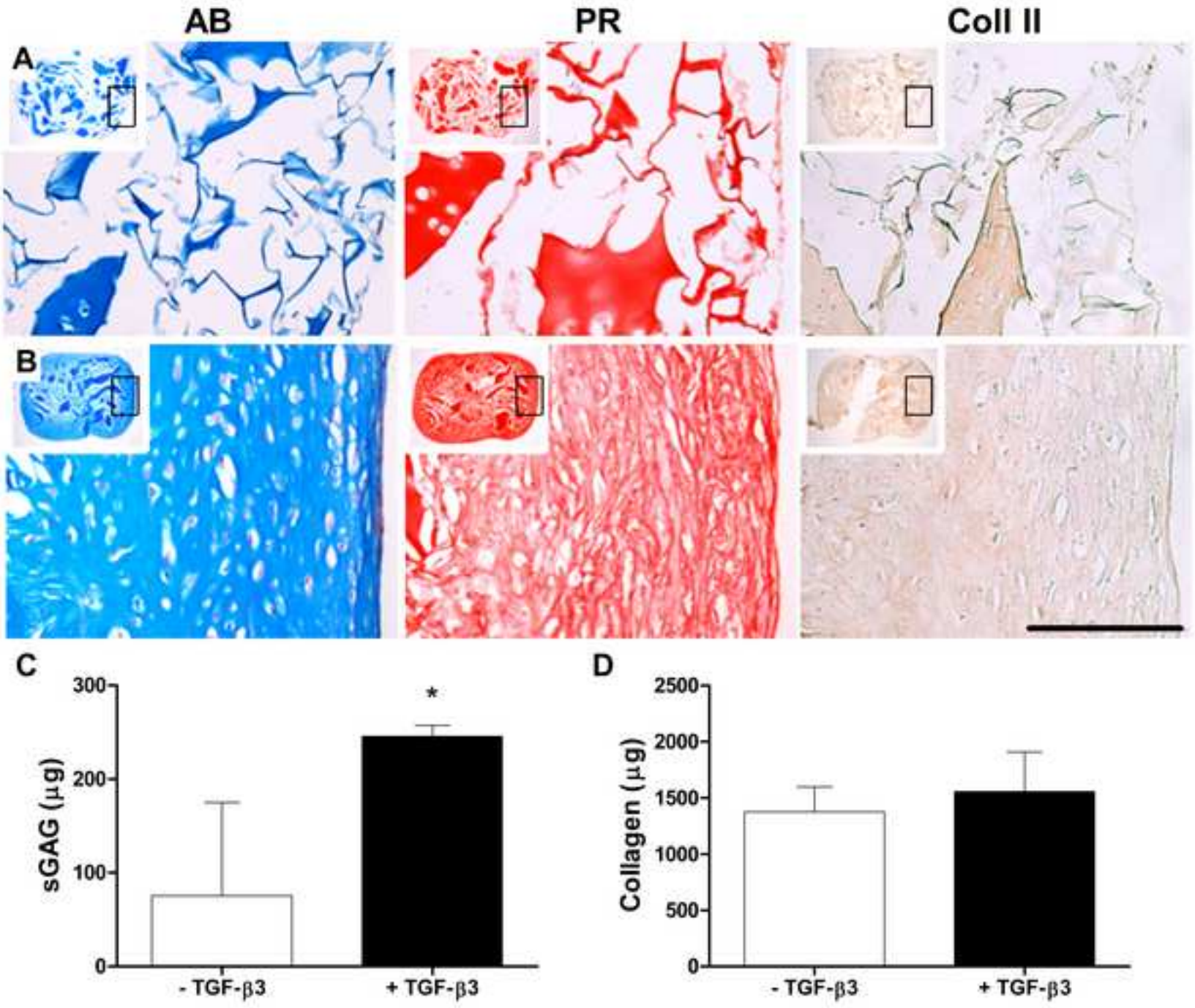

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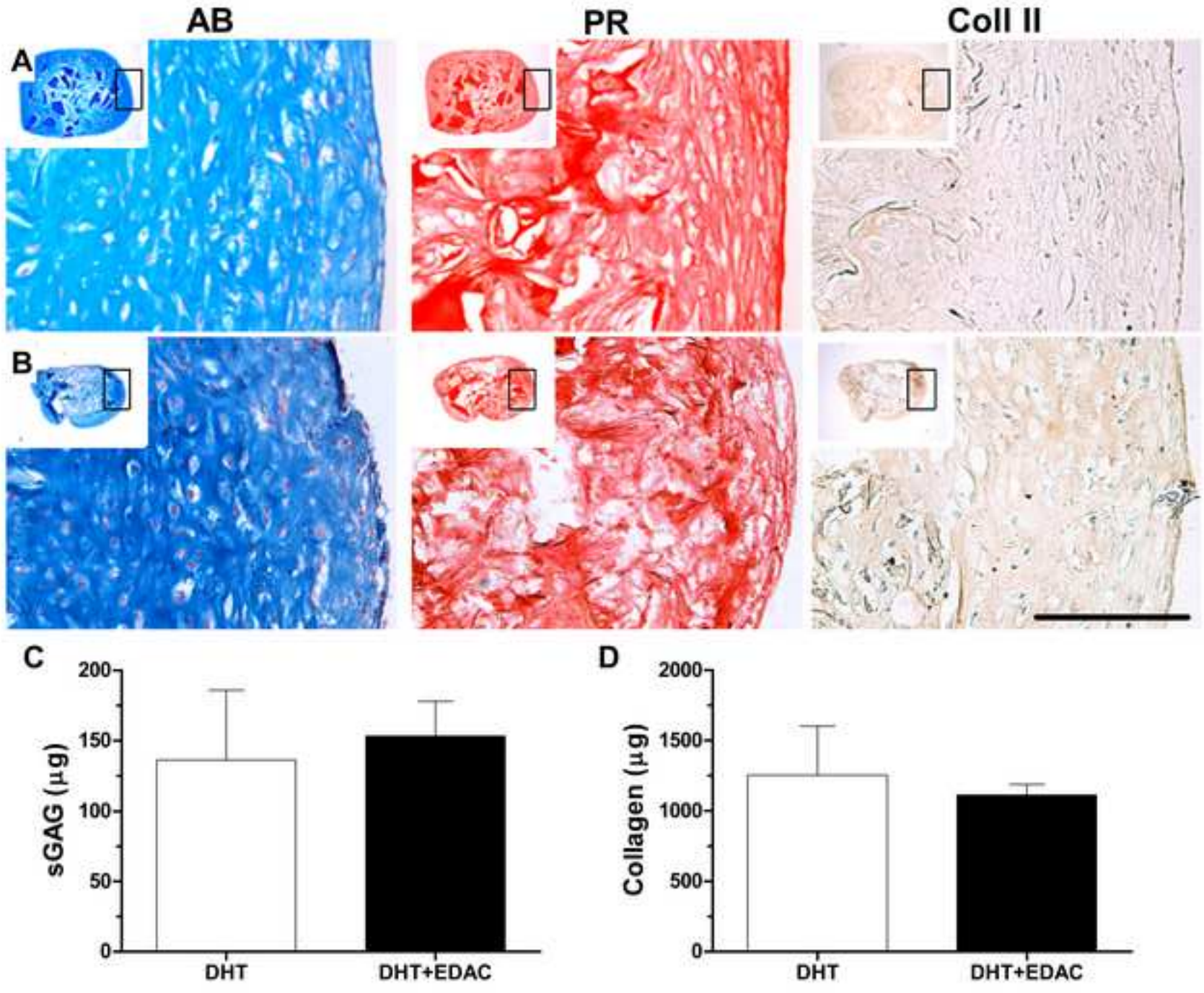

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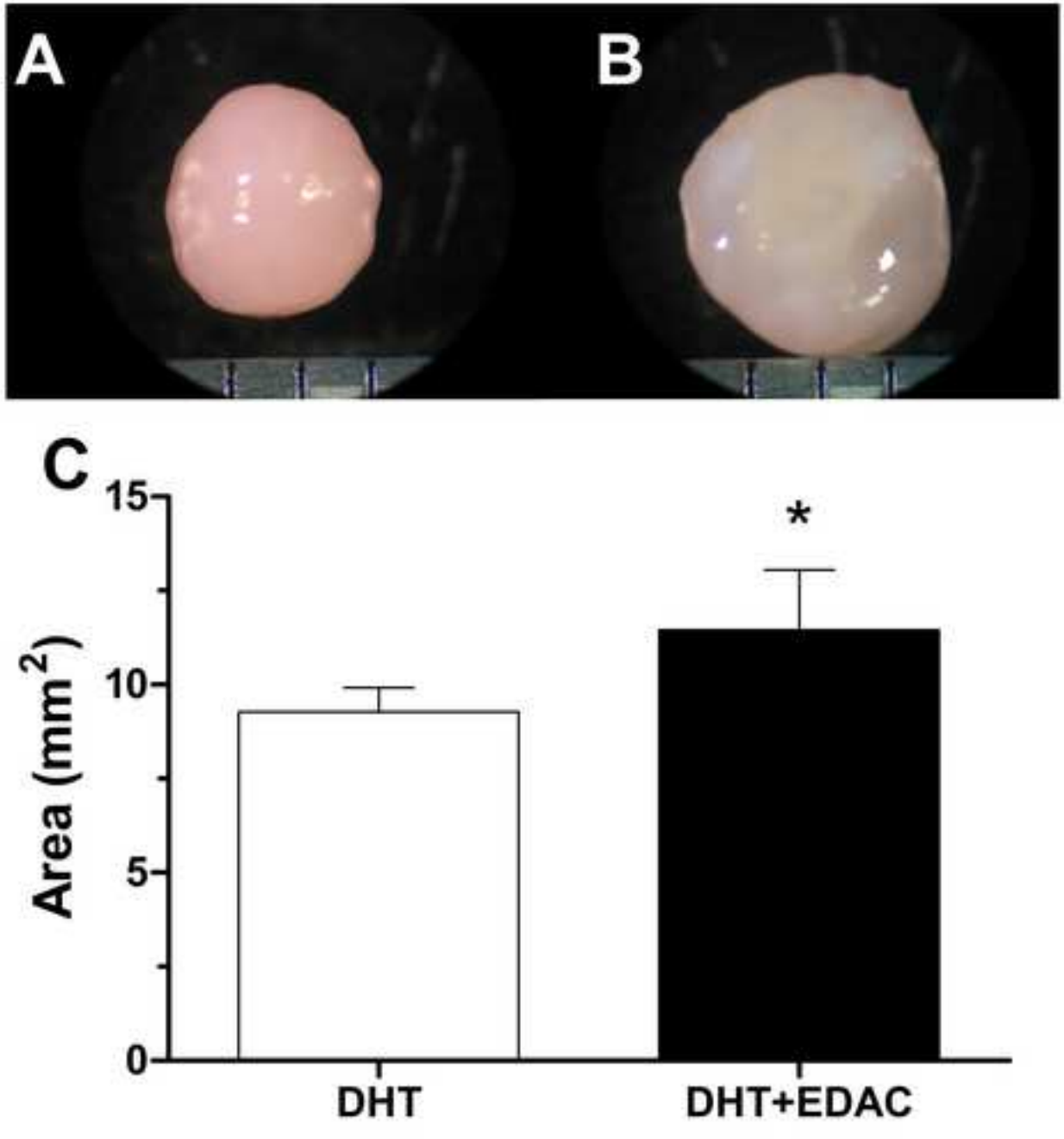

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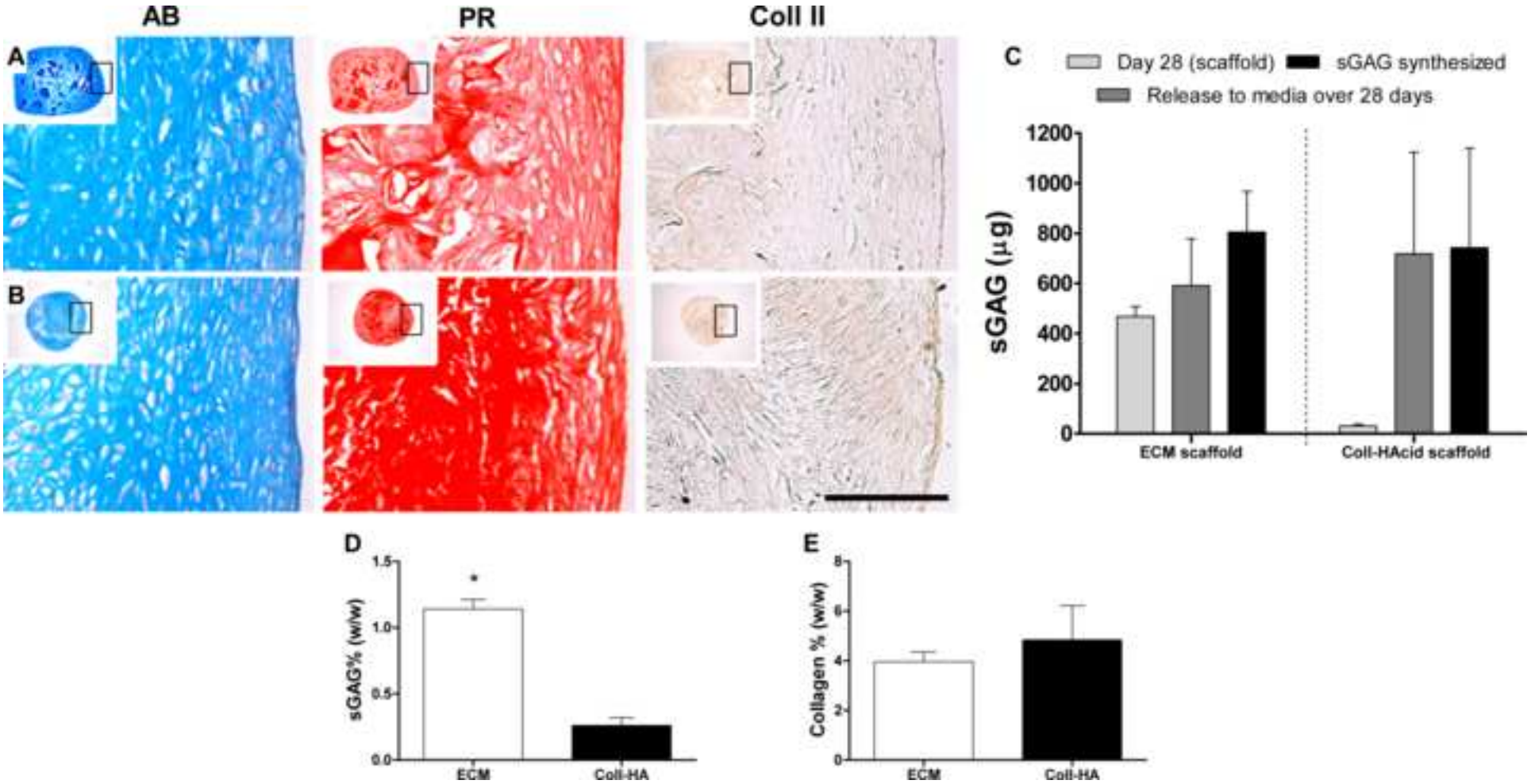

Figure 6

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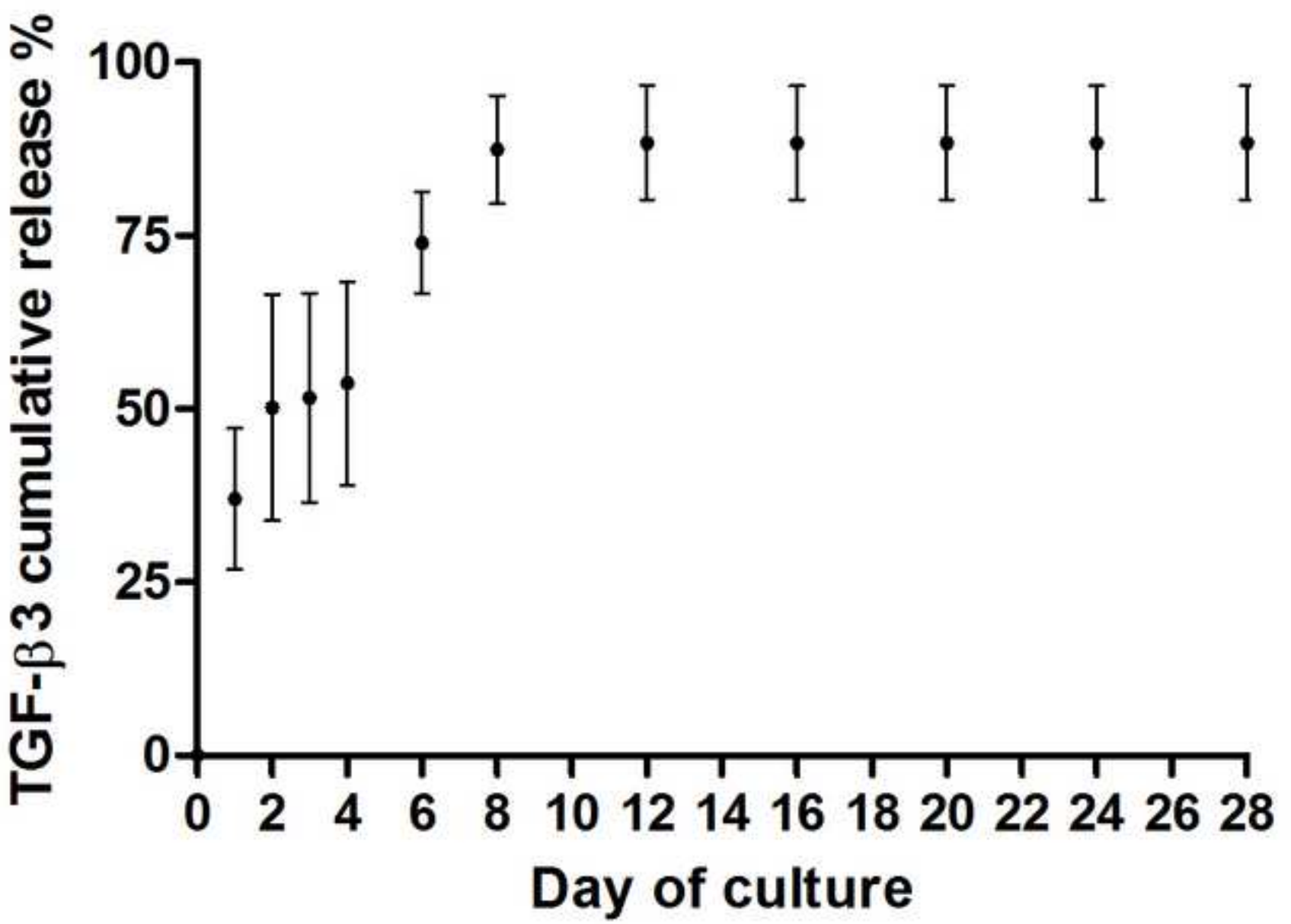

Figure 7

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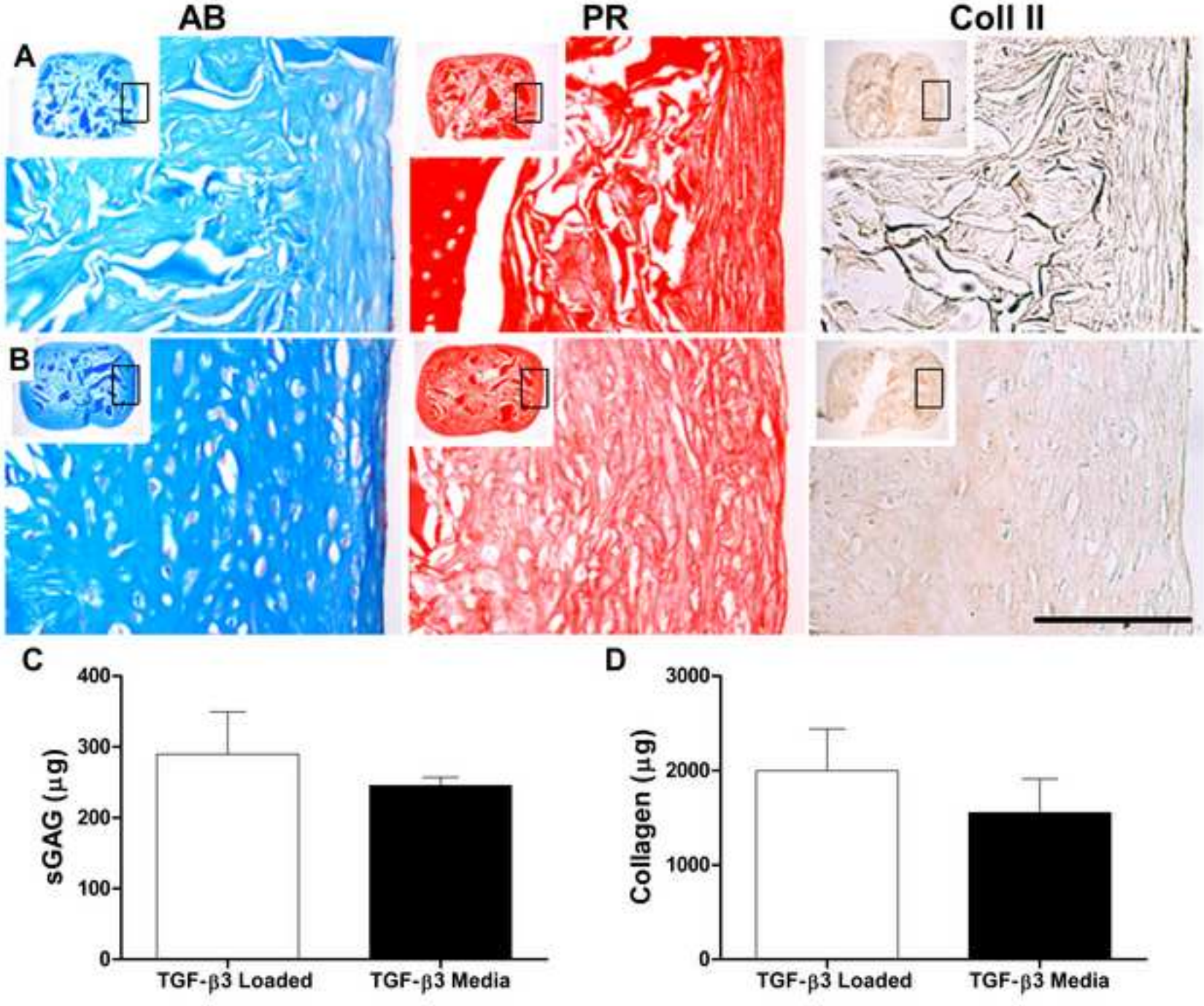


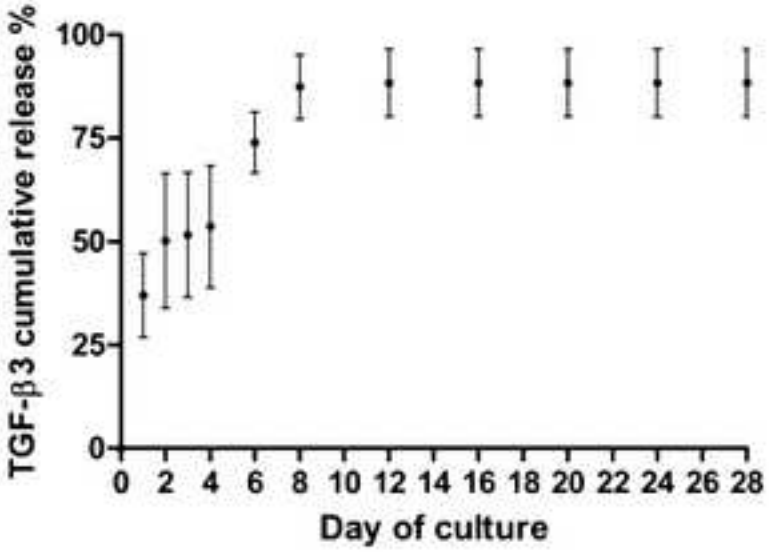
Table 1

	ECM 500	*ECM 1000*
GAG day 0 (µg)	83.0±12.3	253.4±22.5
GAG day 28 (µg)	-	245.5±11.5
Collagen day 0 (µg)	804.0±162.3	1173.9±86.6
Collagen day 28 (µg)	-	1555.5±354.8
Porosity (%)	94.3±1.5	88.2±2.2
Pore size (µm)	104.2±49.2	98.9±37.2
Contraction (%)	-	16.0±4.0

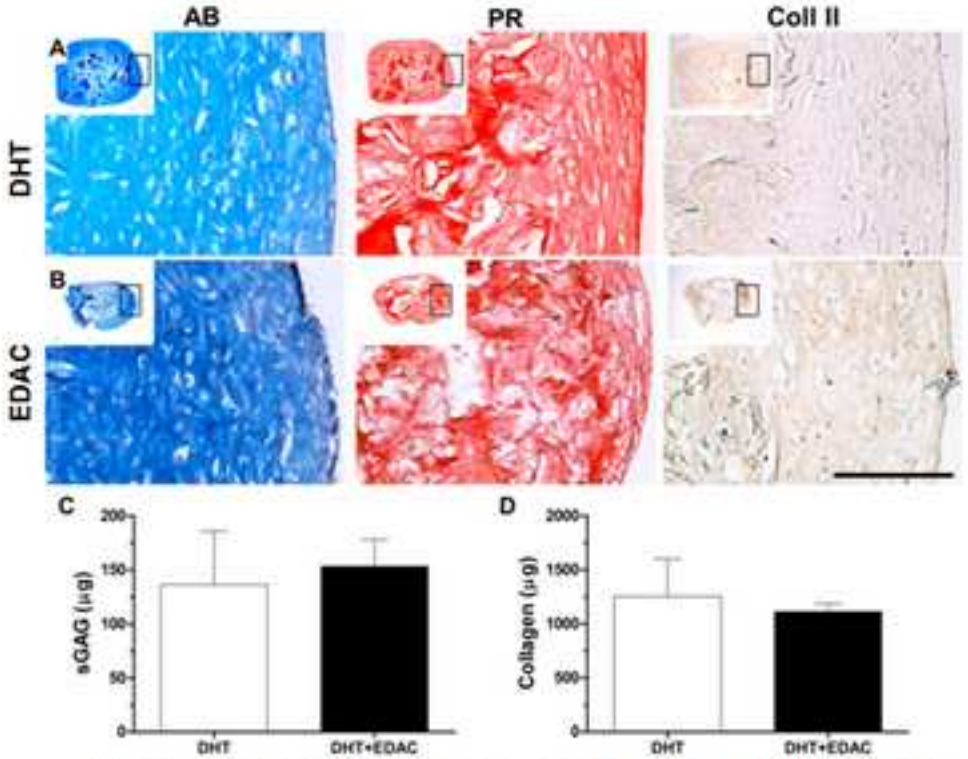
Table 2

	ECM 1000	*Collagen-HA*
GAG day 0 (µg)	253.4±22.5	6.1±8.7
GAG day 28 (µg)	473.5±34.7	29.4±6.0
Collagen day 0 (µg)	1173.9±86.6	468.6±98.8
Collagen day 28 (µg)	1639.3±144.7	542.5±135.3
Porosity (%)	88.2±2.2	98.0±1.0
Pore size (µm)	98.9±37.2	300.1±8.3
Contraction (%)	14.0±3.0	54.0±6.0

*Graphical Abstract



Porous ECM derived scaffold delivered growth factor enhancing chondrogenesis



EDAC crosslinking suppressed contraction without compromising chondrogenesis