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Engineered Microtissues formed by Schiff Base Crosslinking

Restore the Chondrogenic Potential of Aged MSCs

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Abstract

A universal method for reproducibly directing stem cell differentiation remains a major challenge for clinical applications involving cell-based therapies. The standard approach for chondrogenic induction by micromass pellet culture is highly susceptible to interdonor variability. We report here a novel method for the fabrication of condensation-like engineered microtissues (EMTs) that utilizes hydrophilic polysaccharides to induce cell aggregation. Chondrogenesis of MSCs in EMTs is significantly enhanced compared to micromass pellets made by centrifugation measured by type II collagen gene expression, dimethylmethylene blue (DMMB) assay, and histology. MSCs from aged donors that fail to differentiate in pellet culture are successfully induced to synthesize cartilage-specific matrix in EMTs under identical media conditions. Furthermore, the EMT polysaccharides support the loading and release of the chondroinduction factor transforming growth factor β 3 (TGF- β 3). TGF β -loaded EMTs (EMT^{+TGF}) facilitate cartilaginous tissue formation during culture in media not supplemented with the growth factor. The clinical potential of this approach is demonstrated in an explant defect model where EMT^{+TGF} from aged MSCs synthesize de novo tissue containing sGAG and type II collagen in situ.

1. Introduction

Identifying appropriate culture conditions for reproducibly directing stem cell differentiation is key to unlocking the potential of cell-based regenerative medicine therapies. This is especially true for articular cartilage, which is an avascular tissue with limited access to progenitor cells which could drive repair of damaged tissue. Though the use of autologous chondrocytes for treating focal cartilage defects has been demonstrated,^[1] and compared favorably to other treatment methods including microfracture and mosaicplasty,^[2] the procedure is far from ideal. Harvesting chondrocytes from a biopsy of a patient's own cartilage causes donor site morbidity.^[3] Furthermore, expansion of chondrocytes in 2D culture results in loss of cell phenotype and, therefore, a reduced potential to promote healing.^[4] Mesenchymal stem cells (MSCs) are an intriguing alternative cell source for cartilage repair strategies given their relative ease of isolation^[5] and ability to differentiate into chondrocytes,^[6] but they also have limitations. A major barrier to adoption of MSC-based therapies is the reported drop in chondrogenic differentiation capacity with increasing patient age,^[7-9] particularly problematic due to the correlation between increased age and a higher likelihood of cartilage-related pathology.^[10]

The chondrogenic capacity of MSCs could be significantly enhanced by an engineering approach to recapitulate key events in the developmental process of cartilage formation.^[11] During development, MSC chondrogenesis is triggered early on by an event known as condensation.^[12] During condensation, the up-regulation of signaling molecules such as transforming growth factor beta (TGF- β) and N-cadherin induces clustering of cells into high density micromasses.^[13] The condensation period typically lasts for 12 hours,^[14] after which production of hyaluronan, chondroitin sulfate, and other glycosaminoglycans (GAGs) increases the space between cells and induces cartilage-specific matrix production.^[15] MSC condensation is typically mimicked in vitro by culturing cells as micromass pellets formed either by centrifugation^[16, 17] or hanging drop culture^[18] in media containing TGF- β 3. Since it was first demonstrated in the 1990s,^[17] in vitro chondroinduction of MSCs using micromass pellets has become a

standard tool for studying cartilage development,^[19] to evaluate the chondrogenic potential of new stem cell sources,^[20, 21] and also more recently to evaluate the ability of MSCs to heal cartilage defects.^[22] These methods, however, often produce densely compacted structures that inhibit efficient transport of induction factors and other nutrients to the core of the pellet limiting production of cartilage matrix to the pellet periphery.^[7, 23] This limitation is amplified for aged MSCs that are known to express fewer receptors for growth factors (GFs) involved in differentiation,^[24, 25] indicative of a cell population with a reduced sensitivity for directed differentiation. Moreover, the fact that micromass pellets require culture media containing TGF- β 3 necessitates an extensive in vitro culture period which significantly delays the time at which cells could be implanted to treat an injured patient.

The aim of this study was to engineer a method for recapitulating the MSC condensation with the capability of consistently inducing chondrogenic differentiation for a wide range of patient age groups. To achieve this, modified polysaccharides were utilized for organizing MSCs into high density aggregates, or microtissues, by a rapid Schiff base crosslinking reaction. These polysaccharides share structural similarities to those involved in cartilage development which are important for allowing MSCs to deposit the tissue matrix during morphogenesis. We hypothesized that the hydrophilic 3D polysaccharide network would facilitate homogeneous differentiation of densely packed cells within the engineered microtissues (EMTs). Additionally, we demonstrated that TGF- β 3 could be simultaneously encapsulated within the polysaccharide network during microtissue formation and its presence was sufficient to induce chondrogenesis of co-encapsulated MSCs. We showed the significant superiority of EMTs over centrifuged micromass pellets in their ability to induce chondrogenesis of aged MSCs that failed to differentiate using conventional culture methods. Finally, we demonstrated the clinical significance of this approach for treating defects in an in vitro cartilage repair assay.

2. Results and Discussion

2.1. Characterization of Modified Polysaccharides

Hyaluronic acid (HA), chondroitin sulfate (CS), and alginate (Alg) were oxidized by sodium periodate (NaIO_4), which cleaves vicinal hydroxyl groups generating reactive aldehyde groups. The chemical structure of the oxidized polysaccharides (oxPS) were confirmed by ATR-FTIR. Distinct absorption peaks for aldehyde carbonyl stretching vibration ($\nu_{\text{C=O}}$) were observed at 1735 cm^{-1} (**Figure 1A**). Unmodified chitosan is insoluble at physiological pH. Partial amine-substitution by succinylation disrupts the chitosan crystallinity domains, which permits solubility at pH 7.4,^[26] but reduces the number of available amines for Schiff base crosslinking. The degree of substitution of N-succinyl chitosan (sChi) was 34% as calculated from $^1\text{H-NMR}$ spectra (Figure 1B). Interaction between modified polysaccharides was verified by quartz crystal microbalance with dissipation monitoring (QCM-D) which showed that oxPS were able to form durable multilayers upon alternating adsorption with sChi, which was not the case with the native polysaccharides (Figure S1).

2.1 Engineered Microtissues (EMTs) by Schiff Base Crosslinking of Modified Polysaccharides

Human bone marrow mesenchymal stem cells (MSCs) rapidly formed condensation-like 3D microtissues as the result of Schiff base crosslinking between modified polysaccharides. Cells were suspended at $20 \times 10^6\text{ cells mL}^{-1}$ in (sChi) and $10\ \mu\text{L}$ of the mixture was subsequently mixed with a $5\ \mu\text{L}$ droplet of either oxidized hyaluronic acid (oxHA), oxidized chondroitin sulfate (oxCS), or oxidized alginate (oxAlg) (**Scheme 1**). Within 25 minutes of mixing, Schiff base crosslinking between the primary amine groups present in sChi and the free aldehyde groups in the oxidized molecules resulted in the entrapment of cells within a polysaccharide network (**Figure 2A**). Initial experiments were carried out to optimize the formation process of engineered microtissues that included testing the volume ratios of the Schiff base pairs, their concentrations, and the ideal cell seeding density (data not shown). Engineered

condensations formed by Schiff base crosslinking and micromass pellets formed by centrifugation were cultured in chondrogenic induction media containing TGF- β 3 for 21 days. Gross morphology of samples was observed via brightfield microscopy and macro-lens photography (Figure 2B). After 21 days, all samples grew in diameter and shared characteristic cartilage features appearing opaque and white with shiny surfaces (Figure 1B, bottom).

2.2 Enhanced MSC Chondrogenesis in EMTs

Chondrogenic induction was quantified by the dimethylmethylene blue assay (DMMB), which was used to assess the amount of sulfated glycosaminoglycans (sGAG) produced. DMMB values were normalized to the amount of DNA measured per sample during culture of MSCs in centrifuged pellets and in engineered condensations made with sChi and either oxHA, oxCS, or oxAlg. By day 21, MSCs cultured in microtissues formed with sChi and oxAlg had synthesized 4x more sGAG per cell than MSCs cultured in centrifuged pellets (Figure 2C; oxAlg, 189.5 μg sGAG/ μg DNA vs. Centrif., 47.1 μg sGAG/ μg DNA, $p < 0.001$) and more than double the amount synthesized in oxHA microtissues (81.3 μg sGAG/ μg DNA, $p < 0.01$). Levels of dsDNA quantified by PicoGreen remained constant for all samples during the 3 weeks in culture and no statistically significant differences were measured (Figure 2C). MTS assay indicated a slightly higher activity of cells in microtissues made with sChi and oxAlg when compared with other sample types, though these differences were not statistically significant (Figure 2E, Day 1, Centrif. vs. oxAlg, $p = 0.054$).

The diminished chondrogenic response in the presence of HA was unexpected considering reports of improved MSC chondrogenesis in HA-based hydrogels.^[27] However, looking further into the specific role that HA plays during condensation may explain the reduced chondrogenic induction potential observed here. While small amounts of HA are needed to facilitate condensation,^[28] an excess of HA has been shown to inhibit cell aggregation.^[29] Higher HA concentrations possibly lead to

outcompeting with endogenously produced HA for CD44 binding sites on MSC surfaces,^[30] which would hinder chondrogenesis. One possible explanation for the enhanced cartilage matrix production with oxAlg may be due to increased crosslinking with sChi compared to sChi with either oxHA or oxCS. In polysaccharide modifications, HA, CS, and Alg were maximally oxidized with a 2x molar ratio of NaIO₄ to ensure complete cleavage of vicinal hydroxyls. As disaccharide polymers, only one of the sugar groups in HA and CS are susceptible to NaIO₄ oxidation compared to alginate, which can be oxidized at each sugar group (Figure 1). The degree of oxidation is correlated with swelling, crosslinking density, and degradation rates following Schiff base polymerization of polysaccharides,^[31, 32] all of which could in turn impact the mechanical properties of the engineered condensations and the behavioral changes of encapsulated cells. The concentration (w/v) of the various oxPS was selected to remain constant in these studies and sChi with oxAlg was the best combination for promoting the highest levels of MSC chondrogenesis. Based on these results, microtissues formed by sChi and oxAlg were termed engineered microtissues (EMTs) and selected for comparison with the standard controls of centrifuged pellets and further development of the engineered microtissue method.

Histology was used to detect the deposition of cartilage-specific ECM within EMTs and pellets made by centrifugation (**Figure 3**). After 21 days of culture in chondrogenic media, MSCs in both centrifuged pellets and in EMTs exhibited homogeneous deposition of sGAG as visualized by Alcian blue staining (Figure 3A). However, immunohistochemical staining for type II collagen revealed a striking difference in type II collagen deposition. Deposition of type II collagen, an important marker of cartilage,^[33] was limited to the periphery of pellets but was homogeneously distributed over the microtissue structure in EMTs (Figure 3A). In both culture conditions, type I and type X collagen staining was visualized as punctate depositions within the tissues, though to a much lesser extent than type II collagen. Image J was used to quantify the percentage of the tissue cross sectional area that stained positively for type II collagen. On average, EMTs exhibited 80% homogeneity of type II collagen deposition versus less than 25% for the centrifuged pellets (Figure 3B, $p < 0.001$). Furthermore,

evaluation of cartilage matrix deposition over time by Alcian blue staining revealed that MSCs in pellets begin first synthesizing sGAG at the outer surface, while in EMTs the sGAG is first detected at the microtissue core and propagates outward over time (Figure 3C). This visual and quantitative evidence taken together points to an improved efficiency of chondroinduction on a per cell basis within the engineered microtissues.

2.3 Restored Chondrogenic Potential of Aged MSCs in EMT Culture

MSCs from healthy donors were classified into groups according to donor age. Donors above 70 years old were considered 'aged' and donors younger than 55 years old were classified as 'young' (N=6, 3 males and 3 females for each group). MSCs from these two age ranges were cultured as centrifuged pellets or EMTs in chondrogenic media for 21 days. Quantitative data measuring MSC chondrogenesis indicated a clear age-related difference between the two donor groups, both in normalized sGAG production and expression of type II collagen (**Figure 4**). In centrifuged pellets of young donors there was a nearly 7-fold increase in sGAG synthesis compared to the pellets from aged donors (Figure 4A, Centrif.; young, 139.3 $\mu\text{g sGAG}/\mu\text{g DNA}$ vs. aged, 20.4 $\mu\text{g sGAG}/\mu\text{g DNA}$, $p < 0.01$). A similar trend was observed for cells cultured in EMTs that showed a 2-fold increase of sGAG production for young donors over aged donors (Figure 4A, EMT; young, 234.9 $\mu\text{g sGAG}/\mu\text{g DNA}$ vs. aged, 123.6 $\mu\text{g sGAG}/\mu\text{g DNA}$, $p < 0.05$). The age dependent trend was also observed in qPCR measurements of type II collagen expression where fold change was elevated to 5x and 1.9x in young donors compared to aged donors for micromass pellet culture and EMTs, respectively (Figure 4B, Centrif. $p < 0.05$, EMT $p < 0.01$). For all samples there was an up-regulation in expression of aggrecan (Figure 4C) and in type X collagen (Figure 4D) after 21 days in culture, though no statistically significant differences were observed between age groups or culture conditions. These results confirm literature reports of a loss in the ability of MSCs to undergo directed differentiation with increasing age.^[9]

Emphasizing the chondrogenic rescue potential of EMT culture was the fact that EMTs from aged MSCs had similar sGAG production (Figure 4A, Centrif. young vs. EMT aged, $p = 0.951$) and type II collagen expression (Figure 4B, Centrif. young vs. EMT, aged, $p = 0.982$) as micromass pellets from young donors. The biochemical assay and gene expression data was corroborated by histology and immunostaining which highlighted the loss of chondrogenic potential with age in centrifuged pellet culture, as well as the recovery in chondrogenic potential for aged donors cultured in EMTs. Alcian blue staining of centrifuged pellet MSCs from donors younger than 55 years old showed homogeneous sGAG deposition (Figure 4E, Centrif. young), while the same staining for pellets from aged donors was greatly diminished (Figure 4E, Centrif. aged). For EMTs, Alcian blue stained sections showed homogeneous sGAG deposition for both young and aged donors (Figure 4E, EMT). Immunostaining for type II collagen revealed peripheral matrix synthesis in micromass pellets from young donors but was markedly absent in pellets from aged donors (Figure 4F, Centrif.). Type II collagen was homogeneously distributed throughout EMTs from young donors (Figure 4F, EMT young) and, though more heterogeneous, it was also clearly present in EMTs from aged donors (Figure F, EMT aged). The exact mechanism of age-related decline in MSC differentiation potential is not fully understood, but it is believed to be related to lack of telomerase activity in a majority of the cell population.^[34] Furthermore, MSCs from aged donors exhibit significant reduction in expression of MSC markers^[24] as well as reduced response to growth factors such as TGF- β .^[25] It can therefore be deduced that the MSC population from an aged donor would exhibit a decreased response to induction stimuli. Culturing MSCs in engineered microtissues improves cell exposure to necessary stimuli and may serve to overcome this reduced sensitivity.

2.4 EMTs Loaded with TGF- β 3 Induce Chondrogenesis

To investigate whether the enhanced chondrogenic effect in EMTs was dependent on TGF- β 3, EMTs cultured in chondrogenic media with or without the growth factor (GF) were compared after 21

days. DMMB and type II collagen expression were negligible in EMT samples cultured without TGF- β 3 (Figure 5 A and B, $p < 0.001$) and the lack of MSC chondrogenesis without GF was confirmed by histological analysis (Figure 5C). We then postulated that the TGF- β 3 could be preloaded into the polysaccharide matrix at the time of EMT microtissue formation. To test this hypothesis, TGF- β 3 was added to the sChi solution prior to cell resuspension and subsequently encapsulated in the biopolymer network at the time of EMT formation (Scheme 1, EMT^{+TGF}). EMT^{+TGF} were cultured in media lacking the growth factor and the media was changed completely every day for the first week. Release of the GF from EMT^{+TGF} samples was measured by ELISA for a period of 21 days from microtissues seeded with either 25ng, 50ng, or 75ng TGF- β 3 per microtissue. The cumulative release values were normalized to the maximum growth factor concentration observed from the 75ng loading condition, and a dose dependent release profile for the different loading conditions was evident (Figure 5D).

Though the TGF- β 3 release profile followed diffusion-based first order kinetics, it is also possible that some TGF- β 3 is covalently immobilized in the polysaccharide network via Schiff base reaction between its ϵ -lysine amino acids and unreacted free aldehydes present in oxAlg, where it would continue to remain biologically active for stimulation of differentiation.^[35] By day 5, release of TGF- β 3 was complete for all doses. An early burst release of TGF- β 3 is ideal according to others who have shown transient exposure for only the first week of culture to be sufficient for chondroinduction,^[36] and short-term exposure followed by longer culture periods in the absence of TGF- β 3 was even beneficial for the mechanical properties of the produced cartilage.^[37]

Chondrogenic differentiation in EMT^{+TGF} was monitored by DMMB assay and type II collagen gene expression and compared with unloaded controls cultured in media containing TGF- β 3. EMT^{+TGF} loaded with either 50ng or 75ng of TGF- β 3 had higher amounts of deposited sGAG and showed more type II collagen expression than either the control group or samples loaded with only 25ng ($p < 0.05$). This dose response was confirmed in histology, which showed samples loaded with only 25ng TGF- β 3 have diminished sGAG deposition and type II collagen in the ECM (Figure 5G). Samples loaded with 50ng

or 75ng did not differ significantly from one another and appeared similar to samples of unloaded EMTs cultured in full chondrogenic media ($p > 0.95$). This data suggests that for EMTs composed of 200k MSCs, 50ng loading with TGF- β 3 is sufficient to supply enough of the GF to the cells for driving chondrogenic differentiation over 3 weeks.

A major shortcoming of using micromass pellets to generate de novo cartilage for defect repair is the necessitation for extensive in vitro manipulation. While this has been shown to be effective for generating cartilage in in vitro models,^[22] TGF- β 3 is needed as a media supplement during culture. A bolus injection of the GF to the joint capsule would likely not suffice due to its short half-life in vivo.^[38] Here, we demonstrated that TGF- β 3 can be loaded within the polysaccharide network during EMT formation which was sufficient to drive chondrogenesis in serum-free media that was not supplemented with TGF- β 3. These results demonstrate a coupling of key cues in the microtissues, including positioning of TGF- β 3 to optimally direct chondrodifferentiation in situ.

2.5 Cartilage Defect Repair by EMTs in an Explant Model

The translational potential of EMTs was assessed utilizing a cartilage repair assay in bovine explant tissue similar to an approach used by others.^[20, 22] A 4mm diameter biopsy punch was used to take cartilage plugs from the trochlear groove of bovine femurs and defects were made using a 3mm punch through the center of the plugs in the articulating surface (Scheme 1B). MSCs from aged donors were used to make EMTs or centrifuged pellets which were seeded into the defects and subsequently cultured in full chondrogenic media for 4 weeks (Scheme 1). In gross appearance, EMTs grew within the cartilage defects while centrifuged pellets did not (Figure 6A, Centrif. vs EMT). Histology and immunohistochemistry revealed elevated synthesis of cartilage matrix in EMTs. Centrifuged pellets yielded minimal deposition of sGAG and there was a complete absence of staining for type II collagen, the primary phenotypic marker of cartilage (Figure 6B). Using parameters judged to be ideal from earlier

microtissue studies, EMTs loaded with 50ng of TGF- β 3 were seeded in cartilage defects and cultured in serum-free media that was not supplemented with the growth factor. EMT^{+TGF} were induced to produce sGAG and type II collagen containing de novo tissue within the cartilage defects (Figure 6B). There are a few limitations associated with the use of such an in vitro repair model including the absence of soluble factors present in an injured joint that would influence cell behavior (e.g. pro-inflammatory cytokines) and lack of biomechanical stimulation. However, this approach does represent a first step towards clinical translation highlighting the ability for EMT^{+TGF} to induce chondrodifferentiation within a cartilage defect, which eliminates the need for prolonged in vitro culture in defined media.

3. Conclusion

The results presented here outline a systematic approach to engineering a biomimetic environment for directing stem cell differentiation that offers a major advancement over current methods. We utilized a network of modified polysaccharides that polymerized via Schiff base crosslinking in the presence of MSCs and TGF- β 3 that served 3 purposes: 1) to induce rapid accumulation of cells into condensation-like aggregates, 2) to improve homogeneous differentiation of cells within the microtissues structure, and 3) to deliver TGF- β 3 to encapsulated cells for driving chondrogenesis. This approach rescued the chondrogenic potential of MSCs from aged donors that showed reduced sensitivity to induction factors in conventional pellet cultures. In demonstrating the effectiveness of EMTs loaded with TGF- β 3 for driving cartilage matrix production within cartilage defects in vitro, we showed the translational potential of this cartilage repair strategy. An in vivo evaluation of this approach in a subcutaneous animal model is underway and, if successful, would be followed by a large animal cartilage defect model. This engineering method has far-reaching applications beyond the field of cartilage regeneration and we foresee broad applications for improving cell-based therapies to treat patients in a rapidly aging population.^[39]

4. Experimental Section

Modified polysaccharide synthesis: Chitosan (85% deacetylation, 500mPas, Heppe Medical Chitosan GmbH, Halle, Germany) was reacted with succinic anhydride (Sigma) to synthesize N-succinyl chitosan (sChi) according to a previously published protocol.^[32] Briefly, 0.5 g of chitosan was dissolved in 40 mL of 5% (v/v) lactic acid and diluted 1:4 in methanol. 1.5 g of succinic anhydride was added to the solution while stirring and left to react overnight. sChi was precipitated by drop-wise addition of 10 M NaOH and filtered with Whatman filter paper. The precipitate was re-dissolved in Milli-Q water, dialyzed for 3 days, lyophilized, and stored at 4 °C until use. For sChi-FITC synthesis, 4 mg of fluorescein isothiocyanate (FITC, Sigma) and 50 mg of sChi were each dissolved separately in 7 ml of 0.5 M carbonate buffer at pH 9.4, mixed (1:1), and allowed to react overnight at room temperature protected from light. Unreacted free dye was removed by dialysis against Milli-Q water at 4 °C for 4 days. The final product was lyophilized and stored dry at 4 °C until use.

Chondroitin sulfate from shark cartilage (Sigma), hyaluronic acid (800-900 kDa, Novozymes Hyasis, Cambridge, MA, USA), and alginate (20-200 mPas, NovaMatrix, Sandvika, Norway) were oxidized according to Köwitsch et al.^[40]. 100 mg of HA, CS, and alginate were each solubilized in 20 ml of milli-Q water. For complete oxidation of vicinal hydroxyl groups, molar equivalents of sodium periodate (NaIO₄) were calculated for a theoretical 200% oxidation. Corresponding amounts of NaIO₄ were dissolved in 5 ml of milli-Q water and added drop-wise to the stirring polysaccharide solutions. The reactions were carried out overnight protected from light. The oxidized polysaccharides (oxPS) were purified by dialysis for 3 days, lyophilized, and stored dry at 4 °C until use.

Characterization of the modified polysaccharides: Succinylation of sChi was confirmed with $^1\text{H-NMR}$ using a Mercury-vx 300 spectrometer with D_2O as the solvent. Degree of substitution was calculated by taking the integral area ratio of the succinate peak ($\delta = 2.28\text{-}2.33$ ppm, CH_2CH_2 multiplet of succinyl) and the H-2 peak of the D-glucosamine unit in chitosan ($\delta = 2.70$ ppm). Aldehyde formation in oxidized HA, oxidized CS, and oxidized alginate was measured using an infrared spectrophotometer in attenuated total internal reflection mode (Frontier Spectrometer ATR-FTIR, Perkin Elmer, Waltham, MA, USA). Lyophilized materials were milled into a powder with a Pulverisette 7 ball mill (Fritsch, Idar-Oberstein, Germany) using 0.1 mm zirconium beads. 5 mg of powder material was used and measurements were done in triplicate.

Quartz crystal microbalance: Thin film layer-by-layer (LbL) buildup was characterized by quartz crystal microbalance with dissipation (QCM-D E4, Q-Sense AB, Gothenburg, Sweden). In QCM-D measurements, an electric potential is applied to a gold coated quartz crystal causing the crystal to oscillate at its measured resonance frequency. Drops in oscillation frequency due to material deposition onto the surface can be monitored and used to calculate film mass.^[41] For cleaning, gold coated crystals were immersed in surface cleaning solution (Ciba, Basel, Switzerland) for 30 minutes, rinsed with milli-Q water, blown dry under nitrogen, and treated by UV/ozone for 30 minutes. All reagents were solubilized in PBS (pH 7.4) and experiments performed at room temperature. A stable baseline was achieved with 0.5 mL of buffer and then 0.5 mL of sChi (5 mg mL^{-1}) was injected into the flow cell. After 30 minutes the surface was washed with PBS for 5 minutes and either unmodified or oxidized HA, CS, or alginate was injected (0.5 mL, all at 10 mg mL^{-1} in PBS). After 30 minutes another rinsing step was performed. The steps were repeated for two bilayers, followed by 4 hours under D-MEM containing 1% antibiotic-antimycotic (Life Technologies) to further test film stability.

Frequency shifts were reported from measurements of the third overtone with Δf taken as the difference between the oscillation frequency measured before and after injection of a given solution into the flow cell. Changes in areal mass on the crystal surface were calculated using the Sauerbrey relation Equation (1) where n is the overtone number and C is the mass sensitivity constant. Measurements were repeated in triplicate for each pair of sChi with PS or oxPS.

$$\Delta m = -nC\Delta f \quad (1)$$

Cell culture and seeding engineered microtissues: Reagents for cell culture were from Life Technologies (Zug, Switzerland) unless noted. Human mesenchymal stem cells (MSCs) were isolated from femur-derived bone marrow samples that were obtained during surgical hip replacement of otherwise healthy patients after receiving informed consent. The protocol was approved by the ethics board of the Kantonsspital, St. Gallen, Switzerland (ethics committee approval number EKSG08/014/1B). MSC isolation was performed as previously described.^[42] Cells at passage 1 were thawed and expanded in D-MEM/F-12 (1:1) media containing 10% FBS, 1% antibiotic-antimycotic, and 10 ng mL⁻¹ human basic FGF-2 (Peprotech, Rocky Hill, NJ, USA).

For microtissue formation MSCs were trypsinized at passage 3, washed in PBS, and resuspended in a solution of sChi (5 mg mL⁻¹ in PBS) at a cell density of 20 x 10⁶ cells/mL. Drops of 5 μ l each of the corresponding oxPS molecules (10 mg mL⁻¹ in PBS) were distributed onto a petri dish lid and 10 μ l of sChi containing 2.0 x 10⁵ cells were pipetted into each droplet of oxPS. The lid was turned upside down to facilitate mixing and the reaction was continued in an incubator at 37 °C for 15 minutes. For TGF- β 3 loading, either 25ng, 50ng, or 75ng was added to the sChi prior to cell resuspension from a concentrated stock solution. The TGF stock solution was further diluted for the 50ng and 75ng prior to addition to sChi to ensure the same volume (0.2 μ L/EMT) was added for each loading condition. Microtissues were transferred to agarose coated-wells of a 96-well plate with fine-tipped forceps and cultured in

chondrogenic media (D-MEM, 1% penicillin-streptomycin, 50 $\mu\text{g mL}^{-1}$ L-ascorbic acid (Sigma), 1% ITS+ premix (Becton Dickson AG, Allschwill, Switzerland), 40 $\mu\text{g mL}^{-1}$ L-proline (Sigma), 100 ng/mL dexamethasone (Sigma), and 10 ng mL^{-1} TGF- β 3 (Peprotech)). For pellet formation by centrifugation, MSCs (2.0×10^5 per pellet) from the same passage were centrifuged in conical well-plates (ThermoFisher Scientific, Waltham, MA, USA) at 500 xg for 5 minutes and cultured as micromasses in identical media conditions. Media was changed 3 times per week for 21 days.

EMT microtissue fabrication with sChi-FITC: For visualization of the polymer network, EMTs were formed using sChi-FITC (5 mg mL^{-1} in PBS) following the same protocol as above. To visualize cells within the polymer network, nuclei were stained with Hoechst 33342 according to manufacturer's protocol (Life Technologies). They were subsequently washed and imaged under PBS with a laser scanning confocal microscope (LSM 710, Zeiss).

Explant model for in vitro cartilage repair assay: Stifle joints of 6-month-old calves were obtained from a local butcher (Metzgerei Angst, Zurich, Switzerland) and dissected to expose the femoral condyles. Biopsy punches of 4 mm diameter (Polymed Medical Center, Glattbrugg, Switzerland) were used to take plugs from cartilage in the trochlear groove. A 3 mm diameter punch was used to cut a central channel defect through the top 1.5 mm of the plugs. Explants were fixed to the bottom of wells in 12-well plates with a thin layer of 2% (w/v) agarose (Lonza, Basel, Switzerland). EMTs and centrifuged pellets were formed as described above and cultured overnight in chondrogenic media to allow for condensation. The following day, explants were dried by aspiration and EMT or centrifuged pellets were positioned within the defects using fine-tipped forceps. Per defect, 2 EMTs or 3 centrifuged pellets were seeded along the defect length and allowed 30 minutes for β 1-integrin binding.^[43] Explants containing centrifuged pellets and EMTs were cultured in full chondrogenic media while EMT^{+TGF} were cultured in chondrogenic media lacking TGF- β 3. Media was changed 3x per week for 28 days.

Histological and immunohistochemical analyses: For histological studies, EMTs and centrifuged pellets were washed 3x in PBS, submerged in optimum cutting temperature compound (OCT, VWR), and snap frozen for 5 minutes on dry ice. Samples were sectioned to 6 µm thickness using a cryotome (CryoStar NX70, ThermoScientific). For the explant defect model studies, samples were fixed in 4% formaldehyde overnight at 4 °C, dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin. Sections were cut to 6 µm. Alcian blue (8GX, Sigma) staining was performed according to standardized protocol to visualize glycosaminoglycan production. For immunostaining of type I collagen (M-38, Developmental Studies Hybridoma Bank, University of Iowa; dilution of 1:100) and type II collagen (II-I16B3, Developmental Studies Hybridoma Bank, University of Iowa; dilution of 1:20), sections were first washed in PBS, digested with 0.2% (w/v) hyaluronidase (Sigma) for 15 minutes at 37 °C for epitope retrieval, blocked with 5% BSA for 1 hour, and incubated with primary antibody overnight at 4°C. For type X collagen (ab58632, Abcam plc, Cambridge, UK; dilution 1:100) sections were treated the same with the exception of epitope retrieval, which was performed with Digest-All 3 (Life Technologies) for 5 minutes at 37 °C. Sections were then washed and incubated with secondary antibody (IgG goat anti-mouse AlexaFluor 594, Life Technologies) for 1 hour, washed, and covered by a coverslip with mounting media containing 4', 6-diamidino-2-phenylindole (DAPI) for nuclear staining (Vector Laboratories).

RNA isolation and qPCR: Samples were collected (4 per condition) at days 1, 14, and 21, washed with PBS and frozen in liquid nitrogen. Once all samples were collected, RNA was isolated using the NucleoSpin miRNA kit (Macherey-Nagel AG, Oensigen, Switzerland) according to the manufacturer's instructions. RNA concentration was quantified using a plate reader (Tek3 plate, Synergy, BioTek Inc) and reverse transcribed using SuperScript III reverse transcriptase (Life Technologies). Primers were purchased from Microsynth AG (Balgach, Switzerland) and cDNA was amplified by real-time reverse transcription

polymerase chain reaction (qPCR) on a StepOne Plus instrument (Applied Biosystems) with SYBR Green Fast reagents (Applied Biosystems). Fold change was quantified by the $\Delta\Delta C_t$ method using RPL13a as an internal reference due to its previously reported stability during chondrogenesis.^[42] Fold increase was normalized to D1 centrifuged pellets in chondrogenic media. The following primer pairs were designed to overlap adjacent exons to avoid amplification of aberrant genomic DNA: COL2A1 [forward (F), 5'-GGAATTCGGTGTGGACATAGG-3'; reverse (R), 5'-ACTTGGGTCCTTGGGTTTG-3']; ACAN (F, 5'-GAATGGGAACCAGCCTATACC-3'; R, 5'-TCTGTACTTTCCTCTGTTGCTG-3'), COL10A1 (F, 5'-ATTCCTAGTGGCTCCAATGTG-3'; R, 5'-GCCTACCTCCATATGCATTTTG-3') and ribosomal protein L13a (F, 5'-AAGTACCAGGCAGTGACAG; R, 5'-CCTGTTTCCGTAGCCTCATG-3').

GAG/DNA quantification: GAG deposition in samples was quantified by the 1,9-dimethylmethylene blue (DMMB) assay. Samples were harvested on days 1, 7, 14, and 21, washed in PBS, and frozen at -80°C until analysis. All samples were analyzed on the same day. Samples were digested in papain (pH 7.6) at 65°C overnight, shaking at 1000 rpm. Chondroitin sulfate dilutions were used as standards and absorbance was measured at 595nm on a plate reader (Synergy, BioTek Inc). Total dsDNA was quantified using the Quant-IT PicoGreen kit (Life Technologies) according to manufacturer's instructions.

Enzyme linked immunosorbent assay: Growth factor release was quantified by TGF- β 3 ELISA kit from R&D Systems (Abingdon, U.K.) according to manufacturer's protocol. Media was collected every 2 days from EMT^{+TGF} for the first week, and every three days thereafter during which complete change was performed with fresh media not supplemented with growth factor. Data was normalized to the maximum concentration measured following release from EMT^{+TGF} loaded with 75ng TGF- β 3 .

Statistics: SPSS (SPSS for Windows, Rel 20.0.0. Chicago: SPSS Inc.) was used for all statistical operations.

Comparison of results was carried out by multiway analysis of variance (ANOVA) using Tukey's multiple comparison post hoc test for significance. *P* values of less than 0.05 were considered statistically significant results.

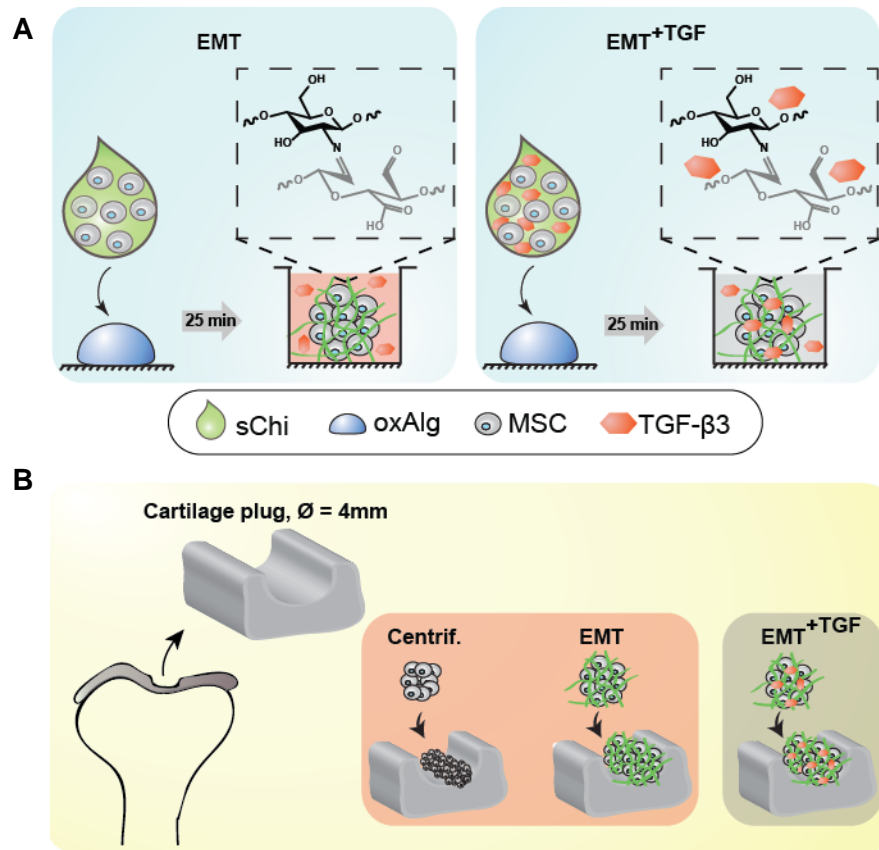
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Scheme 1. (A) Illustration depicting engineered microtissue (EMT) formation via Schiff base crosslinking between modified polysaccharides without (left) and with (right) pre-loading of TGF- β 3 (EMT^{+TGF}). (B) In later experiments, microtissues were used to fill cartilage defects in bovine explant tissue. EMT and centrifuged pellet samples were cultured in full chondrogenic media (red background), while EMT^{+TGF} samples were cultured in media that was not supplemented with TGF- β 3 (grey background).

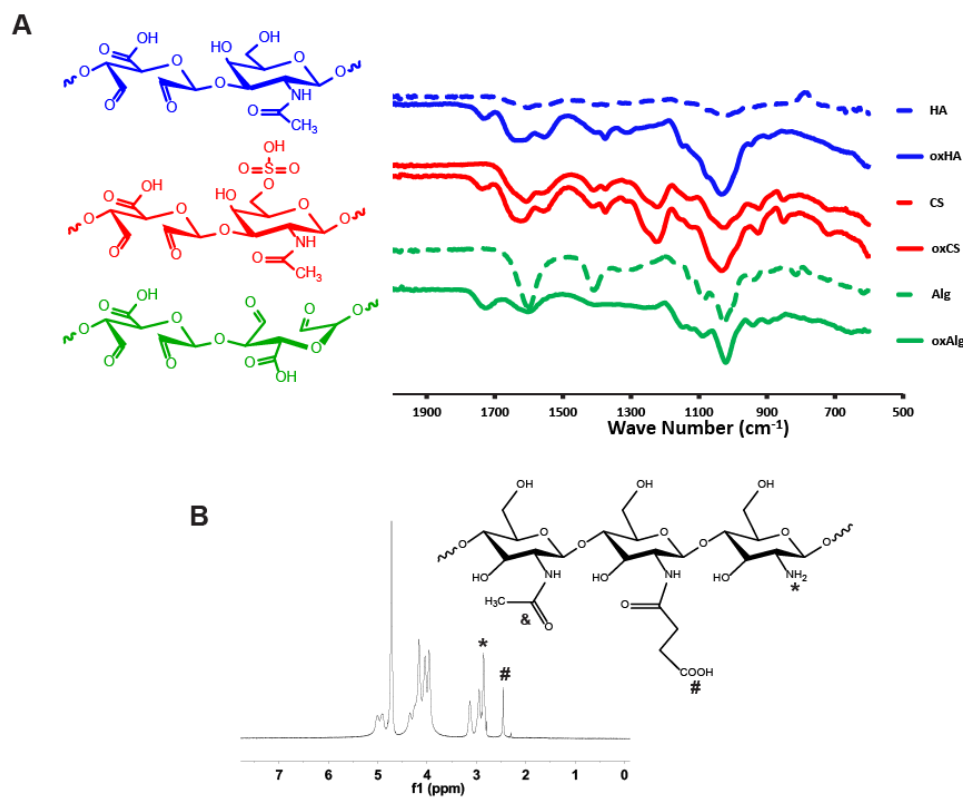


Figure 1. Chemical structures of oxidized polysaccharides. (A) Oxidized hyaluronic acid (oxHA, blue), oxidized chondroitin-6-sulfate (oxCS, red), and oxidized alginate (oxAlg, green, only mannuronic block shown) ATR-FTIR of native and oxidized polysaccharides and. Arrows indicated peaks from carbonyl stretching (uCHO) at 1735 cm^{-1} typical of aldehydes. (B) Chemical structure and ^1H -NMR spectra of N-succinyl chitosan (sChi). Degree of succinylation was 34% as calculated from the integral area ratio of the succinyl peak (#, $\delta = 2.28\text{-}2.33$ ppm) and glucosamine peak (*, $\delta = 2.70$ ppm) in the spectra. Remaining acetyl side groups marked with '&'.

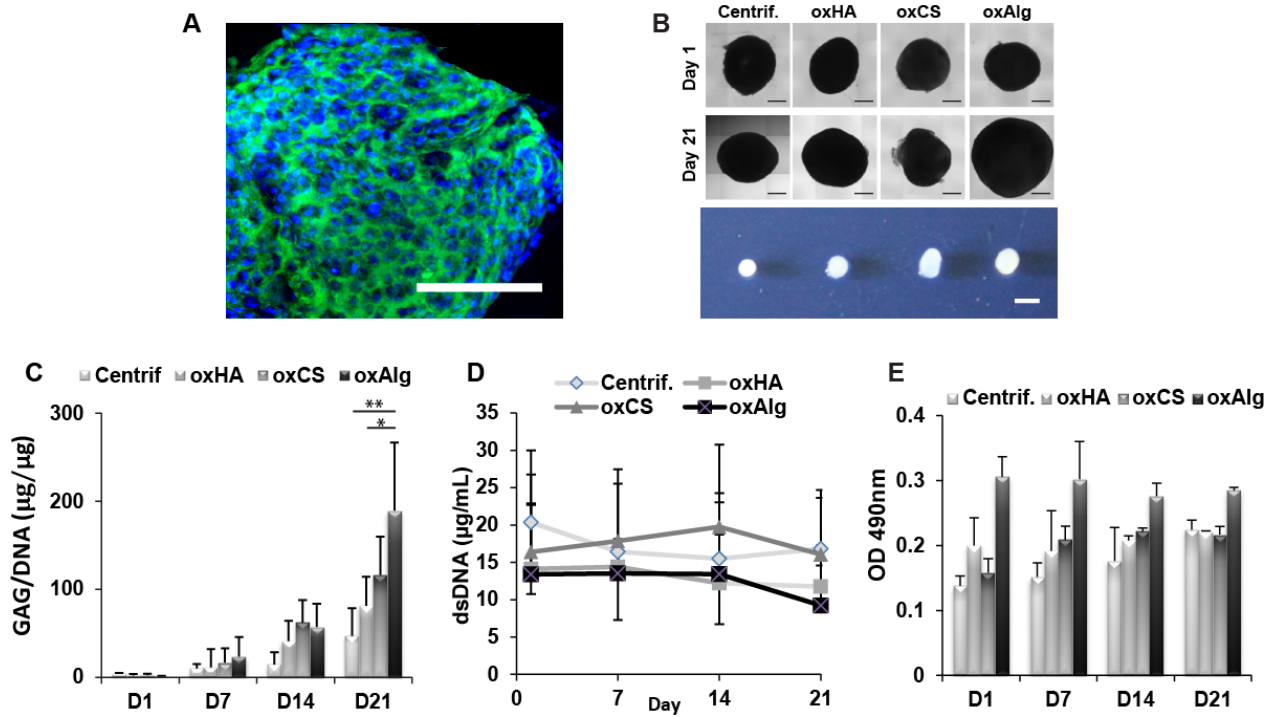


Figure 2. Enhanced chondrogenesis in microtissues made with sChi and oxAlg. (A) Representative engineered microtissue 30 minutes after seeding depicted by maximum intensity projection through a Z-stack taken with confocal microscope. FITC-labeled sChi was used to visualize the polysaccharide network and cell nuclei were labeled with Hoechst. (B) Gross morphology of pellets (Centrif.) and microtissues at day 1 and day 21 shown by brightfield microscopy (top). Macro lens photograph (bottom) shows pellet and microtissues made with oxHA, oxCS, or oxAlg after 21 days culture in chondrogenic media. (C) Quantification of sulfated glycosaminoglycans (sGAG) by DMMB assay normalized to dsDNA content. (D) PicoGreen assay measurements of dsDNA. (E) MTS assay for cell viability. (Plotted are mean values +/- SD; n=5 donors ranging in age from 42 to 74). (*p < 0.01, **p < 0.001). [Scale bars: (A) 250µm and (B) 500µm in brightfield microscopy images and 2mm in photograph.]

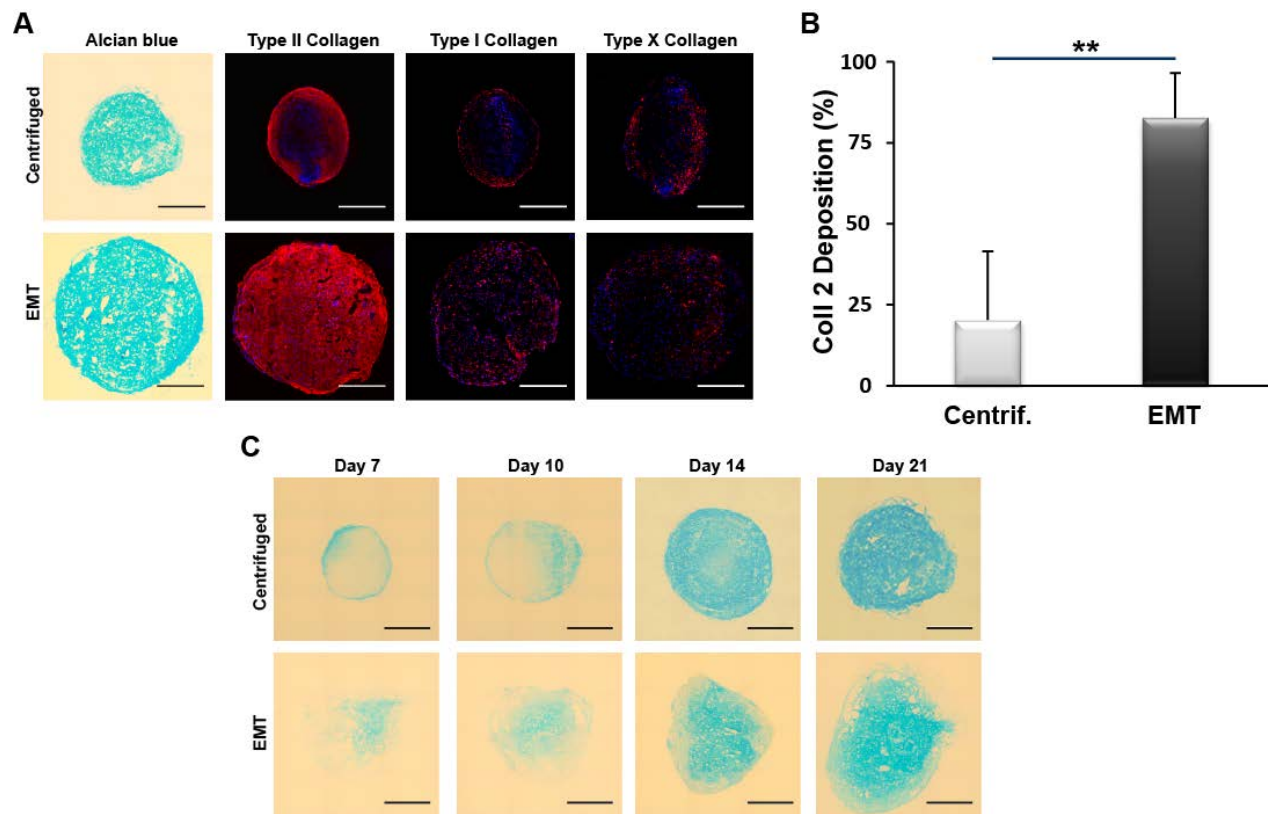


Figure 3. EMTs promote homogeneous deposition of cartilage-specific matrix. (A) Alcian blue staining for glycosaminoglycans (GAGs) and immunohistochemical staining for types II, I, and X collagen in centrifuged pellets and EMTs after 21 days culture in chondrogenic media. DAPI was used to stain nuclei. (B) The percentage of cross-sectional area that stained positively for type II collagen in EMTs vs pellets (Centrif.) at day 21 (Mean +/- SD, N = 5 donors ranging in age from 42 to 74, 3 sections per donor). (C) Alcian blue staining of centrifuged pellets and EMTs over time. (Representative histology images shown are from a single MSC donor, age 50) (**p < 0.001.) (Scale bars = 500 μ m.)

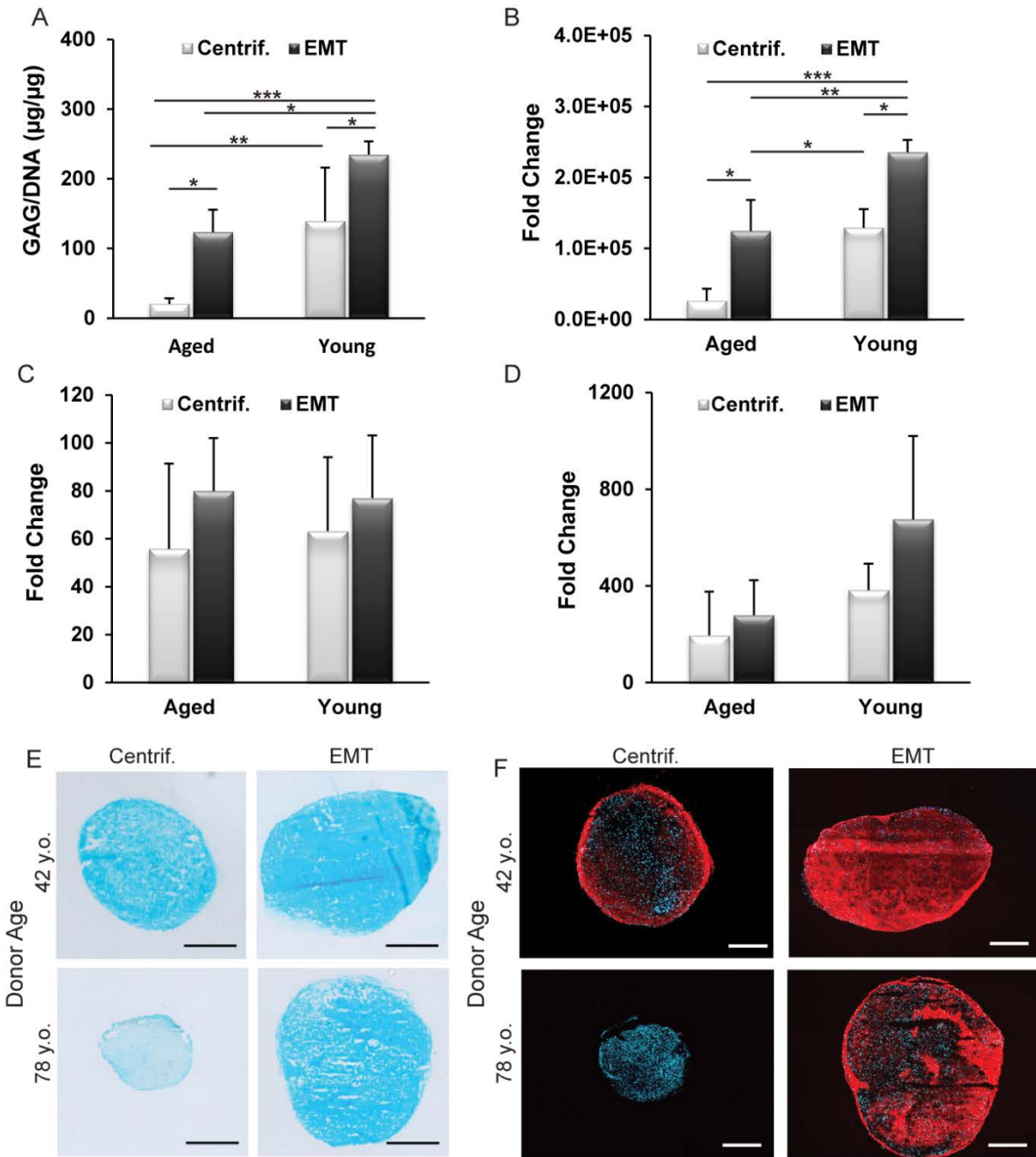


Figure 4. EMT culture restores the chondrogenic potential of aged MSCs. Donors were classified in groups as follows: Aged donors were >70 y.o. and Young donors were <55 y.o. (A) Chondrogenic induction after 21 days culture in chondrogenic media quantified by DMMB normalized to dsDNA and gene expression by qPCR of (B) type II collagen, (C) aggrecan, and (D) type X collagen. (E) Alcian blue histological staining and (F) type II collagen immunostaining confirmed quantitative measures. DAPI was used to stain nuclei. A-D are means +/- SD for N =6 per age group, 3 males and 3 females. B-D Fold change was compared to matched donors harvested on D1 and gene expression was normalized to the RPL13a reference gene. In E and F the same representative donors are shown; Young, 42 y.o. male; Aged, 78 y.o. male. Scale bars = 500µm. (* p < 0.05, **p < 0.01, *** p < 0.001)

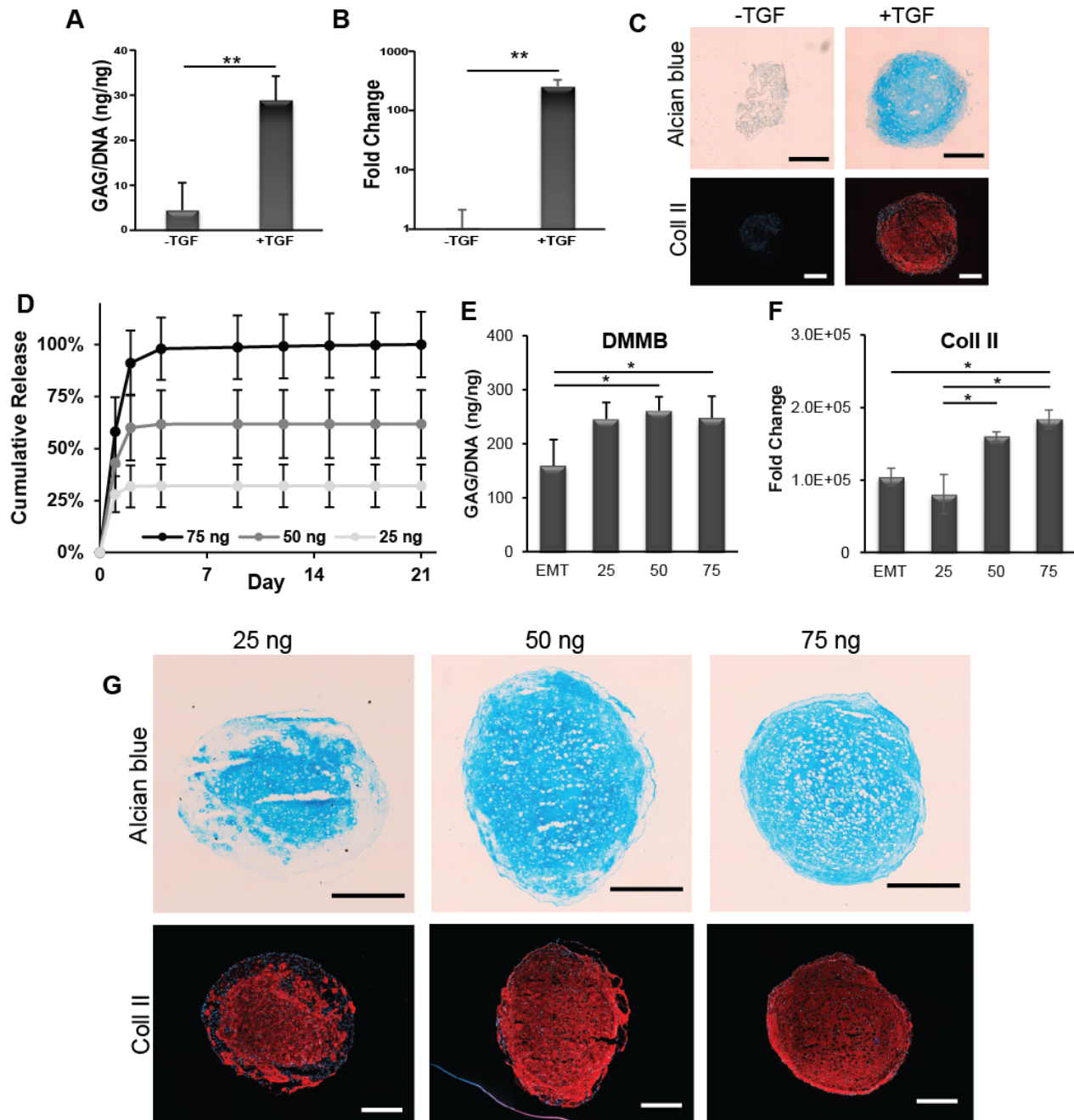


Figure 5. TGF- β 3 loading and release in EMT culture induces chondrogenesis. (A) DMMB and (B) type II collagen expression for cartilage matrix production in EMTs cultured in media with (+TGF) and without (-TGF) growth factor. (C) Alcian blue and immunostaining for type II collagen for EMTs cultured +TGF and -TGF for 21 days. (D) ELISA was used to monitor TGF release over time in microtissues that were loaded with 25 ng, 50ng, or 75 ng TGF- β 3. Data was normalized by the highest value of TGF- β 3 released in the 75ng condition. Plotted are percentage means \pm SEM (N = 3). (E) DMMB and (F) type II collagen gene expression results from microtissues that were loaded with 25, 50, or 75 ng of TGF β 3 at the time of seeding. (G) Histology and immunostaining confirmed results that 25ng was not enough for homogeneous chondrogenesis, but there was little difference between loading with 50ng or 75 ng. (A-B and E-F are means \pm SD for n = 5 donors ranging in age from 42 to 74.) (Histology images shown in C and G are from a single MSC donor, age 50) (Scale bars = 500 μ m.) (*p < 0.05, ** p < 0.01.)

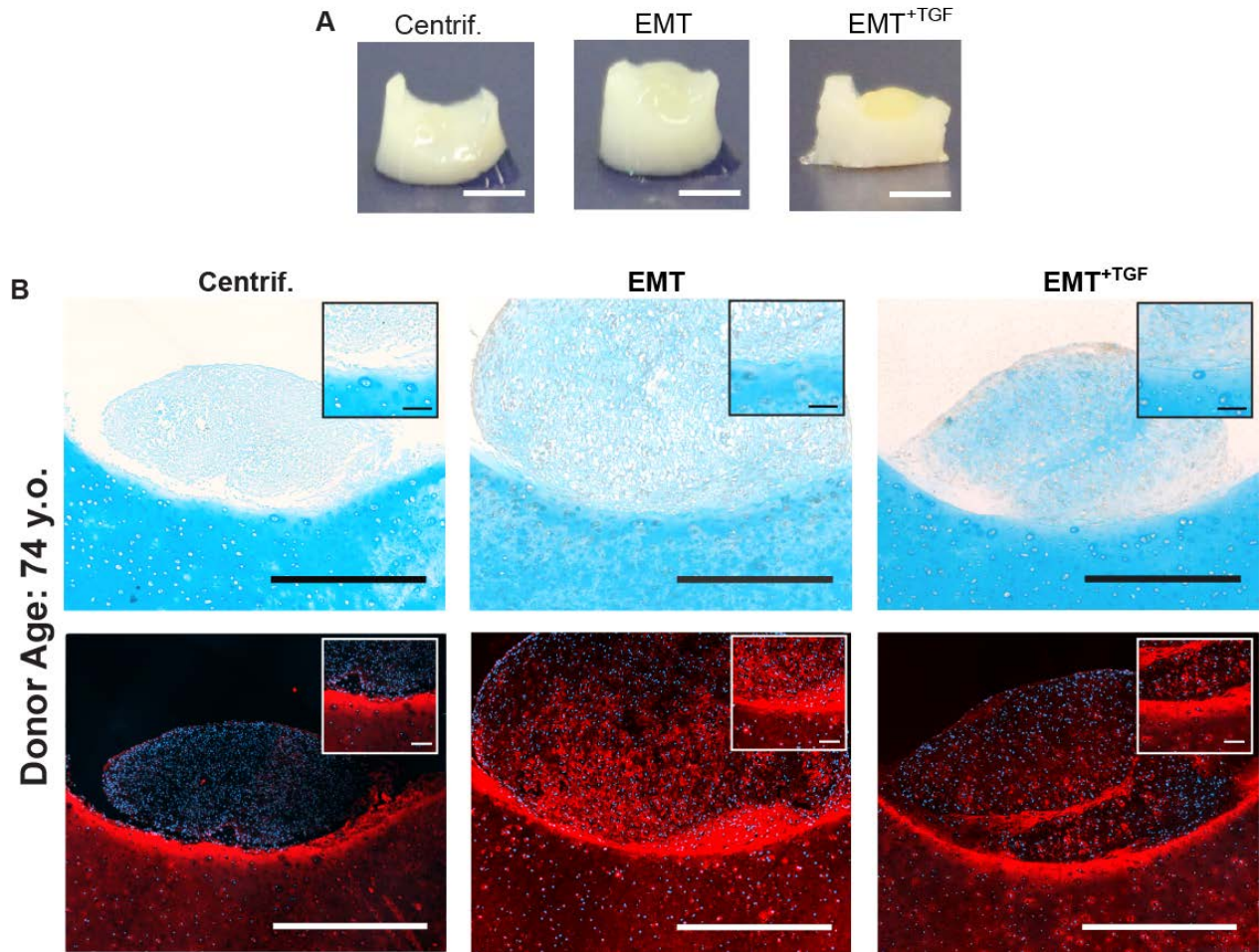


Figure 6. EMTs produce cartilage matrix in an explant model. (A) Photographs show growth of de novo tissue within the defects. (B) Alcian blue (top) and type II collagen (bottom, counterstained with DAPI) stained sections of the filled defects. Inlets are zoomed images at the interface of explant and repair tissue. (Representative images and histology shown are from a single MSC donor, age 74) (Scale bars, A: 2 mm, B: 0.75 mm, inlets: 100 μ m)