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Incorporating β -cyclodextrin into collagen scaffolds to sequester growth factors and modulate MSC activity

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Abstract

The development of biomaterials for a range of tissue engineering applications increasingly requires control over the bioavailability of biomolecular cues such as growth factors in order to promote desired cell responses. While efforts have predominantly concentrated on covalently-bound or freely-diffusible incorporation of biomolecules in porous, three-dimensional biomaterials, opportunities exist to exploit transient interactions to concentrate growth factor activity over desired time frames. Here, we report the incorporation of β -cyclodextrin into a model collagen-GAG scaffold as a means to exploit the passive sequestration and release of growth factors via guest-host interactions to control mesenchymal stem cell differentiation. Collagen-GAG scaffolds that incorporate β -cyclodextrin show improved sequestration as well as extended retention and release of TGF- β 1. We further show extended retention and release of TGF- β 1 and BMP-2 from β -cyclodextrin modified scaffolds was sufficient to influence the metabolic activity and proliferation of mesenchymal stem cells as well as differential activation of Smad 2/3 and Smad 1/5/8 pathways associated with differential osteochondral differentiation. Further, gene expression analysis showed TGF- β 1 release from β -cyclodextrin CG scaffolds promoted early chondrogenic-specific differentiation. Ultimately, this work establishes a novel method for the incorporation and display of growth factors within CG scaffolds via supramolecular interactions. Such a design framework offers opportunities to selectively alter the bioavailability of multiple biomolecules within a three-dimensional collagen-GAG scaffold to enhance cell activity for a range of musculoskeletal regenerative medicine applications.

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Graphical Abstract

Keywords

collagen scaffold; beta-cyclodextrin; guest-host interactions; growth factors

1. Introduction

Collagen-glycosaminoglycan (CG) materials are composed of natural extracellular matrix (ECM) components and have shown great promise as tissue engineering scaffolds. Initially developed for skin regeneration applications, these porous CG scaffolds are produced by the lyophilization of an acidic suspension of type I collagen and glycosaminoglycans [1]. One of the major advantages of CG scaffolds is the ease in which the microstructure, composition, or bioactivity can be adapted to meet the functional demands of a wide variety of tissue engineering applications. For instance, the freezing kinetics can be manipulated to produce anisotropic scaffolds composed of aligned tracks of ellipsoidal pores for tendon repair applications [2–4]. Calcium phosphate nanocrystallites have been incorporated during lyophilization to enhance mesenchymal stem cell (MSC) osteogenic differentiation and bone regeneration [5–10]. These structural modifications have also shown to bias MSC differentiation down chondrogenic vs. osteogenic lineages in the presence of mixed media supplementation [11]. The desire to promote increasingly specific cell behaviors (e.g., proliferation, MSC lineage specification) has motivated efforts to incorporate design elements that alter the bioavailability of growth factors within the scaffold network itself. In addition to covalent immobilization of growth factors to the scaffold via carbodiimide [12] and benzophenone [13] crosslinking chemistries, growth factors have also been covalently bound to polymeric reinforcing cages fabricated by 3D printing that can be incorporated into the scaffold during lyophilization to generate a composite with enhanced mechanical and biomolecular properties [14]. However, while covalent immobilization offers benefits in the form of extended bioavailability, this process is only applicable to biomolecular signals that do not require receptor internalization. Exciting opportunities therefore exist in the space of non-covalent presentation of biomolecular signals.

Recently, *Hortensius et al.* adapted charge-based transient sequestration concepts previously demonstrated in heparin-modified hydrogels [15] and 2D culture surfaces [16], and showed the glycosaminoglycan content of the CG scaffold itself could be manipulated to transiently sequester and release growth factors from the media [17, 18]. However, we examine alternative chemistries to transiently sequester activity-inducing growth factors within the CG scaffold network. Recently, a series of investigation have explored the use of chemical

modifications to a biomaterial to enhance growth factor sequestration or release kinetics. Notably, efforts such as *Benoit et al.* exploiting the incorporation of heparin into PEG hydrogels [19, 20], *Borselli et al.* exploiting VEGF functionalization [21], and *Martino et al.* exploiting the use of fibronectin fragments for cytokine sequestration [22]. However here we explore an alternative promising method for the selective incorporation of growth factors into biomaterial constructs is the use of supramolecular interactions such as those achievable with cyclodextrins. These six, seven, and eight member D-glucopyranose rings (α -cyclodextrin, β -cyclodextrin, and γ -cyclodextrin respectively) [23, 24] are commonly used in pharmaceutical applications to increase drug solubility. Cyclodextrins are known for their ability to form cyclodextrin inclusion complexes (CDIC), where guest-host interactions enable the adsorption of hydrophobic guest molecules into the central cavity (Fig. 1A) [25]. The ability of cyclodextrins to form guest-host complexes is determined by the size of the cyclodextrin cavity (6.0–6.5 angstrom) and the size of the guest molecule or key functional groups within the guest molecule [26]. It is suggested that cyclodextrins can interact with proteins via interactions with hydrophobic amino acids [27, 28]. Thus, cyclodextrins can bind and interact with a variety of molecules, proteins, and peptides. Cyclodextrin chemistries have been demonstrated as a means to generate dynamic, adaptable hydrogel environments [29–33]. Cyclodextrins have also been incorporated into porous biomaterials as a means to alter the release of drug molecules [34, 35]. While cyclodextrins have been shown to have a strong affinity for various growth factors [36], the ability for these molecules to regulate growth factor sequestration and display within porous scaffold biomaterials has not been deeply exploited.

Here, we describe the incorporation of β -cyclodextrin into a CG scaffold variant under development for musculoskeletal repair applications. We hypothesized that incorporation of cyclodextrin into the CG scaffold network would facilitate the formation of CDICs with exogenously added soluble growth factors, leading to growth factor sequestration and enhanced activity on MSCs within the scaffold. We examine sequestration and subsequent release of a model growth factor, transforming growth factor-beta 1 (TGF- β 1), within the scaffold network. We then examine the capacity for cyclodextrin modified CG scaffolds (CGcyclo) to enhance the activity of the chondrogenic growth factor TGF- β 1 or the osteogenic growth factor bone morphogenetic protein-2 (BMP-2) to influence MSC fate. We examine MSC osteochondral lineage specification via activation of the Smad 1/5/8 and Smad 2/3 pathways as well as changes in gene expression profiles.

2. Materials and Methods

All materials and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

2.1 Scaffold fabrication

Scaffolds were fabricated via lyophilization from a precursor collagen suspension. The standard collagen-GAG (CG) suspension was produced from type I collagen from bovine Achilles tendon and chondroitin sulphate from shark cartilage were homogenized in 0.05 M acetic acid at a ratio of collagen to glycosaminoglycan of 11.25:1 [1, 37]. A cyclodextrin-

modified suspension (CGcyclo) were prepared by adding β -cyclodextrin (from a 10 mg/mL stock solution in deionized water) directly to the CG slurry at a final concentration of 37 μ g/mL. Scaffolds were fabricated after pipetting 200 μ L of degassed CG or CGcyclo suspension into wells (6 mm diameter, 7 mm deep), of a polysulfone mold that was subsequently placed on a freeze-dryer shelf (VirTis, Gardner, NY). The CG or CGcyclo suspensions were frozen at -10°C for 2 h and then sublimated at 0°C and 200 mTorr to remove the ice crystals, resulting in a dry porous scaffold.

Scaffolds were then hydrated by immersion in 200 proof ethanol overnight followed by a 24 h rinse in sterile phosphate-buffered saline (PBS) replacing the PBS three times. Each scaffold was then crosslinked using carbodiimide chemistry by immersion in a solution of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS) for 2 h at room temperature in a 5:2:1 molar ratio (EDC:NHS:COOH) in sterile PBS with gentle shaking. After crosslinking, the scaffolds were washed twice with sterile PBS and stored at 4°C .

2.2 SEM imaging of the scaffold microstructure

Critical point drying (CPD) was used to prepare hydrated and cell-seeded scaffolds for SEM imaging. CPD was performed using a Samdri-PVT-3D (Tousimis, Rockville, MD), where the aqueous media in the scaffold was sequentially replaced with ethanol and then liquid CO_2 . Liquid CO_2 was allowed to infiltrate the scaffolds and was then held above 6.895 kPa and 31°C in order to remove the CO_2 as a gas with minimal structural deformation. Samples were then mounted on carbon tape, sputter coated with a gold/palladium mixture, and imaged with a Philips XL30 ESEM-FEG (FEI Company, Hillsboro, OR) at 5 kV with a secondary electron detector.

2.3 Biomolecule sequestration and release

The ability for the conventional CG or CGcyclo scaffolds to sequester and release two model biomolecules, TGF- β 1 and BMP-2, was assessed via ELISA. Briefly, scaffolds were incubated in 5ng/mL recombinant TGF- β 1 or 5ng/mL BMP-2 (R & D Systems, Minneapolis, MN) in sterile PBS with 1% bovine serum albumin (BSA) for 1 h on a gentle shaker; solution without scaffolds were used as controls. Following incubation, scaffolds were removed from the TGF- β 1 or BMP-2 solutions, and the amount of TGF- β 1 or BMP-2 remaining in the seeding solution was determined via DuoSet[®] ELISA (R&D Systems) in order to calculate the fraction of TGF- β 1 or BMP-2 sequestered by the scaffold variants themselves [17].

The elution of TGF- β 1 or BMP-2 from the CG and CGcyclo scaffold variants was subsequently traced over 6 days. Briefly, scaffolds were transferred to fresh PBS with 1% bovine serum albumin media and maintained in the incubator (37°C , 5% CO_2) for up to 6 days. Samples of the media were removed after 1, 6, 12, 24, 72, and 144 h for analysis of TGF- β 1 or BMP-2 release via DuoSet[®] ELISA. Results are reported as the fraction of the total TGF- β 1 or BMP-2 retained by the scaffold at each time point.

2.4 Human mesenchymal stem cell culture and scaffold seeding

Human bone marrow-derived mesenchymal stem cells were purchased from Lonza (Walkersville, MD). Cells from multiple lots, originating from separate donors, were combined to account for any donor-specific responses. The MSCs were cultured at 37°C and 5% CO₂ in a complete MSC growth medium, consisting of low-glucose DMEM with 10 vol % MSC-qualified, USDA-approved fetal bovine serum and 1 vol% antibiotic-antimycotic (Thermo Fisher, Waltham, MA), fed every three days and used at passage 6.

Selected CG and CGcyclo scaffolds were incubated in 5 ng/mL human recombinant BMP-2 or human recombinant TGF-β1 (ProSpec-Tany, Ness-Ziona, Israel) for 1 h in sterile PBS in the same manner as previously described. The scaffolds were then moved into 5% FBS MSC Media for 2 h as cells were lifted and centrifuged in preparation for seeding using a previously established static method [38]. Scaffolds were partially dried with Kimwipes and seeded with 1.0×10^5 MSCs per 24 μL medium (one 10 μL drop on opposite sides of the scaffold) in six well plates, with six scaffolds per well. Cells were allowed to attach for 1 h before submerging in 6 mL of low-serum MSC media, containing 5% FBS, per well. Cell-seeded scaffolds were cultured at 37°C and 5% CO₂ and fed every three days with the low-serum media.

2.5 Cell number and metabolic activity assays

DNA quantification was performed using a previously described method to determine the number of cells present on each scaffold [39, 40]. Briefly, scaffolds to be terminated were washed three times in warmed, sterile PBS to remove dead and unbound cells and placed in papain solution (2.4 mg/mL PBS) for digestion of the scaffold overnight at 60°C. Lysates were then incubated with Hoechst 33258 dye (Invitrogen, Carlsbad, CA) to fluorescently label double-stranded DNA. Fluorescent intensity (352/461 nm excitation/emission) from each sample was read using a fluorescent spectrophotometer (Tecan Infinite F200 Pro, Männedorf, Switzerland) and translated in cell counts through a standard curve of known MSC numbers.

Mitochondrial metabolic activity of the MSCs bound to each scaffold was quantified using the non-destructive alamarBlue[®] assay as previously described [41]. Cell-seeded scaffolds were incubated in 0.75 mL of alamarBlue (Invitrogen, Carlsbad, CA) (1:20 5% FBS MSC Media) and incubated at 37°C for 90 min on a gentle shaker. The reduction of resazurin to resorufin by metabolically active cells was quantified on a fluorescent spectrophotometer through differential absorbance. Relative cell metabolic activity was determined from a standard curve generated with known MSC concentrations and reported as a fraction of initial seeding cell count.

2.6 Protein isolation, SDS-PAGE, and immunoblotting

Upon reaching each time point, (day 1, 3, 6) scaffolds to be terminated were washed three times in warmed, sterile PBS to remove dead and unbound cells. The scaffolds were then blotted briefly and placed in a cold 1:100 dilution each of protease inhibitor cocktail, phosphatase inhibitor cocktail II and phosphatase inhibitor cocktail III in RIPA buffer and placed back on ice for thirty minutes with agitation. The scaffolds were then removed and

lysates were frozen [42]. Protein concentrations were quantified with the Pierce BCA Protein Assay Kit (Thermo Fisher, Waltham, MA) and prepared for gel electrophoresis by diluting 10 µg of protein from each sample with an equal volume of Laemmli buffer. Samples were heated at 90°C for 10 min and loaded into 4–20% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) in duplicate for both Smad 2/3 and 1/5/8 imaging. The gel electrophoresis was run at 150V for 90 min in 1 L of tris-glycine running buffer (25mM NaCl, 192mM glycine, 0.1% SDS, pH~8.3 in DI water) and immediately tank transferred onto nitrocellulose membranes (GE Healthcare, Little Chalfont, UK) at 300mV for 2 hours in 1 L of Towbin's transfer buffer (2.5M Tris base, 19.2mM glycine, 20% methanol in DI water). The nitrocellulose membranes were then cut and blocked in 5% milk in tris-buffered-saline + 0.1% tween 20 (TBST). Membranes were incubated in primary antibody at 4°C overnight in 5% BSA in TBST, washed three times in TBST, incubated in secondary antibody at RT for 1 hour, washed and imaged using the SuperSignal West Pico or Femto Chemiluminescent Substrate (Thermo Fisher, Waltham, MA) and an ImageQuant LAS 4010 system (GE Healthcare, Little Chalfont, UK). Primary antibodies were purchased from Cell Signaling (Danvers, MA): pSmad 2/3 (8828), Smad 2/3 (8685), pSmad 1/5/8 (9511), Smad 1 (6944), β-actin (4967). Secondary antibody used was HRP-linked goat anti-rabbit IgG (1:5000 in TBST). Membranes were stripped using the OneMinute® Western Blot Stripping Buffer (GM Biosciences, Rockville, MD) and re-imaged two additional times, repeating the above procedure. ImageJ was used for band intensity quantification. Results were reported as the ratio of phosphorylated to total protein for each pathway.

2.7 RNA isolation and target gene expression characterization

RNA was extracted from the MSC-seeded scaffolds at D1, D3, D6 using an RNeasy Plant MiniKit (Qiagen, Valencia, CA) and frozen. The isolated RNA was then reverse transcribed to cDNA in a Bio-Rad S1000 thermal cycler, using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA) [2, 43]. Real-time PCR reactions were carried out in triplicate, using 10 ng of cDNA and QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) in a 7900HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA). The primers used were consistent with previous studies and were synthesized by Integrated DNA Technologies (Coralville, IA). The expression level of the following markers was quantified: proteoglycan aggrecan (*ACAN*), cartilage oligomeric matrix protein (*COMP*), SRY-Box9 (*SOX9*), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), which was used as a house keeping gene (Table 1). Results were generated using the Ct method and expressed as fold changes, normalized to the expression levels of MSCs at the time of seeding, D0.

2.8 Statistical analysis

Analysis of ELISA experiments (growth factor sequestration and elution) was performed using a Kruskal-Wallis test (due to non-normal data). Two-way ANOVA was performed on western blot, metabolic activity, cell number and gene expression data sets (independent variables: β-cyclodextrin loading and growth factor incubation), followed by Tukey-honest significant difference *post hoc* tests. For cell experiments, scaffolds at each time point were analyzed for all metrics, however, no statistical analyses was performed between time points. Significance was set at $p < 0.05$. Error is reported in figures as the standard error of the mean, unless otherwise noted.

3. Results

3.1 Scaffold pore microstructure

The pore microstructure of CG and CGcyclo scaffolds was examined by SEM. Both CG and CGcyclo scaffold variants showed an open porous structure, consistent with our previous studies, without any signs of differences as a result of cyclodextrin incorporation (Fig. 2A). Closer examination of CGcyclo scaffolds after carbodiimide crosslinking and subsequent CPD preparation reveals the CGcyclo scaffolds retained characteristic banding pattern of the collagen through the fabrication and SEM preparation processes (Fig. 2B). Additional images were acquired 72 hours after MSC seeding show MSCs spreading out along the struts of the CG scaffolds (Fig. 2C).

3.2 β -cyclodextrin modified scaffolds sequester and retain TGF- β 1 and BMP-2 more efficiently than conventional CG scaffolds

We first examined the retention of TGF- β 1 within the CG vs. CGcyclo scaffold variants from a 5ng/mL loading solution. Initial sequestration within all variants ranged between 45 and 50% of TGF- β 1 in the loading solution, with CGcyclo scaffolds retaining significantly more TGF- β 1 at all time points than standard CG scaffolds (Fig. 3A). We subsequently examined the retention of BMP-2 within the CG vs. CGcyclo scaffold variants from a 5ng/mL loading solution. Initial sequestration within all variants was higher, ranging between 55 and 60% of the BMP-2 in the loading solution. Again, CGcyclo scaffolds retained significantly more BMP-2 within the scaffold network vs. CG scaffolds at all time points (Fig. 3B).

3.3 TGF- β 1- β -cyclodextrin inclusion complexes increase metabolic activity and proliferation

After establishing the binding efficacy of CGcyclo scaffolds, the growth factor's presentation and effect on MSC response was explored. Specifically, the effect of TGF- β 1 on cell number, metabolic activity and protein expression was examined. TGF- β 1 had no effect on cellular proliferation (Fig. 4A) with all four experimental groups increasing from day 1 to day 3 and essentially remaining constant to day 6. Conversely, there was a significant upregulation in cellular metabolic activity in the CGcyclo group with TGF- β 1 at all three time points ($p < 0.05$). Accounting for this equal cell count between all four groups, the distinct upregulation of cellular metabolic activity can be attributed to the enhanced initial retention of TGF- β within the CGcyclo scaffolds (Fig. 4B).

3.4 BMP-2- β -cyclodextrin inclusion complexes increase metabolic activity and proliferation

In parallel with TGF- β 1, the effect of BMP-2 on cell number and metabolic activity was also examined. It was found that BMP-2 has minor effects on cellular proliferation (Fig. 5A). Slightly lower cell numbers on the CGcyclo scaffolds with BMP-2 compared to the CG scaffolds with no BMP-2 on day 1, while this trend was reversed on day 6. While significant, ($p < 0.05$) the magnitudes of these changes are small compared to the significantly lower metabolic activity observed in the CGcyclo scaffolds with BMP-2 compared to the three other groups at all time points. (Fig. 5B).

3.5 TGF- β 1- β -cyclodextrin inclusion complexes activate the Smad 2/3 pathway

The effect of TGF- β 1 inclusion into CGcyclo scaffolds on the activation of the Smad 2/3 and Smad 1/5/8 pathways was evaluated by SDS-PAGE and western blot on days 1 and 3. Samples showed an increased activation of the Smad 2/3 pathway on day 3 when exposed to TGF- β 1 (Fig. 6A). The increased activation of Smad 2/3 was significantly higher in the CGcyclo group with TGF- β 1 compared to both the CG and CGcyclo groups without growth factor ($p < 0.05$). The Smad 1/5/8 pathway was also examined to determine the non-targeted MSC cellular response to TGF- β 1. No significant effects of the incorporation of β -cyclodextrin or TGF- β 1 were observed on the activation of Smad 1/5/8 (Fig. 6B).

3.6 BMP-2- β -cyclodextrin inclusion complexes activate the Smad 1/5/8 pathway

The effect of BMP-2 inclusion into CGcyclo scaffolds on the activation of the Smad 2/3 and Smad 1/5/8 pathways was also evaluated on days 1 and 3. Significant upregulation of Smad 2/3 activation was seen in the CGcyclo scaffolds with BMP-2 compared to the other three groups on day 1 (Fig. 7A) ($p < 0.05$). Additionally, while not significant, there was a general trend of increased activation of the Smad 1/5/8 pathway in the CGcyclo group with BMP-2 compared to the other three groups (Fig. 7B).

3.7 TGF- β 1- β -cyclodextrin inclusion complexes increase tenogenic gene expression

The resultant effect of TGF- β 1 inclusion into CGcyclo scaffolds on MSC chondrogenic gene expression was evaluated on days 1, 3 and 6 (Fig. 8A–C). While the expression levels for *ACAN* were quite low, large increases in expression of *COMP* and *SOX9* were observed in the CGcyclo group with TGF- β 1. The expression levels for *ACAN* were considerably lower throughout the experiment and were found to be significantly downregulated in both the CG and CGcyclo groups with TGF- β 1 on day 6 ($p < 0.05$). Conversely, expression levels for *COMP* were significantly higher in the groups with TGF- β 1 compared to the unsupplemented CG and CGcyclo groups on days 1 and 3, while the CGcyclo group with TGF- β 1 was significantly higher than all three other groups on day 6 ($p < 0.05$). By day 6, the expression levels of *SOX9* were significantly higher in just the CGcyclo group with TGF- β 1 compared to all three other groups ($p < 0.05$).

4. Discussion

Previous research from our group has shown that porous CG scaffolds serve as robust supports for both the stable culture of MSCs and their subsequent differentiation to tendon, cartilage, and osteogenic phenotypes [11, 40, 42, 44]. Here, we examined a novel approach to accelerate this differentiation through the more efficient loading and display of growth factors into the CG scaffolds via β -cyclodextrin sequestration. As previously stated, the driving force for β -cyclodextrin sequestration is hydrophobic interactions. Therefore, it is crucial that the host molecule contains hydrophobic moieties to interact with the interior of β -cyclodextrin. Examination of TGF- β 1 reveals exposed hydrophobic regions through β -strands β -8 and β -9 that form the ‘bow-tie’ link of its two arm domains and through an extended loop connecting α 1 and α 2 helices rich in proline and aromatic residues [45]. It is this region, directly surrounding the α 1 helix, that most likely binds the interior of β -cyclodextrin, as it is more exposed on the tip of the molecule and most extensively

hydrophobic in character. BMP-2 has been found to contain hydrophobic residues throughout the molecule and in particular local density at the bottom of the growth factor's central cleft [46]. The fundamental mechanism for β -cyclodextrin sequestration is applicable to the two growth factors, as it is also known that BMPR-II:BMP-2 complex interactions are dominantly hydrophobic in nature, proven through residue mutations, and TGF- β receptor:ligand complexes result from a combination of hydrophobic and electrostatic interactions [47, 48].

Elucidating the sequestration mechanism for each growth factor to β -cyclodextrin clarifies the results of the work presented. The additional TGF- β 1 and BMP-2 retained by the CGCyclo scaffolds compared to the CG scaffold over the course of the in vitro culture period can be attributed to β -cyclodextrin incorporation. The retention of TGF- β 1 or BMP-2 in the CGCyclo scaffolds makes them an ideal candidate for the control of MSC fate as we have shown here, and also suggest that CGCyclo scaffolds may sequester a broader range of endogenously produced factors as well, which may also enhance cell activity. These results are similar to previous findings by our group using modification to the glycosaminoglycan content of the CG scaffolds (heparin sulfate vs. chondroitin sulfate), finding scaffolds incorporating more highly charged heparin sulfate were able to sequester model biomolecules more efficiently and that sequestration led to significant changes in cell bioactivity [49].

Bone morphogenetic proteins (BMPs) belong to the larger transforming growth factor β (TGF- β) superfamily, which regulate a wide array of cellular functions including cell migration, adhesion, division, and differentiation throughout the human life span [50]. TGF- β 1 and BMP-2 are generally respectively associated with Smad 2/3 and Smad 1/5/8 signaling [51]. Thus, cell metabolic activity and Smad pathway activation were consequently employed as the metrics for increased growth factor loading. Previous work has shown that the minimum effective concentrations for TGF- β 1 and BMP-2 are 0.02 – 2.0 ng/mL and ~20 ng/mL respectively [52, 53]. This difference in threshold concentration may help to explain the lower activity of BMP-2 compared to TGF- β 1 in this study.

TGF- β 1 functionalized CGCyclo scaffolds was found to increase cellular metabolic activity, Smad 2/3 pathway activation, and the expression of chondrogenic markers *COMP* and *SOX9*. This is consistent with previous work where TGF- β 1 has been found to stimulate proliferation and self-renewal of MSCs and inhibit differentiation into osteocytic lineages through Smad 3 signaling [51]. TGF- β 1 has also been found to co-stimulate Smad 2/3 through phosphorylation that collectively associates into a larger transcription factor complex [54, 55]. Phosphorylated Smad 2/3 has then been shown to stimulate tenogenic lineages in conjunction with *COMP*, *ACAN*, and *SOX9* expression [11, 40, 56].

Conversely, BMP-2 functionalized CGCyclo scaffolds was found to lead to a slight drop in cellular metabolic activity and simultaneously generally increase Smad 1/5/8 phosphorylation. The BMP II receptor-mediated phosphorylation of Smad 1/5/8 allows the activated isoforms to associate with Smad 4, translocate to the nucleus, and stimulate transcription of target genes related to bone deposition, making the 1/5/8 signaling pathway critical in MSC osteogenic signaling [57–59]. The upregulation of one such target gene,

runx-related transcription factor 2 (*RUNX2*), has been found to lead to the subsequent expression of alkaline phosphatase (*ALP*) and osteocalcin (*OC*), genes critical for bone formation [53]. The activation of the Smad 1/5/8 osteogenic pathway strongly correlates with the metabolic activity data presented in Fig. 4, as MSCs in the early stages of osteoblastic differentiation rarely divided compared to the more immature MSCs [60, 61].

One additional potential benefit to the incorporation of β -cyclodextrin into the CG scaffold is the possibility of non-specific sequestration of growth factors and other biomolecules from serum or factors that are produced endogenously by cells within the scaffold. Since the sequestration method of β -cyclodextrin is primarily through hydrophobic interactions, this could allow for unoccupied β -cyclodextrin entities within the scaffold to non-selectively sequester various factors from serum, resulting in increased availability within the scaffold and improved cell viability. This could account for the increased cell numbers noticed in the CGcyclo groups on day 6 in Fig. 5. Both CGcyclo groups displayed higher cell numbers than the CG scaffold groups regardless of growth factor treatment. Thus, in addition to using pre-culture exposure to specific growth factors to tailor cell responses, the incorporation of β -cyclodextrin into CG scaffolds could potentially be used to improve general cell viability and proliferation through the non-selective sequestration and presentation of serum factors. Here, efforts could also take advantage of the range of cyclodextrin variants (e.g., α -cyclodextrin, β -cyclodextrin, γ -cyclodextrin) [23, 24] may provide increasing capacity to alter the availability of subsets of biomolecular signals.

5. Conclusions

The work presented demonstrates the ability for β -cyclodextrins to bind growth factors from solution prior to cell-seeding through cyclodextrin inclusion complexes formation as a means to accelerate MSC differentiation with lineage guidance dependent on growth factor bound. This mechanism can effectively lower the *in vitro* concentration of growth factors needed to induce cellular responses and can be potentially utilized for a variety of growth factors, cell types and applications in the hope of driving down patient related costs. This work establishes a new method for the successful incorporation and display of growth factors within CG scaffolds which, in combination with previously developed methods, has the potential to allow for more intricate designs for the selective display of multiple factors.

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Statement of Significance

We describe the incorporation of β -cyclodextrin into a model CG-scaffold under development for musculoskeletal tissue engineering applications. We show β -cyclodextrin modified scaffolds promote the sequestration of soluble TGF- β 1 and BMP-2 via guest-host interactions, leading to extended retention and release. Further, β -cyclodextrin modified CG scaffolds promote TGF- β 1 or BMP-2 specific Smad signaling pathway activation associated with divergent osseous versus chondrogenic differentiation pathways in mesenchymal stem cells . mesenchymal stem cell

Figure 1. β -cyclodextrin guest-host interactions and incorporation into CG scaffolds. (A) Seven-membered ring structure of β -cyclodextrin and spontaneous formation of inclusion complexes. **(B)** Representation of β -cyclodextrin incorporation into CG scaffold for transient sequestration of growth factors from solution.

Figure 2. CG and CGcyclo scaffold microstructure. (A)

Comparative SEM images of the porous CG vs. CGcyclo scaffolds at 200x (scale bar: 500μm), 400x (scale bar: 200μm), and 3000x (scale bar: 30μm). **(B)** High-magnification image of an individual collagen fiber banding within the CGCyclo scaffold microstructure. Scale bar: 1 μm. **(C)** SEM image of hMSC spreading along CG scaffold surface. Scale bar: 40 μm.

Figure 3. Retention of TGF-β1 and BMP-2 within CG versus CGcyclo scaffolds. (A) Retention of TGF-β1 in CG vs. CGcyclo scaffolds for up to 6 days in vitro (5ng/mL loading density). **(B)** Retention of BMP-2 in CG vs. CGcyclo scaffolds for up to 6 days in vitro (5ng/mL loading density). *: $p < 0.05$.

Figure 4. MSC viability in CG scaffolds exposed to TGF-β1. (A)

Number of cells and **(B)** normalized metabolic activity (versus the cells originally seeded into the scaffold) in unmodified CG or β-cyclodextrin CG scaffolds with and without TGF-β1 on days 1, 3, and 6 after seeding. Data expressed as mean ± SEM ($n = 6$). *: $p < 0.05$. ^: $p < 0.05$ versus day 1 within each scaffold group.

Figure 5. MSC viability in CG scaffolds exposed to BMP-2. (A) Number of cells and **(B)** normalized metabolic activity (versus the cells originally seeded into the scaffold) in unmodified CG and β -cyclodextrin CG scaffolds with and without BMP-2 on days 1, 3, and 6 after seeding. Data expressed as mean $\pm$ SEM ($n = 6$). *: $p < 0.05$ between groups. ^: $p < 0.05$ versus day 1 within each scaffold group.

Figure 6. Smad pathway activation in CG scaffolds exposed to TGF-β1
 Relative levels of phosphorylated/total (p/t) (A) Smad 2/3 and (B) Smad 1/5/8 in scaffolds with and without TGF-β1 as determined by immunoblot on days 1 and 3 after seeding. Data expressed as mean ± SEM ($n = 3$). *: $p < 0.05$.

Figure 7. Smad pathway activation in CG scaffolds exposed to BMP-2

Relative levels of phosphorylated/total (p/t) (A) Smad 2/3 and (B) Smad 1/5/8 in scaffolds with and without BMP-2 as determined by immunoblot on days 1 and 3 after seeding. Data expressed as mean $\pm$ SEM ($n = 3$). *: $p < 0.05$.

Figure 8. Changes in pro-chondrogenic gene expression in response to TGF-β1
 Expression levels of (A) *ACAN*, (B) *COMP*, and (C) *SOX9* with and without TGF-β1 exposure on days 1, 3, and 6. Data expressed as mean $\pm$ SEM ($n = 6$). *: $p < 0.05$.

Table 5.1

PCR primer sequences.

Transcript	Sequence	Reference
<i>ACAN</i>	Forward: 5'- TGCATTCCACGAAGCTAACCTT -3' Reverse: 5'- GACGCCTCGCCTTCTTGAA -3'	[62]
<i>COMP</i>	Forward: 5'- GCAACACGGACGAGGACAAG -3' Reverse: 5'- CGCCATCACTGTCCTTCTGG -3'	[63]
<i>SOX9</i>	Forward: 5'- AGCGAACGCACATCAAGAC -3' Reverse: 5'- GCTGTAGTGTGGGAGGTTGAA -3'	[62]
<i>GAPDH</i>	Forward: 5'- CCATGAGAAGTATGACAACAGCC -3' Reverse: 5'- CCTTCACGATACCAAAGTTG -3'	[64]