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Mesenchymal Stem Cell Sheets for Engineering of the Tendon–Bone Interface

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Failure to regenerate the gradient tendon–bone interface of the enthesis results in poor clinical outcomes for surgical repair. The goal of this study was to evaluate the potential of composite cell sheets for engineering of the tendon–bone interface to improve regeneration of the functionally graded tissue. We hypothesize that stacking cell sheets at early stages of differentiation into tenogenic and osteogenic progenitors will create a composite structure with integrated layers. Cell sheets were fabricated on methyl cellulose and poly(N-isopropylacrylamide) thermally reversible polymers with human adipose-derived stem cells and differentiated into progenitors of tendon and bone with chemical induction media. Tenogenic and osteogenic cell sheets were stacked, and the engineered tendon–bone interface (TM-OM) was characterized *in vitro* in comparison to stacked cell sheet controls cultured in basal growth medium (GM-GM), osteogenic medium (OM-OM), and tenogenic medium (TM-TM). Samples were characterized by histology, quantitative real-time polymerase chain reaction, and immunofluorescent staining for markers of tendon, fibrocartilage, and bone including mineralization, scleraxis, tenomodulin, *COL2*, *COLX*, *RUNX2*, osteonectin, and osterix. After 1 week co-culture in basal growth medium, TM-OM cell sheets formed a tissue construct with integrated layers expressing markers of tendon, mineralized fibrocartilage, and bone with a spatial gradient in RUNX2 expression. Tenogenic cell sheets had increased expression of scleraxis and tenomodulin. Osteogenic cell sheets exhibited mineralization 1 week after stacking and upregulation of osterix and osteonectin. Additionally, in the engineered interface, there was significantly increased gene expression of *IHH* and *COLX*, indicative of endochondral ossification. These results highlight the potential for composite cell sheets fabricated with adipose-derived stem cells for engineering of the tendon–bone interface.

Keywords: cell sheets, adipose-derived mesenchymal stem cells, enthesis, tendon–bone interface, tenogenesis

Impact Statement

This study presents a method for fabrication of the tendon–bone interface using stacked cell sheets of tenogenic and osteogenic progenitors differentiated from human adipose-derived mesenchymal stem cells, resulting in a composite structure expressing markers of tendon, mineralized fibrocartilage, and bone. This work is an important step toward regeneration of the biological gradient of the enthesis and demonstrates the potential for engineering complex tissue interfaces from a single autologous cell source to facilitate clinical translation.

Introduction

Injuries involving tendon and ligament are common musculoskeletal injuries requiring surgical intervention. Chronic overuse injuries at fibrocartilaginous insertions of the rotator cuff affect at least 40% of the population older than 60 years and result in more than 300,000 surgeries for repair in the United States annually.[1–3] While there has been success in engineering of tendon, ligament, and bone, the complex tissue interface composed of a functional gradient from soft tissue to hard mineralized tissue poses a challenge to the field of tissue engineering.[4,5] Failure to regenerate the gradient tissue interface results in poor clinical outcomes after surgical repair, resulting in high incidence of long-term pain, reinjury rates, and a loss of stability in repaired rotator cuffs.[5]

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Current strategies for repair of the enthesis utilize combinations of cells, growth factors, and biomimetic scaffolds.[4,5] Phasic and gradient scaffolds have been developed to introduce mechanical and mineral gradation. However, proper integration of layers within scaffolds and engineered grafts with the surrounding tissue remains a challenge, and delamination between layers is a common mode of failure. Scaffolds may also reduce cell density and limit cell–cell interactions necessary for development and maintenance of the enthesis.[5]

Cell sheets have been developed for tissue engineering and regenerative medicine to overcome the limitations of scaffolds and cell injections.[6] Cell sheets are fabricated by growing cells on thermally reversible polymers such as poly(N-isopropylacrylamide) (PNIPAAm) and methylcellulose (MC) to enable harvesting cells while leaving the extracellular matrix (ECM) and cell–cell junctions intact.[7,8] Using this technique, cell sheets can be layered to create cell dense three-dimensional (3D) tissues without addition of materials traditionally used as scaffolds, which may interfere with cell–cell interactions.[9] Because cell surface proteins and ECM remain intact, cell sheets readily adhere to host tissue or other cell sheets and retain better function and viability after transferring than cells treated with proteolytic enzymes and transferred in suspension.[6,10]

In a previous study, our laboratory characterized and compared the results of MC and PNIPAAm fabrication methods and demonstrated the ability of human adipose-derived stromal/stem cells (hASCs) cultured on MC and PNIPAAm substrates to commit to an osteogenic lineage after culturing in osteogenic medium (OM).[11] The PNIPAAm fabrication method results in a two-dimensional cell layer and had more robust mineralization when cultured in OM, indicating that the PNIPAAm fabrication method may be better suited for osteogenesis of hASCs. Cell sheets fabricated with the MC method are thicker due to the presence of collagen type 1 added to promote cell adhesion and result in a quasi-3D matrix that is easier to handle and maintain in culture. Different cell sheet fabrication methods can be explored to optimize differentiation conditions for different tissues.

For regeneration of tissue interfaces, cell sheets can be layered to engineer complex tissues.[9] A recent study by Raju *et al.* demonstrated the feasibility of composite cell sheet structures for 3D tissue regeneration and showed the advantage of using heterogeneous layered cells over a single cell type for anatomical regeneration of a complex tissue with simultaneous regeneration of periodontal ligament and alveolar bone in a mouse model.[12] Other studies have shown the advantages of using multiple cell types, observing increased deposition of ECM and enthesis development similar to native tissue due to heterotypic cell–cell interactions in co-culture.[13,14]

To date, this cell sheet stacking technique has not been demonstrated with cells from a single cell source, requiring procedures to harvest cells from multiple tissues including bone and ligament before cell sheet fabrication or utilizing allogenic cells from one or more donors.[12,14] For clinical translation, a single cell source co-differentiated into a complex tissue is desirable. hASCs are an abundant, multipotent cell source that can be easily harvested with minimally invasive liposuction.[15] hASCs have been differentiated into bone, cartilage, muscle, and tendon and can form all cell types necessary for the regeneration of the enthesis from a single autologous cell source.[16]

The purpose of this study was to conduct a preliminary investigation of a composite cell sheet structure derived from hASCs for repair of the tendon–bone interface. Cell sheets were fabricated with hASCs and differentiated into tenogenic and osteogenic progenitors by treating with chemical induction media before being stacked and cultured in basal growth medium (GM) for up to an additional 2 weeks. We hypothesized that by stacking cell sheets at early stages of progenitor differentiation into tenogenic and osteogenic lineages, we could form an integrated tissue interface expressing tenogenic and osteogenic differentiation markers with formation of fibrocartilage at the boundary between tendon and bone.

Composite cell sheet structures were characterized with histology, quantitative real-time polymerase chain reaction (qRT-PCR), and immunofluorescent staining. Tenogenic differentiation was assessed with expression of scleraxis and tenomodulin. Osteogenic differentiation was assessed with expression of RUNX2, osterix, osteonectin, and mineralization. Upregulated expression of fibrocartilage markers was characterized with *COL2* and *COLX* in addition to regulators of endochondral ossification Indian hedgehog (*IHH*) and parathyroid hormone-related protein (*PTHrP*).

Materials and Methods

Cell culturing

Cryopreserved hASCs were purchased at Passage 0 from Lonza (Walkersville, MD) and LaCell (New Orleans, LA) and cultured as previously described.[11] All tissues were obtained with informed consent under a clinical protocol approved by an Institutional Review Board. Cell cultures were expanded in GM consisting of Dulbecco's modified Eagle's medium-F-12 supplemented with 5% fetal bovine serum and 1% pen strep (Life Technologies, Carlsbad, CA) at 37°C in 5% carbon dioxide (CO_2). hASCs at Passage 2 or 3 were pooled from three donors for all experiments.

Experimental design

Cell sheets fabricated on MC and PNIPAAm (referred to as MC and PNIPAAm cell sheets) were cultured separately in GM and tenogenic or osteogenic chemical induction media. Tenogenic induction media (TM) was formulated with combinations of growth factors identified in the literature to promote tenogenic differentiation, including FGF2, TGF-β1, and BMP-12.[17–21] To make TM, GM with 50 μg/mL ascorbic acid was supplemented with 100 ng/mL FGF2 for 2 days, followed by 10 ng/mL TGF-β1 for 3 days and subsequent supplementation with 10 ng/mL TGF-β1 + 100 ng/mL BMP-12. OM consisted of GM supplemented with 20 mM β-glycerophosphate, 50 μg/mL ascorbic acid, and 10 nM dexamethasone. A preliminary study was conducted to evaluate the effect of tenogenic media on gene expression of *SCX*, *COL1*, and *COL3* in hASCs and optimize tenogenic differentiation conditions.

The experimental design is illustrated in Figure 1, with the timeline for the experiment in Figure 1A and the methods in Figure 1B. MC cell sheets were cultured for 2 weeks, and PNIPAAm cell sheets were cultured 1 week in TM or OM differentiation media before stacking. Therefore, MC cell sheets were fabricated first and PNIPAAm cell sheets were plated 1 week later, as shown in Figure 1A.

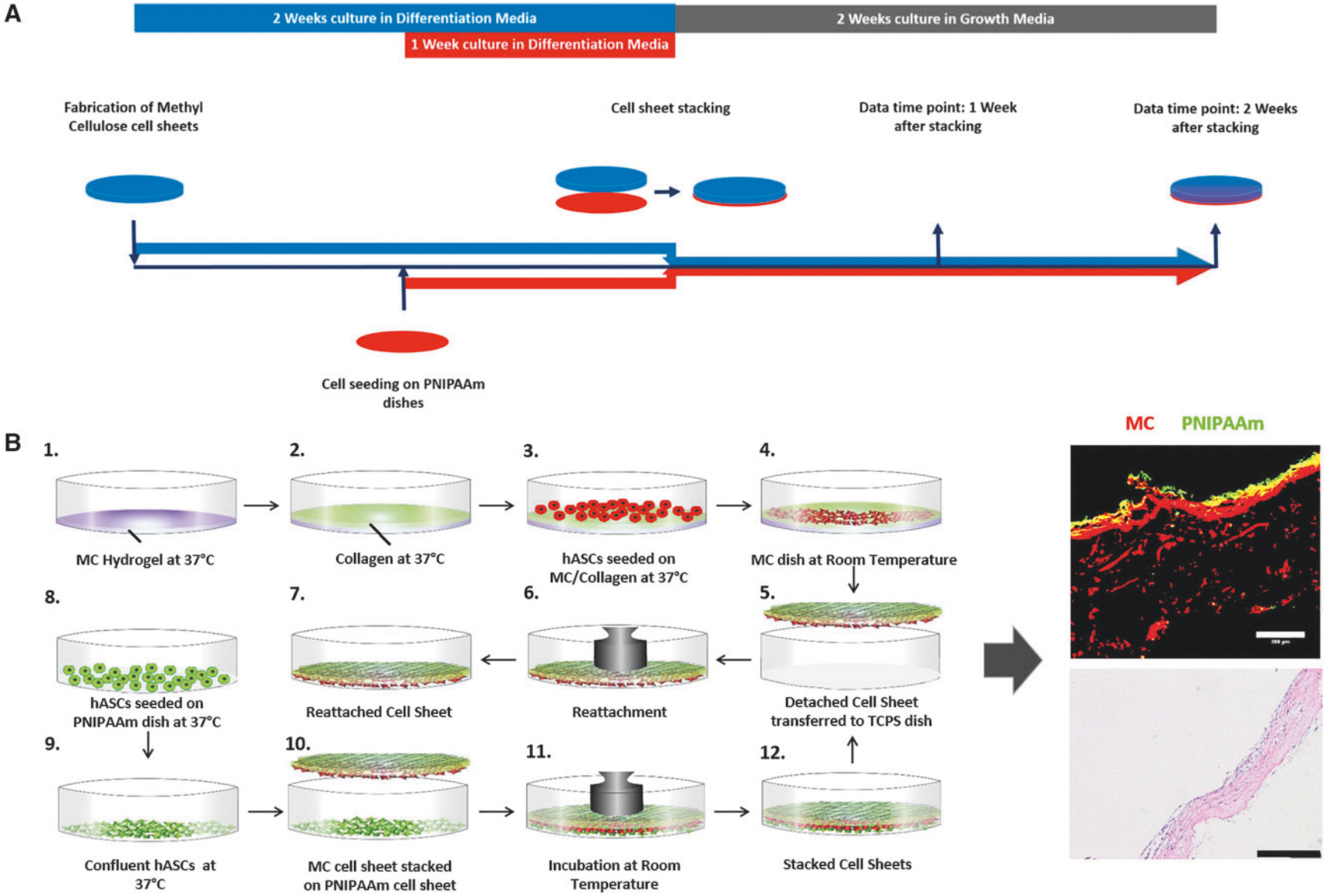

FIG. 1. **(A)** Timeline of cell sheet fabrication, stacking, and sample harvesting with MC cell sheets in *blue* and PNIPAAm cell sheets in *red*. **(B)** Cell sheet fabrication and stacking method with images of resulting cell sheets stained with CellTracker (*top*) and H&E stain of TM-OM stacked cell sheets 1 week after stacking (*bottom*). Scale bars are 200 μm. H&E, hematoxylin and eosin; MC, methylcellulose; OM, osteogenic medium; TM, tenogenic medium. Color images are available online.

Treatment times in differentiation medium were determined based on preliminary results and previously observed differences in the response of hASCs to the 3D environment of MC sheets compared with the planar culture conditions in PNIPAAm cell sheets.[11]

Cell sheets differentiated into tenogenic and osteogenic progenitors were stacked to form an interface after treatment in differentiation media. For the TM-OM group, MC sheets were chosen for tenogenic differentiation due to increased stability in culture and a higher fold change in *SCX* after 2 weeks in TM. PNIPAAm cell sheets were used for osteogenic differentiation because they resulted in more robust mineralization when compared with MC cell sheets.[11]

Stacked MC-PNIPAAm cell sheets were used for all experimental groups and controls to eliminate the process of cell sheet stacking and differences between cell sheet methods as variables. Hereafter, MC-PNIPAAm cell sheet stacks will be referred to according to the culture media conditions. The tenogenic–osteogenic (TM-OM) interface group was compared with GM-GM, OM-OM, and TM-TM stacked cell sheet controls. Cell sheet constructs were co-cultured in GM for an additional 1 or 2 weeks after stacking before samples were harvested for analysis with histology, immunofluorescent staining, and qRT-PCR.

Fabrication and stacking of cell sheets

MC dishes were prepared as previously described.[11] Briefly, a 14% MC solution was prepared by mixing 1.4 g of 15 cp viscosity MC powder in 5 mL of deionized water (90°C), followed by adding 5 mL phosphate buffer serum ($1\times$PBS containing 100 mg/L calcium chloride and magnesium chloride) at room temperature to the solution. After mixing, the solution was left in the refrigerator overnight. MC cell sheets were prepared by coating 35 mm tissue culture polystyrene (TCPS) Petri dishes or 12 well plates with MC solution and ultraviolet sterilized for 45 min before gelation.

Cell sheets were fabricated as shown in Figure 1B. (1) The prepared MC dishes were incubated at 37°C for 45 min to solidify. (2) MC dishes were coated with collagen type I to facilitate cell adhesion. (3) hASCs at Passage 2 or 3 were plated at a cell density of 1×10^6 cells per 35 mm dish and were incubated at 37°C in 5% CO_2 overnight to allow cell adhesion. (4) For formation of cell sheets, MC dishes were removed from the incubator and left at room temperature for 15 min to allow the cell sheets to spontaneously detach.

(5) Detached cell sheets were rinsed with PBS and transferred to a new TCPS dish cell side down using a stainless steel mesh disk to facilitate transfer and keep cell sheets flat as previously described.[11] (6) MC cell sheets were reattached to TCPS dishes to maintain them in culture for a period of 2 weeks. A stainless steel weight (20 g) was added on top of each cell sheet on the stainless steel mesh to increase the contact area and cell adherence.[22] (7) After reattachment, the weights were removed, but metal meshes remained in place to help maintain cell sheet morphology. MC cell sheets were cultured in growth or differentiation medium for 2 weeks before stacking. The media was changed every 2–3 days.

(8) PNIPAAm-coated dishes were purchased in 35 mm Petri dishes and 12 well plates (Nunc UpCell; Thermo Fisher, Waltham, MA). hASCs at Passage 2 or 3 were plated at a cell density of 2.5×10^5 cells per 35 mm dish at 37°C and cultured in growth or differentiation media for 1 week before stacking. (9) PNIPAAm cell sheets were taken from the incubator, and the growth media was immediately removed. (10) Sterile forceps were used to gently detach the MC cell sheet from its culture dish, and the MC sheet was placed on top of the PNIPAAm cell sheet, cell side down, using a mesh disk to facilitate transferring MC cell sheets into PNIPAAm dishes. (11) Stainless steel weights (20 g) were added on top of the cell sheets, on top of a stainless steel mesh disk, and 2 mL GM was added to each dish.

The layered cell sheets were left in the biosafety cabinet at room temperature for 45 min to allow detachment from PNIPAAm dishes and attachment of the PNIPAAm cell sheet to the MC cell sheet. (12) Stacked cell sheets were transferred to a new TCPS dish, repeating steps 5–6 for reattachment and cultured an additional 1 or 2 weeks in GM. Double layer MC-PNIPAAm cell sheets were harvested as a single tissue construct for characterization.

Cell tracker dye was used to visualize cells from MC and PNIPAAm cell sheets after stacking. PNIPAAm cell sheets were stained with Green CellTracker® and MC sheets with Red CellTracker® (Invitrogen, Waltham, MA) before stacking according to the manufacturer's protocol. After formation and stacking, the stained cell sheet stacks were transferred into the incubator and incubated for 7 days in GM before imaging.

Histology

Stacked cell sheets were fixed in 4% paraformaldehyde for 15 min at room temperature and cut into half. Half of each sample was frozen for cryosectioning and half was embedded with paraffin wax. Ten micrometer sections of paraffin-embedded samples were cut and stained with hematoxylin and eosin using a Leica Autostainer ST5010 XL. Ten micrometer sections of frozen samples were prepared for immunofluorescent staining of cross sections.

Immunofluorescent staining

After 1 and 2 weeks co-culture of stacked cell sheets, immunofluorescent staining was performed on whole cell sheet stacks and frozen sections as previously described.[23] Briefly, cell sheet constructs were fixed in 4% paraformaldehyde for 15 min at room temperature, rinsed three times with PBS, and blocked with 1% bovine serum albumin.

Samples were incubated with primary antibodies anti-RUNX2 (1:300; Abcam, Waltham, MA), anti-SCXA (1:200; Abcam), anti-tenomodulin (1:200; Abcam), or anti-collagen II (1:200; Abcam) overnight at 4°C. After rinsing, samples were incubated with secondary antibodies Alexa Fluor® 488-conjugated goat anti-mouse (1:500; Abcam) or DyLight® 650-conjugated goat anti-rabbit (1:200; Abcam) for 1 h at room temperature and counterstained with DAPI (Invitrogen). OsteoImage mineralization assay (Lonza) and ActinGreen 488 ReadyProbes (Invitrogen) were used according to the manufacturer's protocol. Samples were imaged using an Olympus FV10i-LIV Confocal Microscope.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from cell sheets using TRIzol and PureLink RNA Mini Kit (Life Technologies) according to the manufacturer's protocol. Verso cDNA Synthesis Kit (Thermo Fisher) was used for conversion of RNA to cDNA, and PowerUp™ SYBR™ Green Master Mix (Thermo Fisher) was used for qRT-PCR. Primers are listed in Supplementary Table S1. All genes were normalized to expression of a β-actin housekeeping gene, and the $2^{-\Delta\Delta CT}$ method was used to calculate gene expression relative to GM-GM nontreatment controls at each time point.[24]

Statistical analysis

Data are presented as mean ± standard error of the mean. For preliminary tenogenic differentiation data, $N = 4$–6 experimental replicates for at least two independent experiments were used. For cell sheet stacking, $N = 5$–7 experimental replicates from at least two independent experiments were used. Statistical analysis of results was performed. Data were analyzed with one- or two-way analysis of variance for statistically significant differences using GraphPad Prism. Fisher's least significant difference test was used for post comparisons. $p < 0.05$ was considered significant.

Results

hASCs in planar and 3D cell sheet culture were treated with combinations of FGF2, TGF-β1, and BMP-12 to examine potential for commitment into fibroblastic progenitors of tendon and ligament. qRT-PCR was used to quantify gene expression of *SCX*, *COL1*, and *COL3* in cells treated with growth factors compared with GM controls after 1 or 2 weeks in differentiation medium. Preliminary data were used to determine growth factor combinations and culture time for tenogenic cell sheet differentiation before stacking (Fig. 2).

FGF2 + TGF-β1 + BMP-12 (FTB) combinatorial media and sequential delivery of FGF2, TGF-β1, and TGF-β1 + BMP-12 resulted in statistically significant increase in scleraxis after 1 week in planar culture or 2 weeks in MC cell sheets ($p < 0.005$). After 1 week of cytokine treatment in planar culture, cells became confluent and spontaneously detached, making it impossible to maintain tenogenic culture on PNIPAAm dishes for periods longer than 1 week. *COL1* and *COL3* expressions were downregulated with FGF2 + TGF-β1 (FT) and FTB media treatment. There was a significant increase in *COL3* expression after 2 weeks of treatment with the sequential media ($p < 0.0005$).

Based on these results, sequential growth factor delivery was chosen as the TM differentiation medium for cell sheet stacking experiments with 2 weeks of differentiation in MC sheets. After 2 weeks, there was increased expression of *SCX* in hASCs treated with TM and significant increase in *RUNX2* expression ($p < 0.005$) in MC sheets cultured in OM compared with GM controls (Supplementary Fig. S1).

Cell sheets cultured on PNIPAAm were successfully transferred and integrated with MC cell sheets, as shown in Figure 1B. Integration of cell layers into a single 3D construct was visualized with CellTracker dye. Some overlap of cells can be seen at the interface between layers. The bulk of the composite is formed by the MC cell sheet with the PNIPAAm cell sheet forming a compact cell layer on one surface. There was no clear separation between the MC and PNIPAAm cell sheets in histological cross sections of stacked cell sheets after 1 week co-culture (Fig. 1B). Histology and F-actin staining showed increased cell density in tenogenic MC sheets and spindle-shaped cell morphology (Supplementary Figs. S2 and S3).

Cell sheet constructs were analyzed for tenogenic differentiation with expression of the transcription factor scleraxis and the tendon/ligament-specific glycoprotein tenomodulin. Differential gene expression was evaluated with quantitative qRT-PCR compared with GM-GM controls at each time point. Differential gene expression calculated in comparison to GM-GM controls at week 1 can be seen in Supplementary Figure S4.

A transient increase in the expression of tenogenic genes scleraxis and tenomodulin was measured in cell sheets treated with TM 1 week after stacking (Fig. 3). There was a significant increase in the transcription of scleraxis ($p < 0.0005$) in the TM-TM and TM-OM groups after 1 week co-culture in GM with a fold increase of 11.9 ± 3.1 and 18.0 ± 3.2, respectively. There was a significant increase in tenomodulin expression ($p < 0.05$) of 38.0 ± 17.1 in the TM-TM stacked cell sheets group and 37.5 ± 17.9 in the TM-OM stacked cell sheets group 1 week after stacking.

Gene expression results were confirmed with immunofluorescent staining (Fig. 4). Weak positive staining for scleraxis was detected in GM-GM and OM-OM controls with more prominent staining in the cell nuclei of the TM-OM group and strongest positive staining for scleraxis throughout the TM-TM group 1 week after stacking (Fig. 4B). Positive staining for tenomodulin could be seen throughout tenogenic cell sheets, as well as weak positive staining in GM-GM controls (Fig. 4C, E).

Osteogenic differentiation was characterized with gene expression of osterix, osteonectin, and *RUNX2* and immunofluorescent staining for hydroxyapatite and RUNX2 (Figs. 3 and 4). There was a statistically significant increase in the expression of osteonectin in OM-OM cell sheets 2 weeks after stacking with a fold change of 1.4 ± 0.3. Gene transcription of osterix was transiently upregulated in all the groups compared with GM-GM controls.

Clustering of cells could be seen in cell sheets treated with OM and on the surface of TM-OM stacked cell sheets (Fig. 4 and Supplementary Fig. S3). Large clusters of mineralization could be seen with OsteoImage staining of hydroxyapatite formation in OM-OM controls and more diffuse mineralization in the TM-OM group (Fig. 4A). RUNX2 expression was seen in all the groups, consistent with the qRT-PCR results (Figs. 3 and 4B). Positive staining for RUNX2 was most prominent in areas of OM-OM stacked cell sheets with large cell clusters (Fig. 4B). RUNX2 expression in the GM-GM and TM-TM groups was primarily localized to the cytoplasm and not concentrated in cell nuclei.

To determine if stacking resulted in gradient differentiation marker expression, cell sheets were cryosectioned orthogonally to view immunofluorescent staining of cross sections (Fig. 4D, E). There was weak staining for tenomodulin in the GM-GM and OM-OM groups. Tenomodulin was expressed uniformly throughout the TM-TM and TM-OM groups and was observed surrounding the cells. There was no visible gradient of RUNX2 expression in GM-GM, OM-OM, and TM-TM controls. In the TM-OM group, there

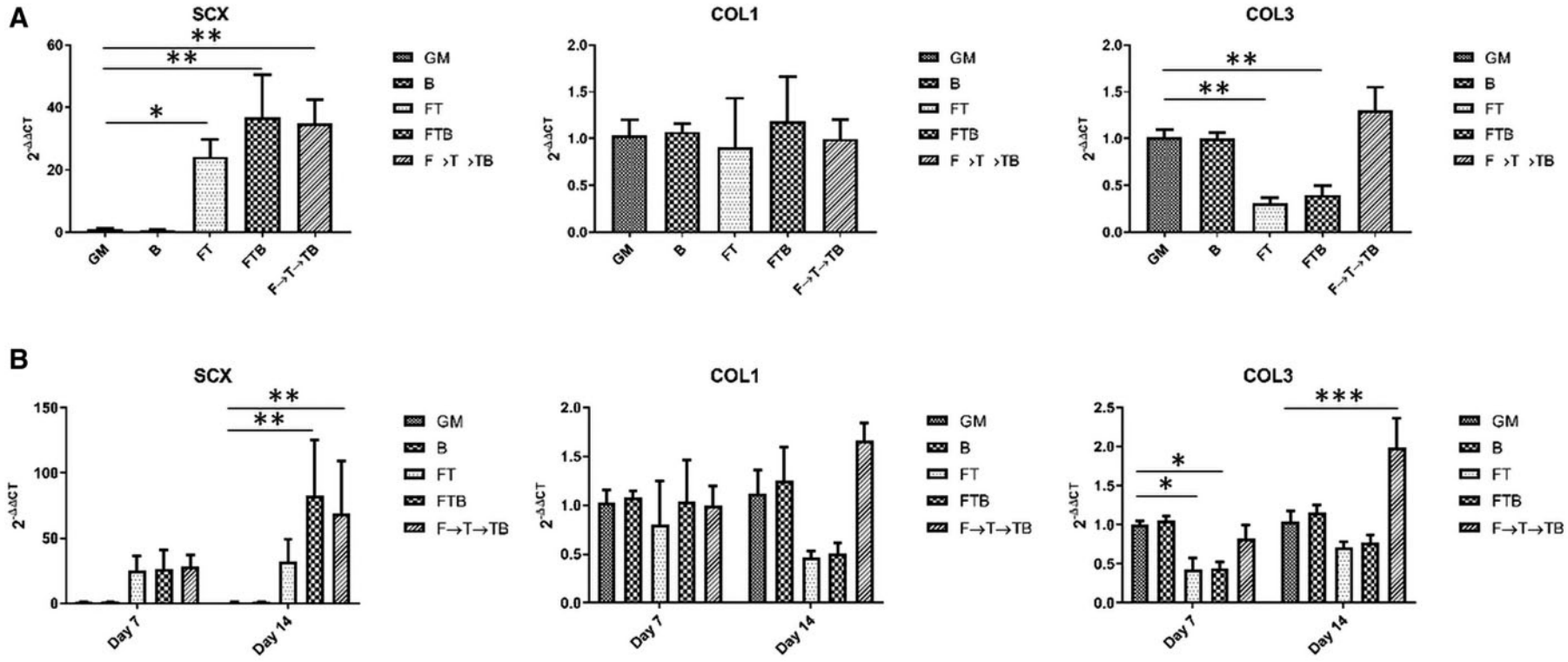

FIG. 2. Preliminary qRT-PCR data for growth factor treatment of hASCs in **(A)** planar PNIPAAm cell sheets and **(B)** MC sheets where GM represents growth medium control; B, BMP-12; FT, FGF2 + TGF-β1; FTB, FGF2 + TGF-β1 + BMP-12; F → T → TB, sequential treatment with FGF2, TGF-β1, and TGF-β1 + BMP-12. *Error bars* represent SEM. Statistically significant differences from GM controls are denoted (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$). GM, growth medium; qRT-PCR, quantitative real-time polymerase chain reaction; SEM, standard error of the mean.

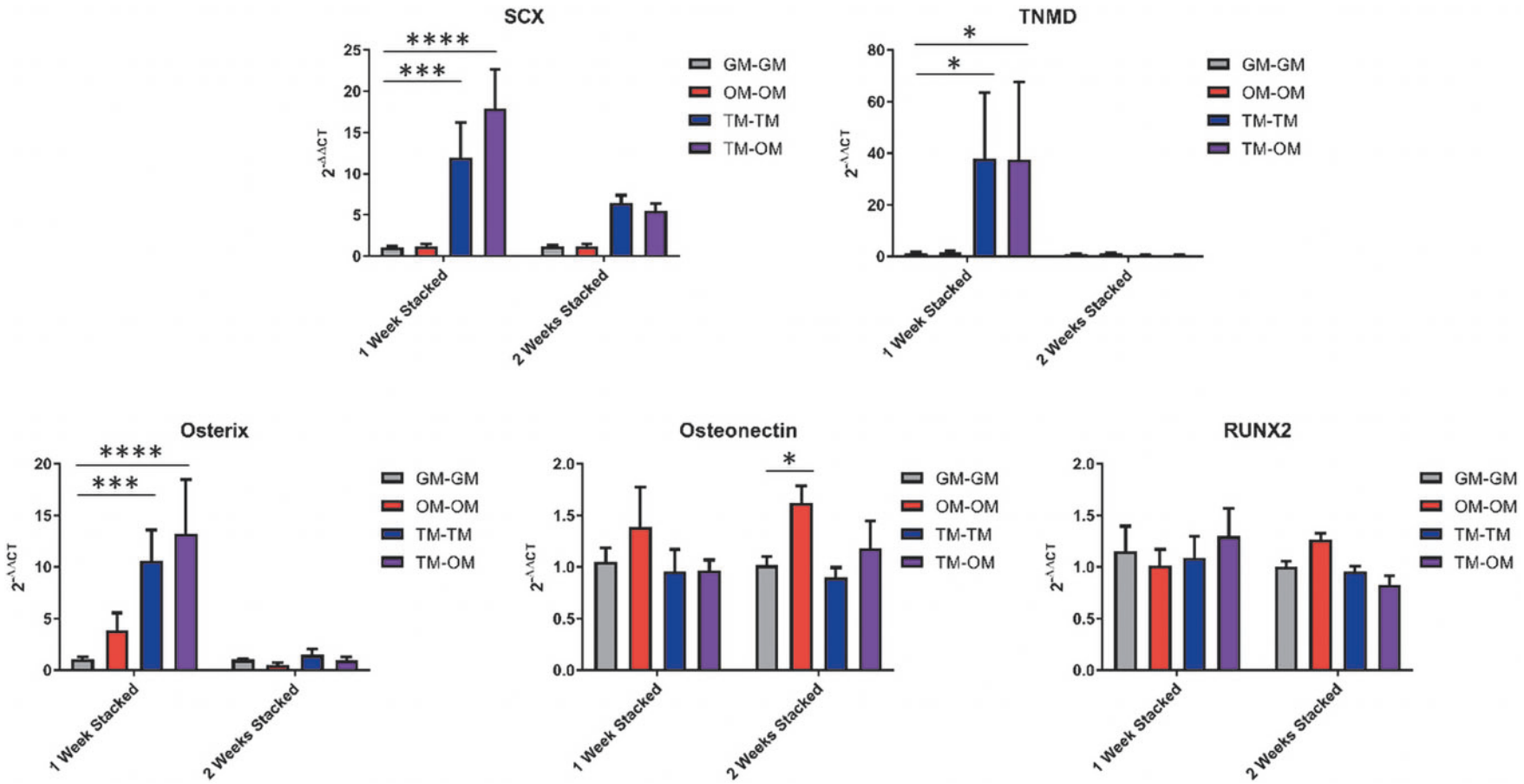

FIG. 3. qRT-PCR data for markers of tendon and bone in cell sheets 1 and 2 weeks after stacking. *Error bars* represent SEM. Statistically significant differences from GM-GM controls are denoted (*$p<0.05$, **$p<0.005$, ***$p<0.0005$). Color images are available online.

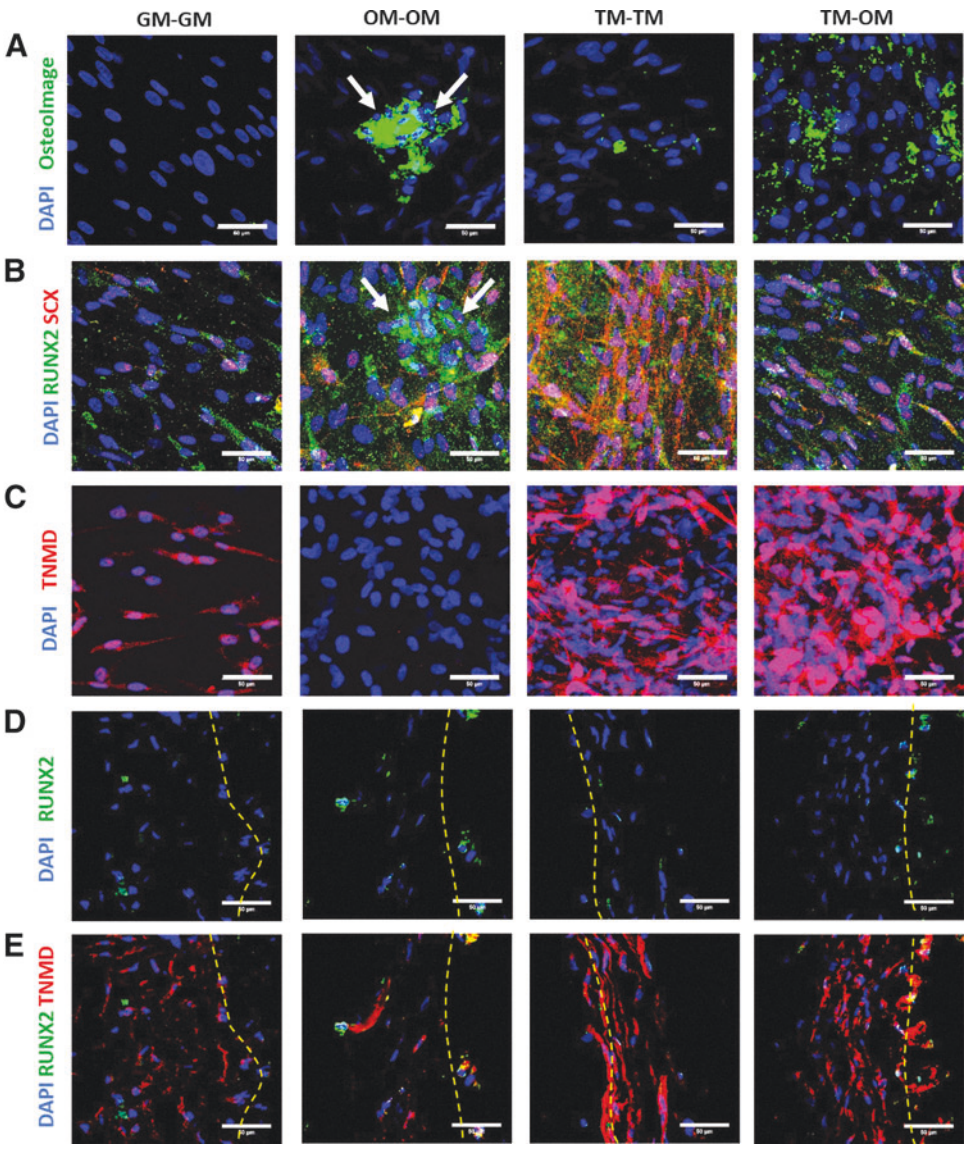

FIG. 4. Stacked cell sheets after 1 week co-culture. Z-stack of cell sheets with immunofluorescent staining of **(A)** hydroxyapatite with OsteoImage, **(B)** RUNX2 and scleraxis, and **(C)** tenomodulin. *White arrows* point to areas of cell clustering. Cross sections of stacked cell sheets after 1 week co-culture with immunofluorescent staining of **(D)** RUNX2 and **(E)** tenomodulin. The *dotted yellow line* marks the approximate boundary between PNIPAAm and MC cell sheets. Scale bars are 50 μm. PNIPAAm, poly(N-isopropylacrylamide). Color images are available online.

was increased RUNX2 expression along the surface where osteogenic progenitors were concentrated in the PNIPAAm cell sheet 1 week after stacking.

qRT-PCR was used to quantify transcription of genes encoding ECM proteins and signaling molecules expressed in calcified fibrocartilage (Fig. 5A). On average, there was increased expression of *COL2* and *COLX* 1 week after cell sheet stacking in all the groups compared with GM-GM controls, with significant upregulation of *COLX* in the TM-TM and TM-OM groups. The OM-OM, TM-TM, and TM-OM groups all had an increase in expression of *IHH* compared with GM-GM controls 1 week after stacking. The TM-OM group had a statistically significant increase in *IHH* expression ($p < 0.005$) with a fold increase of 5.7 ± 1.3 compared with GM-GM controls.

After 2 weeks co-culture in growth medium, there was positive staining for mineralization in the OM-OM, TM-TM, and TM-OM groups (Fig. 5C). The TM-TM and TM-OM groups also had increased expression of *PTHrP*, with a fold change of 4.5 and 3.0, respectively, 1 week after stacking. Upregulation of *PTHrP* was statistically significant ($p < 0.005$) in the TM-TM group compared with GM-GM controls. COL2 expression was confirmed in stacked cell sheets with immunofluorescent staining of cross sections (Fig. 5B). COL2 was expressed uniformly throughout cell sheet constructs. There was weak positive staining in all groups with larger COL2 fibrils visible in the TM-TM and TM-OM groups, consistent with the qRT-PCR results.

Discussion

In this study, hASCs were differentiated into tenogenic and osteogenic progenitor cell sheets and stacked to fabricate a tissue construct with integrated layers expressing markers of tendon, fibrocartilage, and bone. This work demonstrates the potential for regeneration of tendon and ligament-to-bone entheses via cell sheets fabricated from a single autologous cell source.

Tenogenic differentiation of hASCs in the cell sheets was marked by a significant increase in expression of the transcription factor scleraxis (Figs. 2 and 3). Scleraxis is essential for differentiation and maintenance of mature tendon/ligament

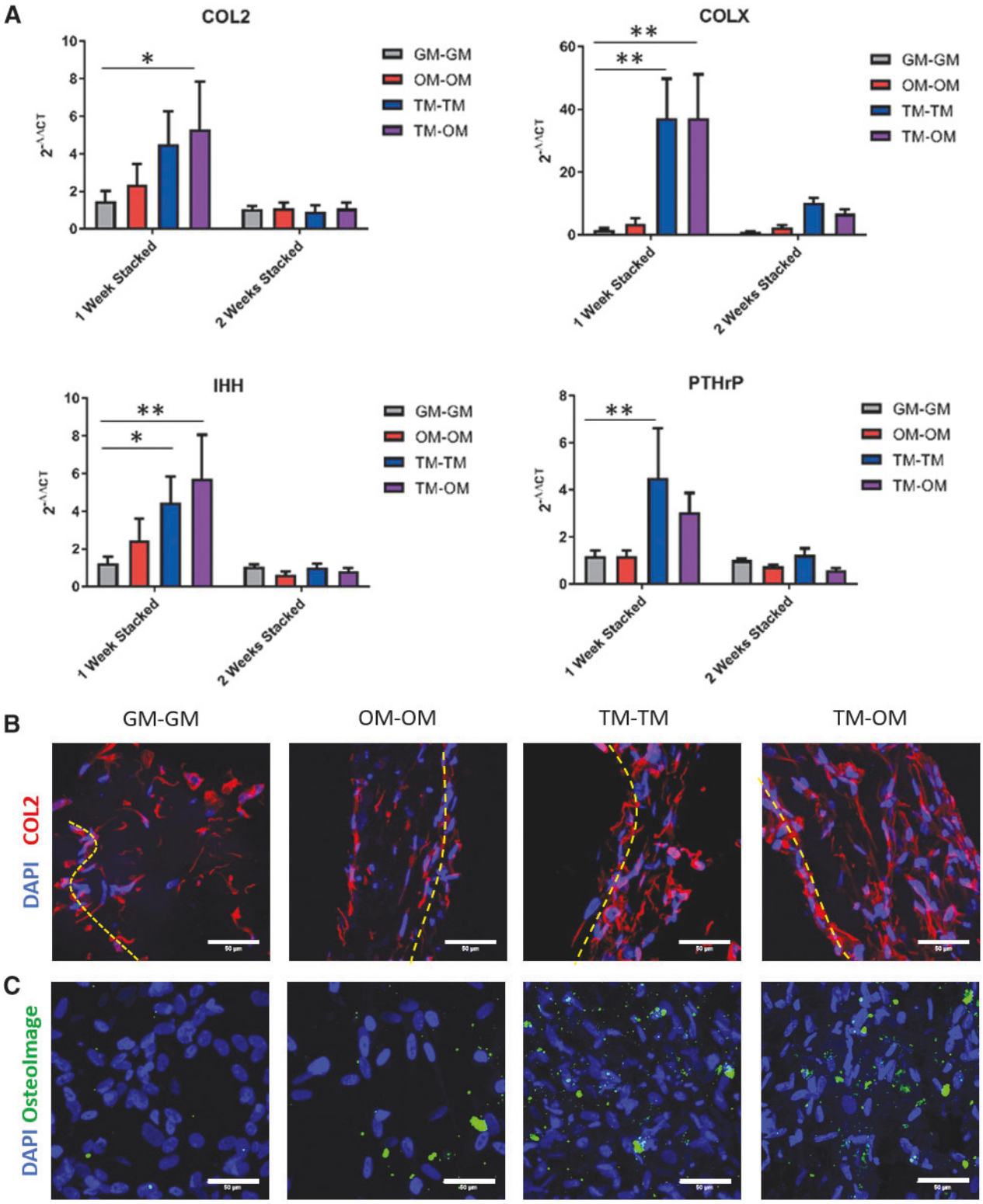

FIG. 5. **(A)** qRT-PCR data for markers of fibrocartilage and mineralized fibrocartilage in cell sheets 1 and 2 weeks after stacking. *Error bars* represent SEM. Statistically significant differences from GM-GM controls are denoted (*$p < 0.05$, **$p < 0.005$). **(B)** Cross sections of cell sheets 1 week after stacking with immunofluorescent staining of CoL2. The *dotted yellow line* marks the approximate boundary between PNIPAAm and MC cell sheets. **(C)** OsteoImage staining of mineralization in Z-stack of cell sheets 2 weeks after stacking. Scale bars are 50 μm. Color images are available online.

and a crucial factor for development of the enthesis.[25–27] Cells were treated with tenogenic differentiation media supplemented with FGF2, TGF-β1, and BMP-12 as individual growth factors or in combination. Unlike previous studies, no upregulation of scleraxis was measured in hASCs supplemented with BMP-12 alone.[19,28] This could be due to the difference in cell culture conditions required for formation of cell sheets compared with planar culture on TCPS used in previous studies. The highest increase in gene expression of *SCX*, *COL1*, and *COL3* in comparison to GM controls was achieved with sequential delivery of all three growth factors, consistent with the findings of previous studies comparing combinatorial and sequential treatment.[17,18,20,21]

Tenogenic cell sheets expressed increased osteogenic transcription factors compared with growth medium controls as well as the expected increase in scleraxis (Fig. 3). There was an increase in gene expression of scleraxis and osterix quantified by qRT-PCR and a single population of cells expressing both scleraxis and RUNX2 visualized by immunofluorescent staining (Fig. 4). This result agrees with previous studies where concurrent expression of tenogenic and osteogenic transcription factors has been observed in tendon and ligament fibroblasts.[29,30]

Takimoto *et al.* observed a population of Scx+/Osx+ fibroblastic cells throughout the periodontal ligament of mice and showed that overexpression of scleraxis in cells cultured in osteogenic conditions inhibits mineralization by downregulating expression of osteogenic genes *Opn* and *Ocn* without affecting Runx2 and Osx levels.[29] Similarly, it was found that the expression of scleraxis antagonizes BMP signaling and inhibits osteogenesis by preventing nuclear translocation of Runx2.[30] These findings are consistent with the results of this study and explain increased or unaltered levels of *OSX* and *RUNX2* in tenogenic samples with immunofluorescence results showing RUNX2 localized to the cytoplasm.

Cell sheets treated with tenogenic differentiation media had increased expression of proteins expressed in fibrocartilage and mineralized fibrocartilage zones of the enthesis including *COL2*, *COLX*, *IHH*, and *PTHrP* (Fig. 5). COL2 was present throughout the TM-TM and TM-OM cell sheets, indicating the potential of composite cell sheets to form fibrocartilage. However, COL2 was not localized to the interface between layers. These results could be attributed to the induction of fibrocartilaginous differentiation induced by compressive loading as a result of cell sheet stacking. Tendons and ligament under compressive loads exhibit fibrocartilaginous morphology, and multiple groups have reported transdifferentiation of tenocytes into fibrochondrocytes with cyclic loading.[31]

Gene expression of fibrocartilaginous proteins *COL2*, *IHH*, and *PTHrP* were increased in tenogenic cell sheets 1 week after stacking. IHH and PTHrP signaling molecules are sensitive to mechanical loading and are known to regulate endochondral ossification during development and maintenance of the enthesis.[32] After 2 weeks co-culture in stacks, both the TM-TM and TM-OM groups exhibited mineralization, indicating endochondral ossification of cell sheets.

There was no significant difference in gene expression between tenogenic only and tenogenic–osteogenic co-culture, in contrast to previous studies that have found a significant increase in the expression of fibrocartilage markers and osteogenic markers as a result of heterotypic cell–cell interactions between fibroblasts and osteoblasts or their progenitors.[13,14] However, the expression of *IHH* and *COL2* was highest in the TM-OM group, and *COL2* was significantly upregulated in the TM-OM group but not in the TM-TM group compared with GM-GM controls. Due to the transient increases in gene expression observed, deviations in gene expression between experimental replicates at the time points measured were high, decreasing the significance of the results. Further study is needed to determine the impact of co-culturing tenogenic and osteogenic progenitors differentiated from hASCs on protein expression and development of the interface.

Cell sheets have been investigated for regeneration of tendon and ligament with cells from a variety of sources, including primary fibroblasts from tendon and ligament and adult mesenchymal stem cells.[3,33–35] To date, the most studied application of cell sheets for tendon/ligament engineering is for regeneration of the periodontal ligament, progressing as far as clinical trials.[36] Cell sheets composed of periodontal ligament cells collected after tooth extraction have been successfully used for regeneration of periodontal ligament and integration of periodontal tissue interfaces with bone, cementum, and dental implants.[12,35–39] More recently, cell sheets have also been applied to regeneration of fibrocartilaginous entheses, such as the tendon–bone enthesis of the rotator cuff resulting in a significant increase in mechanical strength and ultimate failure load with cell sheets composed of human rotator cuff-derived cells.[3]

A variety of strategies implementing cell sheets have been investigated for regeneration of complex tissues such as the osteochondral interface and tendon/ligament–bone interfaces.[3,12,40,41] Wang *et al.* combined cell sheets with scaffolds for regeneration of an osteochondral defect. In this study, osteochondral tissue constructs were fabricated by stacking two layers of cartilage cell sheets on the surface of a polycaprolactone/hydroxyapatite scaffold embedded with bone marrow-derived stem cells.[40] This cell sheet–scaffold hybrid approach resulted in enhanced regeneration of the condyle and decreased inflammatory response in comparison to a biphasic scaffold.

In another study, monolayer periosteal progenitor cell sheets were wrapped around tendon grafts before they were pulled through a bone tunnel, resulting in increased fibrocartilage formation and osteointegration of the tendon.[41] Monolayer layer cell sheets were used to cover the repair site in a rat rotator cuff injury model as a method of delivering cells to the defect site and enhancing tissue regeneration through paracrine signaling.[3]

Raju *et al.* repaired ligament and bone with a two-layered composite consisting of a periodontal ligament cell sheet stacked on a cell sheet of MC3T3-E1 pre-osteoblasts transplanted between the root of a tooth and alveolar bone in a mouse periodontal defect model.[12] To date, composite cell sheets have not been used for repair of fibrocartilaginous entheses. Composite cell sheets could be combined with scaffolds or implanted between the tendon and bone during rotator cuff repair, utilizing a repair strategy similar to the one used by Raju *et al.*

Results of this study were promising, but this experiment was limited in scope to biochemical induction of differentiation and static cell culture *in vitro*. After stacking, cells did not continue down differentiation pathways. A spatial gradient in

RUNX2 expression and upregulated transcription of tenogenic differentiation marker tenomodulin was achieved but not maintained in static culture. These results are not unexpected and consistent with the body of literature indicating the importance of biomechanical stimulation for mature tendon/ligament formation and development of the enthesis.[30,32,42,43]

This study also did not take into consideration cell orientation. Cells and collagen fibrils in cell sheets are randomly oriented, unlike the highly organized physiological tendon–bone enthesis. Despite this, previous studies utilizing cell sheets have resulted in perpendicular fibers connecting ligament to bone after *in vivo* implantation.[12,14,38,44,45] Future *in vivo* studies will need to examine cell and collagen fiber orientation in repaired tissue.

Upregulated expression of fibrocartilage markers seen after stacking cell sheets is encouraging as failure to regenerate fibrocartilage has been linked to negative outcomes in tendon–bone interface repair *in vivo*. Regeneration of the fibrocartilage zones between tendon and bone leads to greater mechanical strength of the repaired enthesis and is a critical factor for long-term success.[3,4] Future studies will determine if this cell sheet stacking technique can be used for regeneration of fibrocartilaginous insertions as a gradient tissue *in vivo*.

Conclusions

In this study, cell sheets fabricated with adult mesenchymal stem cells were differentiated into progenitors of tendon and bone and stacked for *in vitro* fabrication of the tendon–bone interface. This work is an important step toward engineering of a complex tissue interface from a single cell source with composite cell sheets composed of adipose-derived stem cells. Co-culturing tenogenic and osteogenic cells in composite cell sheets resulted in increased expression of tenogenic and osteogenic transcription factors, mineralization, and fibrocartilage markers *COL2* and *IHH* 1 week after stacking. Composite cell sheets have the potential to increase regeneration of fibrocartilage at the enthesis and improve integration of repaired tendon and bone. Future studies will evaluate the impact of composite cell sheets on regeneration of the tendon–bone interface of the rotator cuff *in vivo*.

Authors' Contributions

L.B.: conception and design of study, data acquisition, data analysis and interpretation, writing, and revising the article. A.F.: design of study, data acquisition, writing, and revising the article. D.J.H.: conception of study, data analysis and interpretation, and revising the article.

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No competing financial interests exist.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Table S1

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