Biomaterials - Engineering Cell Behavior

Nanoparticle-Mediated TGF-β Release from Microribbon-Based Hydrogels Accelerates Stem Cell-Based Cartilage Formation *In Vivo*

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Abstract—Conventional nanoporous hydrogels often lead to slow cartilage deposition by MSCs in 3D due to physical constraints and requirement for degradation. Our group has recently reported macroporous gelatin microribbon (μRB) hydrogels, which substantially accelerate MSC-based cartilage formation *in vitro* compared to conventional gelatin hydrogels. To facilitate translating the use of μRB-based scaffolds for supporting stem cell-based cartilage regeneration *in vivo*, there remains a need to develop a customize-designed drug delivery system that can be incorporated into μRB-based scaffolds. Towards this goal, here we report polydopamine-coated mesoporous silica nanoparticles (MSNs) that can be stably incorporated within the macroporous μRB scaffolds, and allow tunable release of transforming growth factor (TGF)-β3. We hypothesize that increasing concentration of polydopamine coating on MSNs will slow down TGF-β3 release, and TGF-β3 release from polydopamine-coated MSNs can enhance MSC-based cartilage formation *in vitro* and *in vivo*. We demonstrate that TGF-β3 released from MSNs enhance MSC-based cartilage regeneration *in vitro* to levels comparable to freshly added TGF-β3 in the medium, as shown by biochemical assays, mechanical testing, and histology. Furthermore, when implanted *in vivo* in a mouse subcutaneous model, only the group containing MSN-mediated TGF-β3 release supported continuous cartilage formation, whereas control group without MSN showed loss of cartilage matrix and undesirable endochondral ossification. The modular design of MSN-mediated drug delivery can be customized for delivering multiple drugs with individually optimized release kinetics, and may be applicable to enhance regeneration of other tissue types.

INTRODUCTION

Hydrogels are attractive scaffolds for cartilage regeneration given their injectability, ease for homogeneous cell encapsulation and tunable chemical and physical properties.[21,40] Conventional hydrogels are nanoporous and have been used as 3D matrices for mesenchymal stem cells (MSCs) or chondrocytes to support new cartilage formation.[19,20] Given one important function of articular cartilage is shock-absorbing and weight bearing, it is critical to accelerate cartilage formation and restore mechanical properties of lost cartilage. However, physical restriction of nanoporous hydrogels often delay the speed of new matrix deposition, and it takes time for degradation before encapsulated cells can deposit new matrix. While a few studies have demonstrated obtaining cartilage with biomimetic strength, they require extremely high initial cell seeding density (i.e. 60 million/mL), dynamic mechanical loading, and prolonged *in vitro* culture with supplemented chondrogenic factors.[9,10,13] To accelerate the speed of cartilage deposition in hydrogels, our group recently reported a gelatin (GEL)-based microribbon (μRB) scaffold as 3D niche for stem cell-based tissue regeneration.[8] Unlike conventional hydrogels, the μRB scaffold is inherent macroporous, exhibit shock-absorbing capacity when subject to cyclic compression,[17] and significantly

accelerate MSC-based cartilage deposition *in vitro*.[8] Using lower density of MSCs (10 M/mL) for encapsulation, gelatin (μRB)-based scaffold led to over 20-fold increase in compressive moduli to 225 kPa after only 3 weeks of culture *in vitro*.[8] In contrast, conventional gelatin hydrogels only showed a slight increase in compressive modulus to ~ 65 kPa. While our study clearly demonstrated the advantage of macroporous μRB-based scaffold over conventional hydrogels as 3D matrix for accelerating MSC-based cartilage regeneration, it was limited to *in vitro* studies only and require supplement of full chondrogenic medium throughout the culture containing transforming growth factor-β3 (TGF-β3). TGF-β has been shown to play a key role in regulating cartilage development and homeostasis by impacting proliferation and phenotype of chondrocytes, the main resident cell type in cartilage.[15,45] When adding into cell culture medium, TGF-β has also been shown to promote MSC chondrogenesis *in vitro*.[1,16] However, TGF-β exhibits short half-life, and requires continuous supplementation. To facilitate translating the use of μRB-based scaffolds for supporting stem cell-based cartilage regeneration *in vivo*, there remains a need to develop a customize-designed drug delivery system with local and sustained release of TGF-β that can be incorporated into μRB-based scaffolds.

Several strategies have been developed to achieve sustained release of TGF-β to enhance chondrogenesis and cartilage regeneration *in vitro* and *in vivo*.[11,27,37,43] Injection of magnetic liposome-containing TGF-β1 into cartilage defects in a rabbit model was reported to significantly improve cartilage regeneration after 12 weeks.[43] In another study, TGF-β was bound to heparin and co-delivered with dexamethasone from PLGA microspheres to enhance chondrogenesis *in vitro* and *in vivo*.[33] However, these systems are usually associated with poor protein loading efficiency due to the hydrophobic nature of particles, and the usage of organic solvents in fabricating PLGA microparticles can reduce bioactivity of encapsulated growth factors. Additionally, the particles used in these studies are physically entrapped within conventional nanoporous hydrogels, which will not be retained in the μRB scaffolds due to the macroporosity.

The main goal of the present study is to design drug delivery components specifically for μRB scaffolds that can be entrapped within μRB scaffold, supporting tunable release kinetics, and retain bioactivity *in vitro* and *in vivo* to accelerate MSC-based cartilage regeneration. TGF-β3 was used as a model protein given its demonstrated efficacy in enhancing MSC chondrogenesis.[22,30] We chose mesoporous silica nanoparticles (MSNs) as the drug delivery vehicles due to their high surface area, high drug loading capacity, commercial availability and demonstrated biocompatibility *in vivo*.[39,48] To allow stable incorporation of MSNs in macroporous μRB scaffold and achieve tunable release kinetics, we chose to coat MSNs with polydopamine, which have been widely used as an adhesive coating [4,23] and for tuning drug release.[2,26] MSNs have been previously used to deliver chemotherapeutic agents, proteins, and therapeutic genetic materials.[48] The drug payloads can be either diffused into the pores and then coated using a polymeric network to prevent the burst release, or chemically conjugated to the particle surface through a chemical reaction.[39] We selected the diffusion loading due to its ease and minimal perturbation to the bioactivity of growth factors.

In this study, we hypothesized that by increasing the concentration of dopamine in the precursor solution, the rate of dopamine polymerization and consequently, the amount of polydopamine coating on the surface of MSNs would increase which can further slow down the diffusion of TGF-β3 through MSNs.[6,49] In addition, we hypothesized that incorporating MSN-mediated TGF-β3 release in μRB scaffold could enhance MSC-based cartilage regeneration *in vivo*. We first characterize the scaffold morphology and release kinetics using MSN coated with varying concentration of dopamine. The bioactivity of released TGF-β3 was first evaluated *in vitro*, and subsequently *in vivo* using a mouse subcutaneous model. Outcomes were evaluated using ELISA, scanning electron microscopy, biochemical assays, mechanical testing, histology, and immunofluorescent staining.

MATERIALS AND METHODS

Fabrication of TGF-β:MSNs

Hydrophilic mesoporous silica nanoparticles (MSNs) were purchased from Degussa AG (Hanau, Germany). For protein loading, MSNs (40 μg/mL) were soaked in TGF-β3 (Peprotech, Rocky Hill, NJ) solution (1 μg/mL) and incubated while mixing (400 rpm, 25 °C) for 30 min to allow the protein molecules diffuse into the mesoporous pores within MSNs. To prepare polydopamine-coated MSNs, 10 mg protein-loaded MSN was dispersed in 1 mL Tris-HCl buffer (pH 8.5) containing dopamine at various concentrations (0.5, 1.0 and 2.0 mg/mL) for 3 hr to allow dopamine polymerization on the surface of MSNs. Polydopamine-coated MSNs were then washed in PBS and centrifuged to remove the unloaded protein, and this process was repeated three times. The amount of unloaded TGF-β3 (in supernatants after each washing

step) was quantified using ELISA. By subtracting unloaded protein from total initial protein, the loading efficiency is defined as as (total protein-unloaded protein)/total protein. The loading efficiency of TGF-β3 in MSNs was calculated to be $43 \pm 6\%$.

μRB Fabrication

Gelatin (GEL) μRBs were fabricated as previously reported.[13,24] To fabricate chondroitin sulfate (CS) μRBs, chondroitin sulfate sodium salt (from bovine cartilage, Sigma-Aldrich) was functionalized with acrylamide and 2.5 mM CRGDS. CS solution (7.5% (w/v)) was spun into a stirring bath of 2-propanol and internally crosslinked with dithiolretinol to fix the μRB shape.

Scanning Electron Microscopy

To verify if polydopamine coating can enable stable incorporation of MSNs onto the surface of gelatin-CS μRB scaffolds, scaffolds before and after MSN incorporation was assessed using a Hitachi S-3400N variable pressure scanning electron microscope (VP-SEM) as previously described.[13]

Cell Encapsulation in 3D μRB Scaffolds

GEL and CS μRBs were mixed in a 1:1 ratio and hydrated in PBS containing 0.1% (w/v) of photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), reaching a final total μRB density of 7.5% (w/v). For groups with MSNs, the TGF-β:MSNs were added to the LAP solution before hydrating the μRBs. In the *in vitro* study, the culture medium of each sample were replaced by 1.5 mL of fresh cohndrogenic medium (containing 10 ng/mL TGF-β3)[52] every other day (15 ng fresh TGF-β3 every other day). Therefore, the amount of TGF-β3 loaded in "MSN + DPA 1" was adjusted in a way that the average amount of released TGF-β3 would be ~ 7.5 ng/day to be able to compare the results with the group with freshly added TGF-β3 to the medium. For the control group containing directly encapsulated TGF-β3, 150 ng of TGF-β3 was added to PBS used to hydrate μRBs. Human MSCs (Lonza, passage 6) were mixed with the hydrated μRB to reach a density of 10 M mL^{-1}, and incubated for 15 mins at 37 °C to ensure full hydration. After incubation, the cell/μRB mixture was loaded into cylindrical molds (4 mm in diameter and 2 mm in height), photocrosslinked by exposing to UV light (365 nm, 4 mW cm^{-2}, 5 min). Crosslinked μRB scaffolds containing MSCs were transferred to 24-well plates for culture.

Quantifying TGF-β3 Release Using ELISA

TGF-β:MSNs were encapsulated within GEL:CS μRB scaffolds as described above and incubated at 37 °C in DMEM with 10% FBS. To determine the protein release kinetics, the supernatant was collected at every time point and replaced completely with fresh medium. TGF-β3 release was determined using an ELISA assay with purified human TGF-β3 as the standard, following the manufacturer's protocol (BioGems).

Cell Culture and Quantification of Cartilage Formation In Vitro

Cell-laden μRB scaffolds were first cultured in growth medium for 24 h and then switched to chondrogenic medium for 21 days before final harvest. Four groups were examined including no TGF-β ($-$T), TGF-β directly encapsulated in μRB scaffolds ($+$T-encap), freshly added TGF-β in medium ($+$T-media), and TGF-β released from MSN encapsulated in scaffolds ($+$T:MSN). A separate *in vitro* study was performed to determine what components from chondrogenic medium are essential for inducing MSC chondrogenesis. Samples were cultured in DMEM supplemented with single or combination of various components from chondrogenic medium. All *in vitro* groups were cultured for 21 days before analysis. After 21 days of culture in chondrogenic medium, scaffolds were harvested and digested in papainase solution. DNA, sGAG, and collagen were quantified as previously reported.[47] To measure mechanical properties of MSC-produced cartilage after 21 days of *in vitro* culture, unconfined compression tests were conducted using an Instron 5944 materials testing system (Instron Corporation) with a 10 N load cell (Interface, Inc.).[13] Compressive moduli of all samples were determined from the 10–20% region of linear fit curve from the stress versus strain plot. Additionally, histology analyses was performed for cartilage and bone markers including Collagen II, and Collagen X, Collagen I, Safronin O, Masson's trichrome and alizarin red S staining (ARS).

For *in vitro* study, each sample was cultured in 1.5 mL of chondrogenic medium (containing 10 ng/mL TGF-β3),[47] which is refreshed every other day (i.e. consuming 15 ng TGF-β3 every other day). This is equivalent to consuming 7.5 ng TGF-β3 per day per sample. To match the total amount of TGF-β3 loaded in scaffolds ($+$T-encap) with the total amount of free TGF-β3 used over 20 days of culture, 150 ng of TGF-β3 was added to PBS used to hydrate μRBs. For T:MSN group, we calculated the amount of MSN with a total accumulated release of 150 ng TGF-β3 after 21 days. Based

on our calculated loading efficiency (43%) and 35% accumulated release *in vitro* after 21 days, MSNs were incubated in solution containing 1000 ng TGF-β3, which led to 1000 ng $\times$ 43% $\times$ 35% = 150 ng.

Evaluating the Efficacy of TGF-β

MSN in Supporting MSC-Based Cartilage Formation In Vivo Using a Mouse Subcutaneous Model

Animal studies were performed in accordance with the guidelines for the care and use of laboratory animals. All protocols were approved by the Stanford Institutional Animal Care and Use committee. For *in vivo* subcutaneous study, all groups were first primed in chondrogenic medium for 14 days *in vitro* to induce chondrogenesis before implantation. Two groups were compared including samples exposed to freshly added TGF-β *in vitro* before implantation, or samples containing TGF-β:MSN for controlled release over time. Scaffolds were prepared as explained above and implanted subcutaneously into nude female mice (NCr nude, 6 weeks old, Taconic). Mice were sacrificed to obtain constructs after 21 days *in vivo*. Four samples per group were evaluated for histology analyses for cartilage and bone markers including Collagen II, and Collagen X, Collagen I, Safronin O, Masson's trichrome and alizarin red S staining (ARS).

Histology and Immunostaining

At each harvesting time point for histology, samples were fixed first in 4% paraformaldehyde for 2 h, before being embedded in optimal cutting temperature compound for cryo-sectioning. For *in vivo* samples, they were also transferred to 16% ethylenediaminetetraacetic acid for two weeks for decalcification before being embedded. For histological staining, sectioned tissue slices were stained with Safronin O (for sulfated glycosaminoglycans), Masson's trichrome (for total collagen), and alizarin red S staining (for mineralization). Staining reagents were obtained from Thermo Scientific (Waltham, MA). For immunostaining, sectioned slices were first treated with blocking buffer consisting of 2% goat serum and 3% bovine serum albumin in 1X PBS, then incubated overnight at 48 °C in primary antibody solution containing rabbit polyclonal antibody against Collagen II (1:100 dilution), Collagen I (1:100 dilution), or Collagen X (1:100 dilution). All primary antibodies were purchased from Abcam (Cambridge, MA). After washing, sectioned tissue slices were subsequently incubated with secondary antibody solution for 1 h at room temperature. The following secondary antibody was used including goat anti-rabbit Alexa 488 (1:200 dilution) (Invitrogen, Carlsbad, CA). Nuclei were counterstained with Hoechst 33342 stain (Thermo Scientific, Waltham, MA) and images were taken using a Zeiss fluorescence microscope as previously described.[8] Sections were stained with all reagents without primary antibody for negative controls.

Statistical Analysis

All experiments were conducted with a minimum of three distinct samples to verify the trend of results. All data are presented as mean $\pm$ standard deviation ($n \geq 3$/group). GraphPad Prism (GraphPad Software) was used to perform a one- or two-way analysis of variance followed by Tukey's multiple comparisons test.

RESULTS

Varying Polydopamine Coating Modulates TGF-β3 Release from MSN

To determine the release kinetics of TGF-β3 from MSNs, TGF-β3 was loaded into MSNs which were then coated with three different concentrations of dopamine (Fig. 1a). The lowest concentration of dopamine (0.5 mg/mL) resulted in burst release of TGF-β3, with all growth factor released by day 7. The highest concentration of dopamine (2 mg/mL) resulted in the slowest release, with only about 20% of the loaded TGF-β3 released after 14 days (Fig. 1b). MSNs coated with intermediate concentration (1 mg/mL) dopamine showed the most desirable release kinetics, with accumulated release up to 30% of the loaded TGF-β3 after 14 days of incubation *in vitro* (Fig. 1b). Based on an initial loading efficiency of 43%, this is equivalent to $\sim$ 7 ng/day release on average, which is comparable to the dosage used in the free TGF-β3 control group. As such, we chose MSNs coated with 1.0 mg/mL dopamine for all further *in vitro* and *in vivo* studies. SEM imaging showed that polydopamine-coated MSNs attached homogenously to the μRB scaffolds (Fig. 1c). In contrast, uncoated MSN failed to be encapsulated inside the μRB scaffolds, and minimal MSN can be observed by SEM (Supplementary Fig. S1). This result is expected as the macroporosity in μRB scaffolds is much larger than the diameter of nanosize MSN, highlighting the importance of using adhesion coating to support stable MSN incorporation.

MSN-Released TGF-β Enhances Cartilage Formation by MSCs In Vitro

To test the efficacy of TGF-β:MSNs in stimulating MSC chondrogenesis *in vitro*, we evaluated four
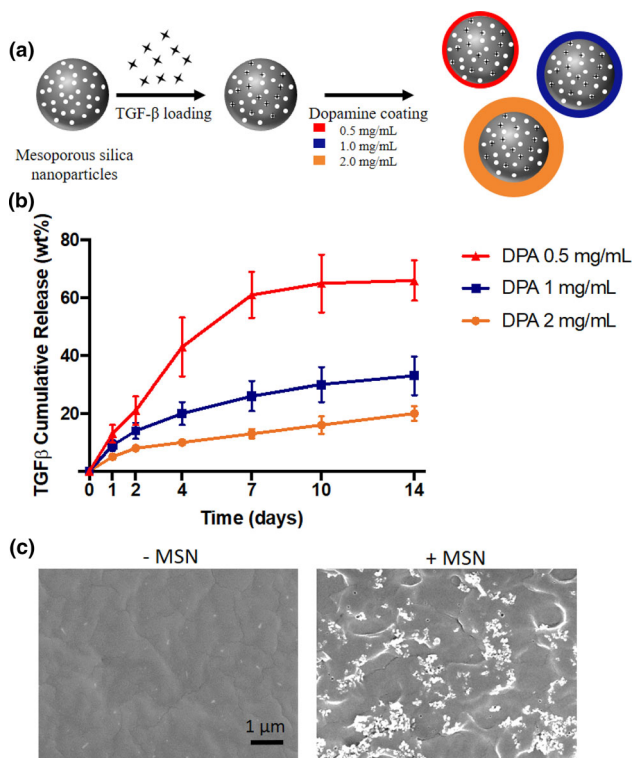
FIGURE 1. Fabrication and characterization of release kinetics of TGF-β MSNs coated with varying concentration of dopamine. (a) Schematic of preparing TGF-β:MSN with varying concentrations of dopamine coating; (b) Accumulated percentage of TGF-β released from MSNs with varying dopamine coating; Data are presented as means ± s.d. (n = 3/group). (c) Scanning electron microscopy images of 3D gelatin/CS μRB scaffolds without (left) and with (right) MSNs coated with DPA1. Scale bar: 1 μm.

groups with MSC encapsulated in gelatin-CS μRB scaffolds: without TGF-β3 ($-$ T), TGF-β3 directly encapsulated in the μRB scaffold ($+$ T encap), TGF-β3 supplemented to the media freshly every other day ($+$ T media), and TGF-β3 loaded into MSNs ($+$ T:MSN). All groups showed high cell viability (Fig. 2a), similar cell number and scaffold background on Day 1 (Supplementary Fig. S2), indicating MSNs are cytocompatible and do not interfere with cell adhesion. After 21 days of culture, scaffolds without TGF-β3 or with directly encapsulated TGF-β3 within the scaffold show very little proliferation or cartilage deposition, as shown both by biochemical assays (Figs. 2b and 2c), mechanical testing (Fig. 2d), and histology (Fig. 3). To characterize the phenotype of newly deposition matrix, we performed staining of Safranin-O (sGAG), Masson's Trichrome (total collagen), Collagen II (articular cartilage marker), Collagen I (fibrocartilage marker) and Collagen X (hypertrophic marker) (Fig. 3). In contrast, TGF-β:MSN group supported robust cartilage deposition to a level comparable to the freshly added TGF-β (posi-

tive control) (Figs. 2 and 3). The groups without TGF-β3 or with encapsulated TGF-β3 exhibited very low compressive moduli at the end of culture (~ 5 kPa) (Fig. 2e), which is similar to Day 1 (Supplementary Fig. S2F). In contrast, TGF-β:MSN group and free TGF-β group exhibited over 50-fold increase in compressive moduli up to ~ 400 to 500 kPa (Fig. 2e). Together, these findings validate that MSN-mediated TGF-β release can support MSC chondrogenesis and robust cartilage deposition *in vitro*.

MSN-Mediated TGF-β3 Release Supports Continuous Cartilage Formation In Vivo

When cultured in PBS *in vitro*, polydopamine-coated MSNs support continuous release of TGF-β3 over long periods for up to 75 days (Fig. 4a). We next evaluated whether TGF-β:MSN can support MSC-based cartilage formation *in vivo*. Two groups were evaluated including gelatin/CS scaffolds with ($+$T:MSN) or without ($-$T:MSN) MSN containing TGF-β. All samples were primed *in vitro* in chondrogenic medium for 14 days prior to subcutaneous implantation, then harvested at day 35 (21 days of in vivo) (Fig. 4b). $-$ T:MSN group also received exogenous TGF-β (10 ng/mL) during *in vitro* priming. After 14 days of *in vitro* culture, both groups showed interconnected sGAG and collagen deposition, high collagen II expression, and minimal hypertrophy (collagen X) or mineralization (ARS) (Fig. 4c). Upon *in vivo* harvest at day 35, -T:MSN groups showed substantial loss of sGAG and robust mineralization, which was accompanied by decreased collagen II and increased collagen I and collagen X staining (Fig. 4d). In contrast, the $+$ T:MSN group showed increased sGAG and collagen production, robust collagen II staining, and low level of mineralization. Together, these results showed *in situ* continued release of TGF-β is necessary for supporting continuous cartilage deposition *in vivo*, and MSN-mediated release was sufficient to support cartilage deposition while reducing undesirable endochondral ossification in a subcutaneous model.

DISCUSSION

Our previous work has shown macroporous gelatin μRB scaffolds are more efficient in accelerating MSC-based cartilage regeneration *in vitro* than conventional gelatin hydrogels.[8] To realize the potential of gelatin μRB scaffolds for supporting MSC-based cartilage regeneration *in vivo*, it is important to develop an *in situ* drug delivery system that is compatible with the macroporous μRB scaffolds. Here we report a drug
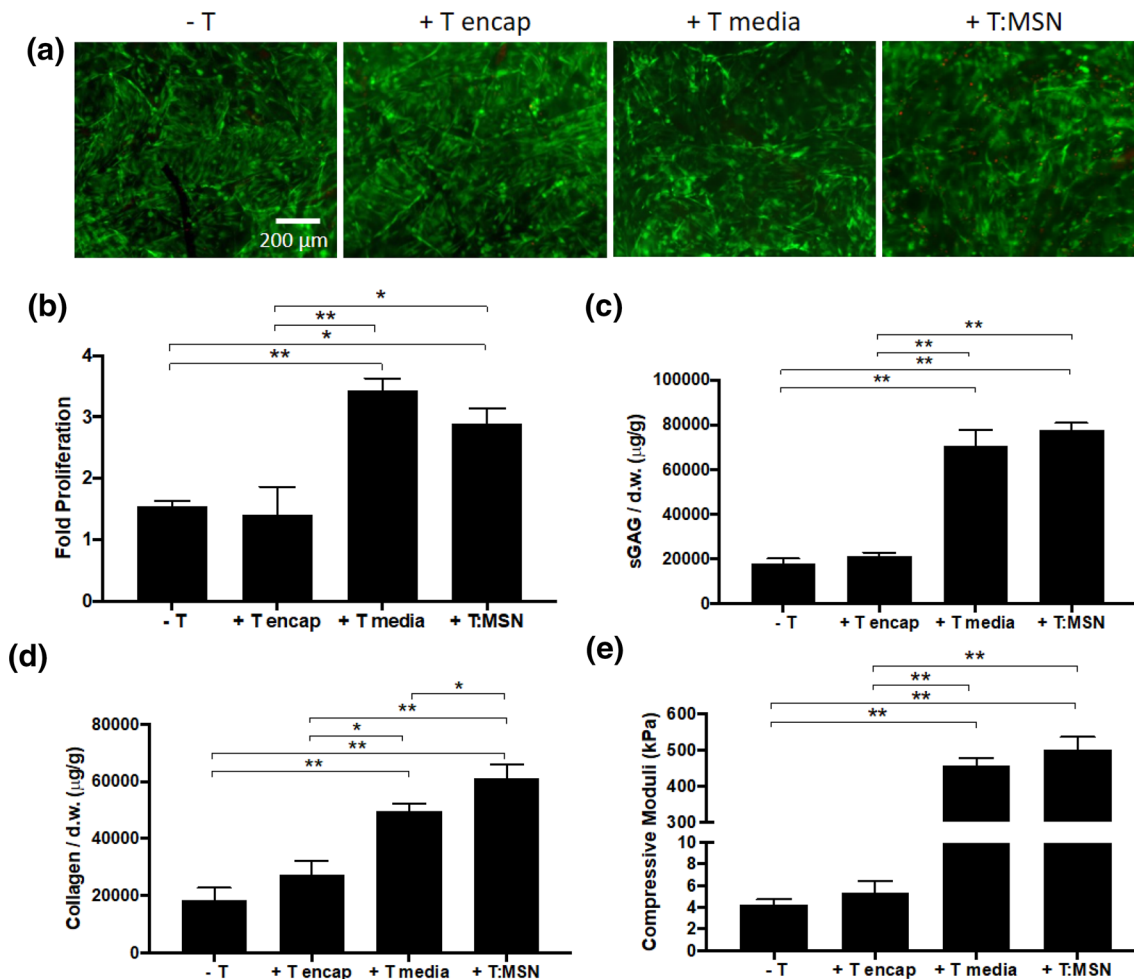
FIGURE 2. TGFβ released from MSNs, but not direct TGFβ encapsulation, support robust cartilage deposition by MSCs *in vitro* to a level comparable to freshly supplemented TGFβ in medium (positive control). A total of four groups were evaluated: – T: no TGFβ in medium, + T encap: TGFβ directly encapsulated in μRB scaffolds, + T media: freshly supplemented TGFβ in medium, + T:MSN: TGFβ-releasing MSNs in μRB scaffolds. (a) Cell viability and morphology at day 1 as shown by live/dead staining, scale bar: 200 μm. (b) fold of cell proliferation at day 21; (c, d) normalized total sGAG and collagen per dry weight at day 21; (e) Compressive moduli of cell-laden μRB scaffolds at day 21. Data is reported as mean $\pm$ SD (n = 3/group). *$p < 0.05$, **$p < 0.001$. Data are presented as means $\pm$ s.d. (n = 3/group).

delivery platform specifically designed for the macroporous μRB scaffold using polydopamine-coated mesoporous silica nanoparticles (MSNs). The polydopamine coating of MSN allows easy and stable incorporation of MSNs into macroporous μRB scaffolds (Fig. 1) and enables tunable release kinetics with retained bioactivity (Figs. 1 and 2). The way polydopamine coating enable tunable TGF-β3 release can be mediated through a combination of several possible mechanisms. First, the adhesive polydopamine coating on the surface of MSNs sets up a physical barrier against the protein diffusion out of the mesopores of MSN. As such, increasing polydopamine concentration is expected to increase the diffusion barrier, thereby slowing down the release, as validated by our *in vitro* release data (Fig. 1b). Second, polydopamine is formed at pH ~ 8.5, but degrades in lower pH than

8.5.[42] Therefore, when cultured in physiological pH (7.4) *in vitro* or *in vivo*, polydopamine coating will slowly degrade over time, allowing protein release. It should be noted that some of the TGF-β3 may interact with dopamine or denature during the coating step, therefore not detectable by ELISA. In this case, these growth factors will probably also not be bioactive for the cells. As such, it is still reasonable to use the trend of release kinetics from ELISA to choose the optimal concentration of dopamine coating (1 mg/mL) that avoids burst release but still allows more sustained protein release over time.

MSNs have been widely used for various drug delivery applications *in vivo* due to their mesoporous structures and high surface areas for loading anti-cancer drugs or biologically active molecules (e.g., proteins and nucleotides).[5,42] In general, the cytotoxi-
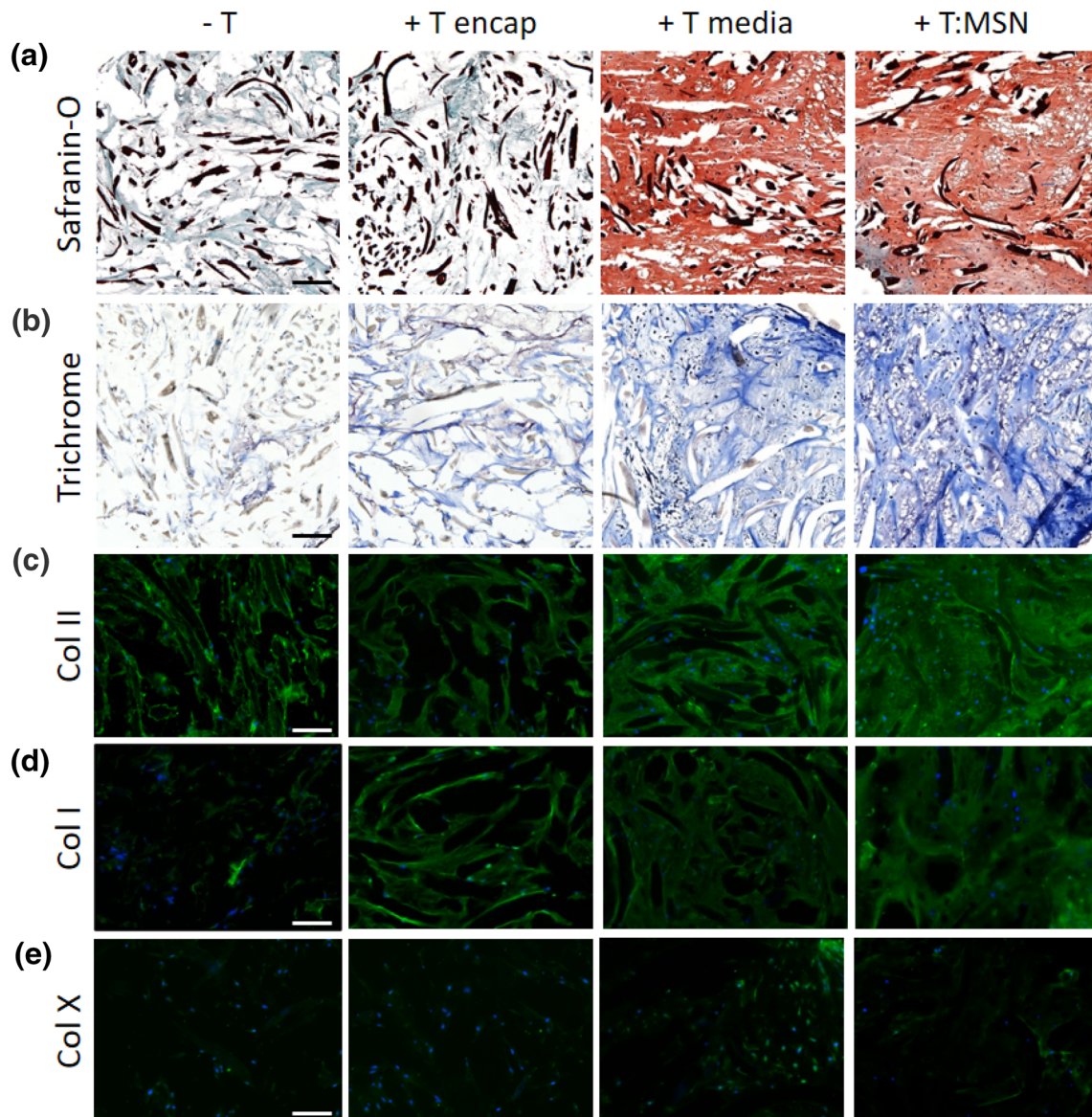
FIGURE 3. Histological staining of MSC-laden μRB scaffolds after 21 days of culture in chondrogenic medium. A total of four groups were evaluated: – T: no TGFβ in medium, + T encap: free TGFβ directly encapsulated in μRB scaffolds, + T media: freshly supplemented TGFβ in medium, + T:MSN: TGFβ-releasing MSNs in μRB scaffolds (n = 3/group). (a) Safranin-O to visualize glycosaminoglycans, (b) Masson's Trichrome to visualize collagen, (c) Collagen II (articular) staining, (d) Collagen I (fibrocartilage) staining, and (e) Collagen X (hypertrophic) staining. Scale bars: 250 μm.

city and biocompatibility of nanomaterials depend on the size, shape, surface chemistry, and charge of particles. Several studies show that when MSNs were used at concentrations lower than 100 μg/mL, minimum cytotoxicity was observed.[18,44] In the current study, we used 25 μg/mL of MSNs in the scaffold, and did not observe any fibrosis or cytotoxicity both *in vitro* and *in vivo*. In addition, it has been shown that in low concentrations (< 100 μg/mL), MSNs could be cleared from the body through excretion with urine.[44]

Our results demonstrate that TGF-β3 released from polydopamine-coated MSNs enhance cartilage depo-sition by MSCs to levels comparable to the control treated with fresh TGF-β3 control *in vitro* (Figs. 2 and 3). Furthermore, only μRB scaffolds containing T:MSN support continuous cartilage deposition *in vivo* (Fig. 4). These results confirm that sufficient amount of TGF-β3 released from MSNs retain bioactivity to achieve the desirable biological outcomes. It is possible that some of the loaded TGF-β3 remain bound to MSNs. Previous studies have shown that even the immobilized TGF-β3 can induce chondrogenic differ-entiation of MSCs.[28,31] As such, both freely released TGF-β3 and MSN-bound TGF-β3 could contribute to
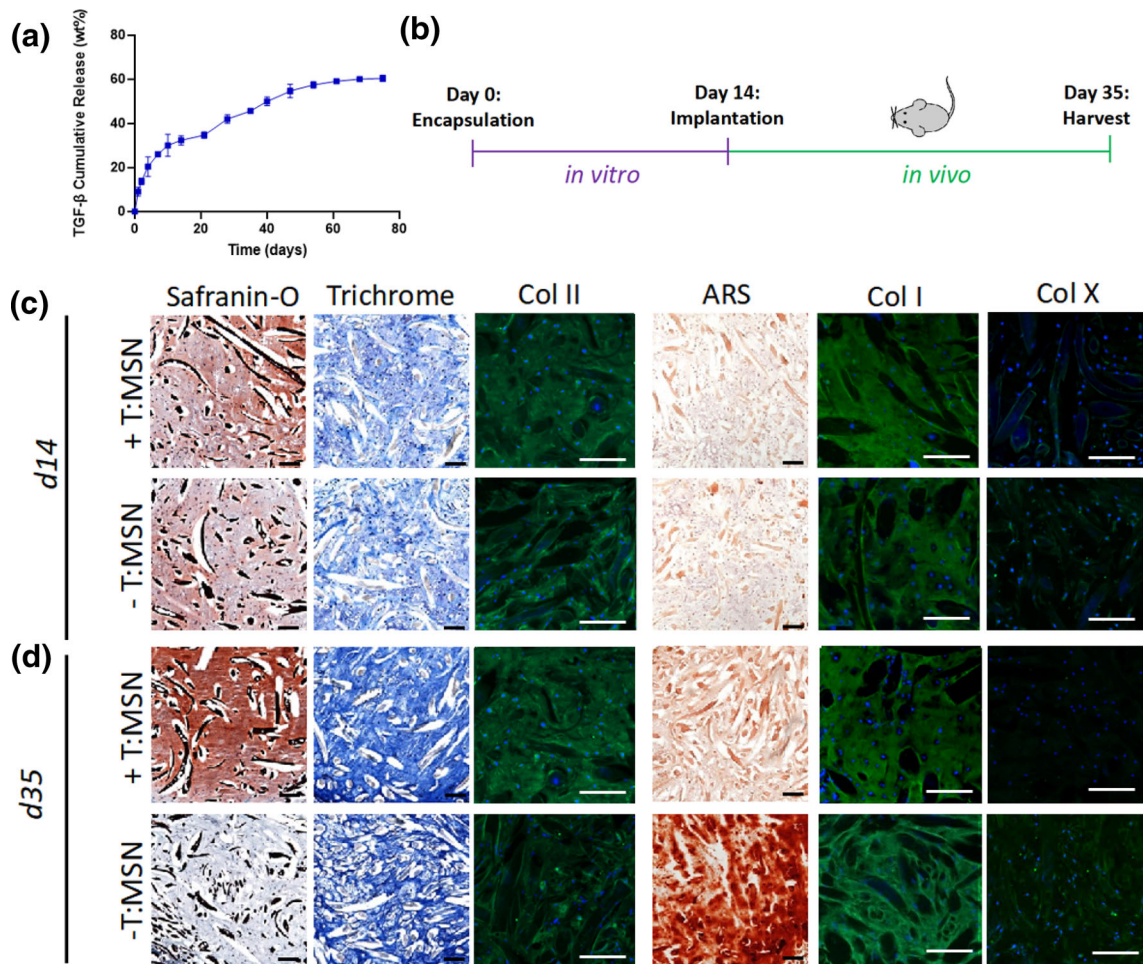
FIGURE 4. *μ*RB scaffolds containing TGF-*β* releasing MSNs support continuous cartilage formation by MSCs and minimizes endochondral ossification in a mouse subcutaneous model. Data are presented as means ± s.d. (*n* = 3/group). (a) TGF-*β* release from MSNs can be detected for up to 75 days *in vitro*. (b) Schematic of *in vivo* experimental design. All samples were primed in chondrogenic medium for 14 days prior to implantation. – T:MSN group also received exogenous TGF-*β* (10 ng/mL) during *in vitro* priming. Histology and immunofluorescence staining of cartilage and bone markers of MSC-laden sample at (c) day 14 (the end of *in vitro* priming) and (d) day 35 (the end of *in vivo* implantation). Safranin-O (glycosaminoglycan), Trichrome: total collagen, ARS: alizarin red S staining for mineralization. Collagen II (articular cartilage marker), Collagen I (fibrocartilage marker), and Collagen X (hypertrophic marker). Scale bars: 250 *μ*m.

the enhanced cartilage deposition observed in our study.

An important finding of the current study is that the continued release of TGF-*β*3 after subcutaneous implantation is essential for maintaining cartilage phenotype and reducing undesirable endochondral ossification (Figs. 4c and 4d). MSC-formed cartilage tissues often shows hypertrophy marker and early bone markers including type X collagen, alkaline phosphatase (ALP) and matrix metallopeptidase 13 (MMP-13),[30,50] which is similar to the phenotype of chondrocytes in growth plates during endochondral ossification. Additionally, some *in vivo* studies have observed the invasion of blood vessels and matrix calcification after human chondrogenic MSCs pellet transplantation into mice.[35,36] This natural behavior is

one of the main concerns with applications of MSCs for articular cartilage regeneration. Further, it has been demonstrated that TGF-*β* signaling can reduce undesirable calcification and catabolic gene expression, such as collagen X and MMP13; while increase collagen II and aggrecan expressions.[15,29,38,45] Interestingly, this is consistent with our observations that TGF-*β*:MSNs group reduced endochondral ossification that was observed in control group without MSNs, possibly due to the sustained release of TGF-*β*.

It is also worth pointing out that mouse subcutaneous model does not mimic the soluble factors or weight-bearing stimuli in the joint, which may explain the observed endochondral ossification.[12,32] Previous studies have shown endochondral ossification is commonly observed in the subcutaneous model, but much

less so in the cartilage defect model.[41,46] We chose the mouse subcutaneous model in the present study as this is the first proof-of-concept *in vivo* study for MSN-mediated drug delivery in macroporous μRB scaffolds. Compared to other more complicated animal models, the subcutaneous model allows testing a larger sample size using less animals, and is commonly used as the first animal model for initial assessment of new biomaterials for supporting cartilage regeneration *in vivo*. Now that we have established MSN-containing μRB scaffolds can enhance MSC-based cartilage regeneration in a mouse subcutaneous model, future studies can validate its efficacy using more disease relevant models such as rat cartilage defect model and other larger animal cartilage defect models.[7]

Another limitation of the current study is the need to prime MSC-seeded μRB scaffolds *in vitro* for 2 weeks prior to *in vivo* implantation. This is because the current study focuses on single factor delivery, yet we found from our *in vitro* screening study that multiple factors in chondrogenic medium are necessary to initiate robust MSC chondrogenesis (Supplementary Fig. S4). To reduce or eliminate the need for *in vitro* priming, future studies are necessary to determine the optimal MSN formulation for co-delivery multiple factors from μRB scaffolds. Given the modular design of polydopamine-coated MSNs (Fig. 1b), different MSNs formulated for delivering various factors can be optimized independently, and mixed and co-encapsulated together in the μRB scaffolds to achieve the sustained release of multiple factors.

While the present paper focuses on cartilage regeneration application, the concept of μRB scaffolds capable of achieving *in situ* drug delivery can be broadly applicable to enhance regeneration of other tissue types. For example, controlled release of bone morphogenetic protein-2 (BMP-2) may be used to accelerate stem cell-based bone formation *in vivo*.[3,25] Moreover, the facile and tunable polydopamine coatings allows customized design of MSNs to achieve temporal release of different growth factors. For example, synovial-derived MSC pellets treated with FGF-2 followed by exposure to a combination of TGF-β1 and IGF-1 showed significantly more cartilage formation compared to other temporal growth factor regimes.[34] Sequential treatment with TGF-β1 followed by IGF-1 has been shown to enhance MSC chondrogenesis compared to control without sequential exposure[51] or TGF-β3 exposure alone.[14]

In summary, here we developed MSN-containing macroporous μRB scaffolds capable of prolonged release of TGF-β3 release to enhance MSC-based cartilage regeneration *in vitro* and *in vivo*. By tuning polydopamine coating, we can achieve tunable release kinetics. TGF-β3 released from MSNs retained its bioactivity comparable to freshly added TGF-β3 control, and led to robust cartilage formation *in vitro*. When implanted *in vivo* in a mouse subcutaneous model, MSN-mediated TGF-β3 release was essential to support continuous cartilage formation while reducing undesirable endochondral ossification. The modular design of MSN-mediated drug delivery allows expanding the platform for delivering multiple soluble factors with individually optimized release kinetics, and can be applicable to enhance regeneration of other tissue types.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (https://doi.org/10.1007/s10439-020-02522-z) contains supplementary material, which is available to authorized users.

CONFLICT OF INTEREST

No potential conflicts of interest exist.

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