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14. ABSTRACT Our primary objective is to demonstrate approaches to create mechanically robust, patient-customized biomaterials for large, load-bearing maxillofacial bone defects. To do this, we are developing approaches to integrate a biomolecule decorated collagen scaffold with micro-scale porosity into a mechanically-robust polymeric frame generated via 3D-printing with macro-porosity. Through the second year of this project we have completed the design, fabrication, as well as mechanical and in vitro osteogenesis testing of multiple elements of the final composite biomaterial design. We identified a PLA-collagen composite that could support robust mesenchymal stem cell (MSC) viability, osteogenic differentiation, and new mineral synthesis while rendering it shape-fitting to improve conformal contact with the wound margin. We have advanced multiple strategies to incorporate biomolecular signals into the collagen scaffold via transient sequestration, covalent attachment, and through included zinc ions that enhance osteogenic activity. Ongoing efforts are completing biomolecule incorporation and release experiments to facilitate long-term (>7 day) biomolecule bioavailability via mineral-based factor sequestration chemistries. Our efforts are essential for our goal to identify a shelf-stable, patient customizable biomaterial that can be seeded with autologous MSCs to regenerate large CMF bone defects.					
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INTRODUCTION:

Severe craniomaxillofacial (CMF) injuries are prevalent after a wide range of acute and chronic injuries, are typically large in size, and are characterized by significant loss of hard and soft tissue. Our primary objective is to demonstrate a novel approach for creating mechanically robust, patient-customized biomaterials for large, load-bearing maxillofacial bone defects. We address a fundamental bottleneck in CMF biomaterial design. Constructs must balance very real considerations regarding mechanical competence and load bearing, the need to fit complex defect geometries unique to each patient, and biotransport of nutrients within the construct during healing. To do this, we are developing approaches to integrate a biomolecule decorated collagen scaffold with micro-scale porosity into a mechanically-robust polymeric frame generated via 3D-printing with macro-porosity. We are first validating the osteogenic potential of growth factor decorated composite (Aim 1). We are evaluating the influence of composite structural properties on mesenchymal stem cell (MSC) osteogenesis in vitro and will subsequently establish whether, and to what extent, selective incorporation and release of the growth factors BMP-2/VEGF, the mineral zinc, or modifications to scaffold glycosaminoglycan (GAG) content enhances osteogenesis. Next, we will examine the quality and kinetics of mandible bone regeneration using the composite in critically-sized mandibular ramus defects in the Yorkshire pig (Aim 2). The larger goal of this program is to identify a shelf-stable, patient customizable biomaterial that can be seeded with autologous MSCs intraoperatively and immediately implanted in order to regenerate large, load-bearing CMF bone defects.

KEYWORDS:

Biomaterial, composite, collagen, growth factor release, osteogenesis

OVERALL PROJECT SUMMARY:

Through the third year of this project, we have made significant progress in addressing tasks and milestones associated with Major Tasks 1 and 2. Our efforts are on track to meet the goals of the entire research project, which are to validate the osteogenic potential of the biomolecule decorated collagen composites via tiered in vitro and in vivo assays. However to complete the in vivo animal component of the proposed work we have requested and received a No Cost Extension (through 9/30/2020).

MAJOR TASK 1: Composite Fabrication

The primary goals associated with Major Task 1 are:

- Fabricate PCL cages for collagen-PCL composites
- Fabricate library of collagen-PCL composites and growth factor modified composites for in vitro testing

Key Milestones associated with Major Task 1 are:

- Biophysical characterization of mineralized collagen scaffolds and PCL-collagen composites
- Biophysical and functional characterization of growth factor loading/elution

MAJOR TASK 2: In vitro osteogenesis assays

The primary goals associated with Major Task 2 are:

- Assess mechanisms by which the mineralized collagen scaffold accelerate MSC osteogenic differentiation and matrix biosynthesis.

Key Milestones associated with Major Task 2 are:

- Identify the degree to which BMP-2, VEGF, and growth factor sequestering chemistries incorporated within the collagen scaffolds increase MSC osteogenic differentiation.

Summary of Results, Progress and Accomplishments with Discussion:

PRIOR WORK (separate from USAMRMC funding). Mineralized collagen scaffolds support MSC osteogenesis. In work separate from this project, we have refined a model CaP nanocrystallite mineralized collagen-GAG scaffold fabricated via lyophilization that promotes MSC osteogenic differentiation in the absence of osteogenic supplements (no BMP-2, osteogenic media). These efforts have focused on mechanistic investigations, where we showed these scaffolds promote osteogenesis via activation of endogenous BMP-2 receptor paths and that they promote enhanced regenerative healing of bone defects in vivo in small animal models (rabbit, rat) [2-6]. However, the compressive modulus of these scaffolds is far too low for application in CMF bone repair and necessitated the composite designs developed by this project. Further, there are opportunities to expand the scope of bone repair via the inclusion of growth factors release technologies.

PRIOR WORK (reported in YR2 annual report). Modification of growth factor bioavailability to improve osteogenesis. Biomaterial implants for craniomaxillofacial bone regeneration require controlling bioavailability of biomolecular cues such as growth factors to

promote desired cell responses. We reported incorporation of β -cyclodextrin into a model collagen-GAG scaffold to passively sequester and release growth factors via guest-host interactions in order to alter mesenchymal stem cell differentiation. We incorporated β -cyclodextrins within the scaffold to improve sequestration as well as extend retention and release of TGF- β 1. We also showed extended retention and release of 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 1/5/8 pathways associated with osteogenic differentiation. Together, this work established a defined method to incorporate and retain growth factors within collagen scaffolds via supramolecular interactions [7].

PRIOR WORK (reported in YR2 annual report). First generation composite biomaterials for CMF repair. A significant challenge to improving the quality and speed of craniomaxillofacial bone regeneration are competing design requirements for a biomaterial platform: porosity required for cell recruitment and adequate biotransport; mechanical strength that is significantly reduced by the inclusion of pores; shape-fitting to improve conformal contact and osseointegration between the implant and the defect. With USMRMC funding, we developed a new class of collagen composite biomaterial. We leveraged 3D printing tools to create macro-scale poly (lactic acid) (PLA) reinforcement frames that can be integrated into the collagen suspension prior to lyophilization, resulting in a multiscale scaffold-fiber composite. A critical advance associated with this work was demonstrating an approach to render the composite shape-fitting to address an unmet clinical need: the need for close conformal contact between biomaterial implant and the surrounding wound site. We showed selective removal of circumferential fiber segments from the PLA frame could yield a composite that was deformable radially yet retained sufficient spring-back capacity to increase the required push-out force. These findings confirm that the addition of even small volume fractions (~10% v/v) of polymeric mechanical reinforcement are sufficient to increase composite mechanical strength and address the current translational limitation of the mineralized collagen scaffold (compressive moduli < 1MPa). We completed biophysical and vitro MSC osteogenic activity characterization (See Appendix; [1]).

Zinc-modified scaffolds promote scaffold osteogenic activity. Implant osteoinduction and subsequent osteogenic activity are critical events that need improvement for regenerative healing of large craniofacial bone defects. While preliminary data was reported in the Year 2 Annual report, in the past year we successfully completed our study of incorporation of zinc ions into the mineralized collagen scaffold to accelerate MSC osteogenesis. Zinc is an essential trace element in skeletal tissue and bone, with soluble zinc being shown to promote osteogenic differentiation of porcine adipose derived stem cells. We augmented the mineral content of a class of mineralized collagen scaffolds under development for craniomaxillofacial bone regeneration via the inclusion of zinc ions to promote osteogenesis in vitro. Zinc sulfate was added to a mineralized collagen-glycosaminoglycan precursor suspension then freeze dried to form a porous biomaterial. We reported biophysical parameters of zinc functionalized scaffolds via imaging (scanning electron microscopy), mechanical testing (compression), and compositional (X-ray diffraction, inductively coupled plasma mass spectrometry) analyses. Zinc-functionalized scaffolds display morphological changes to the mineral phase and altered elastic modulus without substantially altering the composition of the brushite phase or removing the micro-scale pore morphology of the scaffold. These scaffolds also display zinc release kinetics on the order of days to weeks and promote successful growth and pro-osteogenic capacity of porcine adipose derived stem cells cultured within these zinc scaffolds. Taken together, we believe that zinc functionalized scaffolds provide a unique platform to explore strategies to improve in vivo osteogenesis in craniomaxillofacial bone injuries models (See Appendix; [8]).

Scaffold anisotropy may accelerate scaffold osteogenic response. Scaffold-induced bone regeneration becomes more challenging with increasing defect size. In ongoing work, we hypothesized that modifying scaffold pore anisotropy on cell recruitment, viability, and subsequent bone formation. Adapting freeze drying methods we had developed to create anisotropic non-mineralized collagen scaffolds for soft tissues applications[9], we have created a set of anisotropic vs. isotropic scaffold variants (Fig. 1). In the past quarter, we have begun to test the role of pore orientation on human mesenchymal stem cell (MSC) activity. We plan to additionally examine the role of glycosaminoglycan content, notably chondroitin-6-sulfate, chondroitin-4-sulfate, and heparin sulfate on mineralization in order to determine the individual and synergistic effects of pore anisotropy and GAG chemistry on MSC and osteogenic activity (to be reported Y4Q1).

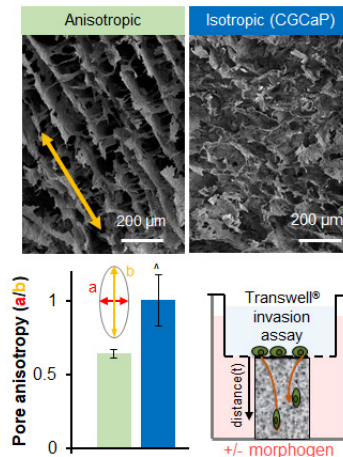


Fig. 1. A new class of anisotropic mineralized scaffold; ongoing work is quantifying MSC invasion, osteogenesis.

Adapting the design of conformal fitting polymeric reinforcement cages to be fabricated with robust PCL polymer. During Year 3 of this project, we reported [1] a polymer fiber reinforcement strategy for the mineralized collagen scaffold. The initial composite used a PLA-fiber array to create conformal fitting design elements for our scaffold. In the past quarter we have adapted the design of the reinforcing cage for the porcine CMF defect model. This requires a more robust PCL-based bioink for comparison to results reported outside of this project that used a high-density PCL reinforcing strategy. We have now fabricated prototype PCL reinforcement cages with conformal-fitting geometries (Fig. 2). Ongoing work is completing testing of these prototypes prior to fabrication of the final devices for future in vivo studies.

Modifying the organic phase of the mineralized collagen scaffold via amniotic membrane matrix. The goal of this project is to develop degradable biomaterials that actively instruct, rather than passively permit, MSC osteogenic differentiation and subsequent bone regeneration. However, the severity of host inflammatory response post-injury can severely limit implant osseointegration and regenerative healing. As a result, there is an unmet clinical need for biomaterials that promote MSC osteogenic differentiation but also modulate the host inflammatory response following injury. The amniotic membrane is derived from placentas and has been recently investigated as an extracellular matrix to prevent chronic inflammation. Herein, we hypothesized that a mineralized collagen-amnion composite scaffold could increase osteogenic activity in the presence of inflammatory cytokines. We have generated a family of mineralized

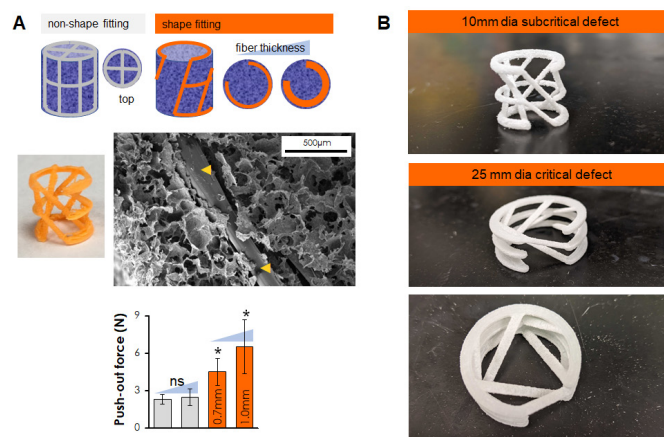


Fig. 2. (A) Previously reported reinforcement caged fabricated from PLA. We described a fiber reinforcement strategy that increased radial deformability to improve conformal fitting with the defect margins (represented as increased Push Out Force) [1]. (B) Adaptation of this design now printed using impregnated PCL, a stated goal of this project.

collagen-amnion scaffolds and completed mechanical and structural characterization (Fig. 3). Ongoing work is completing analysis of gene expression patterns of human MSCs seeded within these scaffolds as a function of IL-1 β inflammatory challenge. We hope that this effort will eventually identify a mineralized collagen-amnion scaffold that provides a beneficial environment to aid craniomaxillofacial bone repair, especially in the course of defects presenting significant inflammatory complications.

Transient regulation of growth factor availability via mineral-based sequestration. We have previously reported the use of covalent (e.g., carbodiimide, light-based crosslinking)[10-14] and supramolecular (e.g.,

proteoglycan, cyclodextrin)[7, 15] biomolecule patterning approaches to augment biological response within the collagen scaffold. However, the majority of these efforts have relied on non-mineralized collagen scaffolds. During the past year, we have been working to facilitate VEGF delivery from the inorganic mineral phase to aid scaffold bioactivity and regenerative capacity in vivo. To do this, we have looked to adapt a nanostructured mineral coating technique reported by *Murphy et al.*[16,17]. This approach used sequential exposure of a microparticle ‘seed’ to simulated body fluid (SBF) to stabilize a proteins (e.g., IL-1RA; bFGF) for extended therapeutic delivery in vivo. We have successfully adapted this method, using the brushite phase of the scaffold as the seed, and achieving >85% initial immobilization of single factors (e.g., VEGF, BMP2), extended retention (>90% after 7 days), and create multi-factor layers (Fig. 4A, B, C). The released factors retain bioactivity: e.g., scaffold-released VEGF activates human umbilical vein ECs like soluble VEGF (Fig. 4D). This has identified a robust strategy to incorporate into the mineralized collagen scaffold that we will be testing in vivo during upcoming porcine trials (in Year 4).

Mineralized collagen scaffolds induce MSC-mediated OPG production that

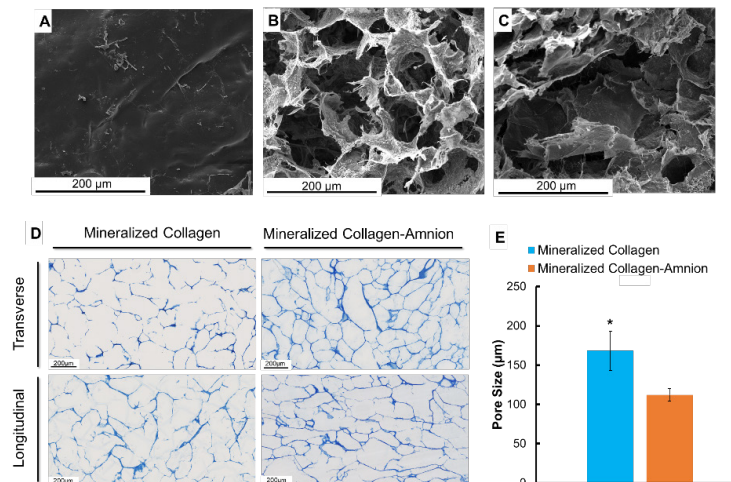


Fig. 3. SEM imaging and pore size analysis of mineralized collagen and mineralized collagen-amnion scaffolds. (A) SEM image of the amniotic membrane. (B) SEM image of a mineralized collagen scaffold. (C) SEM image of a mineralized collagen-amnion scaffold. (D) Representative images of mineralized collagen and mineralized collagen-amnion scaffolds sectioned transversely and longitudinally and stained with aniline blue to determine pore size. (E) Average pore size of mineralized collagen and mineralized collagen-amnion scaffolds. * indicates the mineralized collagen scaffold has a significantly ($p < 0.05$) higher average pore size than the mineralized collagen-amnion scaffold. Data expressed as mean $\pm$ standard deviation ($n=3$).

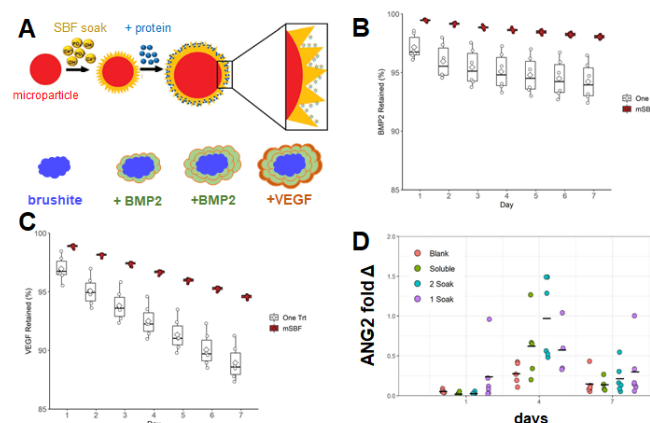


Fig. 4. (A) Adapting a nanostructured mineral coating technique to layer BMP2 (two 40ng/mL layers) and VEGF (one 10 ng/mL layer) within the mineralized scaffold. (B,C) Retention of BMP2, VEGF within the scaffold is enhanced via nanostructured mineral coating. (D) Patterned VEGF drives equivalent (vs. soluble) gene expression in ECs (day 7).

inhibits osteoclast activity. In

collaborative work supported by this project to define the basis for improved bone regeneration capacity of mineralized collagen scaffolds. We showed MSC-osteoprogenitors in this scaffold secrete osteoprotegerin (OPG) a soluble glycoprotein and

endogenous inhibitor of osteoclastogenesis and osteoclast activity[18]. We examined OPG production in the presence of DMH1 (an inhibitor of BMPR signaling) vs. PD98059 (MEK1/ERK1/2 inhibitor). DMH1 increased, but PD98059 reduced, OPG production, suggesting MSCs within mineralized collagen scaffolds produce high levels of OPG independent of osteogenic differentiation but dependent on ERK1/2 signaling [19]. We showed OPG production in the mineralized collagen scaffold is sufficient to inhibit osteoclast activity. We co-cultured human osteoclasts (hOCs) with acellular or MSC-seeded mineralized vs. non-mineralized collagen scaffolds. We quantified hOC proliferation and viability (via a WST-1 assay) and hOC degradative capacity (via direct culture on OsteoAssay Microplates). Mineralized collagen scaffolds reduce hOC viability (Fig. 5A). Moreover, hOC resorptive capacity is dramatically reduced (OsteoAssay microplate degradation, TRAP staining) when hOCs are co-cultures with MSC-seeded mineralized collagen scaffolds (Fig. 5B) [18]. Together, these data indicate the mineralized collagen scaffold directly inhibits (cell-material) hOCs activity. Excitingly, hOC resorptive capacity is maximally reduced in the presence of hMSCs seeded within the mineralized collagen scaffold. These data suggest MSC-mediated OPG production in the mineralized collagen scaffold may be responsible for the observed reduction in hOC activity. It also suggests boosting OPG production would further reduce hOC activity.

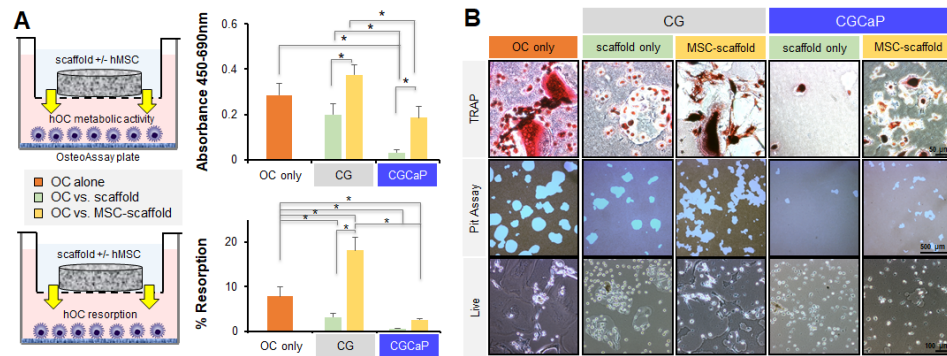


Fig. 5. (A) hOC proliferation and viability (top) vs. pit formation (bottom) due to co-culture with acellular or MSC-seeded non-mineralized (CG) and mineralized (CGCaP) collagen scaffolds. *: $p < 0.05$. **(B)** Representative TRAP staining, resorption pits, and live images of hOCs in single culture (OC only) or cultured with acellular vs. hMSCs-seeded scaffolds. hOC activity is significantly inhibited via MSC-seeded CGCaP scaffolds.

KEY RESEARCH ACCOMPLISHMENTS (cumulative):

The overall goal of this project is to develop a novel composite collagen biomaterial to improve the quality and speed of craniomaxillofacial bone regeneration. The major elements of the project are to demonstrate design modifications to an existing mineralized collagen scaffold in order to:

- Create a polymer-scaffold composite that exhibits improved compressive strength (vs. the scaffold alone) as well as conformal fitting capacity to aid the composite to fit Against the wound margins of irregular defect geometries.
- Demonstrate material and cytokine delivery innovations that accelerate cell infiltration into the scaffold as well as subsequent osteogenic differentiation and angiogenic activity.
- Validate improvements to this scaffold via assessment of the quality and kinetics of new bone formation in a porcine mandibular defect model.

Key accomplishments to date to meet these challenges:

- Demonstrating multi-scale composite reinforcement paradigm for mineralized collagen scaffolds to address acute clinical needs for biomaterials with improved macro-scale compressive strength and conformal fitting within the defect margins [1].
- Confirming incorporation of a polymeric reinforcement frame does not negatively influence the osteogenic capabilities of porcine adipose stem cells within the multi-scale composite [1].
 - NOTE: This finding extends beyond previous publications that examined the activity of human and rabbit bone marrow derived MSCs in the mineralized collagen scaffold alone [2,4] or that only confirmed adMSC viability in one design variant of a collagen-PCL composite [20].
- Developing and characterizing a novel zinc-functionalized variant of the mineralized collagen scaffold at the core of this CMF bone regeneration project. We confirmed zinc can be incorporated into and released from the scaffold microstructure. We showed incorporation of zinc improved scaffold mechanical properties, and that while zinc incorporation alters microscale mineral morphology of the scaffold it does not affect the Brushite phase of the calcium phosphate deposits. We showed zinc-functionalized scaffolds accelerate adMSC proliferation and metabolic health while maintaining osteogenic differentiation and mineral deposition capacity [8].
- In addition to long standing analysis of MSC-osteogenesis within mineralized collagen scaffolds, we have showed the mineralized collagen scaffold may also alter activity of osteoclasts [18,21], and motivates ongoing efforts to better investigate scaffold-osteoclast-MSC interactions in the context of matrix remodeling.
- We demonstrated the use of cyclodextrin-based transient sequestration of growth factors within the scaffold could enhance BMP-2 mediated osteogenic differentiation [7]. While short term growth factor retention may accelerate cell recruitment, cyclodextrin studies are now motivating current exploration of mineral-linked growth factor retention as an alternative means to delivery growth factors within the collagen scaffold.
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- We demonstrated the use of nanostructured mineral-based sequestration of growth factors within the scaffold to enhance the bioavailability of BMP-2 and VEGF (manuscript in preparation). Ongoing efforts will: 1) define the long-term biological efficacy of growth factor retention; and 2) identify the VEGF loading paradigm that efficiently activates

vascular cell activity. These efforts are expected to be early in Year 4 of this project and are necessary prior to in vivo testing of a final scaffold composite in a porcine mandibular defect.

- We completed design and manufacture of a novel PCL polymeric reinforcement cage. The original PCL cage structure detailed in the motivation for this project contained high (>30% vol/vol) polymer. We previously reported a fiber-based design to improve strength and conformal fitting of a composite, but this relied on PLA-fibers. We have completed adaptation of the PLA design to the additive manufacturing technology required to fabricate PCL structures. This PCL design has the dual capacity to improve the compressive strength of scaffold-PCL composites while also providing the opportunity to enhance conformal-fitting capacity of the resulting implants (manuscript in preparation).
- We have adapted the structure of the mineralized collagen scaffold, creating a new class of anisotropic mineralized collagen scaffolds (manuscript in preparation). We are examining the role of scaffold anisotropy (the inclusion of aligned tracks of pores) to aid MSC infiltration into the material as a means to accelerate healing.

CONCLUSION:

Successful biomaterial implants to improve regenerative healing must meet a number of design requirements that are often in conflict with each other. They must be biocompatible, meet micro-scale mechanical needs to promote osteogenesis as well as macro-scale requirements for a mechanically robust implant, be bioresorbable, and conformal fitting within irregular defects to improve osseointegration. Through the third year of this project we have completed the design, fabrication, as well as mechanical and in vitro osteogenesis testing of a conformal fitting mineralized composite for CMF defect repair applications. We have demonstrated multiple means to incorporate and release growth factors from the scaffold. And we have shown incorporation of zinc ions into the scaffold mineral phase is a powerful stimulus to increase MSC proliferation and metabolic health. The ability to increase close contact between the biomaterial implant and the host bone as well as incorporate pro-osteogenic factors (i.e., zinc) is particularly important for improving cell recruitment and subsequent osseointegration between host and implant. Ongoing efforts are completing analysis of cell activity in response to mineral-based sequestration of growth factors as well as the role of scaffold anisotropy in guiding MSC invasion. We will continue evaluation of change in MSC activity in biomolecule and mineral (VEGF, BMP-2, zinc) decorated collagen scaffolds. Over the coming year we expect to submit an additional intellectual property declaration to our campus regarding modification of polymeric reinforcement designs to adapt to more complex defect geometries. We expect this work to generate significant enthusiasm in the regenerative medicine and biomaterials community regarding a new paradigm for orthogonally manipulating multiple design criteria to create biomaterials for large, load bearing craniofacial defects. Impact will be primarily in basic sciences associated with the design of regenerative biomaterials.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. List all manuscripts submitted for publication during the period covered by this report resulting from this project.

(1) Lay Press:

Nothing to report

(2) Peer-Reviewed Scientific Journals:

Accepted/Published:

X. Ren, Q. Zhou, D. Foulad, M.J. Dewey, T.A. Miller, D.T. Yamaguchi, D. Bischoff, B.A.C. Harley, J.C. Lee, 'Nanoparticulate mineralized collagen glycosaminoglycan materials directly and indirectly inhibit osteoclastogenesis and osteoclast activation,' *J. Tissue Eng. Regen. Med.*, 13(5):823-34, 2019.

M.J. Dewey, E.M. Johnson, D.W. Weisgerber, M.B. Wheeler, B.A.C. Harley, 'Shape-fitting collagen-PLA composite promotes osteogenic differentiation of porcine adipose stem cells,' *J. Mech. Behav. Biomed. Mater.*, 95:21-33, 2019.

X. Ren, Q. Zhou, D. Foulad, A.S. Tiffany, M.J. Dewey, D. Bischoff, T.A. Miller, R. Reid, T.-C. He, D.T. Yamaguchi, B.A.C. Harley, J.C. Lee, 'Osteoprotegerin reduces osteoclast resorption activity without affecting osteogenesis on nanoparticulate mineralized collagen glycosaminoglycan scaffolds,' *Sci. Adv.*, 5(6): eaaw4991, 2019.

A.S. Tiffany, D.L. Gray, T.J. Woods, K. Subedi, B.A.C. Harley, 'The inclusion of zinc into mineralized collagen scaffolds for craniofacial bone repair applications,' *Acta Biomater.*, 93:86-96, 2019. PMCID: PMC6615986

Under review/revision

Nothing to report

In preparation

M.J. Dewey, E.M. Johnson, S.T. Slater, D.J. Milner, M.B. Wheeler, B.A.C. Harley, 'Mineralized collagen scaffolds fabricated with amniotic membrane matrix increase osteogenesis under inflammatory conditions,' in preparation, 2019

M.J. Dewey, A.V. Nosatov, K. Subedi, B.A.C. Harley, 'Influence of pore orientation and glycosaminoglycan content on mineralization of porous scaffolds for bone repair,' in preparation, 2019.

R.A. Sun Han Chang, M.E. Kersh, B.A.C. Harley, 'Tough and Tunable Scaffold-Hydrogel Composite Biomaterial for Regenerative Repair of Soft-to-Hard Musculoskeletal Tissue Interfaces,' in preparation, 2019.

A.S. Tiffany, B.A.C. Harley, 'Sequestration and release of bioactive factors via collagen scaffold mineral phase to support craniofacial bone repair,' in preparation, 2019.

(3) Invited Articles:

Nothing to report

(4) Abstracts:
Nothing to report

b. List presentations made during the last year (international, national, local societies, military meetings, etc.).

B.A.C. Harley, '*Engineering complexity via biomaterial design*,' Brown University, Dept. of Biomedical Engineering, 10/2018.

B.A.C. Harley, '*Engineering complexity via biomaterial design*,' Northeastern University, Dept. of Chemical Engineering, 11/2018.

B.A.C. Harley, '*Engineering complexity through biomaterial design*,' Johns Hopkins University, Dept. of Chemical and Biomolecular Engineering, 11/2018.

B.A.C. Harley, '*Multiscale challenges in biomaterials design for regenerative medicine*,' University of Birmingham (U.K.), Dept. of Chemical Engineering, 12/2018.

B.A.C. Harley, '*Engineering complexity through biomaterial design*,' Worcester Polytechnic Institute, Dept. of Biomedical Engineering, 12/2018.

B.A.C. Harley, '*Towards building tissues – engineering complexity through biomaterial design*,' University of Texas at Austin, Dept. of Biomedical Engineering, 2/2019.

B.A.C. Harley, '*Towards building tissues – engineering complexity through biomaterial design*,' Stanford University, Bio-X Frontiers in Interdisciplinary Biosciences Seminar, 3/2019.

B.A.C. Harley, '*Building tissues – at the intersection of materials science & chemical engineering*,' University of Massachusetts at Amherst, Dept. of Chemical Engineering, 4/2019.

M. Dewey (poster and podium), E. Johnson, S. Slater, D. Slater, B. A. C. Harley. 'The Addition of the Amniotic Membrane to Mineralized Collagen Scaffolds for Immunomodulatory Bone Regeneration,' Society for Biomaterials Annual Meeting, 4/2019, Seattle, WA.

R.A. Sun Han Chang (Podium), C.-W. Lee, S. Rogers, B.A.C. Harley, 'Biomimetic triphasic scaffolds for osteotendinous junction regeneration,' Society for Biomaterials Annual Meeting, 2019, Seattle, WA.

B.A.C. Harley, '*Building tissues – engineering complexity through biomaterial design*,' University of Michigan, Dept. of Biomedical Engineering, Ann Arbor, MI, 5/2019.

B.A.C. Harley, '*Engineering porous scaffolds for regenerative medicine*,' Regenerative Medicine Essentials Course, Wake Forest Institute for Regenerative Medicine (WFIRM), Winston-Salem, NC, 6/2019.

B.A.C. Harley, '*Engineering porous scaffolds for regenerative medicine*,' Keynote, 2019 Chinese Society for Biomaterials annual meeting, Dailan, China, 8/2019.

B.A.C. Harley, '*Building tissues – engineering complexity through biomaterial design*,' Boston University, Dept. of Biomedical Engineering, 9/2019.

INVENTIONS, PATENTS AND LICENSES:

Marley Dewey, Brendan Harley, Daniel Weisgerber, 'Adaptable PLA fiber reinforcement for conformal fitting,' submitted, Nov. 2017.

Marley Dewey, Brendan Harley, Rebecca Hortensius, Simona Slater, 'Mineralized Collagen Scaffolds combined with the Amniotic Membrane derived from placentas to address inflammation in Craniomaxillofacial Bone Regeneration,' submitted, July, 2018.

REPORTABLE OUTCOMES:

Multiscale composite supports adMSC osteogenesis and aids implant conformal fitting.

We have integrated a millimeter-scale reinforcing poly(lactic acid) (PLA) frame fabricated via 3D-printing into the mineralized collagen scaffold to form a multi-scale mineralized collagen-PLA composite. We have described modifications to the PLA frame design to increase the compressive strength (Young's Modulus, ultimate stress and strain). We also developed modifications to the frame architecture to render the composite with increased compressive strength in one axis but radial compressibility and conformal fitting capabilities in an orthogonal axis. We completed *in vitro* comparison of the bioactivity of porcine adipose derived stem cells (adMSCs) in the composite versus the mineralized collagen scaffold. We found incorporation of the reinforcing frame does not negatively influence the bioactivity and osseoinductive nature of the mineralized collagen scaffold. We have adapted this design to be fabricated from mineral-impregnated PCL, making these reinforcement strategies broadly applicable for craniofacial bone repair applications. These findings represent the first key element of this project, a composite design to address competing bioactivity, mechanical strength, and shape-fitting design requirements for biomaterials for craniomaxillofacial bone regeneration.

Inclusion of zinc ions into mineralized collagen scaffolds. We have integrated zinc ions into the mineralized collagen scaffold. We have described biophysical properties (porosity, mechanics, mineral content) of the resultant scaffolds. We showed inclusion of zinc accelerated MSC proliferation and metabolic activity. We showed inclusion of zinc does not negatively influence osteogenic differentiation or mineral biosynthesis *in vitro*. These findings represent a key biomolecular adaptation of the mineralized scaffold with the goal of improving craniomaxillofacial bone regeneration.

Alteration of growth factor bioavailability via cyclodextrin chemistry and via mineral-phase patterning. We have used cyclodextrin functionalized collagen scaffolds as a means to exploit the passive sequestration and release of growth factors (TGF- β 1 and BMP-2) via guest-host interactions to control mesenchymal stem cell differentiation. BMP-2 sequestrations confers pro-osteogenic advantages for the scaffold. we report the incorporation of β -cyclodextrin into a model collagen-GAG scaffold. We subsequently described adaptation of a mineral-phase patterning approach to sequester BMP-2 and VEGF within the mineralized scaffold with high affinity and for extended periods of time (>7 days). These findings suggest a design framework to selectively alter the bioavailability of multiple biomolecules within a three-dimensional collagen-GAG scaffold to intensify cell response to exogenously added or endogenously produced growth factors for a range of musculoskeletal regenerative medicine applications.

Future reportable outcomes:

1. Completion of analysis of the role of scaffold anisotropy on osteoprogenitor cell recruitment and osteogenic differentiation.
2. Completion of growth factor dose response curves for MSCs and endothelial cells in mineralized collagen scaffolds as a function of mineral-phase growth factor sequestration.
3. Initiation and completion of tiered *in vivo* craniomaxillofacial bone regeneration studies

OTHER ACHIEVEMENTS:

Degrees obtained by personnel supported by this award:

Nothing to report this year.

Fellowships awarded to personnel supported by this award:

Nothing to report this year.

Additional honors awarded to personnel supported by this award:

Alecandria Tiffany

2019 – 2020; Graduate Research Opportunities Worldwide (GROW) award, National Science Foundation (Research exchange: University of New South Wales, Australia. Supervising PI: K. Kilian).

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APPENDICES:

Please find appended original copies of the following work:

Quad Chart:

Year 2 Quarter 4 Quad Chart

Peer-Reviewed Scientific Journals:

X. Ren, Q. Zhou, D. Foulad, M.J. Dewey, T.A. Miller, D.T. Yamaguchi, D. Bischoff, B.A.C. Harley, J.C. Lee, 'Nanoparticulate mineralized collagen glycosaminoglycan materials directly and indirectly inhibit osteoclastogenesis and osteoclast activation,' *J. Tissue Eng. Regen. Med.*, 13(5):823-34, 2019.

M.J. Dewey, E.M. Johnson, D.W. Weisgerber, M.B. Wheeler, B.A.C. Harley, 'Shape-fitting collagen-PLA composite promotes osteogenic differentiation of porcine adipose stem cells,' *J. Mech. Behav. Biomed. Mater.*, 95:21-33, 2019.

X. Ren, Q. Zhou, D. Foulad, A.S. Tiffany, M.J. Dewey, D. Bischoff, T.A. Miller, R. Reid, T.-C. He, D.T. Yamaguchi, B.A.C. Harley, J.C. Lee, 'Osteoprotegerin reduces osteoclast resorption activity without affecting osteogenesis on nanoparticulate mineralized collagen glycosaminoglycan scaffolds,' *Sci. Adv.*, 5(6): eaaw4991, 2019.

A.S. Tiffany, D.L. Gray, T.J. Woods, K. Subedi, B.A.C. Harley, 'The inclusion of zinc into mineralized collagen scaffolds for craniofacial bone repair applications,' *Acta Biomater.*, 93:86-96, 2019. PMCID: PMC6615986

Polycaprolactone-collagen composite biomaterials for mandible regeneration

USAMRMC 1464004

W81XWH-16-1-0566



PI: **Brendan Harley**

Org: University of Illinois at Urbana-Champaign

Award Amount: \$800,000

Study/Product Aim(s)

Our goal is to demonstrate that integrating a mineralized collagen scaffold within a macroporous PCL construct will generate a multi-scale composite that enhances adipose-derived MSC osteogenesis and subsequent bone regeneration. Our aims:

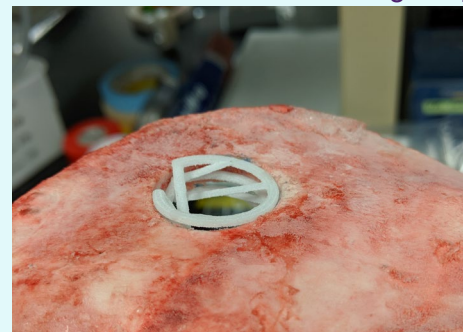
Aim 1: Validate the osteogenic potential of growth factor decorated collagen-PCL composites.

Aim 2: Examine the quality and kinetics of mandible bone regeneration using the collagen-PCL composite.

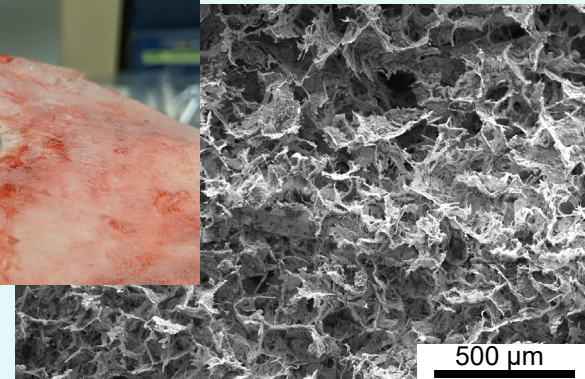
Approach

We a polycaprolactone (PCL) support cage into a mineralized collagen scaffold to form a PCL-collagen composite. We will define the improvement in adipose-derived mesenchymal stem cell osteogenesis in response to the release of growth factors (BMP-2 and VEGF) from the collagen scaffold, as well as regenerative potential of the growth factor decorated collagen-PCL composite in a porcine model of mandibular defect.

Test fit PCL reinforcement cage in porcine cadaveric bone



Mineralized
collagen scaffold



Accomplishments: We validated growth factor incorporation and release strategies as well as the design of a PCL fiber reinforcement cage to incorporate into the collagen scaffold to improve implant conformal fitting.

Timeline and Cost

Activities	CY	16	17	18	19
Fabrication, biophysical characterization of growth-factor decorated collagen-PCL composites					
In vitro osteogenic activity of growth-factor decorated collagen-PCL composites					
Determine kinetics of mandibular healing via growth-factor decorated collagen-PCL composites					
Define improved quality of mandibular healing using growth-factor decorated collagen-PCL composites					
Estimated Budget (\$K)		\$30k	\$200k	\$340k	\$230k

Goals/Milestones

CY16 Goal – Collagen-PCL composite fabrication

- ✓ Fabricate initial library of collagen-PCL composites
- ✓ Finalize campus IACUC approval for growth factor modified composites

CY17 Goal – In vitro osteogenesis assessment

- ✓ Biophysical characterization of composites
- ✓ Define growth factor elution from collagen-PCL composites

CY18 Goal – In vivo bone regeneration assays

- ✓ Quantify degree of enhanced osteogenic potential of adMSCs within BMP-2 and VEGF decorated collagen-PCL composites
- ✓ Finalize design criteria for collagen-PCL composites for *in vivo* implantation and initiate subcritical and critical defect models

CY19 Goal – Quantify quality and kinetics of mandible regeneration

- Identify collagen-PCL variant that displays high quality bone repair.

Comments/Challenges/Issues/Concerns

- We requested a no cost extension to initiate/complete the *in vivo* component of the project (we have retained sufficient budget for the work).

Budget Expenditure to Date

Projected Expenditure: \$725,000

Actual Expenditure: \$656,400.12

Updated: 29 October 2019

RESEARCH ARTICLE

Nanoparticulate mineralized collagen glycosaminoglycan materials directly and indirectly inhibit osteoclastogenesis and osteoclast activation

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Funding information

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Abstract

The ability of the extracellular matrix (ECM) to direct cell fate has generated the potential for developing a materials-only strategy for tissue regeneration. Previously, we described a nanoparticulate mineralized collagen glycosaminoglycan (MC-GAG) material that efficiently induced osteogenic differentiation of human mesenchymal stem cells (hMSCs) and calvarial bone healing without exogenous growth factors or progenitor cell expansion. In this work, we evaluated the interactions between MC-GAG and primary human osteoclasts (hOCs). In the absence of hMSCs, mineralized Col-GAG materials directly inhibited hOC viability, proliferation, and resorption in contrast to nonmineralized Col-GAG, which demonstrated a modest inhibition of resorptive activity only. Cocultures containing differentiating hMSCs with hOCs demonstrated increased hOC-mediated resorption only on Col-GAG while MC-GAG cocultures continued to inhibit resorption. Unlike Col-GAG, hMSCs on MC-GAG expressed increased amounts of osteoprotegerin (OPG) protein, the major endogenous osteoclast inhibitor. Interestingly, OPG expression was found to be antagonized by small mothers against decapentaplegic1/5 (Smad1/5) phosphorylation, an obligate pathway for osteogenic differentiation of hMSCs on MC-GAG, and potentiated by extracellular signal-regulated kinase (ERK1/2) phosphorylation. Collectively, these results suggested that the MC-GAG material both directly inhibited the osteoclast viability, proliferation, and resorptive activity as well as induced hMSCs to secrete osteoprotegerin, an antiosteoclastogenic factor, via a signalling pathway distinct from osteogenic differentiation.

KEYWORDS

bone regeneration, bone resorption, collagen glycosaminoglycan, nanoparticle, osteoclast, osteoprotegerin

1 | INTRODUCTION

The major axis controlling bone homeostasis via osteoclast regulation is mediated by the receptor activator of nuclear factor- κ B (RANK), receptor activator of nuclear factor- κ B ligand (RANKL), and

osteoprotegerin (OPG; Lee et al., 2011; Maxhimer, Bradley, & Lee, 2015). RANK, a tumour necrosis factor superfamily receptor, is essential to osteoclast differentiation and activation upon binding to its cognate ligand, RANKL (Anderson et al., 1997; Kong et al., 1999). Both in vitro and in vivo genetic studies have demonstrated that RANK or RANKL deficiencies result in uncontrolled bone formation (Lee et al., 2011; Li et al., 2000; Yun et al., 2001). As a decoy receptor, OPG is the endogenous negative regulator of RANK/RANKL interactions (Maxhimer et al., 2015). In the absence of OPG, uncontrolled bone resorption and osteoporosis occurs (Simonet et al., 1997; Yun et al., 2001). Thus, the relative quantities of RANKL and OPG have been hypothesized to be important to both the locoregional and organismal balance towards bone formation or resorption (Kadriu et al., 2017). Targeted therapies to this axis are now being investigated for treatment of osteoporosis and other pathologic conditions to yield a net osteogenic state (Faienza et al., 2018; Flick et al., 2003). Similarly, temporary alterations of the RANKL/OPG relative quantities may also be utilized for augmentation of bone regenerative therapies.

In addition to the major regulatory axis, components of the extracellular matrix (ECM) are known to direct changes in osteoclastogenesis and osteoclast activity (Barrow et al., 2011; Kram, Kilts, Bhattacharyya, Li, & Young, 2017; Li et al., 2018). Collagen I, the major species in bone ECM and the basis for the majority of bone ECM-based regenerative materials, is the ligand for the osteoclast-associated receptor (OSCAR), the coreceptor necessary for osteoclast activation (Barrow et al., 2011). Fibronectin has been described to both positively and negatively regulate osteoclast activation by several groups (Gramoun et al., 2010; Li et al., 2018). Glycosaminoglycans (GAGs) such as hyaluronan have also been known to serve antiosteoclastogenic effects via toll-like receptor 4 (TLR4) and inhibition of macrophage-colony stimulating factor (M-CSF) activated signalling pathways (Chang et al., 2007). Additionally, collagen-based coatings that contain sulfated GAGs have been differentially reported to inhibit osteoclast activation (Salbach et al., 2012; Salbach et al., 2012; Salbach-Hirsch et al., 2013; Salbach-Hirsch et al., 2014). Kram et al (2017) demonstrated that expression of the small leucine rich proteoglycans biglycan and fibromodulin by osteoprogenitors attenuated the abilities for osteoclast precursor cells to differentiate through sequestration of RANKL in mouse genetic models. Lastly, mineral content has also been found to have an antiosteoclastogenic effect indirectly through OPG secretion by differentiating osteoprogenitors (Jiao et al., 2015). Thus, ECM components not only have direct effects on osteoclast regulation but also indirectly through differentiation of osteoprogenitors.

Due to the abilities of components of the ECM to differentially direct cell fate, understanding the net effects of individual bone regenerative materials require characterization of interactions with both osteoprogenitors and osteoclast progenitors. We have previously reported that a nanoparticulate mineralized collagen glycosaminoglycan (MC-GAG) material induces efficient osteogenic differentiation of primary human mesenchymal stem cells (hMSCs) and in vivo calvarial healing in a rabbit model in an exogenous growth factor-independent manner (Ren, Bischoff, et al., 2015; Ren, Tu, et al., 2016). We also detailed that an autogenous activation of the canonical

bone morphogenetic protein receptor (BMPR) signalling pathway was necessary for osteogenesis of hMSCs on MC-GAG, whereas the mitogen-activated protein kinase pathways were not required (Zhou et al., 2017). Our current work aims to characterize the effects of MC-GAG on osteoclastogenesis and osteoclast activity.

2 | MATERIALS AND METHODS

2.1 | Fabrication and chemical cross linking of nonmineralized and mineralized collagen scaffolds

Col-GAG and MC-GAG scaffolds were prepared using lyophilization as described previously (Harley et al., 2010; Harley, Leung, Silva, & Gibson, 2007; Weisgerber, Kelkhoff, Caliri, & Harley, 2013). Briefly, microfibrillar, type I collagen (Collagen Matrix, Oakland, NJ) and chondroitin-6-sulfate (Sigma-Aldrich, St. Louis, MO) were combined in suspension in the absence and presence of calcium salts (calcium nitrate hydrate: $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$; calcium hydroxide: $\text{Ca}(\text{OH})_2$, Sigma-Aldrich, St. Louis, MO) in an acetic acid (Col-GAG) or phosphoric acid (MC-GAG) solution. Using a constant cooling rate technique at a rate of $1^\circ\text{C}/\text{min}$, the solution was frozen from room temperature to -10°C using a freeze dryer (Genesis, VirTis). Following sublimation of the ice phase, scaffolds were sterilized via ethylene oxide and cut into 8-mm discs for culture.

Cross linking of scaffolds was performed after rehydration in phosphate-buffered saline for 4 hr using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC, Sigma-Aldrich) and N-hydroxysuccinimide (NHS, Sigma Aldrich) at a molar ratio of 5:2:1 EDC:NHS:COOH where COOH represents the amount of collagen in the scaffold as we previously described (Olde Damink et al., 1996). Scaffolds were washed with phosphate-buffered saline to remove any of the residual chemical.

2.2 | Cell culture

Primary hMSCs (Lonza, Inc., Allendale, NJ) were expanded in proliferation media composed of Dulbecco's Modified Eagle Medium (Corning Cellgro, Manassas, VT) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 2 mM L-glutamine (Life Technologies, Carlsbad, CA), 100 IU/ml penicillin/100 $\mu\text{g}/\text{ml}$ streptomycin (Life Technologies).

2.2.1 | Osteogenic differentiation of hMSCs on Col-GAG and MC-GAG

3×10^5 hMSCs were seeded onto 8-mm discs of Col-GAG and MC-GAG scaffolds in proliferation media. Twenty-four hours after seeding, media was switched to osteogenic differentiation media consisting of 10 mM β -glycerophosphate, 50 $\mu\text{g}/\text{ml}$ ascorbic acid, and 0.1 μM dexamethasone. For inhibitor studies, scaffolds were treated or untreated with dorsomorphin homologue 1 (DMH1; Sigma-Aldrich) or PD98059 (Cell Signaling Technologies, Beverly, MA) separately, all at a concentration of 50 μM . Fresh DMH1 and PD98059 were added to each media change every 3 days.

2.2.2 | Indirect hMSC and hOC cocultures

2×10^5 hMSCs were seeded onto 6-mm Col-GAG and MC-GAG scaffolds in proliferation media. Twenty-four hours after seeding hMSCs, 6×10^4 primary human osteoclast precursors (hOCs; Lonza, Inc., Allendale, NJ) were separately cultured in Osteoclast Precursor Basal Medium (Lonza, Allendale, NJ) supplemented with 33 ng/ml M-CSF, 66 ng/ml of RANKL, 10 mM β -glycerophosphate, 50 μ g/ml ascorbic acid, 0.1 μ M dexamethasone on 24 well Corning Osteo Assay Surface Microplates (Corning, NY), as the lower chamber of the coculture. After 2 hr, Col-GAG and MC-GAG scaffolds were transferred to 8 μ m Transwell inserts (Corning, NY), the upper chamber of the coculture. Media were changed every 3 days for 3 weeks.

2.2.3 | Direct hMSC and hOC cocultures

3.5×10^5 hMSCs were seeded onto 8-mm Col-GAG and MC-GAG scaffolds in proliferation media for 24 hr. 6×10^4 hOCs were cultured in Osteoclast Precursor Basal Medium (Lonza, Allendale NJ) supplemented with 33 ng/ml M-CSF, 66 ng/ml RANKL, 10 mM β -glycerophosphate, 50 μ g/ml ascorbic acid, 0.1 μ M dexamethasone on 24 well Osteo Assay Microplates. Two hours after hOCs were seeded, Col-GAG and MC-GAG scaffolds were transferred to the Osteo Assay Plates and directly cocultured with hOCs. Media were changed every 3 days for 2 weeks.

2.3 | OPG enzyme-linked immunosorbent assay

Supernatants were collected from hMSC only, osteoclast only, or hMSC and hOC cocultures. OPG protein concentrations were determined using the Human OPG DuoSet enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. Briefly, a 96 well microplate was coated with the capture antibody and incubated overnight at room temperature. After blocking, samples were incubated for 2 hr at room temperature with the detection antibody followed by incubation with streptavidin-horseradish peroxidase for 20 min. The reaction was quenched by adding 100 μ l of 2N H_2SO_4 . Plates were read at 450- and 540-nm wavelengths on the Epoch microplate reader (BioTex, Winooski, VT).

2.4 | Micro-CT imaging

Scaffolds were fixed using 10% formalin and mineralization was quantified by microcomputed tomographic (micro-CT) imaging using Scanco 35 (Scanco Medical AG, Bruttisellen, Switzerland) in triplicate for each time point. Scans were performed at medium resolution with a source voltage of 70 E (kVp) and I (μ A) of 114. The images had a final element size of 12.5 μ m. Images were analysed using software supplied from Scanco (Image Processing Language version 5.6) and reconstructed into three-dimensional (3D) volumes of interest. Optimum arbitrary threshold values of 20 (containing scaffold and mineralization) and 80 (containing mineralization alone) were used uniformly for all specimens to quantify mineralized areas from surrounding unmineralized scaffold.

Analysis of 3D reconstructions was performed using Scanco Evaluation script #2 (3D segmentation of two volumes of interest: solid dense in transparent low-density object) and script #6 (bone volume/density only bone evaluation) for volume determinations.

2.5 | Western blot

Lysates were prepared from scaffolds at 0, 4, 14, and 24 days of culture using sodium dodecyl sulfate (SDS) sample buffer and equal amounts were subjected to 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA). Western blot analysis was carried out with antibodies against OPG, RANKL, phosphorylated small mothers against decapentaplegic 1/5 (p-Smad1/5), total Smad5, phosphorylated extracellular regulated kinase 1/2 (p-ERK1/2), total ERK1/2, and β -actin followed by 1:4,000 dilutions of streptavidin-horseradish peroxidase-conjugated IgG antibodies (Bio-Rad, Hercules, CA) and an enhanced chemiluminescent substrate (Thermo Scientific, Rockford, IL). For detection of p-Smad1/5 and total Smad5, 10 μ g of lysate was loaded per lane. For detection of OPG, RANKL, p-ERK1/2, total ERK1/2, and β -actin, 20 μ g of lysate was loaded per lane. All antibodies were obtained from Cell Signaling Technologies (Beverly, MA) with the exception of RANKL, OPG, and β -actin antibodies, which were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Imaging analysis was carried out using ImageJ (NIH, Bethesda, MD).

2.6 | WST-1 assay

Culture media was supplemented with cell proliferation reagent water soluble tetrazolium-1 (WST-1; Roche, Basel, Switzerland) at a 1:10 concentration. Scaffolds were incubated for 3–4 hr at 37°C in a humidified atmosphere with 5% CO_2 . Absorbance of the incubation medium was measured at 450 and 690 nm (Epoch spectrophotometer, BioTek, Winooski, VT).

2.7 | TRAP staining

hOCs were detected using Leukocyte tartrate-resistant acid phosphatase (TRAP) Kit 387-A (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, cultured cells were fixed with formaldehyde for 5 min at room temperature, washed, and air-dried. After staining, TRAP-positive multinucleated cells were observed under a phase-contrast microscope at 20 $\times$ magnification and digitally photographed.

2.8 | Resorption pit assay

Activity of hOCs in single culture or cocultured with scaffolds with and without hMSCs was evaluated for resorption pit formation on Osteo Assay microplates. At the completion of the culture period, culture media was aspirated and 500 μ l of 10% bleach solution was added for 5 min at room temperature. The wells were washed with distilled water and allowed to dry at room temperature for 3–5 hr. Pits were observed using a standard microscope and digitally photographed.

Percentage of resorption for the whole well of the culture at magnification 2× was calculated by ImageJ.

2.9 | Statistical analysis

All statistical analyses were performed using SPSS Version 24 (Chicago, IL). Data points were composed of duplicates of at least three independent experiments, unless otherwise indicated. Mean measurements of mRNA expression were analysed for statistical significance by analyses of variance (ANOVA) followed by post hoc tests using the Tukey criterion. A value of $p < 0.05$ was considered significant.

3 | RESULTS

3.1 | hMSCs undergoing osteogenic differentiation induce expression of OPG in a differential manner on nonmineralized versus MC-GAG materials

We have previously demonstrated the capabilities of MC-GAG to induce in vitro hMSC osteogenic differentiation and mineralization as well as in vivo bone healing beyond that of a nonmineralized Col-GAG

control material (Lee et al., 2015; Ren, Tu, et al., 2016; Ren, Weisgerber, et al., 2016; Ren, Bischoff, et al., 2015; Weisgerber, Caliri, & Harley, 2015; Zhou et al., 2017). To evaluate the role of MC-GAG in the regulation of osteoclast activation during osteoprogenitor differentiation, bone marrow-derived primary hMSCs (CD105 + CD166 + CD29 + CD44 + CD14 – CD34 – CD45 –) were cultured in osteogenic differentiation medium and expression of OPG protein was evaluated (Figure 1a,b). Over the course of osteogenic differentiation, OPG protein expression was significantly increased in MC-GAG at Days 3, 14, and 24 when compared with hMSCs differentiated on Col-GAG as assessed by western blot analysis (Figure 1a).

To understand the coordination of osteoclast differentiation and hMSCs undergoing osteogenic differentiation on Col-GAG and MC-GAG, we devised an indirect in vitro coculture assay. hMSCs seeded on Col-GAG and MC-GAG were cultured in the upper chamber of Transwell inserts in the absence or presence of primary hOCs plated in the lower chamber. Cocultures were induced to simultaneously undergo osteogenic and osteoclastogenic differentiation with RANKL (66 ng/ml), M-CSF (33 ng/ml), β -glycerophosphate, and dexamethasone for 3 weeks, and western blot analysis of the cultures were performed. In the presence of osteoclasts, the expression of phosphorylated Smad1/5 (p-Smad1/5) increased significantly in both

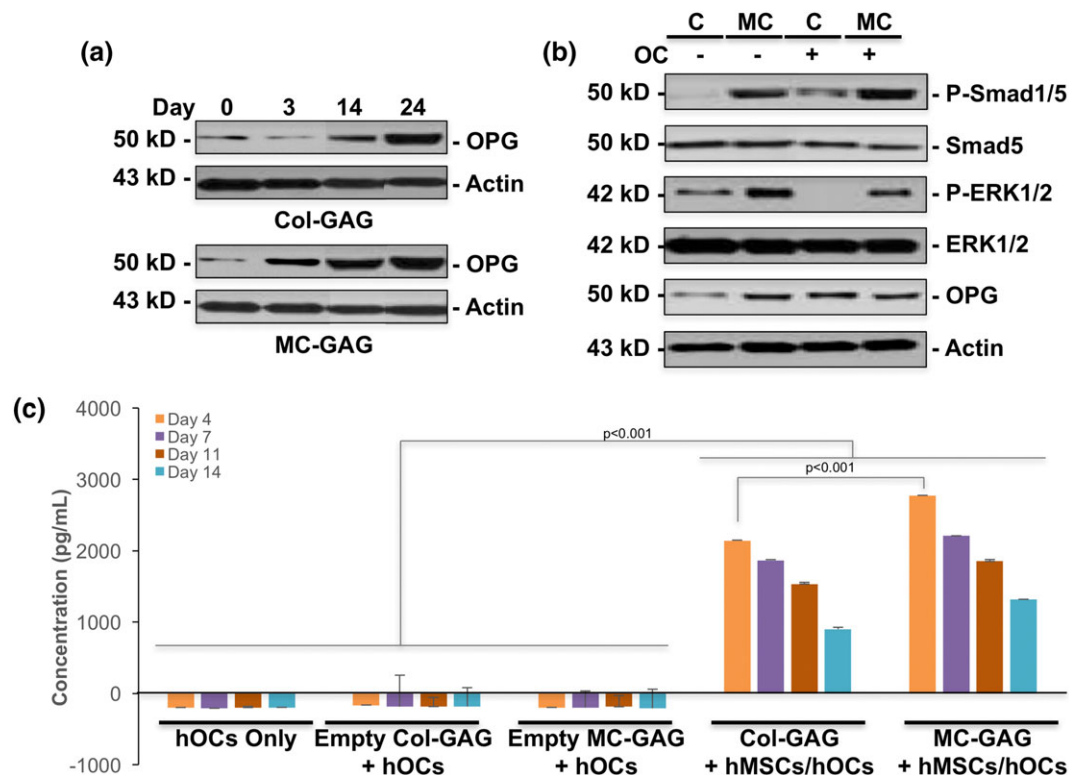


FIGURE 1 OPG is expressed and secreted at higher levels by hMSCs on MC-GAG compared with Col-GAG in the absence and presence of differentiating hOCs. (a) Western blot of primary hMSCs cultured on Col-GAG or MC-GAG materials for 0, 3, 14, and 24 days in osteogenic differentiation medium for OPG and β -actin. (b) Western blot for p-Smad1/5, total Smad5, p-ERK1/2, ERK1/2, OPG, and β -actin of hMSCs differentiated on Col-GAG or MC-GAG for 3 weeks in the absence and presence of differentiating primary hOCs. (c) OPG ELISA of hOCs only, hOCs cocultured with empty Col-GAG (Empty Col-GAG + hOCs), empty MC-GAG (Empty MC-GAG + hOCs), hMSCs differentiated on Col-GAG (Col-GAG + hMSCs/hOCs), or hMSCs differentiated on MC-GAG (MC-GAG + hMSCs/hOCs) for 4, 7, 11, and 14 days. Bars represent mean concentrations in pg/mL, errors bars represent standard deviation. Significant post hoc comparisons following ANOVA indicated with p values. OPG: osteoprotegerin; hMSC: human mesenchymal stem cell; MC-GAG: mineralized collagen glycosaminoglycan; hOC: human osteoclast; ANOVA: analysis of variance [Colour figure can be viewed at wileyonlinelibrary.com]

Col-GAG and MC-GAG scaffolds (Figure 1b). Additionally, the expression of OPG also increased for hMSCs on Col-GAG in the presence of hOCs but not on MC-GAG in the presence of hOCs whereas ERK1/2 phosphorylation was decreased in hOC cocultures.

To determine the effect of hOCs on OPG secretion, OPG ELISAs were performed (Figure 1c). Significant differences in secreted OPG were found between cultures on ANOVA ($F(15,48) = 172.56$, $p < 0.001$). OPG was undetected in the absence of hMSCs (hOC only, empty Col-GAG/hOCs, or empty MC-GAG/hOCs) when compared with cocultures with hMSCs at every time point on post hoc comparisons ($p < 0.001$). Between cocultures of hOCs with differentiating hMSCs on Col-GAG versus MC-GAG, OPG was elevated in MC-GAG cocultures particularly at Day 4 ($p < 0.001$). At later time points, OPG trended higher in MC-GAG cocultures but was no longer found to be significant.

3.2 | hOCs augment hMSC mineralization on MC-GAG

To understand the effects of primary human osteoclast differentiation on hMSCs on Col-GAG and MC-GAG, we evaluated mineralization in hMSCs undergoing osteogenic differentiation in the absence and presence of hOCs (Figure 2a and 2b). Empty scaffolds, scaffolds seeded with hMSCs, or cocultures with hMSCs on scaffolds in Transwell inserts and hOCs in the lower chamber were evaluated with micro-CT scanning after 3 weeks of culture in concurrent osteogenic and osteoclastogenic differentiation medium. Overall, differences in mineralization were found to be present ($F(5,8) = 22.44$, $p < 0.001$). Post hoc comparisons between groups did not demonstrate statistically significant differences

between mineralization on empty scaffolds or scaffolds cultured with hMSCs as single cultures on either material, an expected result at 3 weeks of culture. However, in the presence of hOCs, a significant increase in new mineral formation was seen in MC-GAG when compared with empty scaffolds ($p = 0.02$) or scaffolds cultured with hMSCs in single culture ($p = 0.004$). Although cocultures of hOC and hMSCs cultured on Col-GAG demonstrated a qualitative increase in mineralization, this difference did not reach statistical significance. These data suggest that hOCs increase hMSC osteogenic differentiation on MC-GAG scaffolds via soluble factors in a paracrine fashion.

3.3 | MC-GAG diminishes hOC activation and resorption directly and indirectly

Next, osteoclasts were evaluated in a direct coculture system in order to account for both direct and indirect effects on osteoclast activity (Figure 3a–c). hMSCs were cultured on Col-GAG or MC-GAG materials that were then cocultured directly with primary hOCs 24 hr after seeding. The cocultures were differentiated simultaneously in osteogenic differentiation medium supplemented with M-CSF (33 ng/ml) and RANKL (33 ng/ml).

At 14 days of culture, hOC proliferation and viability on the plate were assessed with WST-1 assays and found to demonstrate significant differences between the conditions by ANOVA ($F(4,10) = 26.38$, $p < 0.001$; Figure 3a). Post hoc comparisons between groups demonstrated that hOC viability and proliferation were significantly diminished in the presence of empty MC-GAG ($p < 0.001$) but not Col-GAG scaffolds when compared with hOCs alone. When comparing the differences between empty versus

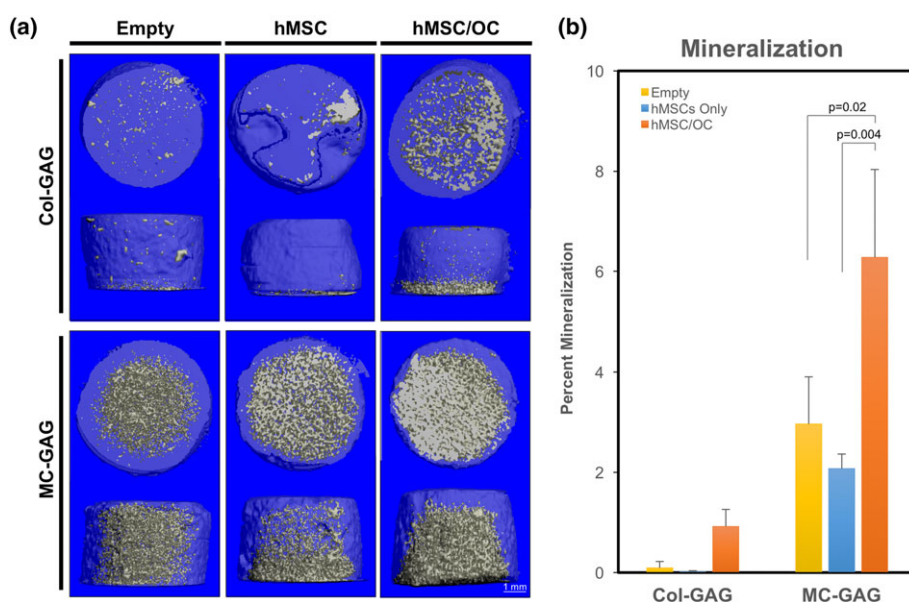


FIGURE 2 hMSC mineralization on Col-GAG and MC-GAG is increased in the presence of differentiating hOCs. (a) Representative micro-CT images and (b) quantitative analysis of empty scaffolds, scaffolds cultured with hMSCs only, or scaffolds cultured with hMSCs in coculture with hOC (hMSC/OC) for 3 weeks. Significant post hoc comparisons following ANOVA indicated with p values. hMSC: human mesenchymal stem cell; MC-GAG: mineralized collagen glycosaminoglycan; hOC: human osteoclast; ANOVA: analysis of variance; micro-CT: microcomputed tomographic [Colour figure can be viewed at wileyonlinelibrary.com]

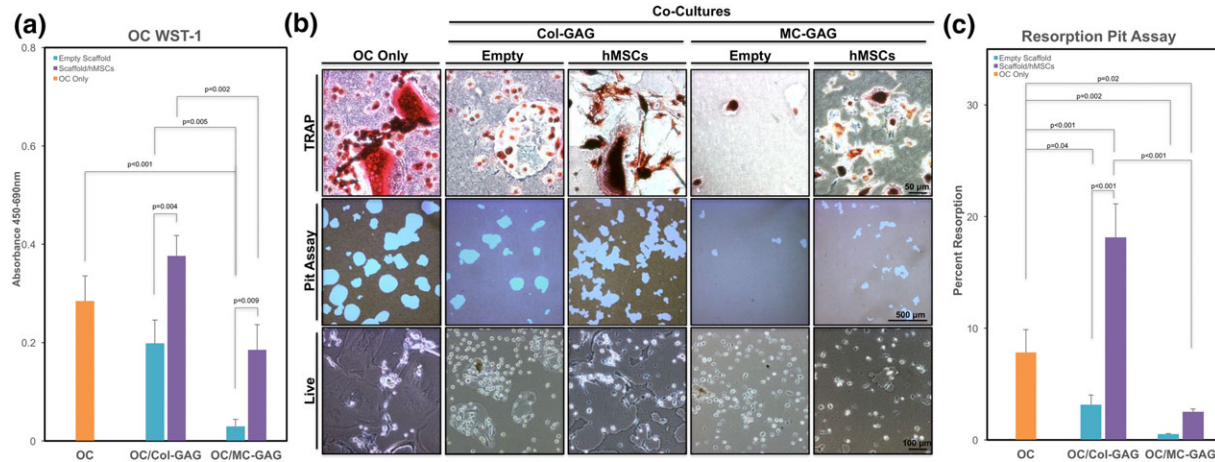


FIGURE 3 Empty MC-GAG and MC-GAG with differentiating hMSCs diminish the viability, proliferation, and resorption of hOCs. (a) WST-1 proliferation and viability assays of primary hOCs in single culture (OC only), cocultured with empty Col-GAG or MC-GAG (empty scaffold), or cocultured with Col-GAG or MC-GAG loaded with hMSCs (scaffold/hMSCs) after 14 days. (b) TRAP staining (upper row), resorption pits (middle row), and live images (lower row) of hOCs in single culture (OC only) or cocultured with empty or hMSCs-loaded Col-GAG or MC-GAG. (c) Quantitative analysis of pit assays as percentage of total area of well in differentiated osteoclasts without hMSCs (OC only) and osteoclasts cocultured with Col-GAG and MC-GAG as empty scaffolds (empty scaffold) or scaffolds loaded with differentiating hMSCs (scaffold/hMSCs). Significant post hoc comparisons following ANOVA indicated with p values. hMSC: human mesenchymal stem cell; MC-GAG: mineralized collagen glycosaminoglycan; hOC: human osteoclast; ANOVA: analysis of variance; WST-1: water soluble tetrazolium-1; TRAP: tartrate-resistant acid phosphatase [Colour figure can be viewed at wileyonlinelibrary.com]

hMSC-seeded materials, osteogenic differentiation of hMSCs clearly demonstrated an increase in hOC viability and/or proliferation on both Col-GAG ($p = 0.004$) and MC-GAG ($p = 0.009$) materials. However, the increase in hOC viability and proliferation in the presence of hMSCs on MC-GAG was significantly lower compared with hMSCs on Col-GAG ($p = 0.002$).

To evaluate hOC differentiation and activity, TRAP staining and resorption pit assays were performed for each coculture condition and corresponding controls (Figure 3b). Both TRAP staining and resorption were diminished in coculture with either empty Col-GAG or MC-GAG. Additionally, live images demonstrated qualitatively small rounded cells as opposed to large, differentiated multinucleated osteoclasts. When cocultured with differentiating hMSCs on Col-GAG, TRAP staining and resorption pits increased. Simultaneously, an increase in larger, multinucleated cells was clearly evident in live cell imaging. When cocultured with differentiating hMSCs on MC-GAG, TRAP staining and resorption pits increased; however, this was qualitatively less compared with hOC single culture or hOCs cocultured with hMSCs on Col-GAG.

To objectively evaluate the differences in hOC activity, percentages of the area of resorption were quantified for the different conditions (Figure 3c). Significant differences were found between the conditions on ANOVA ($F(4,10) = 53.98$, $p < 0.001$). On post hoc comparisons, both coculture conditions of hOC with empty Col-GAG or MC-GAG materials demonstrated decreased resorptive activity ($p = 0.04$ and $p = 0.002$, respectively). Similar to the WST-1 results, addition of hMSCs to the materials resulted in augmentation of resorptive activity. In the presence of hMSCs differentiated on Col-GAG, hOC-mediated resorption was significantly higher than in the presence of empty Col-GAG ($p < 0.001$) as well as hOC single cultures

($p < 0.001$). In the presence of hMSCs differentiated on MC-GAG, the recovery of hOC-mediated resorption was significantly less compared with that mediated by hMSCs differentiated on Col-GAG ($p < 0.001$) or hOC single cultures ($p = 0.02$).

3.4 | OPG expression on MC-GAG is upregulated by ERK1/2 activation and antagonized by canonical BMPR signalling

We previously reported differential regulation of osteogenic differentiation and mineralization of primary hMSCs on Col-GAG and MC-GAG (Ren, Bischoff, et al., 2015; Ren, Weisgerber, et al., 2016; Zhou et al., 2017). MC-GAG demonstrated an autogenous activation of the BMPR signalling in hMSCs that greatly surpasses Col-GAG. In both materials, BMPR signalling was essential for mineralization; however, Col-GAG also requires MEK1/ERK1/2-mediated signalling for mineralization whereas MC-GAG-mediated mineralization was completely independent of ERK1/2 phosphorylation.

To characterize the mechanism responsible for differential OPG expression by osteogenically differentiated hMSCs cultured on MC-GAG versus Col-GAG, we utilized DMH1 and PD98059 small molecule inhibitors for the canonical BMPR and MEK1/ERK1/2 signalling pathways, respectively (Figure 4). As we previously reported, in the presence of DMH1, phosphorylation of Smad1/5 was partially decreased at all time points in either material on western blot analyses (Figure 4a). Due to the massive upregulation of Smad1/5 phosphorylation in MC-GAG, a smaller inhibition was noted compared with Col-GAG. No differences in total Smad5, total ERK1/2, RANKL, or actin were detected. In contrast, OPG expression was increased in

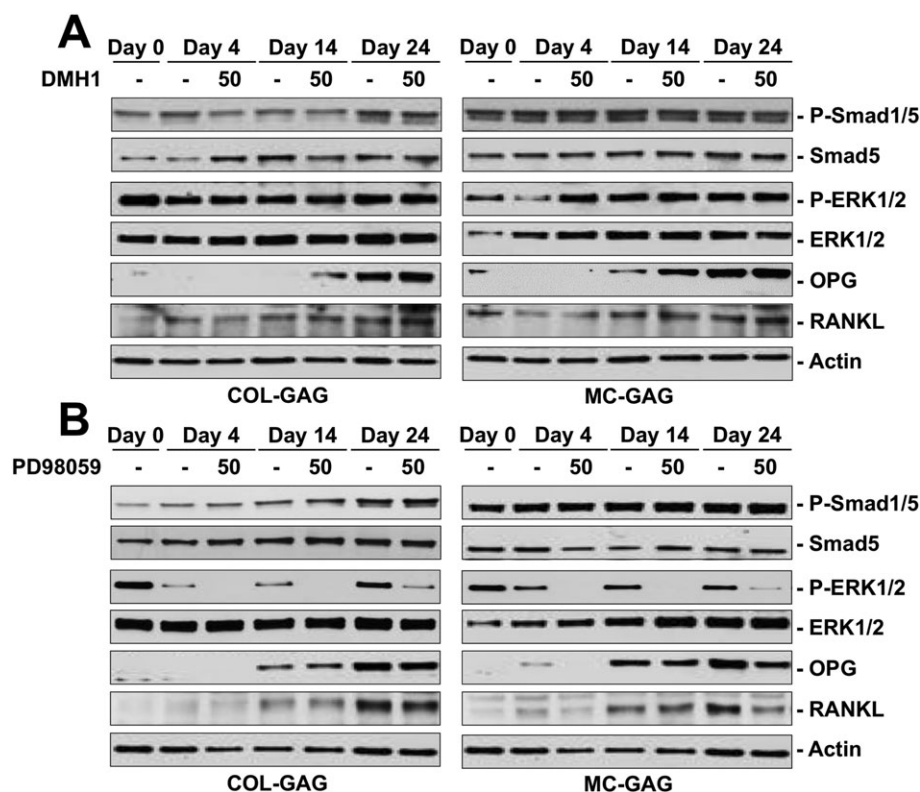


FIGURE 4 Western blot of intracellular signalling molecules expressed by hMSCs cultured on Col-GAG and MC-GAG in the absence and presence of DMH1 or PD98059. Western blot of phosphorylated Smad1/5 (P-Smad1/5) and total Smad (Smad5), phosphorylated ERK1/2 (P-ERK1/2) and total ERK1/2 (ERK1/2), OPG, RANKL, and β -actin in hMSCs cultured on Col-GAG and MC-GAG at day 0, 4, and 24 days of culture with and without (a) 50 μ M DMH1 or (b) 50 μ M PD98059. OPG: osteoprotegerin; hMSC: human mesenchymal stem cell; MC-GAG: mineralized collagen glycosaminoglycan; DMH1: dorsomorphin homologue 1; RANKL: receptor activator of nuclear factor- κ B ligand

the presence of DMH1 on both Col-GAG and MC-GAG, particularly at the Day 14 time point. A small elevation in phosphorylated ERK1/2 could also be seen in the presence of DMH1 specifically in MC-GAG at day 4.

To determine the contribution of the ERK1/2-mediated pathways, primary hMSCs osteogenically differentiated on Col-GAG or MC-GAG were treated with PD98059 (Figure 4b). As expected, PD98059 decreased phosphorylated ERK1/2 at all time points in either material, whereas no effects were noted for phosphorylated Smad1/5, total Smad5, total ERK1/2, or actin. Unlike DMH1, OPG protein expression was decreased in MC-GAG in the presence of PD98059 at all time points, whereas no differences were detected on Col-GAG. Similarly, RANKL expression was also downregulated, particularly at Day 24, in the presence of PD98059 on MC-GAG whereas no differences were detected on Col-GAG. In combination, these data suggest that the necessary signalling mechanisms for osteogenic differentiation are both distinct and antagonistic to the mechanisms responsible for osteoclast regulation in hMSCs differentiated on MC-GAG.

4 | DISCUSSION

In this work, we investigated the effect of nanoparticulate MC-GAG on osteoclastogenesis and osteoclast activity. Through activation of

osteogenic differentiation, MC-GAG and Col-GAG, its nonmineralized counterpart, induced an elevation of OPG protein expression by osteoprogenitors (Figure 1a). Early in differentiation, MC-GAG surpassed Col-GAG in the amount of total OPG protein and the amount of secreted OPG (Figure 1c). The quantity of OPG protein expression was potentiated by the addition of differentiating osteoclasts in coculture (Figure 1b). With respect to differentiating hMSCs, the presence of hOCs on either material augmented the canonical BMPR signalling pathway as seen by an increase in phosphorylated Smad1/5 (Figure 1) as well as mineralization particularly on MC-GAG (Figure 2). When the interactions between materials and hOCs were investigated, hOCs cultured in the presence of either Col-GAG or MC-GAG demonstrated a significant decrease in osteoclast-mediated resorption in the absence of hMSCs (Figure 3c). Whereas Col-GAG only affected resorption but not viability or proliferation, MC-GAG appeared to diminish both osteoclast activity and viability/proliferation (Figure 3a and 3c). The addition of differentiating hMSCs on either material improved hOC viability/proliferation significantly (Figure 3a). However, whereas osteogenic differentiation of hMSCs on Col-GAG augmented osteoclast resorption (Figure 3c), hOCs in the presence of MC-GAG continued to demonstrate diminished resorptive activity suggesting an indirect inhibitory effect of differentiating osteoprogenitors on MC-GAG. To mechanistically understand the indirect effects induced by MC-GAG on osteoclasts through

osteogenic differentiation of hMSCs, the canonical BMPR and ERK1/2 pathways were inhibited with respective small molecule inhibitors (Figure 4). Inhibition of the canonical BMPR pathway resulted in an increase in OPG protein expression by hMSCs cultured on both Col-GAG and MC-GAG materials. In contrast, inhibition of ERK1/2 phosphorylation downregulated both OPG and RANKL expression on MC-GAG whereas Col-GAG was not affected. These results suggest several conclusions regarding the direct and indirect influences of MC-GAG on hOC viability, proliferation, and activity: (a) Col-GAG and MC-GAG have direct inhibitory effects on osteoclast resorptive activity; (b) MC-GAG possesses an additional intrinsic ability to directly diminish osteoclast viability and proliferation that is not present in Col-GAG; (c) indirectly, the addition of hMSCs undergoing osteogenic differentiation improves osteoclast viability or proliferation on either Col-GAG or MC-GAG; (d) differentiating hMSCs on MC-GAG continue to inhibit the resorptive activity of hOCs whereas Col-GAG augments hOC-mediated resorption, correlating to the increased protein expression and secretion of OPG from hMSCs on MC-GAG; (e) although the canonical BMPR signalling pathway is essential for osteogenic differentiation of hMSCs cultured on either Col-GAG or MC-GAG, the MEK1/ERK1/2 pathway regulates OPG and RANKL expression on MC-GAG but not Col-GAG.

The combination of these conclusions suggests differing models in the interactions between Col-GAG and MC-GAG with osteoprogenitors and osteoclast progenitors (Figures 5 and 6). With respect to osteoprogenitors, we previously reported that the necessary mechanism for osteogenic differentiation was an autogenous activation of the canonical BMPR signalling pathway through elevated

Smad1/5 phosphorylation for both Col-GAG and MC-GAG (Figure 5, Mechanism 1; Figure 6, Mechanism 1; Ren, Wei, et al., 2015; Ren, Tu, et al., 2016; Zhou et al., 2017). Whereas Col-GAG also required activation of the ERK1/2 pathway, MC-GAG was independent of ERK1/2 for mineralization. With respect to osteoclast progenitors, both Col-GAG and MC-GAG had the ability to directly diminish osteoclast activation and resorptive activity (Figure 5, Mechanism 2A; Figure 6, Mechanism 2), thus suggesting that the activation of osteoclasts is diminished in the presence of collagen and glycosaminoglycan in the form of chondroitin-6-sulphate. Unlike Col-GAG, the direct inhibition of osteoclasts by MC-GAG materials is also accompanied with a diminished viability and proliferation of osteoclast precursors, suggesting a role for nanoparticulate mineral content in decreasing osteoclast activity and proliferation. In the presence of differentiating osteoprogenitors, differing net effects on osteoclast activation are observed between Col-GAG and MC-GAG (Figure 5, Mechanism 2B; Figure 6, Mechanism 3). Although both materials induce OPG expression by osteoprogenitors, the quantity of OPG expression is higher in hMSCs differentiated on MC-GAG, which is correlated to a net effect of continued inhibition of osteoclast-mediated resorption (Figure 5, Mechanism 2B). Although differentiating osteoprogenitors on either material improves the viability and/or proliferation of osteoclasts, a net effect of increased resorptive activity is only observed in the presence of Col-GAG (Figure 6, Mechanism 3).

Direct inhibitory effects of Col-GAG and MC-GAG on hOC-mediated resorption are likely to be related to glycosaminoglycan content. Similar to our results, chondroitin sulphate has been previously reported to inhibit resorptive abilities of osteoclasts, but not the

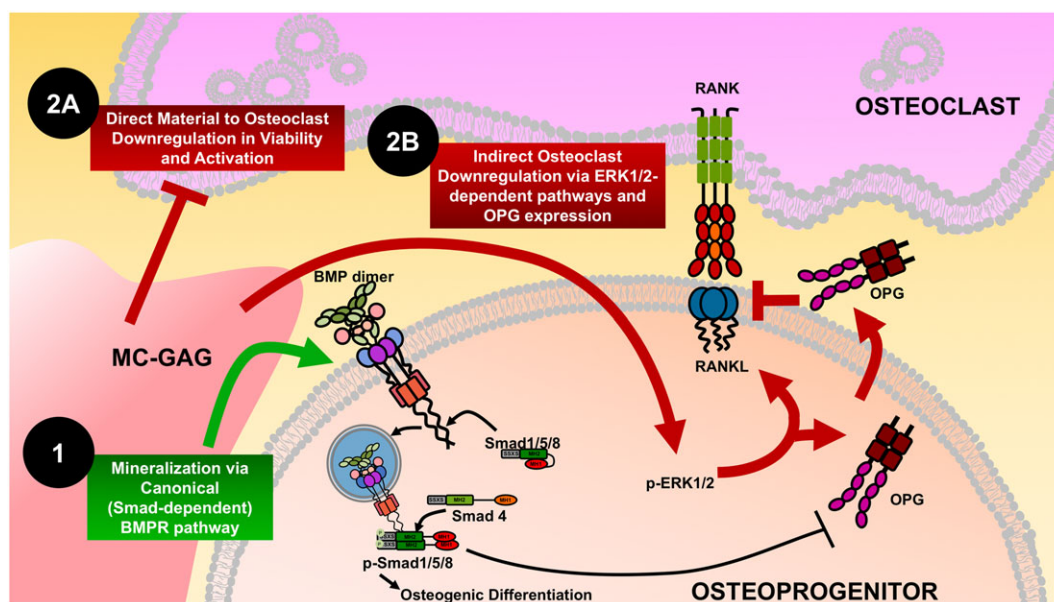


FIGURE 5 Mechanisms induced by MC-GAG on osteoprogenitors and osteoclast progenitors. MC-GAG induces osteogenic differentiation of primary hMSCs via an autogenous activation of the canonical BMPR signalling pathway with phosphorylation of Smad1/5/8 (Mechanism 1, green arrow). MC-GAG directly inhibits viability, proliferation, and resorptive activity of osteoclasts even in the absence of differentiating hMSCs (Mechanism 2A, red lines). MC-GAG also upregulates OPG expression through an ERK1/2 dependent pathway, correlating to an indirect inhibition of resorptive activity but not viability or proliferation in coculture with differentiating hMSCs (Mechanism 2B, red arrows and lines). OPG: osteoprotegerin; hMSC: human mesenchymal stem cell; MC-GAG: mineralized collagen glycosaminoglycan; BMPR: bone morphogenetic protein receptor [Colour figure can be viewed at wileyonlinelibrary.com]

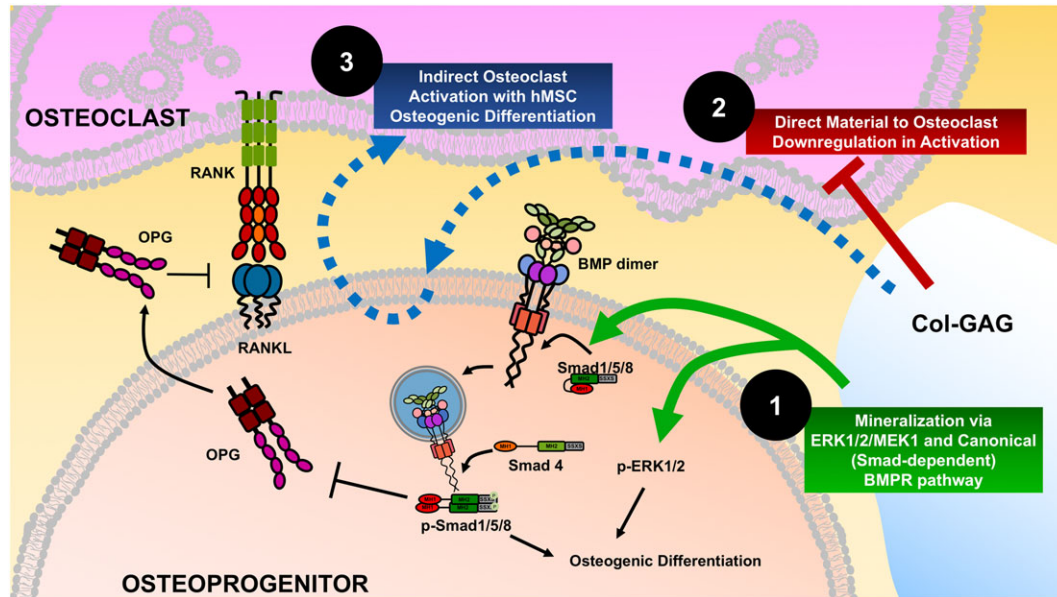


FIGURE 6 Mechanisms induced by Col-GAG on osteoprogenitors and osteoclast progenitors. Col-GAG induces osteogenic differentiation of primary hMSCs via both an autogenous activation of the canonical BMPR signalling pathway with phosphorylation of Smad1/5/8 and phosphorylation of ERK1/2 (Mechanism 1, green arrows). Col-GAG directly inhibits resorptive activity of osteoclasts even in the absence of differentiating hMSCs (Mechanism 2, red line). Although OPG expression is present in hMSCs differentiated on Col-GAG, a net activation of osteoclast mediated resorption occurs through unclear mechanisms (Mechanism 3, blue dashed line). OPG: osteoprotegerin; hMSC: human mesenchymal stem cell; BMPR: bone morphogenetic protein receptor [Colour figure can be viewed at wileyonlinelibrary.com]

viability, proliferation, or maturation in two-dimensional cultures (Cantley, Rainsford, & Haynes, 2013; Salbach, Kliemt, et al., 2012). The mechanism of this effect has been suggested to be related to the sulfation status of GAGs such that a dose dependent suppression of osteoclast activation occurs in highly sulfated GAGs (sGAGs; Salbach, Kliemt, et al., 2012). Two reasons for sGAG-mediated osteoclast suppression have been reported in the literature. Similar to reports on growth factor sequestration by sGAGs in tendon regeneration (Hortensius & Harley, 2013), sGAGs have also been reported to prevent RANKL from interacting with its cognate receptor (Ling, Murali, Stein, van Wijnen, & Cool, 2010). Second, separate from RANKL sequestration, a direct inhibitory effect of sGAGs on osteoclast adherence and spreading has also been reported (Baud'huin et al., 2011). Both effects are likely to be important in our current system. In terms of RANKL sequestration, we have found that the inhibitory effects on osteoclasts can be overcome with increases in RANKL concentration. We have also found that both Col-GAG and MC-GAG-mediated osteoclast inhibition require direct contact of the material with cells such that indirect cultures (via Transwell inserts) demonstrate no statistically significant effects on resorption (unpublished observations, J. C. L.).

MC-GAG also demonstrated an additional direct effect of reducing hOC viability and proliferation. As the major difference in composition between Col-GAG and MC-GAG is the presence of nanoparticulate calcium phosphate in the latter, one potential explanation would be negative regulation by calcium or phosphate ion-induced signalling pathways. High levels of extracellular calcium ion have been identified

to induce osteoclast apoptosis dependent on L-type voltage gated calcium channels but not the calcium sensing receptor (Lorget et al., 2000). Similarly high-extracellular phosphate concentrations have also been identified to inhibit osteoclastogenesis as well as induce osteoprogenitors to upregulate OPG, thereby acting as both a direct and indirect inhibitor of osteoclastogenesis and osteoclast activation (Kanatani, Sugimoto, Kano, Kanzawa, & Chihara, 2003). Conversely, both calcium and phosphate ions are known to be activators of osteogenic differentiation (Barradas et al., 2012; Dvorak et al., 2004; Shih et al., 2014). Thus, bone regenerative materials that include mineral content are likely to be able to utilize the dichotomy of osteogenic activation and osteoclast inhibition imparted by calcium and phosphate ion signalling.

A second inhibitory effect on osteoclast activity was also produced indirectly by osteoprogenitors differentiated on MC-GAG. The correlation of increased OPG expression by osteoprogenitors on MC-GAG suggests that the antiresorptive effect may be due to an alteration in the relative equilibrium between RANKL and OPG within this system. The balance of RANKL and OPG has been suggested to be responsible for the net effect towards bone formation or resorption on the locoregional or organismal environment (Gori et al., 2000; Kadriu et al., 2017). Tanaka, Mine, Ogasa, Taguchi, and Liang (2011) have demonstrated that the RANKL/OPG ratio changes over time following injury with a low ratio in the early stages, favouring bone formation, followed by a higher ratio, favouring bone resorption and remodelling, later after injury. The increase OPG secretion in MC-GAG was higher than Col-GAG only early in differentiation (Day 4). Thus, further

in vivo studies evaluating the effects of MC-GAG on local osteoclast differentiation may be necessary to understand the ultimate net effects of MC-GAG on OPG over time.

Although OPG is generally considered a marker of osteogenic differentiation, mechanisms governing OPG expression in osteoprogenitors on Col-GAG and MC-GAG are clearly distinct from the necessary pathways for mineralization. Whereas both Col-GAG and MC-GAG requires Smad1/5 phosphorylation for hMSC mineralization, the canonical BMPR pathway antagonized OPG expression such that inhibition by DMH1 resulted in higher levels of OPG. Both OPG and RANKL expression in hMSCs depended upon ERK1/2 phosphorylation for MC-GAG but not Col-GAG. These data suggested that osteogenic differentiation and expression of osteoclast regulatory proteins may be separately modulated by specific ECM compositions in regenerative materials. Future materials-based regenerative strategies for controlled augmentation of osteogenesis with concurrent, temporary downregulation of osteoclastogenesis are underway.

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CONFLICT OF INTEREST

There are no conflicts of interest to declare among any of the authors.

AUTHOR CONTRIBUTIONS

X. R., Q. Z., and D. F. performed all of the experiments. X. R. also contributed portions of the manuscript. M. J. D. and B. A. C. H. fabricated the materials, contributed expertise in material science, and contributed critical review of the manuscript. D. B., T. A. M., and D. T. Y. contributed bone biology expertise, input in interpretation of the study, and critical review of the manuscript. J. C. L. designed and directed the experiments and wrote the manuscript.

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Shape-fitting collagen-PLA composite promotes osteogenic differentiation of porcine adipose stem cells

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ABSTRACT

Craniomaxillofacial bone defects can occur as a result of congenital, post-oncologic, and high-energy impact conditions. The scale and irregularity of such defects motivate new biomaterials to promote regeneration of the damaged bone. We have recently described a mineralized collagen scaffold capable of instructing stem cell osteogenic differentiation and new bone infill in the absence of traditional osteogenic supplements. Herein, we report the integration of a millimeter-scale reinforcing poly (lactic acid) frame fabricated via 3D-printing into the mineralized collagen scaffold with micron-scale porosity to form a multi-scale mineralized collagen-PLA composite. We describe modifications to the PLA frame design to increase the compressive strength (Young's Modulus, ultimate stress and strain) of the composite. A critical challenge beyond increasing the compressive strength of the collagen scaffold is addressing challenges inherent with the irregularity of clinical defects. As a result, we examined the potential for modifying the frame architecture to render the composite with increased compressive strength in one axis or radial compressibility and shape-fitting capacity in an orthogonal axis. A library of mineralized collagen-PLA composites was mechanically characterized via compression testing and push-out test to describe mechanical performance and shape-fitting capacity. We also report in vitro comparison of the bioactivity of porcine adipose derived stem cells in the mineralized collagen-PLA composite versus the mineralized collagen scaffold via metabolic activity, gene expression, and functional matrix synthesis. The results suggest that incorporation of the PLA reinforcing frame does not negatively influence the osteoinductive nature of the mineralized collagen scaffold. Together, these findings suggest a strategy to address often competing bioactivity, mechanical strength, and shape-fitting design requirements for biomaterials for craniomaxillofacial bone regeneration.

1. Introduction

Cranio-maxillofacial defects (CMF) are bone defects that will not heal without surgical intervention due to the defect being too large to heal naturally by the body (Spicer et al., 2012). CMF defects are associated with a range of trauma, from birth defects such as cleft palate to surgical resection of head and neck cancers and high energy impacts such as battlefield injuries. The gold standard for treating such defects remains either permanent implants or biologic grafts such as patient-derived autograft tissue or allograft tissue, typically from a cadaveric donor (Depeyre et al., 2016). Allografts offer advantages in terms of

ready supply and do not require a second surgery, but can exhibit significantly different osteogenic capacity and the possibility of enhanced immune response (Elsalanty and Genecov, 2009). While autografts do not lead to enhanced immune response and are naturally osteoconductive, their limited availability, need for a secondary surgical defect, and reduced healing capacity with age motivate a range of tissue engineering efforts (Thompson et al., 2015). Biomaterial approaches seek to address these concerns via an implantable material that provides the appropriate signals to promote cell recruitment, bioactivity, and eventual regenerative repair of the CMF defect. However, biomaterial solutions are not without challenges. Successful implants to

Abbreviations: PLA, poly(lactic acid); CMF, cranio-maxillofacial; PCL, poly(caprolactone); pASC, porcine adipose derived stem cell

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improve regenerative healing must be biocompatible, meet micro-scale mechanical needs to promote osteogenesis as well as macro-scale requirements for a mechanically robust implant, be bioresorbable, and fit irregular defect margins. Such materials must be porous and support cell recruitment and bioactivity, be osteoconductive or osteoinductive in nature, and promote remodeling and the formation of vascular networks (Bose et al., 2012; Cunniffe et al., 2010). A poorly integrated bone replacement material can result in graft resorption, limited osteointegration, and the possibility of the material being lost or actively ejected from the defect (Nail et al., 2015; Zhang et al., 2014a).

One class of biomaterial that has received recent attention have been collagen-based scaffolds and mineralized collagen composites (Donzelli et al., 2007; Farrell et al., 2006; Harley et al., 2010b; Kanungo et al., 2008; Murphy et al., 2010; Tierney et al., 2008). Porous collagen scaffolds offer an open pore structure to facilitate rapid cell attachment and invasion (Harley et al., 2008; O'Brien et al., 2005), and while they are mechanically weak, they have been shown to promote osteogenic differentiation and new mineral formation, typically through the addition of exogenous stimuli such as osteogenic media or BMP-2 (Curtin et al., 2012; Farrell et al., 2007). Recently, efforts in our lab and a number of other investigators have begun to demonstrate the potential of collagen-mineral composites. Here, nanocrystallite calcium phosphate mineral can be incorporated into or coated onto the collagen scaffold network to more closely approximate the compositional features of bone (Al-Munajjed et al., 2009; Caliri and Harley, 2014; Cunniffe et al., 2010; Curtin et al., 2012; Harley et al., 2010a; Weisgerber et al., 2015b). We have recently described the development of a calcium phosphate mineralized collagen scaffold containing glycosaminoglycans for CMF bone regeneration (Lee et al., 2015; Ren et al., 2016a; Weisgerber et al., 2015a). Notably, the mineralized collagen scaffold can promote mesenchymal stem cell (MSC) osteogenic differentiation and mineral biosynthesis in vitro (Lee et al., 2015; Ren et al., 2015; Weisgerber et al., 2015b), as well as new bone formation in vivo in both rabbit calvarial and porcine mandibular defects (Ren et al., 2016b, 2016c; Weisgerber et al., 2018), all in the absence of supplementary BMP-2. However, while these mineralized collagen scaffolds provide appropriate pore size and endogenous signals to promote osteogenic differentiation and matrix biosynthesis, their porous nature results in sub-optimal mechanical properties as stand-alone implants. Recently, we described fabrication approaches to integrate macroporous (millimeter scale) polycaprolactone (PCL) polymer frame to create a mineralized collagen-PCL composite (Weisgerber et al., 2016a). The mineralized collagen-PCL composite possessed enhanced mechanical stiffness 6000-fold and promoted both bone marrow mesenchymal stem cell and porcine adipose derived stem cell (pASC) osteogenic differentiation in vitro (Ren et al., 2015; Weisgerber et al., 2016a). Further, the collagen-PCL composite induced rapid bone in-fill in vivo in a large animal mandibular defect model in adolescent Yorkshire pigs (Weisgerber et al., 2018). However, two concerns noted with the current generation mineralized collagen-PCL composite was the slow degradation rate of the PCL polymer as well as the potential for poor fitting of the implant into an irregularly-shaped defect. Recent efforts by Grunlan et al. described an osteoconductive shape-memory biomaterial that possessed radial expansion capacity to improve conformal fitting between implant and defect margin (Nail et al., 2015; Zhang et al., 2014a), motivating efforts to redesign the polymeric reinforcement mesh of our mineralized collagen scaffold to improve the potential for conformal fitting within the defect margin.

In this manuscript, we describe the development and in vitro testing of a unique mineralized collagen-poly (lactic acid) (PLA) composite as a next-generation biomaterial for complex CMF defect repair that can support shape-fitting. PLA has been used extensively as a degradable polymeric biomaterial for a range of tissue engineering applications (Naderi et al., 2011), suggesting its use here to form a collagen-PLA composite biomaterial. We hypothesized that a low volume fraction frame architecture composed of mechanically-robust PLA could be

incorporated into the mineralized collagen scaffold to form a composite with enhanced compressive strength. Further, this work seeks to demonstrate that modifications to the PLA frame geometry could render the collagen-PLA composite capable of returning to its original shape after being radially-compressed to enhance conformal fitting with defect margins. We first describe integration of the PLA frame into the mineralized collagen scaffold as well as the mechanical performance (ultimate stress, ultimate strain, elastic modulus, push-out strength) of the resultant mineralized collagen-PLA composite. We subsequently evaluate the functional capacity of the composite, examining attachment, proliferation, osteogenic differentiation, and mineral biosynthesis of porcine adipose derived stem cells in the mineralized collagen-PLA composite relative to the PLA cage or mineralized collagen scaffold alone.

2. Materials and methods

2.1. 3D printing poly (lactic acid) cages

PLA cages were fabricated by extrusion printing of PLA filaments at 185 °C using an Ultimaker 2 + 3D printer (Ultimaker, Geldermalsen, Netherlands) utilizing a 0.25 mm diameter nozzle. PLA cages were designed using the Fusion 360 (Autodesk, San Rafael, California) design program, with each design stored as a .stl file for fabrication. Two classes of collagen-PLA composites were used for this project. Mechanical testing was performed using cylindrical PLA cages (10 mm dia., 10 mm high) created in four distinct cage architectures of increasing complexity so as to examine the effect of PLA fiber thickness (0.7 mm vs. 1.0 mm) or the inclusion of design elements to alter compressive strength or space-fitting (angled vs. straight fibers; open vs. closed cage; Fig. 1A). These specimens are of the size used previously for in vitro testing of mineralized collagen scaffolds in a porcine mandibular defect model (10 mm dia., 10 mm thick cylindrical implants). However, the large volume of these implants made a comprehensive cell bioactivity study in vitro impractical in regards to the number of required adipose derived stem cells. As a result, in vitro cell bioactivity and osteogenic assays to examine whether the inclusion of a PLA frame reduced the osteogenic capacity of the mineralized collagen scaffold were performed using a smaller cage design (6 mm dia., 6 mm high; Fig. 1A) fabricated with the same volume fraction of PLA (10.7% v/v) as in the larger composites tested mechanically (6 – 10.4% v/v PLA).

2.2. Fabrication of mineralized scaffolds and composites

Scaffolds were fabricated by lyophilization of a mineralized collagen precursor suspension in a manner previously described (Weisgerber et al., 2015a). Briefly, the mineralized collagen suspension was prepared by homogenizing (1.9 w/v%) bovine type I collagen (Collagen Matrix, Oakland, NJ) and (0.84 w/v%) chondroitin-6-sulfate (Sigma-Aldrich, St. Louis, MO), and calcium salts ($\text{Ca}(\text{OH})_2$, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, Sigma-Aldrich), in phosphoric acid (Sigma-Aldrich). The mineralized collagen precursor suspension was then stored at 4 °C and degassed before use. Scaffolds were then fabricated via lyophilization using a Genesis freeze-dryer (VirTis, Gardener, NY) (Harley et al., 2010a). The mineralized collagen suspension was pipetted into individual wells created in a polysulfone mold. Scaffolds were then fabricated by freezing the suspension via cooling from 20 °C to –10 °C at a constant rate of 1 °C/min followed by a temperature hold at –10 °C for 2 h. The frozen suspension was then sublimated at 0 °C and 0.2 Torr, resulting in a porous scaffold network.

Mineralized collagen-PLA composites were fabricated by pipetting the precursor suspension into wells, gently lowering the PLA cages into the mold, then adding remaining slurry to cover the cages completely (Fig. 1B) (Weisgerber et al., 2016a). Two classes of composites were fabricated to tests described in this manuscript. **Mechanical assays:** Large collagen-PLA composites were fabricated using PLA cages

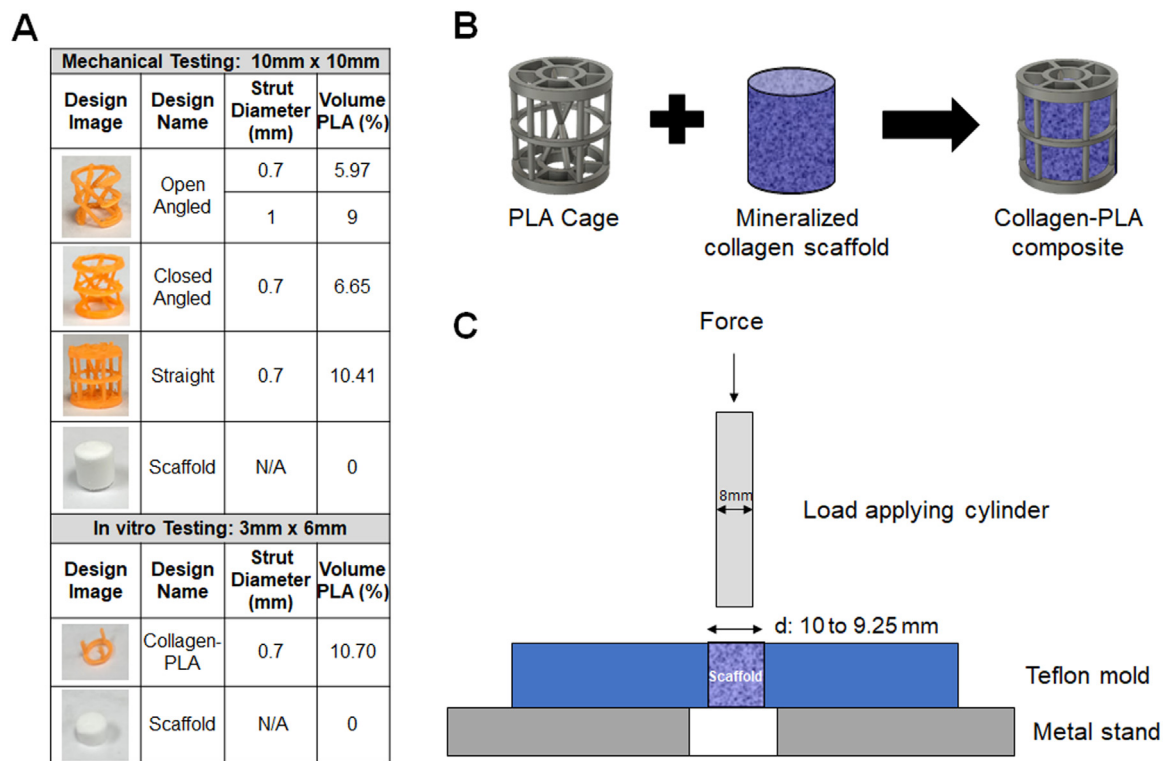


Fig. 1. (A) Outline of designs used for mechanical and in vitro testing. (B) Schematic of mineralized collagen-PLA composite design. (C) Push out test schematic.

(10 mm dia., 10 mm high) placed into 11.9 mm dia., 10 mm high polysulfone wells with 1.2 mL suspension. **In vitro bioactivity assays:** Smaller collagen-PLA composites, to reduce the number of cells required for in vitro experiments, were fabricated using smaller PLA cages (6 mm dia., 3 mm high) placed into 6 mm dia., 3 mm high polysulfone wells with 100 μ L suspension. Both classes of collagen-PLA composites were designed to have comparable PLA volume fractions (< 11%) (Fig. 1A).

After fabrication, all scaffolds and composites were sterilized via ethylene oxide treatment for 12 h utilizing an AN74i Anprolene gas sterilizer (Andersen Sterilizers Inc., Haw River, NC) in sterilization pouches (Ren et al., 2016b; Weisgerber et al., 2015b, 2016b). All subsequent handling steps leading to studies of cell activity were performed in a sterile manner.

2.3. ESEM imaging

Environmental scanning electron microscope was used to examine the integration of the PLA fibers and mineralized collagen scaffold structures in the collagen-PLA composites. Imaging was performed using an FEI Quanta FEG 450 ESEM (FEI, Hillsboro, OR), on composites cut with a razor blade prior to imaging to reveal the internal collagen-PLA microstructure.

2.4. Mechanical behavior of scaffold, cage, composites under compression

Stress-strain curves of the non-hydrated mineralized collagen scaffolds, all PLA cage designs, and all mineralized collagen-PLA composites under compression were generated using an Instron 5943 mechanical tester (Instron, Norwood, MA) using a 100 N load cell under dry conditions. Briefly, 8 samples of each design type were compressed to failure at a rate of 2 mm/min with the ultimate stress and strain and Young's Modulus determined from the stress-strain curves using conventional analysis methods for low-density open-cell foam structures such as the collagen scaffolds (Gibson and Ashby, 1997; Harley et al., 2007a; Kanungo and Gibson, 2009a, 2009b; Kanungo et al., 2008).

While hydrating and carbodiimide crosslinking collagen scaffolds reduces the modulus of the scaffold versus non-hydrated specimens, we have previously shown the relative effects of scaffold architecture are maintained for dry vs. hydrated specimens and that the performance of non-hydrated and hydrated collagen-fiber composites is largely unaffected by scaffold hydration level due to the dominant influence of the fiber architecture on composite mechanical performance (Harley et al., 2007a; Mozdzen et al., 2016, 2017; Weisgerber et al., 2016b).

2.5. Measuring conformal fit via push out testing

The contribution of shape-fitting PLA fiber designs to the conformal fitting of the mineralized collagen-PLA composite within a cylindrical defect was examined via mechanical push out test (Nganga et al., 2011; Seong et al., 2013) performed using an Instron 5943 mechanical tester (Instron, Norwood, MA). Tests were performed on 8 non-hydrated open-angled design specimens, measuring 0.7 mm and 1 mm in strut diameter using a 100 N load cell. Cylindrical mineralized collagen-PLA specimens were compressed radially with forceps, inserted into 10 mm or 9.25 mm dia. cylindrical holes in a Teflon base, then were released, allowing them to expand and fill the hole prior to testing. The 9.25 mm dia. cylindrical defect was the smallest hole the specimens could be press-fit into. The Teflon base plate was clamped on the mechanical tester, with a push out test performed on the specimens using an 8 mm diameter metal rod to push the composites until exit of the bottom of the Teflon base at a rate of 2 mm/min (Fig. 1C).

2.6. Hydration and crosslinking of designs

PLA cages, mineralized collagen scaffolds, and mineralized collagen-PLA composites were all hydrated prior to use with cells. Briefly, samples were soaked for two hours in 100% ethanol, then underwent multiple PBS washes to fully hydrate the scaffold (Weisgerber et al., 2015b). The scaffolds and composites were subsequently crosslinked via carbodiimide chemistry in a PBS solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Sigma-Aldrich) and

Table 1
Antibodies used for Western Blots.

Protein	Blocking	Primary antibody	Secondary antibody
β-actin (45 kDa)	5% dry milk	1:1000 in 5% dry milk (Cell Signaling Technologies, Rabbit mAB, 4967 L)	1:5000 in TBST, Anti-rabbit IgG, HRP linked antibody (Cell Signaling Technologies, 7074S)
SMAD1/5/9 (52–60 kDa)	5% dry milk	1:1000 in 5% dry milk (Abcam, Rabbit mAB, ab66737)	
p-SMAD1/5/9 (60 kDa)	5% dry milk	1:1000 in 5% dry milk (Cell Signaling Technologies, Rabbit mAB, 13820S)	
SMAD2/3 (52–60 kDa)	5% dry milk	1:1000 in 5% dry milk (Cell Signaling Technologies, Rabbit mAB, 8828S)	
p-SMAD2/3 (52–60 kDa)	5% dry milk	1:1000 in 5% dry milk (Cell Signaling Technologies, Rabbit mAB, 8685S)	
ERK1/2 (42–44 kDa)	5% dry milk	1:1000 in 5% dry milk (Cell Signaling Technologies, Rabbit mAB, 9102S)	
p-ERK1/2 (44–42 kDa)	5% dry milk	1:1000 in 5% dry milk (Cell Signaling Technologies, Rabbit mAB, 9101S)	
p38 (40 kDa)	5% dry milk	1:1000 in 5% dry milk (Cell Signaling Technologies, Rabbit mAB, 8690S)	
p-p38 (43 kDa)	5% dry milk	1:1000 in 5% dry milk (Cell Signaling Technologies, Rabbit mAB, 9215S)	
AKT (60 kDa)	5% dry milk	1:1000 in 5% dry milk (Cell Signaling Technologies, Rabbit mAB, 9272S)	
p-AKT (60 kDa)	5% dry milk	1:1000 in 5% dry milk (Cell Signaling Technologies, Rabbit mAB, 4060S)	

N-hydroxysulfosuccinimide (NHS, Sigma-Aldrich) at a molar ration of 5:2:1 EDAC:NHS:COOH (carboxylic acid groups on the collagen backbone) (Caliari and Harley, 2011b, 2014; Olde Damink et al., 1996; Veilleux et al., 2004). Scaffold and composites were washed multiple times in sterile PBS, then soaked in cell culture media for 42 h prior to seeding with cells.

2.7. Porcine adipose derived stem cell (pASC) culture and seeding

Porcine adipose derived stem cells (pASCs, a gift of Dr. M. Wheeler, Department of Animal Science, UIUC, Urbana, IL) were expanded at 37 °C and 5% CO₂ in complete mesenchymal stem cell growth media (low glucose DMEM, 10% mesenchymal stem cell fetal bovine serum, and 1% antibiotic-antimycotic) that did not include any osteogenic supplements (Mónaco et al., 2009). pASCs show robust osteogenic capabilities in response to osteogenic media (Bionaz et al., 2015) or in mineralized collagen scaffolds in the absence of osteogenic supplements (Weisgerber et al., 2016a), and were used at passage 4 throughout all experiments here. pASCs were seeded onto PLA cages as well as into collagen scaffolds and collagen-PLA composite using a previously described static-seeding method (Caliari et al., 2012; O'Brien et al., 2005). Briefly, a total of 7.5×10^4 pASCs in 40 μL of growth media were seeded onto each cage, scaffold, or composite in costar® 24 Well plate ultra-low attachment surface (Corning, Corning, NY). First, 3.75×10^4 pASCs (in 20 μL of growth media) were seeded on one side of the cylindrical sample and left for 30 min for cells to initiate attachment; cages, scaffolds, and composites were subsequently flipped over and another 3.75×10^4 cells (in 20 μL of growth media) were seeded on the opposite side. Samples were then incubated for an additional 1.5 h at 37 °C to let cells more fully attach to the specimens. Additional growth media was added to each well, with pASC seeded samples then cultured for the remainder of the experiment (up to 28 days) at 37 °C and 5% CO₂ and in the presence of complete mesenchymal cell growth media (replaced every 3 days) that lacked any osteogenic supplements.

2.8. Measurement of pASC metabolic activity

The metabolic activity of 6 of each pASC seeded constructs (cage, scaffold, composite) was quantified over 28 days (days 1, 4, 7, 14, and 28) using a non-destructive alamarBlue® assay (Caliari and Harley, 2014). Cell seeded samples were incubated in an alamarBlue® solution (Invitrogen, Carlsbad, California) at 37 °C under gentle shaking for 2 h.

The fluorescence of the reduced resazurin byproduct, resorufin, by the metabolically active pASCs was measured using a F200 spectrophotometer (Tecan, Mannedorf, Switzerland) at 540(52) nm excitation and 580(20) nm emission. The metabolic activity of each sample is reported as the normalized activity generated using a standard prepared from the initial number of cells seeded onto each construct (7.5×10^4).

2.9. Measurement of pASC cell number

The total number of pASCs on the cage, scaffold, and composite at days 1, 7, 14, and 28 was quantified using DNA quantification using methods previously described (Caliari and Harley, 2011a). Three samples and one unseeded control of each design type were rinsed in PBS three times to remove dead or unattached cells, and then placed in a papain solution (Sigma-Aldrich) at 60 °C for 24 h to digest samples and lyse cells. Hoechst 33258 (Invitrogen) was used to fluorescently label double-stranded DNA and was read at an excitation of 360 nm and emission of 465 nm utilizing a fluorescent spectrophotometer (Tecan). Total cells per construct was reported as a normalized value, with background fluorescence first removed for each sample using a blank control construct at each time point, and those results then normalized using a standard generated on day 0 from the initial number of cells seeded onto each construct (7.5×10^4) with known cell number to determine cell number in each sample.

2.10. Western Blot analysis

Protein lysates from three of each seeded cage, scaffold, and composites were collected at days 1, 4, 7, and 14 using a mixture of Phosphate Inhibitor Cocktail (Sigma-Aldrich), Phosphatase Inhibitor Cocktails 2 and 3 (Sigma-Aldrich), and a RIPA lysis buffer (Grier et al., 2017). Total protein content was assessed using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) and a Pierce™ Bovine Serum Albumin Standard Pre-Diluted Set (Thermo Fisher Scientific). Western blot analysis was performed using 5 μg of protein lysate per lane, using primary antibodies listed in Table 1, followed by a 1:5000 dilution of anti-rabbit HRP-IgG (Cell Signaling Technologies, Danvers, MA). β-actin was used as a loading control throughout. Western blots were imaged using a SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and Image Quant LAS 4010 (GE Healthcare Life Sciences, Little Chalfont, United Kingdom).

Table 2
Primers used for TAQMAN gene expression analyses.

Gene	Catalog Number
<i>GAPDH</i>	Ss03375629_u1
<i>COL1A2</i>	Ss03375009_u1
<i>BGLAP</i>	Ss03373655_s1
<i>BMP2</i>	Ss03373798_g1
<i>Osterix</i> (LOC404701)	Ss03373734_s1
<i>MMP9</i>	Ss03392100_m1

2.11. RT-PCR analysis

RNA was extracted from 5 of each pASC seeded constructs (cage, scaffold, composite) at days 1, 4, 7, and 14 utilizing an RNeasy Plant Mini kit (Qiagen, Valencia, CA) and was reverse transcribed to cDNA utilizing a Bio-Rad S1000 thermal cycler and a QuantiTect Reverse Transcription kit (Qiagen) using previously described methods (Caliari and Harley, 2011a). Real-time PCR reactions were performed in duplicate (10 ng of cDNA) using the QuantiTect SYBR Green PCR kit (Qiagen) or Taqman fast advanced master mix and Taqman gene expression assays (Applied Biosystems, Foster City, CA) Quantstudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific), with results normalized to pASC expression profiles from cell-seeded composites at day 0.

Taqman gene expression assays (Table 2) are pre-validated by Applied Biosystems. However, RUNX2 was not available in Taqman gene expression assays for porcine cells, so additional primers for SYBR Green analyses were synthesized by Integrated DNA Technologies (Coralville, IA) using sequences previously reported in the literature (Table 3), with *GAPDH* used as a housekeeping gene. Primers for SYBR Green analyses were validated via RT-PCR against multiple concentrations of forward and reverse primer (20uM, 10uM, 5uM, 2.5uM) using pASCs to verify one single melting peak and the lowest CT value. Primer efficiency was then calculated from RT-PCR with the optimal primer concentration against multiple cDNA concentrations (10 ng/well, 5 ng/well, 2.5 ng/well, 1 ng/well, 0.5 ng/well, 0.1 ng/well), with 100% efficiency found if plotting delta CT vs. log(cDNA) led to a straight line (slope < 0.1). In this manner, only RUNX2 was validated for SYBR Green analysis of porcine cells used in this study. The delta-delta CT method was utilized to generate results, and all results were expressed as fold changes normalized to the day 0 standard.

2.12. Micro-CT analysis

Quantification of new mineral formation in the constructs at day 28 (cage, scaffold, composite) and day 0 unseeded controls was performed via microcomputed tomographic (micro-CT) imaging using the MicroXCT-400 (Zeiss, Oberkochen, Germany). Three seeded samples and three day 0 unseeded samples were fixed in 10% formalin and stored at 4 °C prior to analysis. Scans were performed using a 1x resolution lens, 40 V, 8 W, with the same binning, exposure time, and source and detector positions. All images had a final pixel size of 15.75 μm, and brightness and contrast were the same for each sample. Total mineral content was analyzed from z-stacks of 2D micro-CT images using a custom Matlab program developed to quantify the fill

Table 3
Primers used for SYBR Green gene expression analyses.

Gene	Primer Sequence (5'-xxx-3')	Citation
<i>GAPDH</i>	Forward: GGACCTCTGGGTATGGCTTTC	(Nygard et al., 2007)
	Reverse: TGG TAA CAT CAA TAC GAT TTC TGA	
<i>RUNX2</i>	Forward: CTC AGT GAT TTA GGG CGC ATT	(Lee et al., 2016)
	Reverse: AGG GGT AAG ACT GGT CAT AGG	

fraction and mineral intensity in scaffold, composite, and control samples as a function of radial, angular, and depth position (Weisgerber et al., 2018). A value of 215 was set for the threshold of every sample in the program, with a depth of 140. We report the degree of new mineral formation in the scaffold and composite groups along three axes of the cylindrical specimens (depth: 0 – 1 bottom to top; radius: 0 – 1470 center to edge; angle: 0 – 360°) for a volume of interest inside the PLA frame of the composites so as to avoid any contribution of the frame itself on the measurement of mineral formation. A radius of 100 (2 mm) was used on the scaffold and composite due to the interference of PLA struts at a greater radius, and a radius of 120 (2 mm) was used for control samples due to their larger size. Results for the mineralized scaffold and mineralized collagen-PLA composite were normalized to the day 0 unseeded scaffold.

2.13. Histology of designs

After micro-CT analysis, each of the three samples were rinsed in PBS, embedded in Tissue-Tek® O.C.T. freezing compound (Sakura Finetek, Netherlands), then stored at –80 °C. 14 μm thick histology specimens were cut from frozen block using a motorized Microm HM 550 cryostat (Thermo Fisher Scientific) and placed onto glass slides. Samples were H&E stained using Gill II Hematoxylin solution (Leica, Wetzlar, Germany) and with a Eosin solution made from Eosin Y powder (Fisher Scientific, Pittsburgh, PA), and were Alizarin Red stained with a solution made from Alizarin Red S powder (Sigma-Aldrich), and Von Kossa stained using a kit (ab150687, Abcam, Cambridge, UK), then imaged using a NanoZoomer Digital Pathology System (Hamamatsu, Japan). All images taken were analyzed qualitatively.

2.14. Statistics

Statistical analysis utilized OriginPro software (Northampton, MA). Significance was set to $p < 0.05$. Data was first tested for normality via the Shapiro-Wilk test, then tested for equal variances between samples with a Browne-Forsythe test. If multiple samples analyzed (2+) were normally distributed and had equal variances, a one-way ANOVA was performed with a tukey post-hoc test. If samples were normally distributed without equal variances, a *t*-test with a Welch correction was used. If two samples were normally distributed then a *t*-test was always used. If data was not normally distributed, a Kruskal-Wallis test was performed (Necker, 2010). The power was also checked after running either ANOVA or *t*-tests, and if found to be less than 0.8 then there was deemed no significance ($p < 0.05$) between samples tested. Outliers were removed using the Grubbs test. The number of samples per experimental group for each type of assay was informed by previous studies using collagen scaffolds and composites (Caliari and Harley, 2014; Grier et al., 2017; Mozdzen et al., 2016; Weisgerber et al., 2016a, 2016b): compressive tests ($n = 8$), push out tests ($n = 6$), cell number ($n = 3$), metabolic activity ($n = 6$), western blot ($n = 3$), gene expression ($n = 5$), and micro-CT ($n = 3$). Error bars are reported as mean ± standard deviation.

3. Results

3.1. Determining PLA incorporation in mineralized collagen-PLA composites

ESEM images of both open angled and straight composites architectures showed full incorporation of the PLA into the mineralized collagen scaffold architecture with no evidence of delamination (Fig. 2).

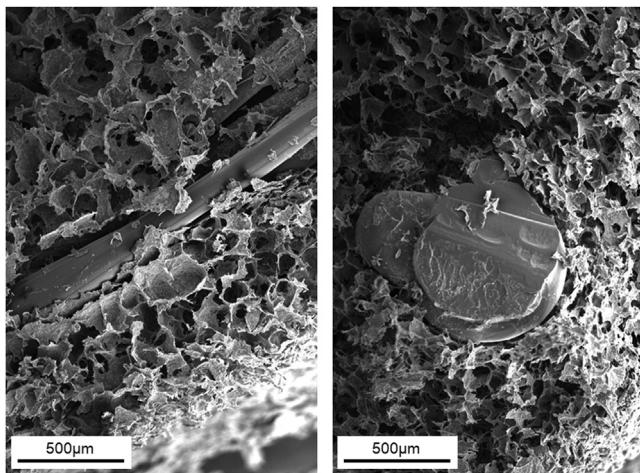


Fig. 2. Environmental Scanning Electron Microscope images of cross-sections of composite designs with 3D printed PLA struts within mineralized collagen scaffold.

3.2. The compressive strength of the mineralized collagen scaffold is significantly enhanced via the inclusion of PLA reinforcing fibers

The compressive mechanical behavior of four mineralized collagen-PLA composite designs were chosen to span a range of experimental design parameters. We compared a 0.7 mm dia. straight cage and composite PLA design with hypothesized highest axial compressive strength to angled composite designs of different fiber diameter (0.7 mm vs. 1.0 mm dia.) and different shape-fitting ability (open vs. closed). For the 0.7 mm dia. PLA fiber variant, we examined the effect of angle of the PLA fibers ('straight' fibers parallel to the direction of compressive loading vs. 'angled' fibers at $\sim 40.7^\circ$ off the axis of compressive loading (37.9, 40.6, 43.5) with the goal of increasing axial composite compliance). We subsequently examined the effect of the diameter of angle strut (0.7 mm vs. 1 mm) on the open angled design. Lastly, and again using 0.7 mm dia. PLA fiber variants, we examined the effect of removing a section of the circumferential PLA fiber bands at the periphery of the reinforcing cage ('open' vs. 'closed') to enhance the radial compressibility of the composites. We also tested the 0.7 mm dia.

straight cage (vs. the 0.7 mm dia. straight composite) as well as the non-reinforced mineralized collagen scaffold (Fig. 3). The 0.7 mm straight cage and 0.7 mm straight composite had significantly ($p < 0.05$) higher ultimate stresses and Young's Moduli than all open and closed angled composites, and the scaffold had a significantly ($p < 0.05$) lower ultimate stress and Young's Moduli compared to all other tested designs. No significant difference in ultimate stress and Young's Moduli was found between the 0.7 mm straight composite and the 0.7 mm straight cage. However, increasing the strut diameter of the open angled design from 0.7 mm to 1 mm led to a significant ($p < 0.05$) increase in ultimate stress.

3.3. Mineralized collagen-PLA composites generated using open fiber designs show conformal fitting and enhanced push-out resistance in a model cylindrical defect

We subsequently compared the push-out behavior of two open angled mineralized collagen-PLA composites (0.7 mm and 1.0 mm fiber diameter; both originally 11.9 mm in diameter) in 10 mm and 9.25 mm cylindrical channels in a Teflon plate (Fig. 4). Both open-angle composite designs could be radially compressed and press-fit into the model defects. The shape-fitting capacity was subsequently assessed as there was increased push-out resistance in smaller diameter defects, and we observed that both open-angled composite designs demonstrated a significantly ($p < 0.05$) higher maximum load at push-out in the 9.25 mm diameter defect versus the 10 mm diameter defect. Mineralized collagen-PLA composites fabricated using a closed PLA frame design could not be press-fit into the channels (not shown). No significant difference in maximum load was observed between the 0.7 mm vs. 1 mm fiber diameter designs in either the 10 mm or 9.25 mm channels.

3.4. Tracing the metabolic activity and proliferation of pASCs

We then evaluated the metabolic activity of porcine ASCs seeded collagen-PLA composites versus the PLA cage and mineralized collagen scaffold alone (Fig. 5A). There was a significant ($p < 0.05$) increase in metabolic activity for all samples with time. ASC-seeded PLA cages showed significantly ($p < 0.05$) lower metabolic activity than either the mineralized collagen scaffold or the mineralized collagen-PLA composite. The ASC metabolic activity was largely the same between

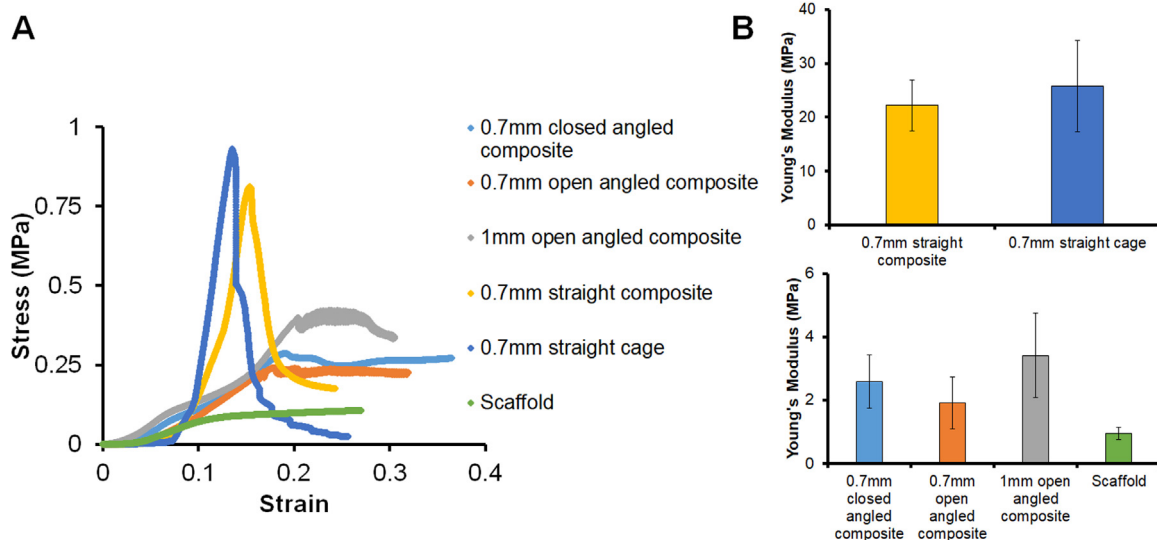


Fig. 3. Mechanical compression testing of PLA cage, scaffold, and various collagen-PLA composite designs. (A) Representative images of stress-strain curves comparing all experimental conditions, PLA cage vs. collagen-PLA composite, collagen scaffold vs. collagen-PLA composite, and between all composite designs. (B) Young's modulus and Ultimate stress for all scaffold, cage, and composite designs. * indicates significantly ($p < 0.05$) higher value than scaffold. Data expressed as mean $\pm$ standard deviation ($n = 8$).

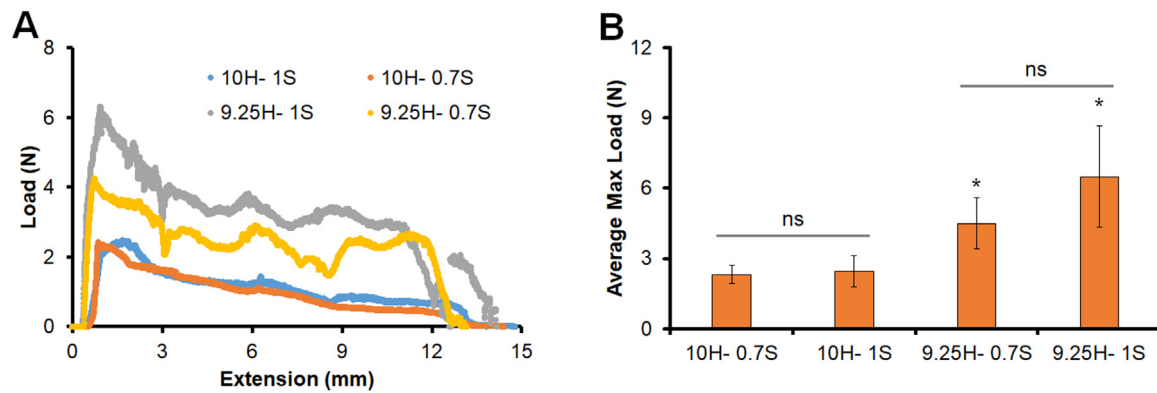


Fig. 4. Push out testing of open angled composites. (A) Representative load-extension curves for open-angle composites. Data shown for four testing conditions. Push-out test from a 10 mm dia. defect of composites fabricated from open-angle PLA cages with fiber strut diameters of 0.7 mm vs. 1.0 mm (10H-1S, 10H-0.7S). Push-out test from a 9.25 mm dia. defect of composites fabricated from open-angle PLA cages with fiber strut diameters of 0.7 mm vs. 1.0 mm (9.25H-1S, 9.25H-0.7S). (B) Average maximum load reached during push out test for all 4 test conditions. * indicates significantly ($p < 0.05$) higher average maximum load reached than 10 mm hole. ns indicates no significance between samples. Data expressed as mean $\pm$ standard deviation ($n = 8$).

the scaffold alone and the mineralized collagen-PLA composite, and both scaffold and mineralized collagen-PLA composite showed greater than 3.5-fold expansion in metabolic activity over the course of the experiment

We also examined ASC proliferation by quantifying cell numbers in all groups (Fig. 5B). While the total number of cells increased significantly with time (days 7, 14, 28 vs. day 1) for all conditions (PLA cage alone, collagen scaffold, collagen-PLA composite) the effect was largest in the scaffold and collagen-PLA composite groups. Not surprisingly, the PLA cage alone consistently showed significantly ($p < 0.05$) reduced cell number compared to both the collagen scaffold or mineralized collagen-PLA composite.

3.5. Evaluating signal transduction and pro-osteogenic signaling

We subsequently examined expression signatures for SMAD1/5/9, AKT, ERK1/2, p38, and SMAD2/3 in the scaffold vs. mineralized collagen-PLA composite to confirm the addition of the PLA cage did not alter the pro-osteogenic signatures previously demonstrated for the mineralized collagen scaffold (Supp. Fig. 1). We focused analysis on the collagen scaffold alone versus the collagen-PLA composite as the PLA cage alone group showed significantly reduced cell expansion (and faint β -actin bands, not shown). Faint β -actin bands observed at early timepoints (day 1, 4) are likely due to low cell numbers at early timepoints. Importantly, we observed no appreciable change in the overall

signature of SMAD1/5/9, ERK1/2, and SMAD2/3 activity as a result of the inclusion of the PLA-reinforcing cage into the mineralized collagen scaffold (collagen scaffold vs. collagen-PLA composite groups). While largely similar, we observed faster upregulation of AKT activity in the mineralized collagen-PLA composite (significant increase by day 4). Interestingly, while P38 activity decreased significantly ($p < 0.05$) in the collagen scaffold over the course of the 14 day experiment, it increased significantly ($p < 0.05$) in the collagen-PLA composite. Moreover, while the kinetics of the response were different, both the collagen scaffold and the collagen-PLA composite showed reduction in SMAD2/3 activity over the 14 day experiment.

3.6. Gene expression analysis of cage, scaffold, and collagen-PLA composite

We subsequently examined the expression profiles for a suite of genes (*RUNX2*, *BMP2*, *BGLAP*, *Osterix*, *COL1A2*, *MMP9*) associated with osteogenic gene expression and matrix remodeling previously benchmarked in our mineralized collagen scaffold (Ren et al., 2016a, 2016b, 2016c; Weisgerber et al., 2016a). Inclusion of the PLA reinforcing frame did not appreciably alter osteogenic signature of the mineralized scaffold (Fig. 6). There was no significant ($p < 0.05$) differences in *RUNX2* expression levels between scaffold and collagen-PLA composite groups across all timepoints, with *RUNX2* upregulated at days 1, 4, and 7 in both groups. *BMP2* expression was downregulated at early timepoints (day 1 and 4) but upregulated by day 14, however, with only the

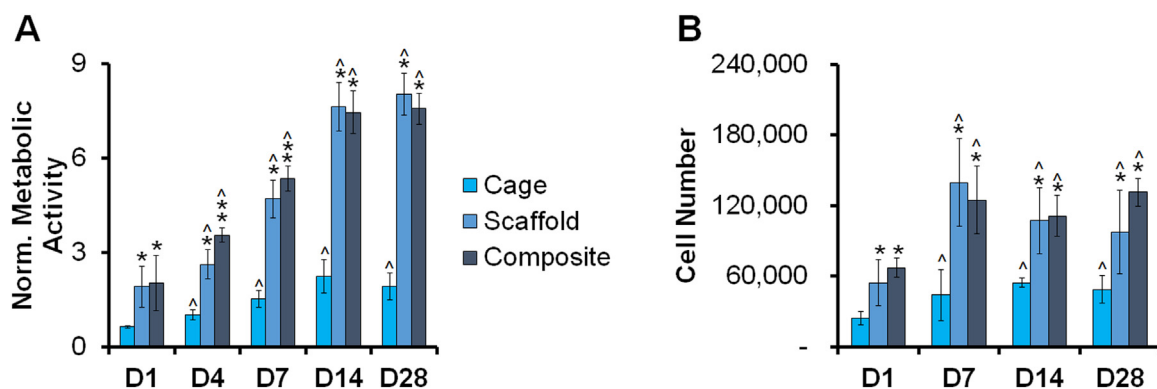


Fig. 5. Viability of pASCs within PLA cages, mineralized collagen scaffolds, and mineralized collagen-PLA composites. (A) Metabolic activity of pASC seeded constructs (AlamarBlue®) over the 28 day experiment. Data normalized against original seeding density and expressed as mean $\pm$ standard deviation ($n = 6$). (B) Total number of pASC cells per construct (Hoechst assay). Data expressed as mean $\pm$ standard deviation ($n = 3$). * indicates significant ($p < 0.05$) higher metabolic activity of one group to another on the same day. ** indicates significantly ($p < 0.05$) higher metabolic activity of one group to another on the same day. ^ indicates significantly ($p < 0.05$) higher metabolic activity of one group compared to same group on day 1.

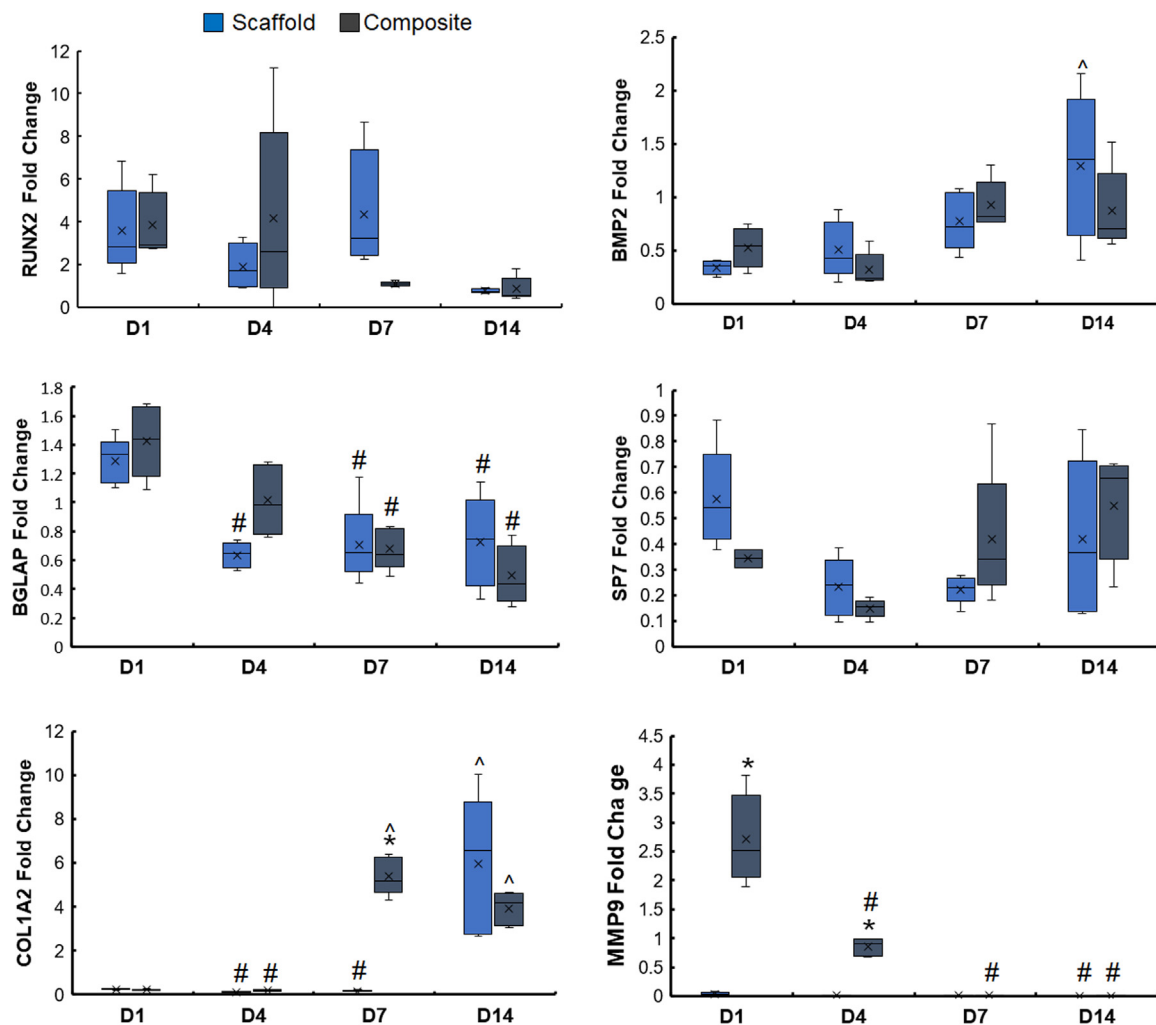


Fig. 6. Comparing osteogenic differentiation profiles after adding PLA reinforcing frame to the mineralized collagen scaffold. Gene expression profiles of RUNX2, BMP2, BGLAP, Osterix, COL1A2, and MMP9 for pASCs within the mineralized collagen scaffold vs. collagen-PLA composite. * indicates significant ($p < 0.05$) higher gene expression between the two groups on the same day. ^ indicates significantly ($p < 0.05$) higher gene expression of one group compared to the same group on day 1. # indicates significantly ($p < 0.05$) lower gene expression of one group compared to the same group on day 1. Data expressed as mean $\pm$ standard deviation ($n = 5$).

scaffold showing significantly increased ($p < 0.05$) expression over the experiment. Osteocalcin (*BGLAP*), upregulated in both the scaffold and collagen-PLA composite at day 1 showed decreased expression over later timepoints. There was no significant ($p < 0.05$) difference among days and groups fold change of Osterix (*SP7*), which was down-regulated at all timepoints. Interestingly, while *COL1A2* was down-regulated at early timepoints we observed a marked, significant rise in expression at later timepoints, with the collagen-PLA composite showing the fastest increase in expression. While expression of *MMP9* was largely absent in the mineralized collagen scaffold, the collagen-PLA composite showed significantly ($p < 0.05$) greater fold change of *MMP9* at early timepoints (days 1 and 4).

3.7. Micro-CT analysis of cage, scaffold, and mineralized collagen-PLA composite

We subsequently performed micro-CT analysis on ASC-seeded constructs at 28 days of culture to quantify the extent of new mineral formation on the surface of the PLA cages as well as within the mineralized collagen scaffolds and mineralized collagen-PLA composites (Fig. 7). Not surprising given the stiff PLA, there was some degree of mineralization on the surface of the PLA cage as observed qualitatively with intense white CT images. Overall, the mineralized collagen-PLA

composite showed increased mineral formation compared to the mineralized collagen scaffold alone. Looking at the distribution of mineral through the sample depth, the composite showed significantly ($p < 0.05$) higher mineral content towards the top of the specimens. Examining radial patterns of mineral formation, the mineralized collagen-PLA composite showed significantly ($p < 0.05$) higher micro-CT intensity that the scaffold alone. We also examined mineral formation as function of angular position around the specimens, finding all specimens showed largely angularly symmetrical mineral formation.

3.8. Histological evaluation of scaffold and collagen-PLA composite

We then performed histological evaluation of matrix remodeling and mineral formation via Hematoxylin and Eosin, Alizarin Red staining, and Von Kossa staining (Fig. 8). H & E stains showed good cellular distribution within the scaffold and mineralized collagen-PLA composites. Both scaffolds and composites showed strong Alizarin Red staining at day 28 of culture, with no visible differences between scaffold and composite, and a stronger red than the day 0 control. Von Kossa staining indicated nuclear red stained cells mostly at the edge of the scaffold and mineralized collagen-composite but some cells are visible within the center of the constructs. Notably, the unseeded control scaffolds and composites show reduced remodeling and mineral

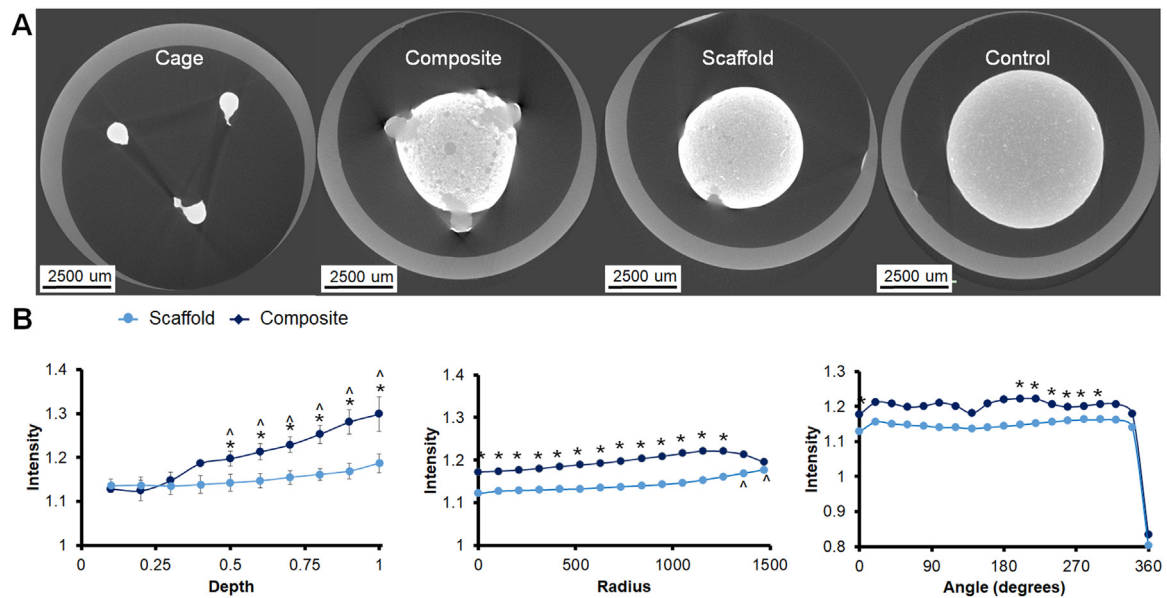


Fig. 7. Micro-CT analysis of mineral formation. (A) Representative CT images of cage, composite, scaffold at day 28, and day 0 unseeded control. Regions of white indicate a greater mineral intensity. (B) Analysis of mineral intensity within collagen-PLA composite vs. scaffold alone as a function of depth, radius, and angle. Significance is indicated above markers for the composite and below markers for the scaffold. * Composite has a significantly ($p < 0.05$) higher intensity than the scaffold. ^ indicates significantly ($p < 0.05$) higher intensity of one group compared to the same group at the first data point. Data expressed as mean $\pm$ standard deviation ($n = 3$).

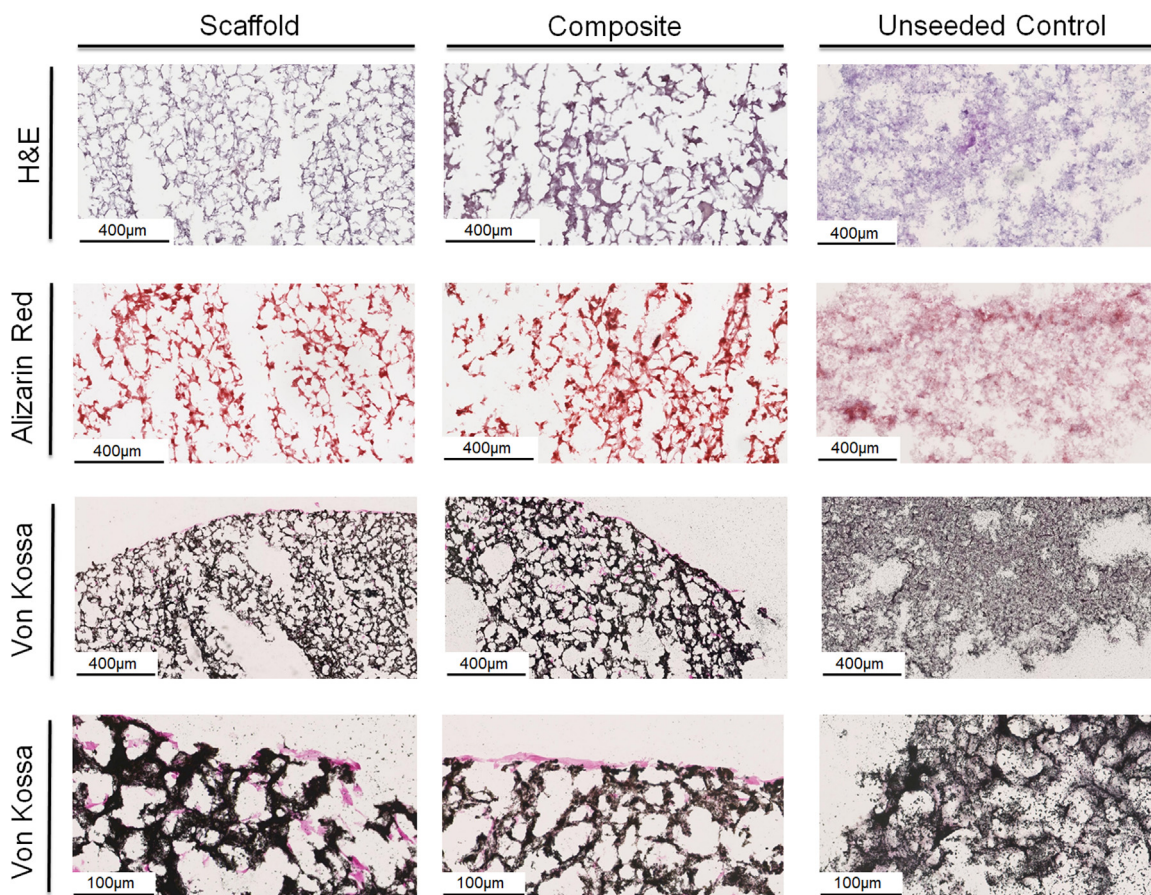


Fig. 8. Histological evaluation of mineralized collagen scaffold, collagen-PLA composite, and Day 0 unseeded control. Hematoxylin & Eosin stain shows robust cell penetration within the scaffold and composite. Alizarin Red demonstrates increased calcium deposition in the composite and scaffold compared to the control. Von Kossa staining indicates calcium deposits and cell nuclei are visible throughout the composite and scaffold. All stains suggest significant matrix remodeling in both the mineralized collagen-PLA composite and mineralized collagen scaffold compared to unseeded controls.

formation compared to both the cell-seeded scaffold and mineralized collagen-composite.

4. Discussion

In this study, we describe the development, mechanical testing, and profiling of the in vitro bioactivity of a shape-fitting mineralized collagen-PLA composite for applications in CMF defect regeneration. Here, a significant challenge to improving the quality and speed of cranio-maxillofacial bone regeneration are competing design requirements for a biomaterial platform: *porosity* required for cell recruitment and adequate biotransport; *mechanical strength* that is significantly reduced by the inclusion of pores; shape-fitting to improve conformal contact and osseointegration between the implant and the defect. The latter is particularly important in the context of CMF bone defects, where developmental, post-oncologic, and high-energy impacts can result in a wide range of defect morphologies and irregular defect geometries. Our efforts here are built upon our recent development of a class of mineralized-collagen scaffolds that promote osteogenic differentiation of human and rabbit marrow MSCs as well as porcine adipose-derived stem cells in the absence of osteogenic supplements (BMP-2, osteogenic media) (Caliari and Harley, 2014; Weisgerber et al., 2015b). While exogenous BMP-2 can enhance the effect, the mineralized collagen scaffold *natively instructs osteogenesis* (Caliari and Harley, 2014; Lee et al., 2015; Ren et al., 2015, 2016b, 2016c; Weisgerber et al., 2015b). These mineralized scaffolds promote activation of canonical (SMAD1/5/8) (Ren et al., 2015) and SMAD-independent (ERK1/2, AKT, p38 MAPK) BMP receptor signaling pathways (Ren et al., 2016c) leading to robust mineral formation in vitro and enhanced bone regeneration in vivo (Weisgerber et al., 2018). However, a significant challenge for the scaffold is the porosity (> 85%) that support high cell bioactivity significantly reduces scaffold mechanical strength. To address significant challenges associated with clinical translation of these collagen scaffolds for musculoskeletal tissue engineering applications, we are exploring the integration of reinforcing structures created via 3D-printing into the scaffold. Recently we have added polycaprolactone cages to increase the compressive strength of mineralized collagen scaffolds (Weisgerber et al., 2016a) and sinusoidally-crimped PLA fibers to increase the tensile strength of collagen scaffolds for tendon repair (Mozdzen et al., 2016, 2017). However, a unique need for CMF repair is not only mechanical reinforcement but also improved shape-fitting capacity to improve initial osseointegration into irregular defects. Here, we address this need via a unique composite biomaterial design.

Herein we describe inclusion of a low volume fraction (~10% v/v) PLA reinforcement frame into the mineralized collagen scaffold to form a collagen-PLA composite. For this study, PLA was selected rather than polycaprolactone (PCL) used in a previous scaffold-polymer composite (Weisgerber et al., 2016a) due to its shorter degradation time (order 6 months compared order years for PCL). CMF bone defects show significant early healing on the timeframe of months (Bose et al., 2012), suggesting the need for a composite that more closely matches this degradation time (Harley and Yannas, 2007b). Notably, the collagen-PLA composite shows enhanced and customizable compressive strength via modification of PLA fiber diameter and orientation (Fig. 3). These findings are consistent with recent results from our lab integrating polymeric fibers structures fabricated via 3D printing into collagen scaffolds to increase compressive or tensile properties (Mozdzen et al., 2016, 2017; Weisgerber et al., 2016a) and is consistent with a larger attempt at forming bioactive nano-scale or micro-scale composites for tissue engineering applications (Arce et al., 2016; Sethu et al., 2017; Xing et al., 2017; Yang et al., 2015). A critical advance associated with this work is demonstrating an approach to render the composite shape-fitting. Poor osseointegration into the surrounding mandibular bone is a key contributing factor to poor healing (Lan Levensgood et al., 2010; Wise et al., 2010; Zhang et al., 2014b), suggesting that improving conformal fitting between the micro-scale pore architecture of the

collagen scaffold and the wound margins is critical. Push-out tests in model cylindrical defects of decreasing diameter demonstrated that the selective removal of circumferential fiber segments could yield a composite that was deformable radially yet retained sufficient spring-back capacity to increase the required push-out force (Fig. 4). Increasing the strut diameter of the open angled design from 0.7 mm to 1 mm increased ultimate stress, suggesting that increasing the diameter of PLA struts further may increase the compressive strength of the open angled design. However, the low volume fraction of PLA in this first-generation composite (~10% v/v) suggests increasing PLA volume fraction may be a useful design consideration, a hypothesis consistent with recent findings in the literature (Gregor et al., 2017) as well as data here showing an increase in ultimate stress due to an increase (0.7–1 mm) in the polymer strut diameter. Regardless, the addition of even small volume fractions (~10% v/v) of polymeric mechanical reinforcement are sufficient to increase composite mechanical strength and address the current translational limitation of the mineralized collagen scaffold (compressive moduli < 1 MPa) (Weisgerber et al., 2016a).

In addition to examining the changes in mechanical performance of the mineralized collagen-PLA composite, we also compared the bioactivity of porcine adipose stem cells in the mineralized collagen-PLA composite versus the mineralized collagen scaffold alone to assess any consequences of incorporating the PLA frame. The composition of native bone has long motivated efforts to create mineralized collagen biomaterials (David et al., 2015; Harley et al., 2010a; Kanungo et al., 2008; Kruger et al., 2011). Indeed, recent efforts in our lab have identified a nanocrystallite mineralized collagen scaffold (Caliari and Harley, 2014; Harley et al., 2010a; Lee et al., 2015; Weisgerber et al., 2015b, 2013) that natively instructs osteogenic differentiation of human and rabbit marrow MSCs as well as porcine adipose derived stem cells in the absence of osteogenic supplements (Caliari and Harley, 2014; Weisgerber et al., 2015b) via activation of canonical BMP receptor signaling pathways (Ren et al., 2016c; Zhou et al., 2017). We also demonstrated this mineralized collagen scaffold variant promotes rapid bone regeneration in rat and rabbit calvarial defects as well as porcine mandible defects, finding the scaffold alone (without exogenous MSCs or BMP2) promotes rapid bone in-fill (Lee et al., 2015; Ren et al., 2016b; Weisgerber et al., 2018). In this manuscript, we established that PLA frame could be incorporated into the collagen scaffold during lyophilization (Fig. 2), and that the addition of PLA to form the composite did not adversely affect the ability of the mineralized collagen scaffold to support adipose derived stem cell viability and proliferation (Fig. 5). Notably, while metabolic activity and cell number increased steadily in the scaffold and composite, the relatively low surface area of the PLA cage alone did not support comparable cell viability, suggesting the PLA frame alone is not sufficient to maintain cell growth and proliferation.

Gene and protein expression patterns within the scaffold and collagen-PLA composite suggested that both promoted osteogenic differentiation and functional mineral formation in vitro. Signal transduction pathways suggest the collagen-PLA composite provides a supportive environment for ASC osteogenic differentiation. Notably, SMAD1/5/9, essential in early activation of BMP receptor pathways in mineralized collagen scaffolds (Weisgerber et al., 2016a), activity was near 100% expression throughout the experiment. Further, we observed increased activity at later timepoints for AKT and p38, both of which are involved in osteoblast differentiation and bone formation (Matsushita et al., 2009; Thouverey and Caverzasio, 2015). SMAD2/3, which can work with or against BMP signaling depending on the differentiation stage (Song et al., 2009), was active throughout the experiment in both the scaffold and collagen-PLA composite. Analysis of gene expression also suggested the inclusion of the PLA reinforcing frame did not significantly alter ASC activity. *RUNX2*, a major transcription factor regulating osteogenic differentiation from mesenchymal stem cells, was upregulated throughout days 1 through 7, indicating osteogenic lineage commitment of pASC (Dalle Carbonare et al., 2012). BMP is an essential

element of osteogenic commitment in vivo and in vitro (James, 2013), with our results confirming *BMP2* was upregulated at later timepoints, suggesting osteogenesis and bone formation was continuing. Further, *BGLAP* (osteocalcin) was upregulated at day 1 and downregulated at later timepoints while *Osterix*, a bone specific transcription factor that regulates late stages of bone formation (Hayrapetyan et al., 2015), was downregulated across all timepoints in the scaffold and composite. *COL1A2*, a marker for the type I collagen alpha 2 chain, an important structural element of bone, was upregulated at day 7 and 14 collagen-PLA composites while *MMP9* was upregulated at early timepoints in the collagen-PLA composite, suggesting early stages of matrix remodeling (Hayrapetyan et al., 2015; James, 2013). Critically, there were minimal differences in gene expression and protein activity between the scaffold and collagen-PLA composite across all days, strongly suggesting the addition of PLA reinforcement frame to the mineralized collagen scaffold did not negatively impact differentiation of osteoblasts and mineralization of the construct.

While analysis of bone regeneration requires in vivo assessment, we employed a series of histology and three-dimensional imaging approaches to quantify cell-mediated matrix remodeling and new mineral synthesis, finding inclusion of the PLA reinforcing frame in the PLA-collagen composite does not negatively influence in vitro metrics of ASC activity. Notably, Alizarin Red and Von Kossa analyses of mineralized collagen scaffold and composites after 28 days in culture demonstrated increased definition of mineral deposits compared to unseeded controls. Subsequent analysis of mineralization patterns via Micro-CT suggested that the presence of the PLA reinforcing frame not only did not reduce mineral formation, but may have an effect of augmenting new mineral formation (Fig. 7). Here, we applied an algorithm previously developed to quantify new bone infill into cylindrical scaffold specimens in all 3-axes of a cylindrical coordinate system (depth, radial, angular) (Weisgerber et al., 2018), finding the general trend of increased mineral content in the collagen-PLA composites. Ongoing efforts are employing cell-tracing algorithms to examine the kinetics of stem cell penetration into the composite as well as to explore additional fiber morphologies to facilitate conformal fitting of more complex craniomaxillofacial defect geometries. And while beyond the scope of this work which had the goal to validate the use of three-dimensional printing to generate PLA-reinforced collagen scaffolds that have enhanced compressive strength and shape-fitting capacity, future work will test the osseointegration and regenerative capacity of these composites using a porcine mandibular defect model that we have recently described for testing non-shape fitting implant designs (Weisgerber et al., 2018). Ongoing efforts provide the opportunity to more rigorously define increased push-out strength in cadaveric bone defects rather than defects in Teflon plates, which allowed this study to demonstrate shape-fitting, but not necessarily predict push out force required in vivo. Further, while this study evaluated cellular response using short term RT-PCR and Western Blot assays (days 1–14), ongoing efforts in line with previous work performed for the mineralized scaffold alone (Ren et al., 2015, 2016a, 2016c, 2018; Zhou et al., 2017) are tracing cell response over longer times 2 – 8 weeks) to more accurately define osteogenic activity. While micro-CT is particularly useful with comparing osteogenic activity in vivo by comparing response within the scaffold to the surrounding bone (Weisgerber et al., 2018), the lack of surrounding reference bone and the short timeframe of these in vitro experiments did not provide sufficient variations to compare.

5. Conclusions

We describe the design, fabrication, and testing of a conformal fitting mineralized collagen-PLA composite for CMF defect repair applications. The composite demonstrated similar mineralization, cell viability, and osteogenesis to the mineralized collagen alone, which had previously demonstrated excellent biocompatibility and osteogenesis. Further, we demonstrate a library of PLA reinforcement designs to:

increase compressive strength of the collagen scaffold, provide radial compression and spring-back to facilitate shape-fitting within a cylindrical defect; or both. The ability to increase close contact between the biomaterial implant and the host bone is particularly important for improving cell recruitment and subsequent osseointegration between host and implant.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jmbbm.2019.03.017.

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CELL BIOLOGY

Osteoprotegerin reduces osteoclast resorption activity without affecting osteogenesis on nanoparticulate mineralized collagen scaffolds

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The instructive capabilities of extracellular matrix–inspired materials for osteoprogenitor differentiation have sparked interest in understanding modulation of other cell types within the bone regenerative microenvironment. We previously demonstrated that nanoparticulate mineralized collagen glycosaminoglycan (MC-GAG) scaffolds efficiently induced osteoprogenitor differentiation and bone healing. In this work, we combined adenovirus-mediated delivery of osteoprotegerin (AdOPG), an endogenous anti-osteoclastogenic decoy receptor, in primary human mesenchymal stem cells (hMSCs) with MC-GAG to understand the role of osteoclast inactivation in augmentation of bone regeneration. Simultaneous differentiation of osteoprogenitors on MC-GAG and osteoclast progenitors resulted in bidirectional positive regulation. AdOPG expression did not affect osteogenic differentiation alone. In the presence of both cell types, AdOPG-transduced hMSCs on MC-GAG diminished osteoclast-mediated resorption in direct contact; however, osteoclast-mediated augmentation of osteogenic differentiation was unaffected. Thus, the combination of OPG with MC-GAG may represent a method for uncoupling osteogenic and osteoclastogenic differentiation to augment bone regeneration.

INTRODUCTION

Materials inspired by bone-specific extracellular matrix (ECM) components have generated great enthusiasm in regenerative technologies because of their abilities to instruct osteoprogenitor differentiation. In addition, recent attention to the effects on osteoclast activation has suggested the potential for modulation of resorption within the host microenvironment via alterations of the receptor activator of nuclear factor κ B (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG) axis (1).

The RANK/RANKL/OPG axis serves an important role in osteoclast regulation and bone homeostasis (1, 2). The activation of RANK, a tumor necrosis factor superfamily receptor originally identified in T lymphocytes and osteoblasts, via its cognate ligand RANKL is required for osteoclast differentiation and activation (3, 4). Murine genetic models have shown that both RANK and RANKL deficiencies resulted in osteopetrosis because of a complete absence of osteoclasts (5, 6). In the craniofacial skeleton, RNA interference using small interfering RNAs (siRNAs) specific for RANK has been shown to fuse patent cranial sutures in ex vivo cultures (2). OPG, the soluble decoy receptor for RANKL, is the major endogenous negative regulator of the pathway. In contrast to the RANK- and RANKL-deficient mice, OPG knockouts exhibit profound osteoporosis (6, 7). Because of the direct relationship between the RANK/RANKL/OPG

axis and osteoclast activation, targeted therapies against the axis are under investigation for fracture healing and other conditions requiring a net osteogenic state (8). Similar to other immune cells such as lymphocytes, in vivo activation of osteoclasts proceeds with two signals. While the RANK/RANKL/OPG axis is undeniably the major signal, co-stimulation via receptors such as osteoclast-associated receptor (OSCAR) contributes to homeostasis and may be deranged in pathological circumstances (9, 10).

The importance of osteoclast homeostasis in normal bone physiology suggests that bone regeneration is likely to be affected by the regulatory mechanisms of osteoclast activity. Components of ECM-based materials have been reported to both affect osteogenic differentiation and negatively or positively regulate osteoclastogenesis (11). As the most abundant protein within bone ECM, most ECM-inspired materials for bone regeneration are based on collagen I (11). However, the ligands for OSCAR, a costimulatory molecule for osteoclast maturation, are collagens I, II, and III (9). Thus, collagen-based materials intrinsically provide costimulation for osteoclast activation, potentially lowering the threshold for resorption. Collagen-based osteoclast costimulation is likely able to be offset with the negative osteoclast regulatory effects of certain glycosaminoglycan (GAG) species, as well as the inorganic components of bone ECM. Salbach-Hirsch and colleagues (12) extensively evaluated sulfated GAG (sGAG) coatings and found an increased osteogenic differentiation of primary murine mesenchymal stem cells and simultaneously diminished osteoclast activity, in part through sequestration of OPG. The mechanism for which GAGs or sGAGs influence osteoclastogenesis remains to be elucidated, although their work suggested that sulfation status may differentially affect osteoclast activity. Inorganic ions, specifically calcium and phosphate, have been the most extensively described ECM components that contribute to osteoclast action. High levels of extracellular calcium ions, such as during bone remodeling, negatively regulate podosomal

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assembly and resorption via entry through the L-type voltage-gated calcium channels (13). Similarly, high levels of extracellular inorganic phosphate inhibit RANKL-induced osteoclast activity in a manner dependent on the sodium phosphate cotransporter (14). Recently, the signaling mechanisms induced by inorganic ions in the context of materials have been characterized in greater detail, and further modulation may be of great utility in regenerative technologies (15, 16). Using a combination biphasic silicified and calcified collagen material, Jiao *et al.* (17) demonstrated the up-regulation of OPG gene expression during osteogenic differentiation that was not present to the same degree in monophasic silicified collagen scaffolds or calcified collagen scaffolds. Signaling pathways responsible for osteogenesis and osteoclast inhibition of the biphasic silicified and calcified collagen material were regulated by different intracellular mediators.

Our group previously demonstrated that a nanoparticulate mineralized collagen glycosaminoglycan (MC-GAG) scaffold induced efficient mineralization of bone marrow-derived primary human mesenchymal stem cells (hMSCs) and primary rabbit bone marrow stromal cells via autogenous activation of the bone morphogenetic protein receptor (BMPR) signaling pathway through phosphorylation of small mothers against decapentaplegic-1/5 (p-Smad1/5) (18–22). Furthermore, we demonstrated that MC-GAG scaffold induced *in vivo* rabbit calvarial regeneration without the addition of exogenous growth factors or progenitor cells (21). We recently reported that MC-GAG scaffold demonstrated both direct and indirect inhibitory effects on osteoclast viability, proliferation, and activation (23). In comparison to its nonmineralized collagen glycosaminoglycan (Col-GAG) scaffold counterpart, MC-GAG scaffolds directly reduced viability and proliferation of primary osteoclast precursors. MC-GAG scaffolds also induced hMSCs to express higher levels of OPG during osteogenic differentiation via intracellular signaling pathways distinct from those governing osteogenic differentiation. Combining these findings suggests two concepts: (i) ECM-based materials for skeletal regeneration must account for both osteogenesis and osteoclastogenesis and (ii) differences in regulatory mechanisms evoked by nonmineralized versus mineralized collagen scaffolds may provide a route to disconnect physiologic osteoblast and osteoclast coupling. In this work, we evaluate the addition of an adenovirus-mediated OPG (AdOPG) expression in primary hMSCs with MC-GAG scaffolds to promote osteogenesis and augment osteoclast inhibition.

RESULTS

AdOPG transduction of primary hMSCs differentiated on Col-GAG and MC-GAG does not affect cell viability or proliferation

Recently, we reported that MC-GAG induces hMSCs to up-regulate OPG at higher levels than Col-GAG, suggesting a potential role for MC-GAG in simultaneously regulating the host resorptive capacity in the local microenvironment during bone regeneration. However, the amount of OPG protein expressed by hMSCs differentiated on MC-GAG was only significantly higher within the first 10 days of differentiation. Thus, we hypothesized that a prolongation of OPG expression may augment the antiresorptive capabilities of MC-GAG.

In an effort to augment anti-osteoclastogenic activities induced by MC-GAG, primary bone marrow-derived hMSCs (CD105⁺CD166⁺CD29⁺CD44⁺CD14[−]CD34[−]CD45[−]) were transduced with AdOPG. Control and AdOPG-transduced hMSCs were cultured in osteogenic

differentiation medium for 7 and 14 days and evaluated for infection efficiency, OPG expression, and effects on cell viability and proliferation (Fig. 1). Using the coexpressed red fluorescent protein (RFP) as an indicator, AdOPG transduction resulted in a 35% infection efficiency based on cell counting with a maximum amount of protein expression on Western blot analysis at a multiplicity of infection (MOI) of 200 (Fig. 1B).

Before evaluating long-term scaffold mineralization, the viability and proliferation of hMSCs were confirmed by measuring the mitochondrial dehydrogenase activity using the WST-1 (water-soluble tetrazolium salt 1) colorimetric assay. hMSC viability and proliferation were not found to be significantly different in control versus AdOPG-infected cells in two-dimensional (2D) cultures after 3 weeks of transduction (Fig. 1C). To confirm that cells were viable in long-term 3D cultures, we seeded control and AdOPG on Col-GAG and MC-GAG and differentiated them for 8 weeks in osteogenic differentiation medium (Fig. 1D). Again, hMSC cell viability and proliferation were found to be equivalent between control and AdOPG hMSCs on Col-GAG or MC-GAG with no statistically significant differences between the materials.

AdOPG transduction changes RANKL/OPG homeostasis in primary hMSCs differentiated on Col-GAG and MC-GAG

Because of the known importance of the OPG to RANKL-relative protein ratios in influencing bone homeostasis, we next evaluated the relative expression of OPG to RANKL in control and AdOPG-infected cells. Control and AdOPG-infected cells were induced to undergo osteogenic differentiation on Col-GAG and MC-GAG for 14 days, and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed to assess OPG and RANKL gene expression (Fig. 2, A and B). No statistically significant differences were found in OPG or RANKL gene expression between control cells on either material. In the presence of AdOPG, OPG gene expression increased more than 30-fold in cells cultured on either scaffold, while no differences in RANKL expression was noted.

Protein expression was next evaluated using both Western blot and enzyme-linked immunosorbent assay (ELISA) of OPG and RANKL expression over 56 days (Fig. 2, C to F). On Western blot analysis, we detected two dominant isoforms of RANKL including a band at 35 kDa, as well as a higher-molecular weight band near 45 kDa, which may reflect expression of different splice variants, similar to reports by several other investigators (24, 25). Densitometry was used to quantify the relative expression of OPG and RANKL, and the RANKL/OPG protein expression ratio over 56 days was evaluated (Fig. 2E). As Western blots were performed on the total protein of the scaffolds, the RANKL/OPG ratio derived represented the total cellular and scaffold-bound protein. A statistically significant difference was found in the RANKL/OPG protein expression ratios between the groups [$F(3,24) = 19.35$, $P < 0.001$]. In both Col-GAG and MC-GAG, AdOPG expression lowered the RANKL/OPG protein expression ratio as expected ($P < 0.001$). In control hMSCs, Col-GAG displayed a higher RANKL/OPG ratio compared to MC-GAG ($P < 0.001$).

In addition to scaffold-bound protein, we evaluated RANKL and OPG in culture supernatants using ELISAs to calculate the secreted RANKL/OPG ratio (Fig. 2F). Similar to total protein RANKL/OPG ratios, a statistically significant difference was found [$F(3,20) = 60.56$, $P < 0.001$]. Unlike the total protein of the scaffolds, the secreted RANKL/OPG ratio was higher in MC-GAG compared to Col-GAG

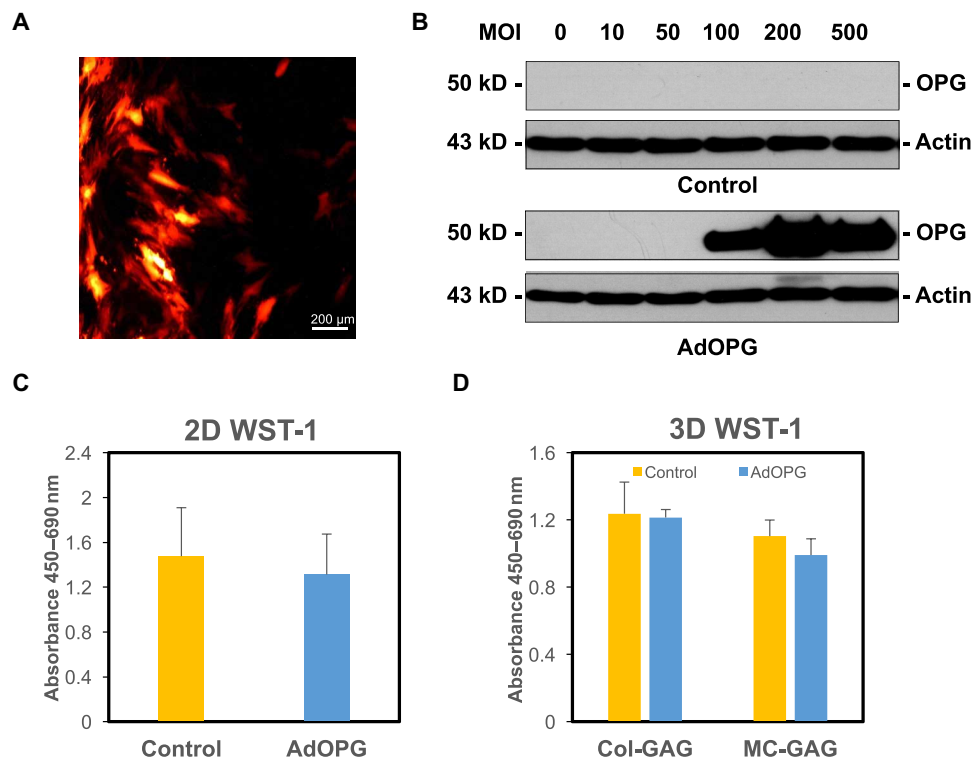


Fig. 1. AdOPG transduction of primary hMSCs differentiated on Col-GAG and MC-GAG does not affect cell viability or proliferation. (A) Photomicrograph of AdOPG-transduced primary hMSCs in 2D cultures after 7 days. (B) Western blot of primary hMSCs transduced with control or AdOPG viruses for 7 days on 2D cultures at varying MOI. (C and D) WST-1 proliferation and viability assays of primary hMSCs transduced with control or AdOPG viruses at (C) 3 weeks in 2D cultures and (D) 8 weeks on Col-GAG or MC-GAG. Mean values are shown in bars, with error bars representing SD.

($P < 0.001$) in control cells. Expression of AdOPG diminished the RANKL/OPG ratio compared to control cells on MC-GAG ($P < 0.001$), whereas less of an effect was observed for Col-GAG. Together, AdOPG significantly reduces the RANKL/OPG ratio at both the levels of gene and protein expression. For the alterations in RANK/OPG protein ratios, the difference is primarily manifested in cellular or scaffold-bound protein rather than secreted protein.

AdOPG does not affect hMSC mineralization on Col-GAG or MC-GAG

Two reports have suggested that OPG may have capabilities to induce osteogenic differentiation and mineralization in osteoprogenitor cells separate from its effects on osteoclasts and osteoclast precursors (26, 27). To evaluate whether AdOPG directly affects mineralization in our system, we evaluated control and AdOPG-infected hMSCs undergoing osteogenic differentiation on Col-GAG and MC-GAG for expression of osteogenic markers, activation of osteogenic signaling pathways, and matrix mineralization (Fig. 3). At 14 days of culture, no significant differences in runt-related transcription factor 2 (RUNX2) or osteopontin (OPN) gene expression were found between control and AdOPG cells on Col-GAG or MC-GAG (Fig. 3, A and B).

We previously showed that both Col-GAG and MC-GAG depended on the activation of the BMPR signaling pathway via p-Smad1/5 for osteogenic differentiation and matrix mineralization (22). Thus, to evaluate the contribution of AdOPG to the activation of intracellular signaling pathways that contribute to mineralization on Col-GAG and MC-GAG, we performed the Western blots analyses

of control and AdOPG-infected hMSCs undergoing osteogenic differentiation on Col-GAG or MC-GAG on total protein lysates over 8 weeks (Fig. 3, C and D). No significant differences in p-Smad1/5 activation were detected in the absence or presence of AdOPG. MC-GAG, as we previously demonstrated, induced substantially more p-Smad1/5 compared to Col-GAG.

We also quantified matrix mineralization using microcomputed tomography (μ CT) analysis (Fig. 3, E and F). Again, no significant differences between control and AdOPG hMSCs were detected on either Col-GAG or MC-GAG. Similar to our previous reports, MC-GAG demonstrated more mineralization than Col-GAG with or without AdOPG.

Indirect osteoclast cocultures augment mineralization in hMSCs undergoing mineralization on MC-GAG in the absence or presence of AdOPG

To understand the effects of MC-GAG on human osteoclasts (hOCs), we used two coculture techniques: indirect and direct. Indirect cocultures were performed to isolate the effects of hOCs on hMSCs and vice versa without the confounding effects of scaffold resorption from direct contact and to understand the paracrine effects between the two cell types (Fig. 4A). Direct cocultures were devised for the purposes of understanding the net effects of the system with cells and materials in direct contact with each other. For indirect cocultures, Col-GAG or MC-GAG scaffolds were cultured in an 8- μ m Transwell insert with and without hMSCs seeded on the materials (upper chamber). In the lower chamber, human primary pre-osteoclasts were seeded on a calcium phosphate-coated plate

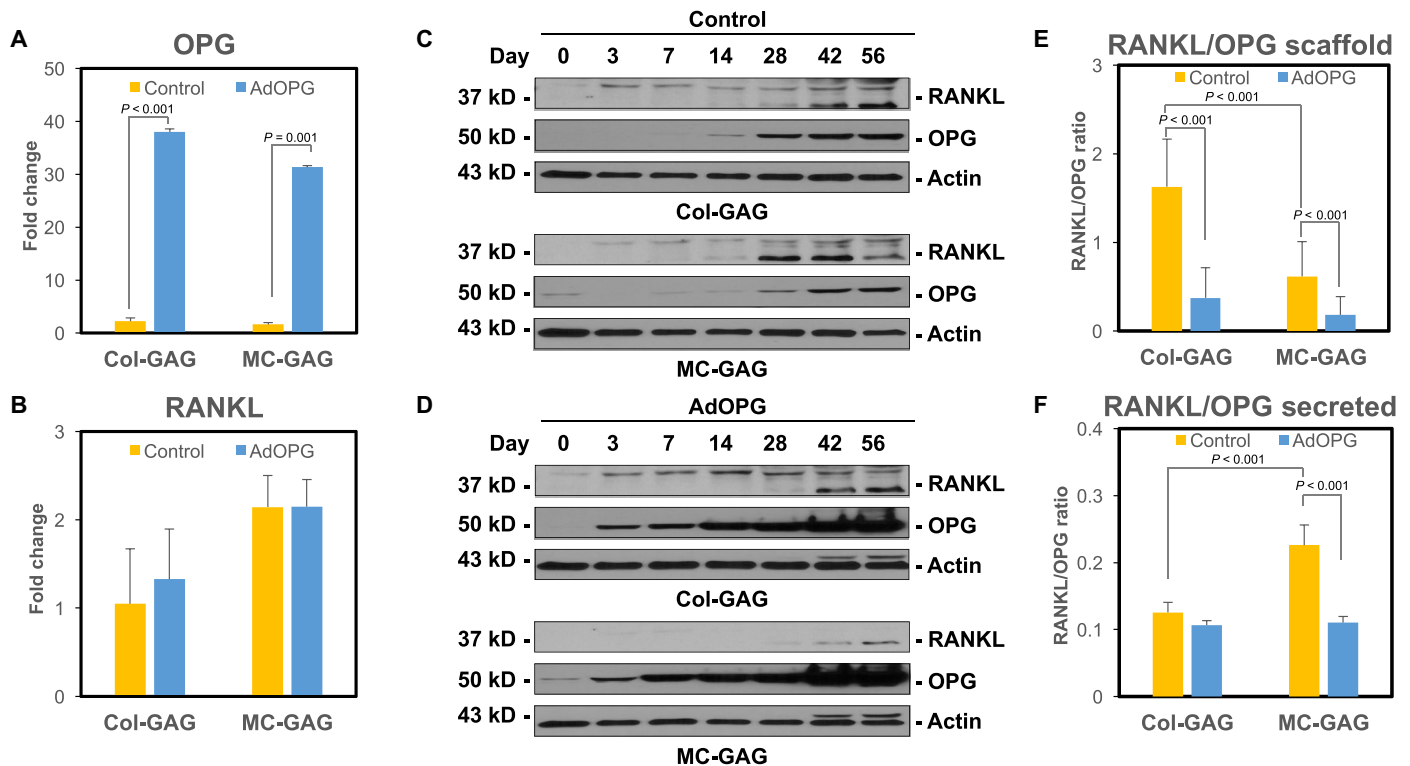


Fig. 2. AdOPG transduction changes RANKL/OPG homeostasis in primary hMSCs differentiated on Col-GAG and MC-GAG. qRT-PCR of control or AdOPG-transduced primary hMSCs cultured on Col-GAG or MC-GAG materials for 14 days in osteogenic differentiation medium for (A) OPG and (B) RANKL ($n = 3$). Western blot of (C) control or (D) AdOPG-transduced primary hMSCs cultured on Col-GAG or MC-GAG materials for 56 days in osteogenic differentiation medium for RANKL, OPG, and β -actin. Average RANKL/OPG protein expression ratio based on (E) densitometric analysis of RANKL and OPG Western blot bands or (F) ELISA of culture supernatants over 8 weeks. Mean values are shown in bars, with error bars representing SD. Significant post hoc comparisons following analysis of variance (ANOVA) indicated with P values.

where resorptive activity may be evaluated. Cocultures were concurrently differentiated with medium supplemented with RANKL, macrophage colony-stimulating factor (M-CSF), β -glycerophosphate, ascorbic acid, and dexamethasone.

In the indirect coculture system, the effects on hMSCs were first evaluated. After 21 days of culture, we subjected the Transwell inserts (upper chambers) to WST-1 assay (Fig. 4B). Statistically significant differences in viability and proliferation were found between hMSCs of the different groups [$F(7,18) = 81.36$, $P < 0.001$]. Empty Col-GAG or MC-GAG scaffolds without hMSCs cocultured with osteoclasts displayed no evidence of cell viability or proliferation as expected ($P < 0.001$ compared to any other conditions). No differences were found between hMSCs cultured without osteoclasts (hMSC only) or control hMSCs cocultured with osteoclasts (control hMSC/OC) on either material. With AdOPG, hMSCs cultured on MC-GAG demonstrated a decrease in viability and proliferation compared to Col-GAG in a statistically significant fashion ($P = 0.03$).

To determine the amount of soluble OPG in the coculture system, we performed ELISAs over the entirety of the coculture period and compared them to an osteoclast-only negative control (Fig. 4C). Differences between the cultures were found to be statistically significant [$F(4,15) = 552.37$, $P < 0.001$]. Osteoclasts did not display any significant amount of OPG secretion as expected. In control cells, hMSCs on MC-GAG produced significantly more endogenous OPG compared to Col-GAG at day 7 ($P < 0.001$) and day 10 ($P = 0.02$). Multiple comparisons of any control time point versus

any AdOPG-infected time point for either material displayed significantly higher amounts of OPG in AdOPG-infected cells ($P < 0.001$ for all conditions).

We evaluated mineralization after 3 weeks of coculture using μ CT scanning (Fig. 4, D and E). Overall, differences in mineralization were found to be present [$F(7,26) = 26.48$, $P < 0.001$]. An increase in osteogenic differentiation occurred in control hMSC cocultures with differentiating hOCs (control hMSC/OC) compared to hMSC single cultures (hMSC only) on MC-GAG materials ($P < 0.001$). Although a mild increase in mineralization was evident qualitatively and quantitatively on Col-GAG in cocultures versus single cultures, this difference did not reach statistical significance. In MC-GAG, the increase in mineralization for cocultures with AdOPG compared to hMSC-only single cultures remained significant ($P = 0.02$). In combination, indirect cocultures of differentiating hMSCs with hOCs resulted in positive regulation of osteogenic differentiation manifested by mineralization, particularly on MC-GAG. This increase in mineralization is largely unaffected by AdOPG transduction.

Indirect cocultures with AdOPG-transduced hMSCs on MC-GAG diminish hOC resorptive activity

In the same indirect cocultures, we also evaluated the effects on osteoclasts (Fig. 4, F and G). Following removal of the Transwell inserts, the lower chamber consisting of hOCs was subjected to WST-1 assay and found to have statistically significant differences on analysis of variance (ANOVA) [$F(6,21) = 9.23$, $P < 0.001$]. An increase in

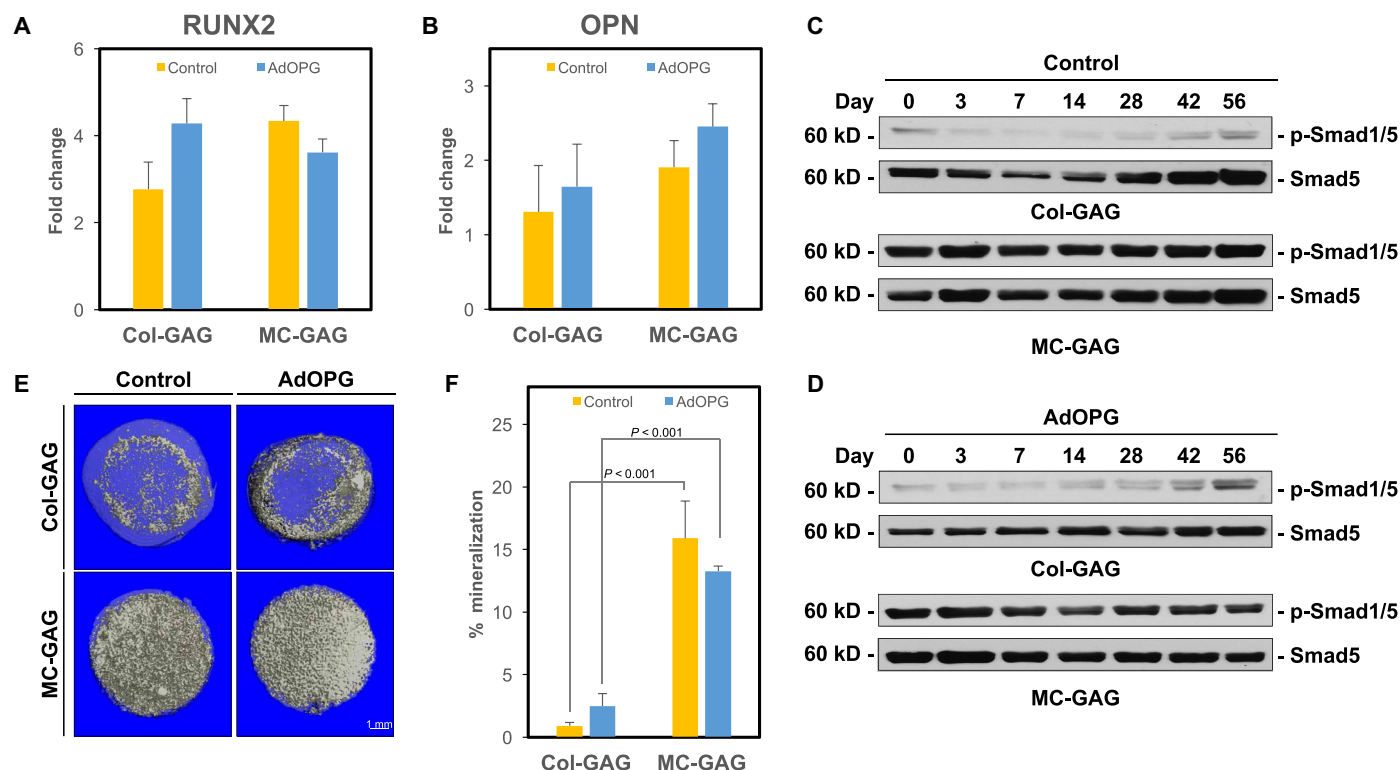


Fig. 3. AdOPG does not affect hMSC mineralization on Col-GAG or MC-GAG. qRT-PCR of control or AdOPG-transduced primary hMSCs cultured on Col-GAG or MC-GAG materials for 14 days in osteogenic differentiation medium for (A) RUNX2 and (B) OPN ($n = 3$). Western blot of (C) control or (D) AdOPG-transduced primary hMSCs cultured on Col-GAG or MC-GAG materials for 56 days in osteogenic differentiation medium for Smad5 and p-Smad1/5. (E) Representative μ CT images and (F) quantitative analysis of control or AdOPG-transduced primary hMSCs cultured on Col-GAG or MC-GAG for 8 weeks ($n = 3$). Bars represent means, and error bars represent SD. Significant post hoc comparisons following ANOVA indicated with P values.

viability and proliferation of hOCs occurred in cocultures with control hMSCs on Col-GAG or MC-GAG ($P = 0.02$ and $P < 0.001$, respectively). AdOPG-transduced hMSCs diminished viability and proliferation of hOCs compared to control hMSCs on MC-GAG ($P = 0.03$). Minimal differences in hOC viability were detected on Col-GAG with hMSCs transduced with AdOPG.

Resorptive activity of the hOCs was also characterized and found to have significant differences via ANOVA [$F(6,17) = 15.34$, $P < 0.001$; Fig. 4G]. hOC-mediated resorption significantly increased in cocultures of hMSCs on Col-GAG ($P = 0.001$) or MC-GAG ($P = 0.002$) compared to hOC single cultures. In AdOPG-transduced hMSCs, hOC-mediated resorption diminished on both materials compared to control hMSCs; however, only the decrease in MC-GAG reached statistical significance ($P < 0.001$). In combination, these data suggest that differentiating hMSCs increase the viability, proliferation, and resorptive capabilities of hOCs on either nonmineralized or mineralized Col-GAG materials. While cocultures with AdOPG-transduced hMSCs mildly reduced hOC viability, proliferation, and resorption on Col-GAG, MC-GAG demonstrated a significantly greater effect.

Direct contact of AdOPG-transduced hMSCs on Col-GAG and MC-GAG with osteoclasts diminishes the proliferation and resorption activity of osteoclasts

Next, the effects on osteoclasts were evaluated in a direct coculture system. hOCs were first plated on a calcium phosphate-coated plate. Two hours after seeding, hMSCs seeded on Col-GAG or MC-GAG were transferred to each well in direct contact with hOCs. Cocul-

tures were simultaneously differentiated with medium supplemented with RANKL, M-CSF, β -glycerophosphate, ascorbic acid, and dexamethasone. After 14 days of culture, we removed the respective scaffolds and subjected the osteoclasts to WST-1 assay (Fig. 5A). Statistically significant differences between the cultures were noted [$F(6,14) = 22.48$, $P < 0.001$]. In the presence of empty Col-GAG, the viability and proliferation were not significantly different from osteoclasts differentiated alone (OC only). As we previously reported, empty MC-GAG materials did reduce osteoclast viability and proliferation (23). Between control versus AdOPG-transduced hMSCs on either material, both viability and proliferation were diminished in the presence of AdOPG.

We assessed osteoclast differentiation and activity using tartrate-resistant acid phosphatase (TRAP) staining and resorption pit assays, respectively (Fig. 5, B and C). Under all conditions, with the exception of the negative control, TRAP activity was detected, indicating that osteoclasts were present. Osteoclast activity, detected by resorption pits of the inorganic crystalline calcium phosphate coating of the plate, demonstrated significant differences between the groups. In the presence of empty Col-GAG, a mild decrease in resorption was elicited, which was rescued with the addition of control hMSCs. In MC-GAG, a significant decrease in resorptive abilities was seen, which was also improved with the addition of control hMSCs. In both cases, osteoclast resorption was completely inhibited when transduced with AdOPG.

Quantification of the total resorption pit areas demonstrated significant differences in resorption activity between the conditions

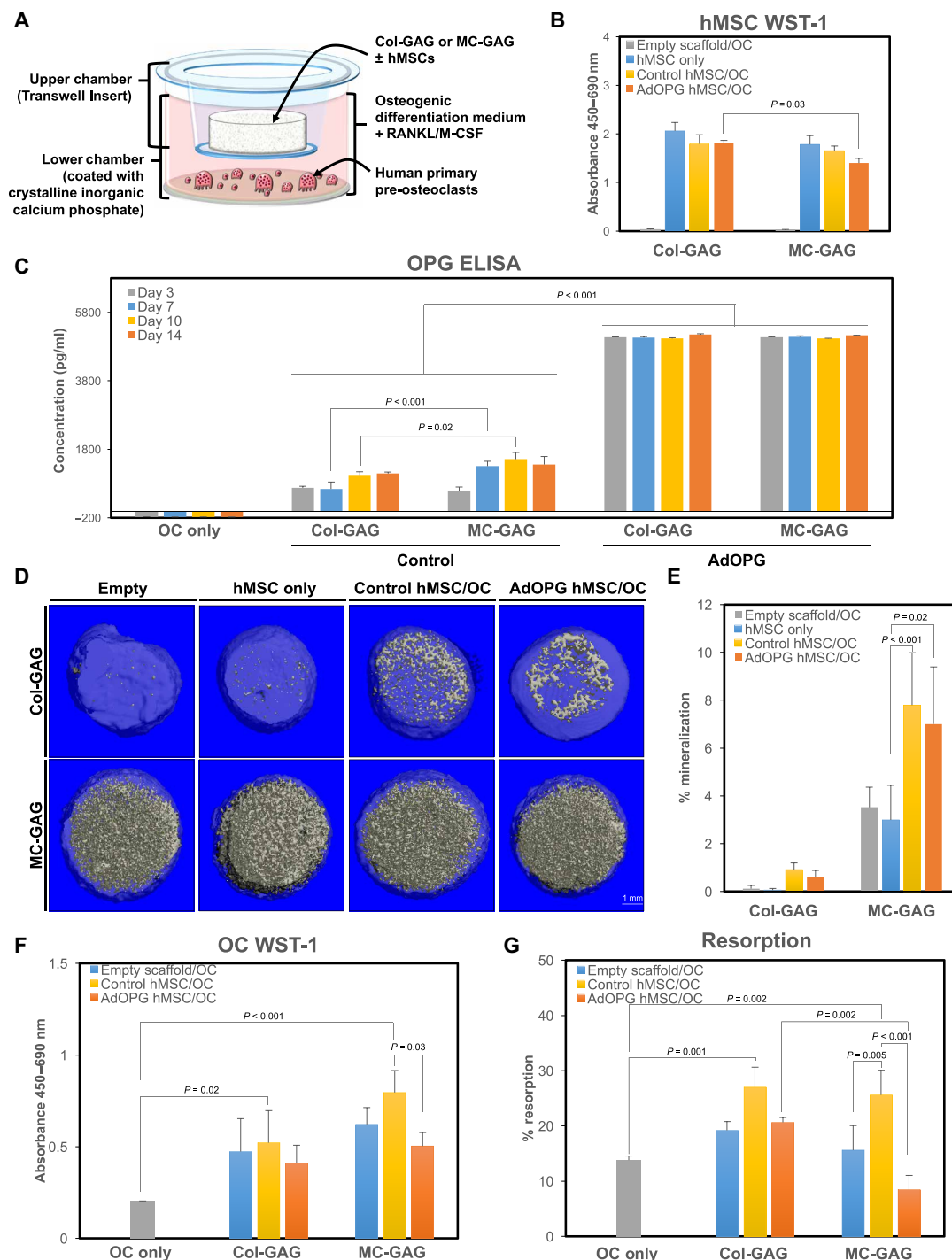


Fig. 4. Osteoclast cocultures indirectly augment mineralization in hMSCs undergoing mineralization on MC-GAG. (A) Schematic diagram of coculture design indicating the placement of differentiating hMSCs on Col-GAG or MC-GAG within Transwell insert and lower chamber consisting of primary pre-osteoclasts cultured on a plate coated with calcium phosphate to allow for the detection of resorptive pit activity. (B) WST-1 assays of hMSCs in single culture (hMSC only) or cocultured (control hMSC/OC and AdOPG hMSC/OC, respectively) on Col-GAG or MC-GAG for 21 days. Empty cell-free scaffolds cocultured with osteoclasts shown for control (empty scaffolds/OC). (C) OPG ELISA of hMSC/OC coculture medium (days 3, 7, 10, and 14) with control and AdOPG-transduced hMSCs on Col-GAG and MC-GAG. Differentiated osteoclast-only (OC only) culture shown at the left as a control. (D) Representative μ CT images and (E) quantitative analysis of empty scaffold (empty), hMSCs without osteoclasts (hMSC only), control hMSCs cocultured with osteoclasts, or AdOPG-transduced hMSCs cocultured with osteoclasts on Col-GAG or MC-GAG for 21 days. (F) WST-1 assays of primary pre-osteoclasts in single culture (OC only), cocultured with empty scaffolds, or cocultured with control or AdOPG-transduced hMSCs (control hMSC and AdOPG hMSC, respectively) in medium supplemented with RANKL and M-CSF on Col-GAG or MC-GAG for 14 days. (G) Quantitative analysis of pit assays as the percentage of total area of well in differentiated osteoclasts without hMSCs (OC only) and osteoclasts cocultured with Col-GAG and MC-GAG as empty scaffolds, scaffolds with control hMSCs, and scaffolds with AdOPG-transduced hMSCs. Significant post hoc comparisons following ANOVA indicated with P values.

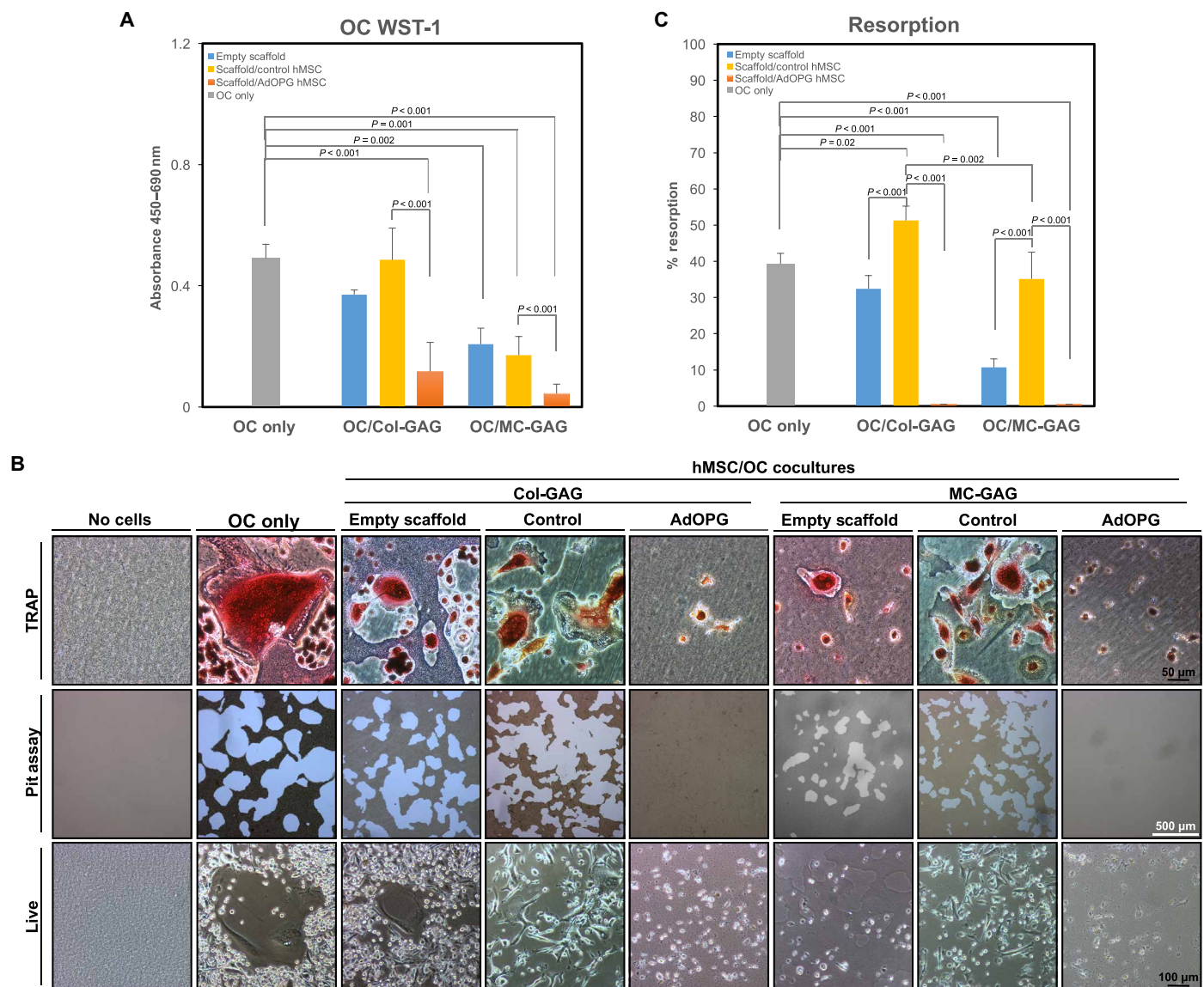


Fig. 5. AdOPG-infected hMSCs on Col-GAG and MC-GAG diminish the proliferation and resorption activity of osteoclasts. (A) WST-1 proliferation and viability assays of primary pre-osteoclasts in single culture (OC only), cocultured with empty scaffolds, or cocultured with control or AdOPG-transduced hMSCs (control hMSC and AdOPG hMSC, respectively) in medium supplemented with RANKL and M-CSF on Col-GAG or MC-GAG for 14 days. (B) TRAP staining (top row), resorption pits (middle row), and live images (bottom row) of negative control without cells (no cells), osteoclast only without hMSCs or scaffolds (OC only), and osteoclasts cocultured with Col-GAG or MC-GAG as empty scaffolds (empty scaffold), with control hMSCs (control), or with AdOPG-transduced hMSCs (AdOPG). (C) Quantitative analysis of pit assays as percentage of total area of well in differentiated osteoclasts without hMSCs (OC only) and osteoclasts cocultured with Col-GAG and MC-GAG as empty scaffolds, scaffolds with control hMSCs, and scaffolds with AdOPG-transduced hMSCs. Significant post hoc comparisons following ANOVA indicated with *P* values.

[$F(6,14) = 88.22$, $P < 0.001$; Fig. 5C]. In post hoc comparisons, no statistically significant differences were seen between osteoclasts cultured alone (OC only) and osteoclasts cocultured with empty Col-GAG, whereas osteoclasts cultured with empty MC-GAG were significantly less active ($P < 0.001$). In cocultures with control hMSCs differentiated on Col-GAG or MC-GAG, resorption increased compared to empty scaffolds ($P < 0.001$ for both), although the quantity of resorption continued to be lower in MC-GAG compared to Col-GAG ($P = 0.002$). In the presence of AdOPG, resorption was completely eliminated for either material compared to scaffolds with control hMSCs ($P < 0.001$ for both).

AdOPG transduction augments mineralization and hMSC expression of p-Smad1/5, Runx2, and p-ERK1/2 when directly contacting osteoclasts

Direct contact of hMSCs differentiated on Col-GAG and MC-GAG with hOCs allows for investigation of the net effects of positive and negative regulation including resorption on mineralization. Empty Col-GAG and MC-GAG scaffolds, scaffolds seeded with control hMSCs, or AdOPG-transduced hMSCs were directly cocultured with hOCs and concurrently differentiated for 14 days. Scaffolds were assessed for mineralization and activation of intracellular mediators known to be involved in osteogenic differentiation (Fig. 6).

Unlike indirect cocultures, direct cocultures with hOCs resulted in a decrease in mineral content on both Col-GAG and MC-GAG with control hMSCs when compared to empty scaffolds (Fig. 6, A and B). The decrease in mineralization is in concordance with the increase in hOC activity (Fig. 5, B and C) seen in the presence of hMSCs, as well as the presence of hOCs directly on the scaffolds (fig. S1). When hMSCs transduced with AdOPG on Col-GAG and MC-GAG were directly cocultured with hOCs, mineralization was significantly improved, resulting in a net osteogenic state.

To compare the osteogenic mechanisms activated in hMSCs in the direct coculture system, intracellular mediators known to be up-regulated in osteogenic differentiation were evaluated on Western blot analysis (Fig. 6C). Unlike single cultures with hMSCs, direct cocultures resulted in an up-regulation of p-Smad1/5, p-ERK1/2 (phosphorylated extracellular regulated kinase 1/2), and Runx2. These data suggest that hOCs simultaneously positively regulate hMSC osteogenesis while actively resorbing mineralized volumes. These activities may be separated using an endogenous secreted decoy receptor for RANKL.

DISCUSSION

In this work, we investigated the feasibility of combining OPG with a mineralized collagen scaffold for affecting osteogenic differentiation with concurrent osteoclastogenic inhibition. Using an adenoviral vector, we demonstrated that OPG expression did not affect the viability or proliferation of primary hMSCs and that expression could be detected at even 8 weeks following transduction. With respect to endogenous RANKL and OPG, hMSCs in MC-GAG scaffolds demonstrated a significantly lower RANKL/OPG cellular and scaffold-bound protein expression ratio compared to nonmineralized Col-GAG scaffolds, albeit a higher RANKL/OPG-secreted protein ratio. In the presence of AdOPG, the RANKL/OPG cellular and scaffold-bound protein expression ratios were significantly lowered from baseline. While AdOPG mildly reduced the secreted RANKL/OPG protein ratio in Col-GAG, AdOPG significantly diminished the secreted RANKL/OPG ratio in MC-GAG. Osteogenic differentiation of control versus AdOPG-transduced hMSCs did not show significant differences in terms of expression of osteogenic genes, p-Smad1/5, or quantitative matrix mineralization in the absence of osteoclasts. In the presence of differentiating primary hOCs in indirect cocultures, mineralization was increased beyond hMSC single cultures, particularly in hMSCs cultured on MC-GAG scaffolds. Augmented mineralization by hOCs persisted even with AdOPG transduction in indirect cocultures, suggesting that paracrine effects were responsible for the positive regulation. AdOPG transduction mildly affected hOC-mediated resorption in the presence of Col-GAG in indirect cultures, whereas resorption is significantly reduced in the presence of MC-GAG. Upon direct contact of differentiating osteoclasts with Col-GAG or MC-GAG materials, resorptive activities decreased but could be partially rescued with the addition of hMSCs. In the same direct cocultures with AdOPG-transduced hMSCs, the resorptive activities of osteoclasts were completely abrogated. The decrease in hOC resorptive activity by AdOPG resulted in an increase in activation of intracellular osteogenic mediators on both Col-GAG and MC-GAG scaffolds. These results suggest several conclusions: (i) hMSC osteogenic differentiation on MC-GAG scaffolds is largely unaffected by the addition of OPG; (ii) osteoclast and hMSCs cocultures positively regulate the activities of each other;

(iii) OPG is largely sequestered on the scaffold, and thus, anti-osteoclastogenic effects are most evident in direct cocultures; and (iv) direct osteogenic and osteoclastogenic coupling may be separated to augment bone regeneration, where we observe that hMSCs on MC-GAG scaffolds combined with OPG augment osteoclast activity inhibition.

Two other groups have suggested that bone regeneration with OPG is a viable strategy. Su *et al.* (28) expressed OPG in rabbit periodontal ligament stem cells seeded on β -tricalcium phosphate materials for regeneration of alveolar defects and demonstrated improved bone healing. Liu *et al.* (29) reported using adenoviral expression of OPG in rat BMSCs implanted on hydroxyapatite scaffolds for repair of osteoporotic mandible defects. Similar to our current report, they reported long-lasting OPG expression, even after 6 weeks for their *in vivo* experiments with a statistically significant improvement in mineralization and decrease in osteoclast cell density. Our current work now contributes to the concept of OPG expression in primary human MSCs and its effect on primary hOC differentiation and activity. Our work also demonstrates that the expression of OPG does not diminish osteoclast-induced mineralization, suggesting that separate processes within the osteoclast control the paracrine stimulation of osteoprogenitors versus the resorptive activity of the osteoclast.

In part, the concept of blocking osteoclastogenesis is contrary to some of the lessons learned in the genetics literature. In OPG knockout mice, a recent study documented that the release of negative regulation in osteoclastogenesis coincided with a simultaneous increase in bone formation, which was mediated through the suppression of sclerostin expression (30). Conversely, Runx2/Cbfa1 (core-binding factor $\alpha 1$) knockout mouse studies indicated that maturational arrest in osteoblast differentiation also resulted in the absence of osteoclasts (31, 32). Although this phenomenon was related to the expression of RANKL by osteoblasts downstream of Runx2, Runx2^{-/-} mice with a soluble RANKL transgene did not result in complete rescue (31). The combination of these genetic studies indicates that a direct relationship exists between bone formation and resorption. Our current study reflects the positive coordination of osteogenic and osteoclastogenic differentiation with observed dual increases when both cell types are allowed to interact in coculture (Figs. 4 and 5). Thus, it is clear that systemic or permanent deficiencies of any of these factors are likely not necessary or useful for bone regeneration.

In normal physiology and pathology, there are many examples of transient changes in the relative abundance of positive or negative regulators of osteogenesis or osteoclastogenesis. For osteoclast activation and activity, the relative balance between RANKL and OPG has been suggested to be responsible for the net effect toward bone formation or resorption on the locoregional or organismal environment (33, 34). In an effort to better understand fracture healing, Kon *et al.* (35) evaluated gene expression of the RANK/RANKL/OPG axis and proinflammatory cytokines in a mouse model. While OPG was constitutively expressed, RANKL was up-regulated in response to injury. Expressed in a different manner, Tanaka and colleagues (36) have demonstrated that the RANKL/OPG ratio changes over time following injury with a low ratio in the early stages, favoring bone formation, followed by a higher ratio, favoring bone resorption, later after injury. In our current work, there was a clear difference in the total endogenous OPG secreted (Fig. 4C), the endogenous RANKL/OPG protein ratio (Fig. 2E), and hOC resorptive

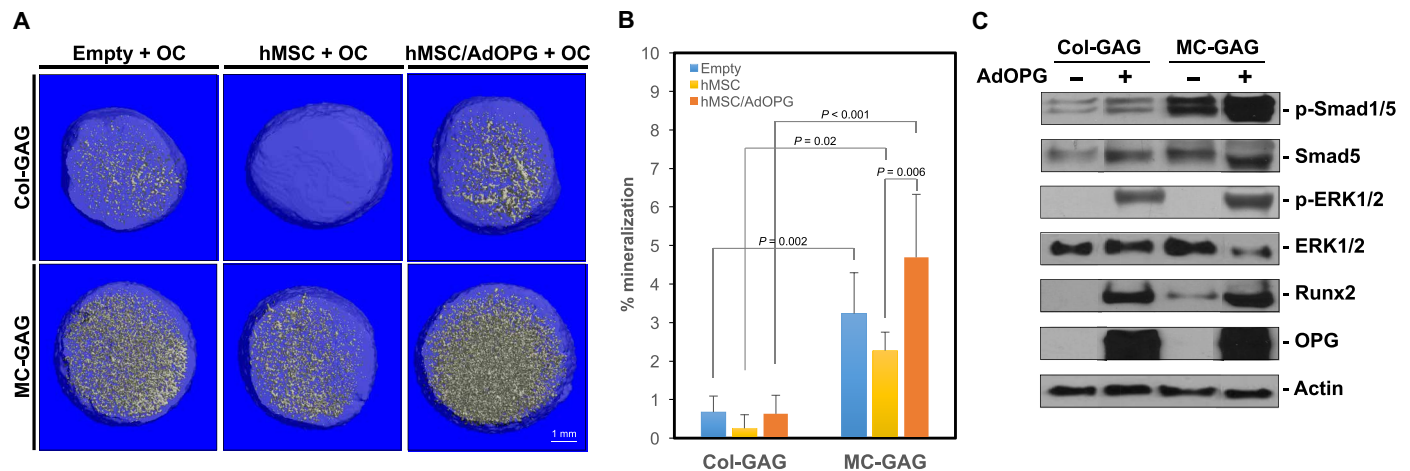


Fig. 6. Direct cocultures of osteoclasts with AdOPG-infected hMSCs on Col-GAG and MC-GAG increase p-Smad1/5, Runx2, p-ERK1/2, and mineralization. (A) Representative μ CT images and (B) quantitative analysis of direct cocultures of osteoclasts with empty scaffold (empty + OC), control hMSCs (hMSC + OC), or AdOPG-transduced hMSCs (hMSC/AdOPG + OC) on Col-GAG or MC-GAG for 14 days. Significant post hoc comparisons following ANOVA indicated with *P* values. (C) Western blot of control of AdOPG-transduced primary hMSCs differentiated on Col-GAG or MC-GAG materials cocultured with osteoclasts for 14 days.

activity (Fig. 5C) between control hMSCs cultured in nonmineralized Col-GAG versus MC-GAG scaffolds. The addition of AdOPG increases the total quantity of OPG protein and diminishes the total cellular and scaffold-bound RANKL/OPG ratio corresponding to the decrease in osteoclast resorptive activity in both materials. The differences in hOC resorptive abilities between indirect and direct cocultures suggest that most of the OPG remain sequestered on the scaffold, potentially pointing toward the ability for spatial control of modulatory factors in the presence of Col-GAG materials. The addition of OPG decreased osteoclast resorption without losing the osteogenic effects of hOCs (Fig. 5, D and E). As the osteogenic stimulation of hOCs is evident in indirect cultures, paracrine signals are likely the main positive regulatory signals from hOCs.

Despite the bidirectional positive regulation of osteogenic and osteoclastogenic differentiation in coculture, the net effects of the direct cocultures were tipped toward resorption in the presence of endogenous hMSCs (Fig. 6, A and B), suggesting that the endogenous levels of OPG cannot overcome the osteoclast activation present in cocultures. In the presence of AdOPG, not only did mineralization improve Smad1/5 phosphorylation and Runx2 protein expression both increased. As this increase was not detected in single cultures of hMSCs transduced with AdOPG, these effects are most likely to be attributable to hOC-induced osteogenic effects. Similar to the paracrine effects on mineralization, cocultures of AdOPG-transduced hMSCs on MC-GAG with hOCs may diminish hOC-mediated resorptive capacities but do not mitigate the osteoinductive signals from hOCs.

One of the potential benefits of using OPG as a temporary measure in decreasing osteoclast activity is that its function is a nonsignaling, decoy receptor for the major osteoclast growth factor (1). Although we used an adenoviral vector for OPG expression because of its high efficiency and high protein production for the purposes of proof of concept, a nongenetic method for OPG delivery would likely be better suited for clinical translation for safety reasons. In addition, we detected a high level of OPG protein even at 8 weeks following transduction. This may represent a second drawback in using a genetic methodology for OPG delivery, as the remodeling phase of bone

regeneration should be well underway at 8 weeks. Future work on the augmentation of osteoclast inhibition on MC-GAG materials would likely require refinement of the quantity and timing of OPG release.

In all, controlled coordination of both positive and negative processes is essential to the success of regenerative technologies. While most of the attention within the bone regeneration field has been focused on improving osteogenesis, consideration for diminishing osteoclastogenesis has only been recently investigated. Our current work details the ability of adenoviral expression of OPG in combination with MC-GAG to augment the inhibition of osteoclast resorptive activity without diminishing the positive paracrine effects on osteogenic differentiation. These findings contribute the potential utility of a strategy that capitalizes on a transient disturbance in osteoclast resorption to allow for the establishment of a critical mass of regenerated bone, suggesting new avenues for material development.

MATERIALS AND METHODS

Fabrication and chemical cross-linking of nonmineralized and mineralized collagen scaffolds

Col-GAG and MC-GAG scaffolds were prepared using lyophilization, as described previously (37–39). Briefly, microfibrillar, type I collagen (Collagen Matrix, Oakland, NJ) and chondroitin-6-sulfate (Sigma-Aldrich, St. Louis, MO) were combined in suspension in the absence and presence of calcium salts (calcium nitrate hydrate, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$; calcium hydroxide, $\text{Ca}(\text{OH})_2$; Sigma-Aldrich, St. Louis, MO) in an acetic acid (Col-GAG) or phosphoric acid (MC-GAG) solution. Using a constant cooling rate technique at a rate of $1^\circ\text{C}/\text{min}$, the solution was frozen from room temperature to -10°C using a freeze dryer (Genesis, VirTis). Following sublimation of the ice phase, scaffolds were sterilized via ethylene oxide and cut into 8-mm disks in diameter and 4 mm in height for culture.

Cross-linking of scaffolds was performed after rehydration in phosphate-buffered saline (PBS) for 4 hours using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC; Sigma-Aldrich) and *N*-hydroxysuccinimide (NHS; Sigma-Aldrich) at a molar ratio of 5:2:1 EDAC:NHS:COOH, where COOH represents the amount of

collagen in the scaffold as we previously described (40). Scaffolds were washed with PBS to remove any of the residual chemical.

Cell culture

Primary hMSCs (Lonza Inc., Allendale, NJ) were expanded in proliferation medium composed of Dulbecco's modified Eagle's medium (DMEM; Corning Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 2 mM L-glutamine (Life Technologies, Carlsbad, CA), and penicillin (100 IU/ml)/streptomycin (100 µg/ml; Life Technologies).

2D culture

hMSCs of passage 3 to 5 were plated at 5000 cells per well in 12-well plates, grown until 80 to 90% confluent, and then transduced with and without an AdOPG and RFP in DMEM at a MOI of 200 and polybrene (4 µg/ml; Sigma-Aldrich, St. Louis, MO). Twenty-four hours after transduction, hMSCs were subjected to differentiation medium consisting of proliferation medium plus 10 mM β-glycerophosphate, ascorbic acid (50 µg/ml), and 0.1 µM dexamethasone. Cell cultures were evaluated on day 7 after transduction for morphological changes, transduction efficiency, and Western blot.

Osteogenic differentiation of hMSCs on Col-GAG and MC-GAG

hMSCs (3×10^5) were seeded onto 8-mm discs of Col-GAG and MC-GAG scaffolds in proliferation medium. The cells were resuspended in growth medium, and half of the suspension was used to seed one side of the scaffold. After incubation for 15 min, the scaffold was turned upside down and the other half of the suspension was used to seed the opposite side. One millimeter of growth medium was then added to each well. Twenty-four hours after seeding, the medium was switched to osteogenic differentiation medium consisting of 10 mM β-glycerophosphate, ascorbic acid (50 µg/ml), and 0.1 µM dexamethasone.

Indirect hMSC and hOC cocultures

hMSCs (2×10^5) were seeded to 6 mm of Col-GAG and MC-GAG scaffolds in proliferation medium. Twenty-four hours after seeding hMSCs, primary hOC precursors (6×10^4 ; Lonza Inc., Allendale, NJ) were cultured in Osteoclast Precursor Basal Medium (Lonza, Allendale, NJ) supplemented with M-CSF (33 ng/ml), RANKL (66 ng/ml), 10 mM β-glycerophosphate, ascorbic acid (50 µg/ml), and 0.1 µM dexamethasone for concurrent hMSC and hOC differentiation on 24-well Corning Osteo Assay Surface Microplates (Corning, NY). After 2 hours, Col-GAG and MC-GAG scaffolds were transferred to 8-µm Transwell inserts (Corning, NY) and cocultured with hOCs. Medium was changed every 3 days for 3 weeks.

Direct hMSC and hOC cocultures

hMSCs (3.5×10^5) were seeded to 8 mm of Col-GAG and MC-GAG scaffolds in proliferation medium. Twenty-four hours after seeding hMSCs, hOCs (6×10^4) were cultured in Osteoclast Precursor Basal Medium (Lonza Inc., Allendale, NJ) supplemented with M-CSF (33 ng/ml), RANKL (66 ng/ml), 10 mM β-glycerophosphate, ascorbic acid (50 µg/ml), and 0.1 µM dexamethasone on 24-well Osteo Assay Microplates. After 2 hours, Col-GAG and MC-GAG scaffolds were transferred to the Osteo Assay Microplates and directly cocultured with hOCs. Medium was changed every 3 days for 2 weeks.

Quantitative real-time reverse transcription polymerase chain reaction

RNeasy kit (Qiagen, Valencia, CA) was used to extract total RNA from scaffolds at 0, 3, and 14 days of culture. Gene sequences for 18S, Runx2, OPN, OPG, and receptor activator of RANKL were ob-

tained from the National Center for Biotechnology Information gene database, and primers were designed (table S1). qRT-PCR was performed on the Opticon Continuous Fluorescence System (Bio-Rad Laboratories Inc., Hercules, CA) using the QuantiTect SYBR Green RT-PCR Kit (Qiagen). Cycle conditions were as follows: reverse transcription at 50°C (30 min); activation of HotStarTaq DNA polymerase/inactivation of reverse transcriptase at 95°C (15 min); and 45 cycles of 94°, 58°, and 72°C for 15, 30, and 45 s, respectively. Results were analyzed and presented as representative graphs of triplicate experiments.

Enzyme-linked immunosorbent assay

Supernatants were collected from hMSC only, osteoclast (OC) only, or hMSC and hOC cocultures. OPG protein concentrations were determined using the Human OPG DuoSet ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, a 96-well microplate was coated with the capture antibody and incubated overnight at room temperature. After blocking, samples were incubated for 2 hours at room temperature with the detection antibody, followed by incubation with streptavidin-horseradish peroxidase (HRP) for 20 min. The reaction was quenched by adding 100 µl of 2 N H₂SO₄. Plates were read at 450- and 540-nm wavelengths on the Epoch Microplate Reader (BioTex, Winooski, VT).

µCT imaging

Scaffolds were fixed using 10% formalin, and mineralization was quantified by µCT imaging using Scanco 35 (Scanco Medical AG, Brüttisellen, Switzerland) in triplicate for each time point. Scans were performed at medium resolution with a source voltage *E* of 70 kVp and with a current *I* of 114 µA. The images had a final element size of 12.5 µm. Images were analyzed using software supplied from Scanco (Image Processing Language version 5.6) and reconstructed into 3D volumes of interest. Optimum arbitrary threshold values of 20 (containing scaffold and mineralization) and 80 (containing mineralization alone) were used uniformly for all specimens to quantify mineralized areas from surrounding unmineralized scaffold. Analysis of 3D reconstructions was performed using Scanco Evaluation script no. 2 (3D segmentation of two volumes of interest: solid dense in transparent low-density object) and script no. 6 (bone volume/density-only bone evaluation) for volume determinations.

Western blot

Total protein lysates were prepared from scaffolds at 0, 3, 14, 28, 42, and 56 days of culture by incubating scaffolds morselized using scissors in 3× SDS reducing sample buffer. Lysates were then incubated at 95°C for 5 min and centrifuged in 0.2 µm of Spin-X filters (Corning Costar, Corning, NY) at 14,000 rpm for 5 min. Protein concentration was measured, and equal amounts were subjected to 4 to 20% SDS-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA). Western blot analysis was carried out with antibodies against OPG, RANKL, p-Smad1/5, total Smad5, p-ERK1/2, total ERK1/2, and β-actin, followed by 1:4000 dilutions of HRP-conjugated immunoglobulin G antibodies (Bio-Rad, Hercules, CA) and an enhanced chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL). For the detection of p-Smad1/5 and total Smad5, 10 µg of lysate was loaded per lane. For the detection of OPG, RANKL, p-ERK1/2, total ERK1/2, and β-actin, 20 µg of lysate was loaded per lane. All antibodies were obtained from Cell Signaling Technology (Danvers, MA), with the exception of RANKL, OPG, and β-actin antibodies from

Santa Cruz Biotechnology (Santa Cruz, CA). Imaging analysis was carried out using ImageJ (NIH, Bethesda, MD). The RANKL/OPG-relative protein ratios were calculated by quantifying the densitometry of all RANKL and OPG normalized to actin using ImageJ (NIH, Bethesda, MA).

WST-1 assay

Culture medium was supplemented with cell proliferation reagent WST-1 (Roche, Basel, Switzerland) at a 1:10 concentration. Scaffolds were incubated for 3 to 4 hours at 37°C in a humidified atmosphere with 5% CO₂. Absorbance of the incubation medium was measured at 450 and 690 nm (Epoch spectrophotometer, BioTek, Winooski, VT).

TRAP staining

hOCs were detected using the Leukocyte TRAP Kit 387-A (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, cultured cells were fixed with formaldehyde for 5 min at room temperature, washed, and air-dried. After staining, TRAP-positive multinucleated cells were observed under a phase-contrast microscope at 20× magnification and digitally photographed.

Resorption pit assay

Activity of hOCs in single culture or cocultured with scaffolds with and without hMSCs was evaluated for resorption pit formation on Osteo Assay Microplates. At the completion of the culture period, culture medium was aspirated, and 500 µl of 10% bleach solution was added for 5 min at room temperature. The wells were washed with distilled water and allowed to dry at room temperature for 3 to 5 hours. Pits were observed using a standard microscope and digitally photographed. The percentage of resorption for the whole well of the culture at magnification 2× was calculated by ImageJ.

Statistical analysis

All statistical analyses were performed using SPSS version 24 (Chicago, IL). Data points were composed of duplicates of at least three independent experiments, unless otherwise indicated. Mean measurements of mRNA expression were analyzed for statistical significance by ANOVA, followed by post hoc tests using the Tukey's criterion. A value of $P < 0.05$ was considered significant.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/5/6/eaaw4991/DC1>

Supplementary Materials and Methods

Table S1. Primer sequences.

Fig. S1. Osteoclasts migrate into Col-GAG and MC-GAG scaffolds in the presence of control hMSCs in direct cocultures.

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Competing interests: J.C.L., B.A.C.H., X.R., and T.A.M. are inventors on a patent application related to this work filed by the University of California, Los Angeles (no. PCT/US2019/016709, filed 05 February 2019). All other authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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Osteoprotegerin reduces osteoclast resorption activity without affecting osteogenesis on nanoparticulate mineralized collagen scaffolds

Xiaoyan Ren, Qi Zhou, David Foulad, Aleczandria S. Tiffany, Marley J. Dewey, David Bischoff, Timothy A. Miller, Russell R. Reid, Tong-Chuan He, Dean T. Yamaguchi, Brendan A. C. Harley and Justine C. Lee

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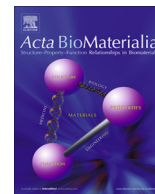
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Full length article

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ABSTRACT

Implant osteoinduction and subsequent osteogenic activity are critical events that need improvement for regenerative healing of large craniofacial bone defects. Here we describe the augmentation of the mineral content of a class of mineralized collagen scaffolds under development for craniomaxillofacial bone regeneration via the inclusion of zinc ions to promote osteogenesis *in vitro*. Zinc is an essential trace element in skeletal tissue and bone, with soluble zinc being shown to promote osteogenic differentiation of porcine adipose derived stem cells. We report the development of a new class of zinc functionalized scaffolds fabricated by adding zinc sulfate to a mineralized collagen-glycosaminoglycan precursor suspension that was then freeze dried to form a porous biomaterial. We report analysis of zinc functionalized scaffolds via imaging (scanning electron microscopy), mechanical testing (compression), and compositional (X-ray diffraction, inductively coupled plasma mass spectrometry) analyses. Notably, zinc-functionalized scaffolds display morphological changes to the mineral phase and altered elastic modulus without substantially altering the composition of the brushite phase or removing the micro-scale pore morphology of the scaffold. These scaffolds also display zinc release kinetics on the order of days to weeks and promote successful growth and pro-osteogenic capacity of porcine adipose derived stem cells cultured within these zinc scaffolds. Taken together, we believe that zinc functionalized scaffolds provide a unique platform to explore strategies to improve *in vivo* osteogenesis in craniomaxillofacial bone injuries models.

Statement of Significance

Craniomaxillofacial bone defects that arise from traumatic, congenital, and post-oncologic origins cannot heal on their own and often require surgical intervention. We have developed a class of mineralized collagen scaffolds that promotes osteogenesis and bone regeneration. Here we describe the inclusion of zinc sulfate into the mineralized collagen scaffold to improve osteogenesis. Zinc functionalized scaffolds demonstrate altered crystallite microstructure but consistent Brushite chemistry, improved mechanics, and promote zinc transporter expression while supporting stem cell viability, osteogenic differentiation, and mineral biosynthesis.

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1. Introduction

Craniomaxillofacial bone injuries arise from a diverse set of congenital defects as well as oncologic or traumatic injuries.

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Trauma-related craniomaxillofacial injuries, both those experienced by civilians [1–3] as well as high-energy impact injuries experienced by Warfighters [4,5], present particular clinical challenges. Trauma-related craniomaxillofacial bone injuries are often large and complex in size and geometry, and cannot be repaired with external fixtures alone [6]. Most often clinical treatment utilizes autografts, bone taken from a secondary site in the patient with the defect, or allografts, bone taken from a human donor [7–9]. Autografts are limited by the size of the defect area as well as concerns associated with secondary site morbidity. Bone allografts raise concerns about disease transmission, rejection, and

lot-to-lot variability that may impact graft integration and healing [10]. Thus, there is a clinical need for alternative solutions to address critically sized craniomaxillofacial defects.

Strategies to improve regenerative healing of such bone defects often seek to employ a biomaterial template. The collagen and calcium phosphate mineral nano-crystallites associated with the bone extracellular matrix have inspired a wide range of collagen-mineral composite biomaterials [11–13]. Our laboratory has recently developed a mineralized collagen-glycosaminoglycan scaffold that promotes osteogenesis and mineral deposition *in vitro* without the addition of exogenous factor (e.g. BMP2) [14,15]. Here, calcium salts added to a suspension of collagen and glycosaminoglycans in phosphorous acid lead to precipitation of a brushite form of calcium phosphate into the collagen network that is subsequently lyophilized to form a porous foam. A portion of the calcium and phosphate ions incorporated into the mineralized scaffolds are released during culture and are believed to accelerate osteogenesis [14]. Craniofacial bones rely on intramembranous ossification processes, and the mineralized collagen scaffold has been previously shown to promote osteogenesis *in vitro* and also to enhance regenerative healing of craniofacial bone injuries (rabbit calvarial; sub-critical sized porcine mandible) [16–18]. However, as with most tissue engineering biomaterials, the need to enhance osteogenic capacity increases with the size and complexity of craniomaxillofacial trauma. These challenges are heightened given the increasing technical and regulatory challenges associated with the delivery of growth factors to enhance bone healing (e.g., BMP2) [12].

Zinc is an essential trace element found primarily in skeletal tissue and bone [19,20]. Zinc is an important mediator of bone development and growth. Hojyo et al. found that mice with a knockout for Zrt- and Irt-like protein 14 (ZIP14) – a zinc transporter – had shorter long bones and exhibited dwarfism compared to wildtype mice [21]. Similarly, zinc deficiency in humans is related to stunted bone development and dwarfism [22]. *in vitro* studies have shown that zinc ions improve stem cell osteogenesis and increase mineral deposition *in vitro* [23,24]. Recent work by Bertels et al. identified a critical dose range (0.04–0.08 mM) of zinc sulfate that when added to osteogenic media enhanced adipose derived stem cell mineral nodule formation in two-dimensional culture [25]. Importantly, the response was biphasic, with zinc sulfate concentrations above 0.08 mM detrimental to cell growth.

In this manuscript we describe the incorporation of zinc into the mineralized collagen scaffold via addition of zinc sulfate into the mineralized collagen suspension prior to lyophilization. We report dose-dependent influences of zinc incorporation on scaffold mechanical and structural properties, composition and organization of scaffold mineral phase, rate of zinc release, and resultant changes in *in vitro* activity of adipose derived stem cells.

2. Materials and methods

2.1. Scaffold fabrication

Mineralized collagen-glycosaminoglycan (*Mineralized*) and mineralized collagen-glycosaminoglycan-zinc scaffolds (*Zinc*) were fabricated via lyophilization from mineralized collagen and mineralized collagen zinc precursor suspensions. The mineralized collagen suspension was created as previously described by homogenizing type I collagen (1.9 weight per volume, Sigma Aldrich, St. Louis, Missouri USA), chondroitin-6-sulfate (0.84 weight per volume, Sigma Aldrich), and calcium salts (calcium hydroxide and calcium nitrate, Sigma Aldrich) in mineral buffer solution (0.1456 M phosphoric acid/0.037 M calcium hydroxide) [14,26]. The mineralized collagen zinc suspension was prepared

in the same manner, with zinc sulfate (Sigma Aldrich) added simultaneously with the calcium salts. Three mineralized collagen zinc scaffold variants were created, inspired by data from a previous study from our group [14] that quantified the release rates of calcium and phosphate ions from mineralized collagen scaffolds. We created three zinc scaffold variants estimated to generate soluble zinc ion concentrations within the scaffold below and above the 0.08 mM zinc ion concentration found by Bertels et al. [25] to enhance adipose derived stem cell activity in two-dimensional culture. The final zinc scaffold variants contained 0.5 mg/mL (1× Zinc), 2.5 mg/mL (5× Zinc), or 5 mg/mL (10× Zinc) of added zinc sulfate. Precursor suspensions were stored at 4 °C and degassed prior to lyophilization.

Scaffolds were subsequently fabricated via lyophilization using a Genesis freeze-dryer (VirTis, Gardener, New York USA) [27]. Briefly, 100 µL of precursor suspension was pipetted into each well of a custom 144-well polysulfone mold (6 mm diameter, 7 mm tall wells). Scaffolds were then fabricated by freezing the suspension via cooling from 20 °C to –10 °C at a constant rate of 1 °C/min followed by a temperature hold at –10 °C for 2 h. The frozen suspension was then sublimated at 0 °C and 0.2 Torr, resulting in a porous scaffold network. After fabrication, all scaffolds used for cell culture were sterilized via ethylene oxide treatment for 12 h utilizing an AN74i Anprolene gas sterilizer (Andersen Sterilizers Inc., Haw River, North Carolina USA) in sterilization pouches [14,18,28]. All subsequent handling steps leading to studies of cell activity were performed in a sterile manner.

2.2. Scanning electron microscopy and pore size quantification

Dry scaffolds specimens were cut into semi-cylinders and placed on carbon tape, sputter coated with gold/palladium, and imaged using a Philips XL30 ESEM-FEG (FEI Company) at 5 kV with a secondary electron detector. Alternatively, critical point drying (CPD) was performed using a Samdri-PVT-3D (Tousimis, Rockville, Maryland USA) to prepare hydrated and cell-seeded scaffolds prior to SEM imaging [29]. Briefly, scaffolds were placed in formalin for 24 h and then the aqueous media in hydrated scaffolds was sequentially replaced with ethanol and then liquid CO₂. The specimens were then held above 6.895 kPa and 31 °C to remove the CO₂ as a gas with minimal structural deformation.

Quantification of scaffold pore size was performed using a previously described stereology method [30,31]. Briefly, cylindrical scaffold specimens were embedded in glycomethacrylate (Polysciences Inc., Warrington, Pennsylvania USA) then sectioned (10 µm slices) in both transverse and longitudinal planes. Sections were stained with aniline blue (Thermo Fisher Scientific Inc, Waltham, Massachusetts USA) then imaged using a NanoZoomer Digital Pathology System (Hamamatsu, Japan). A linear intercept macro was then employed to calculate pore size and pore aspect ratio from histology specimens [32,33].

2.3. X-ray diffraction & unit cell analysis

Scaffolds were prepared for X-ray diffraction (XRD) compositional analysis by freeze-drying the mineralized collagen or mineralized collagen zinc precursor suspensions directly in XRD sample holders. Pure zinc sulfate was used as a positive control. Powder XRD data were collected on a Rigaku MiniFlex 600 diffractometer (SN BD63000258-03). Measurements employed Ni-filtered Cu K α (λ = 1.54178 Å) radiation from a copper sealed tube with 40 kV voltage and 15 mA current in the Bragg-Brentano geometry. Diffraction patterns were measured over the range of 3–100° 2 θ by step scanning at a rate of 1 s/0.02°. Scaffolds were prepared for unit cell analysis using the same method described above, but with a silicon standard added to each precursor suspension prior

to freeze-drying. The silicon standard provided a calibrant to correct the patterns for sample displacement effects. All patterns were corrected and analyzed for comparison of unit cell parameters between scaffold variants, with unit cell refinements performed using TOPAS (Version 4.2. Bruker AXS, Inc., Madison, Wisconsin, USA).

2.4. Quantification of Zn, Ca, and P in freeze-dried scaffolds

Freeze-dried scaffold specimens (samples between 0.5 and 14 mg) were analyzed via inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectrometry (ICP-OES). ICP-MS was used to detect zinc in our scaffolds, while ICP-OES was used to detect calcium and phosphorus. Dry samples were transferred to a digestion tube, then digested with concentrated nitric acid (67–70%) followed by automated sequential microwave digestion in a CEM Mars 6 microwave digester (CEM Microwave Technology Ltd., North Carolina, USA). The final product was a clear aqueous digest which was diluted to a volume of 25 mL using DI water. This solution was then introduced to inductively coupled plasma-mass spectrometer (NexION™ 350D ICP-MS, PerkinElmer, USA) for the elemental analysis in a standard mode. Digestion and ICP-MS/ICP-OES analysis parameters are in [Supplemental Tables 1–3](#).

2.5. Zinc ion release

The release of zinc ions from mineralized zinc (vs. mineralized) scaffolds was monitored for up to 28 days in culture. Hydrated scaffolds (6 mm diameter, 3 mm height) were placed in 1 mL phosphate buffered saline (PBS) + 1% bovine serum albumin at 37 °C for up to 28 days. Aliquots were collected every day for the first 7 days of release followed by every 3 days until day 28, with media replaced after each aliquot collection. Zinc ion release was quantified using ICP-MS. A volume of 0.5 mL of the sample was transferred to a digestion tube. Sample degradation and zinc analysis were performed as described above.

2.6. Scaffold mechanical properties

Stress–strain curves of the non-hydrated scaffolds were generated using an Instron 5943 mechanical tester (Instron, Norwood, MA) using a 100 N load cell under dry conditions. Samples were compressed at a rate of 2 mm/min with the Young's Modulus determined from the stress–strain curves using conventional analysis methods for low-density open-cell foam structures such as the collagen scaffolds [34–38].

2.7. Porcine derived adipose stem cell culture

Primary porcine derived adipose stems cells (pASCs) were used for all *in vitro* cell experiments and were a gift from Dr. Matthew Wheeler (University of Illinois). pASCs were chosen as they have been shown to have robust and well defined osteogenic capabilities [39]; pASCs were also used to identify a dose range of zinc ions to be added into cell culture media (0.04–0.08 mM) to enhance osteogenesis. Further, we have previously used a porcine mandibular defect model to evaluate the regenerative capacity of our mineralized collagen scaffolds [17,25,39]. Cells were expanded in T175 flasks (Fisher Scientific, Hampton, New Hampshire USA) until passage 6 and cultured in complete mesenchymal stem cell growth media (Low glucose DMEM + L-glutamine, 10% fetal bovine serum, 1% antibiotic–antimycotic) at 37 °C and 5% CO₂ until confluent. Once confluent, pASCs were seeded (100,000 cells/scaffold for 7-day experiment, 80,000 cells/scaffold for 56-day experiment) onto mineralized and zinc-functionalized scaffolds using a static

seeding method used in our laboratory previously [14,29,40]. Cell seeded scaffolds were cultured in mesenchymal stem cell growth media at 37 °C and 5% CO₂ for up to 56 days (cell activity), with media changed every 3 days.

2.8. Cell metabolic activity & cell number

The metabolic activity and total cell number in scaffold specimens was measured using alamarBlue and Hoechst assays, respectively, via fluorescent spectrophotometer (Tecan Infinite F200 Pro, Männedorf, Switzerland). Cell seeded scaffolds were incubated in a 10% alamarBlue solution (Invitrogen, Carlsbad, California USA) for 90 min at 37 °C under moderate shaking. The active ingredient in the alamarBlue reagent, resazurin, is reduced to a compound that is highly fluorescent when added to metabolically active cells [14,41]. The relative cell metabolic activity was determined from a standard curve generated with known pASC concentrations and reported as a fraction of initial seeding cell count and normalized by the initial seeding density (i.e. experimental value of 1 indicate the metabolic activity of the number of cells originally seeded onto the scaffold). The total number of pASCs within a scaffold specimen was quantified via a Hoechst assay. Briefly, cell-seeded scaffolds were rinsed in PBS to remove dead, unattached cells [31] then digested in papain (4.8 mg/mL, Sigma Aldrich) at 60 °C for 18–24 h. Lysates were then incubated with Hoechst 33258 dye (Invitrogen) to fluorescently label double-stranded DNA that could be quantified via a fluorescent spectrophotometer, then translated into cell count through a standard curve of known pASC numbers.

2.9. RNA isolation and gene expression characterization

RNA was isolated from scaffolds using 1% beta-mercaptoethanol lysis solution and the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands), then was reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen) and a BioRad S1000 thermal cycler (BioRad, Hercules, California USA). Real-time PCR reactions were carried out in duplicate, using 10 ng of cDNA and either QuantiTect SYBR Green (BioRad) or Taqman (ThermoFisher, Waltham, Massachusetts USA) primers. Primers were chosen because they're validated for both selectivity and efficiency during PCR; however, the difficulty in finding validated primers for all targets in porcine cells with one system led to the use of both SYBR Green and Taqman systems. A Quantitect SYBR green PCR kit (Qiagen) or Taqman PCR kit (ThermoFisher) – depending on primer type – along with an Applied Biosystems 7900HT Fast Real-Time PCR System was used to perform the real-time PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene, with both Taqman and SYBR green primers for GAPDH used to compare between primer types. Gene expression profiles were obtained for Collagen 1 (Col1A), osteocalcin (OCN), bone morphogenic protein 2 (BMP2), transcription factor Sp7 (Osterix), runt-related transcription factor 2 (RunX2), zinc transporter 7 (ZnT7), Zrt-and Irt-like protein 1 (ZIP1), and ZIP13 ([Table 1](#)).

Table 1
PCR primers and supplier.

Transcript	Supplier	Assay ID
GAPDH	ThermoFisher (Taqman)	Ss03375629_u1
COL1A2	ThermoFisher (Taqman)	Ss03375009_u1
BGLAP	ThermoFisher (Taqman)	Ss03373655_s1
BMP2	ThermoFisher (Taqman)	Ss03373798_g1
OSTERIX	ThermoFisher (Taqman)	Ss03373734_s1
ZNT7	ThermoFisher (Taqman)	Ss03819782_s1
GAPDH	Bio-Rad (SYBR Green)	qSscCED0017494
RUNX2	Bio-Rad (SYBR Green)	qSscCID0002170
ZIP1	Bio-Rad (SYBR Green)	qSscCED0016809
ZIP13	Bio-Rad (SYBR Green)	qSscCED0011005

Taqman primers (ThermoFisher) were used for Col1A, OCN, BMP2, Osterix, and ZnT7 while SYBR green primers (BioRad) were used for RunX2, ZIP1, and ZIP13.

2.10. Micro computed tomography

The mineral content of mineralized collagen vs. mineralized collagen zinc scaffolds was assessed via micro-computed tomography (micro-CT). Cell-seeded and unseeded control scaffolds were examined after 28 and 56 days in culture and compared to an unseeded control scaffold (day 0). All samples were fixed in 10% formalin (Polysciences, Warrington, Pennsylvania USA), with scans performed using a MicroXCT-400 (Zeiss, Oberkochen, Germany). Scans consisted of 721 images performed at 40 kV and 8 W. Total particle count and particle size (area) were obtained using the “analyze particles” function in ImageJ. Image stacks were imported into ImageJ, converted to 16-bit binary images, and converted into black and white images using the Shanbhag automatic thresholding method [42,43]. Within the analyze particles settings, we set the particle size as “0-Infinity” (pixels²; ~364 pixel is 6 mm) and circularity as “0.00–1.00.” This allows the counting of particles at any size and any circularity (i.e. shapes other than spheres will be counted).

2.11. Statistics

All statistical analyses were done in Origin Pro. All experiments were performed with at least $n = 6$ samples per group, except for gene expression analysis which was performed with $n = 5$ samples per group. Normality and equal variance were determined using Shapiro-Wilk and Brown-Forsythe, respectively. One-way ANOVA was used for normal data with equal variance followed by Tukey-HSD post-hoc analysis. A t -test with Welch correction was used for normal data without equal variance. Finally, Kruskal-Wallis with post-hoc analysis was used for non-normal data. Significance reported for $p < 0.05$. All error bars reported are $\pm$ standard error unless otherwise noted.

3. Results

3.1. Addition of zinc sulfate alters the morphology of the mineral phase

Scanning electron microscopy (SEM) analysis revealed that all scaffolds (Mineralized; 1× Zinc, 5× Zinc; 10× Zinc) displayed an open pore network (Fig. 1A). Stereology analysis revealed a minimal influence of zinc incorporation on scaffold pore size (Table 2), with the 10× Zinc scaffolds having significantly reduced pore size

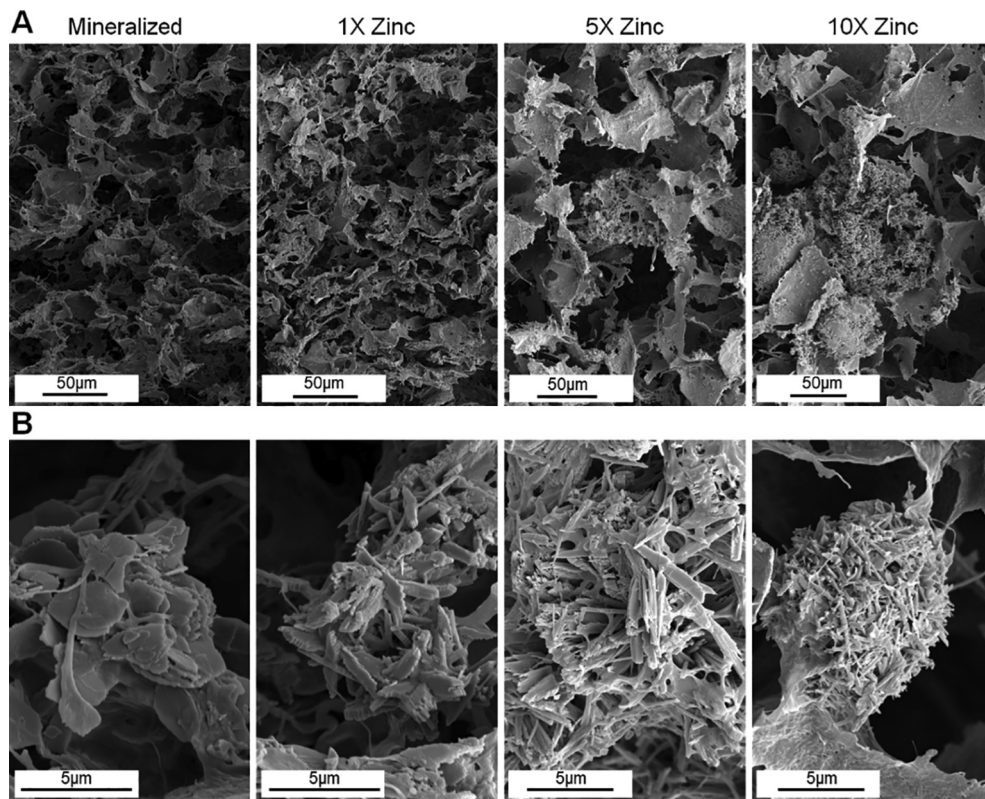


Fig. 1. Scanning electron microscopy images of mineralized zinc scaffolds show alterations in crystal microstructure. (A) There are no noticeable changes to pore structure across scaffold variants, but there are noticeable changes in crystal aggregates in 5× and 10× zinc groups. (B) The addition of zinc alters the brushite microstructure within the scaffolds from plate-like crystals to elongated needle-like structures. (Top: Magnifications – 800×, 800×, 600×, 600×; Bottom: Magnifications – 12,000×, 12,000×, 10,000×, 8000×; 16-bit Tiff images; Cropped to size only).

Table 2

Pore size of zinc functionalized scaffold normalized to mineralized scaffolds ($62 \pm 1 \mu\text{m}$).

	Mineralized	1× Zinc	5× Zinc	10× Zinc
Normalized Pore Diameter	1.00	0.93	0.98	0.85
Standard Error ($\pm$)	0.02	0.01	0.02	0.01

than the other scaffold variants. We subsequently examined the effect of zinc incorporation on the morphology of the resultant mineral content. The fabrication conditions for the mineralized collagen suspension result in the formation of a brushite mineral phase within the scaffold [14], under higher magnification the mineralized collagen scaffold control in this experiment exhibits the conventional plate-like mineral architecture of brushite mineral (Fig. 1B). However, incorporation of zinc ions leads to the formation of elongated, needle-like mineral precipitates, with crystal elongation increasing with increasing concentrations of zinc sulfate added to the precursor suspension (Fig. 1B).

3.2. Addition of zinc sulfate does not alter composition of the mineral phase

Due to the observed differences in the micron-scale morphology of the mineral deposits in the zinc functionalized scaffolds vs. the conventional mineralized collagen scaffold, we performed in depth compositional analysis of all scaffold variants. Despite the morphological differences, X-ray diffraction (XRD) indicates brushite is present in all scaffold variants (Fig. 2A). The XRD pattern associated with the zinc functionalized scaffolds is different from the pattern observed for pure zinc sulfate, and there are peaks present in the zinc sulfate spectra that are not present in the zinc functionalized scaffolds (Fig. 2B; red arrows). Further, unit cell analysis of brushite crystals within the scaffolds revealed the brushite lattice parameters (a, b, and c) as well as the beta angle

of brushite ($\beta = 116.4^\circ$) do not deviate with increasing amounts of zinc sulfate (Fig. 2C).

3.3. Zinc-functionalized scaffolds can be tuned to alter mechanical properties and the release of zinc ions

We subsequently quantified the efficiency of zinc incorporation, the effect of zinc inclusion on scaffold mechanical properties, and release kinetics of zinc ions from the scaffold. Inductively coupled plasma mass spectrometry (ICP-MS) indicated that ~50% of zinc added to the mineralized collagen suspension was present in the freeze-dried scaffold (Fig. 2D). Furthermore, the amount of calcium (Ca) and phosphorus (P) precipitated into the mineralized scaffold was not altered by the inclusion of zinc ions (Fig. 2E). Unconfined compression tests revealed that inclusion of zinc ions impacted scaffold elastic modulus, with 5× Zinc and 10× Zinc scaffolds showing significantly higher elastic modulus than the traditional mineralized and 1× Zinc scaffolds (Fig. 2F). The zinc ions incorporated into the scaffold mineral phase are released from the scaffold over extended culture, and this was previously observed for the calcium and phosphate components of the mineral phase [14]. ICP-MS analysis revealed steady release of zinc ions through 28 days in culture (37 °C) into a model culture media (PBS + 1% BSA). This release was dependent on the dose of zinc incorporated into the scaffold (Fig. 3), with significant differences between all groups beginning at Day 4.

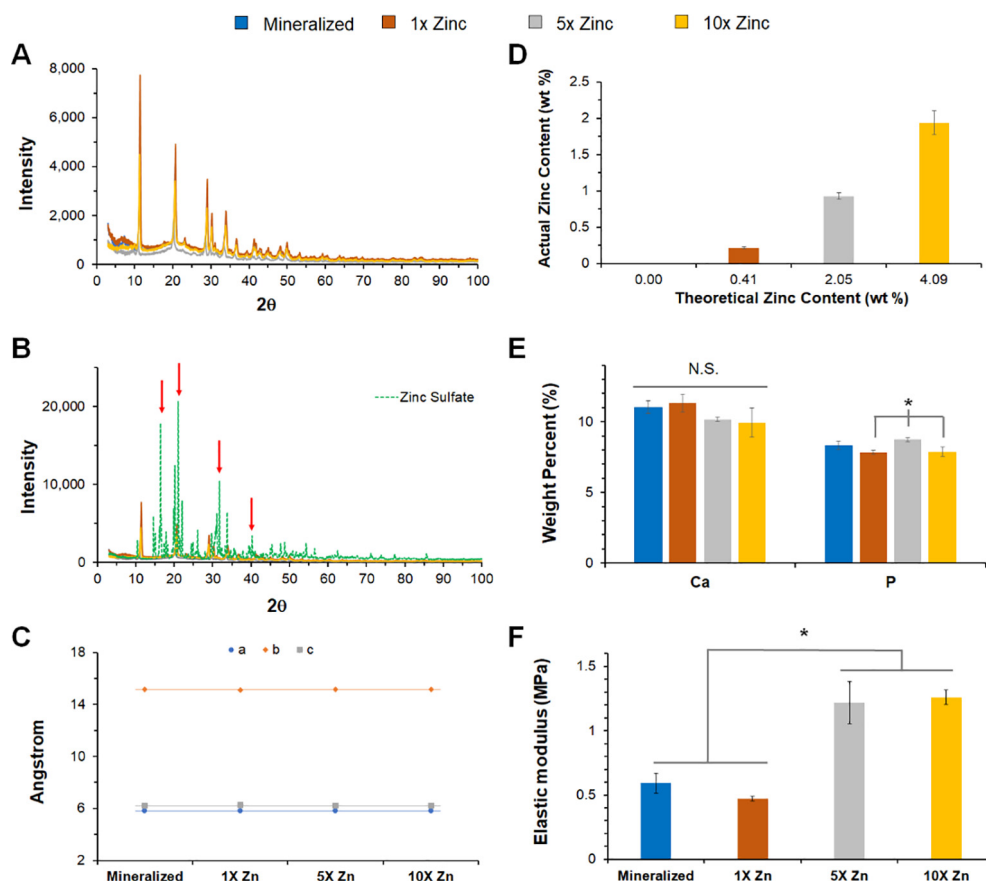


Fig. 2. Compositional and mechanical analysis of zinc-functionalized mineralized scaffolds. (A) X-ray diffraction patterns of mineralized and zinc functionalized scaffolds indicate brushite is present in all variants. (B) X-ray diffraction data from 2A overlaid with zinc sulfate diffraction pattern. Additional peaks present in zinc sulfate are not present in zinc functionalized scaffolds (red arrows). (C) Unit cell analysis of brushite within each scaffold variant, indicating that increasing zinc concentration does not change unit cell parameters. (D) ICP-MS analysis of zinc in scaffolds. Approximately 50% of zinc added into slurry is in scaffolds. All groups are significantly different from one another at $p < 0.05$. (E) ICP-OES analysis of calcium and phosphorus content in each scaffold demonstrate no relationship between inclusion of zinc and the overall amount of calcium phosphate in the scaffold. (F) Elastic modulus for each scaffold variant. *: significance reported at $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

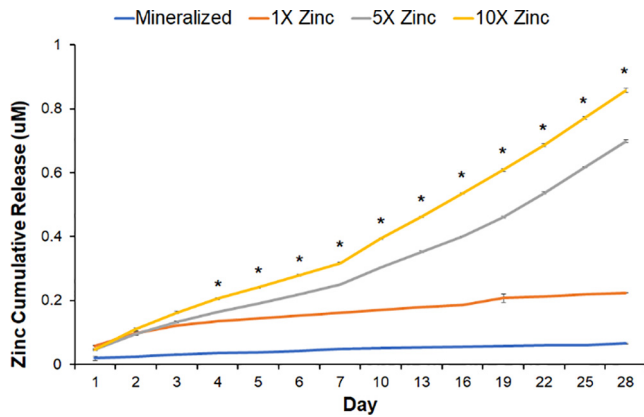


Fig. 3. Zinc ion release. Zinc ion release quantified using ICP-MS for all scaffold variants: Mineralized, 1× Zinc, 5× Zinc, and 10× Zinc. *: significance between all groups reported at $p < 0.05$.

3.4. Zinc sulfate addition does not negatively impact cell activity at early time points

We subsequently examined the attachment and activity of porcine adipose derived stem cells (pASCs) within the zinc functionalized scaffold variants. We observed cell attachment and spreading on all scaffold variants after 7 days in culture (Fig. 4A, white arrowheads). We compared the number of attached pASCs and the metabolic activity of the cell seeded constructs over 7 days between groups to confirm that inclusion of zinc ions was not detrimental to stem cell activity. While there were some significant differences in cell number and metabolic activity between groups (notably increased metabolic activity for 5× and 10× Zinc scaffolds at Day

4), there was no significant differences in metabolic activity or cell number between groups by Day 7 (Fig. 4B). We decided to remove the 1× Zinc variant from subsequent long-term *in vitro* experiments because the metabolic activity observed for this group did not differ from the mineralized scaffold at any timepoint, the cell number for this group was lower than the mineralized scaffold at all timepoints, and there was no difference in elastic modulus when this group was compared to the mineralized scaffold.

3.5. Zinc functionalized scaffolds support long term pASC activity *in vitro* as well as signatures of osteogenic differentiation in the absence of osteogenic supplements

We subsequently performed an additional 8-week *in vitro* experiment, with cell-seeded scaffolds maintained in growth (not osteogenic) media. All scaffolds supported pASC proliferation and metabolic health. The 5× Zinc variant had the highest metabolic activity by Day 56 compared to the mineralized and 10× Zinc groups (Fig. 5A). The 5× Zinc variant had the greatest pASC expansion, and this was especially noticeable at Days 14, 28, and 56 (Fig. 5B). Curiously, we observed a significant decrease in cell number after Day 7 in the 10× Zinc variant (Fig. 5B). This effect was not seen in the short term cell activity screen (Fig. 4); further, cell number and activity in the 10× Zinc variant rebounded by Day 56, and while not as strong as the 5x Zinc variant were consistent with results observed with our conventional mineralized collagen scaffold. Analysis of gene expression profiles displayed that all scaffold variants supported osteogenic activity of the seeded pASCs, despite the lack of osteogenic supplements (Fig. 6). Notably, zinc functionalized scaffolds supported increased levels of *COL1A2* (5× Zinc: Day 4), *BMP2* (10× Zinc: Day 1) and *RUNX2* (5× Zinc:

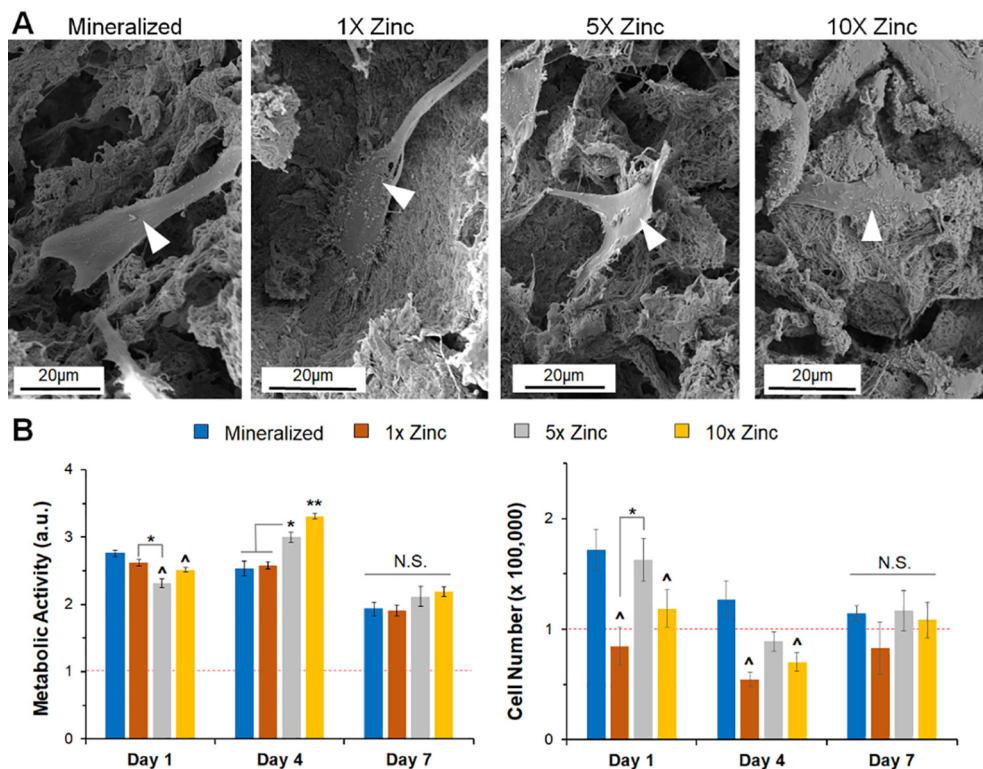


Fig. 4. Short-term cell morphology and activity on mineralized and zinc scaffolds. (A) SEM images of seeded scaffolds show healthy cell spreading and morphology on all variants. (B) Metabolic activity for porcine derived adipose stem cells on all scaffold variants. No significant differences were seen between mineralized and 1× zinc groups for any timepoint. (C) Cell number showed no significant impact of zinc concentration on cell number by Day 7. (Magnifications – 2,400×, 2,500×, 2,400×, 2,500×; 16-bit Tiff images; Cropped to size only). *: significance at $p < 0.05$ for indicated groups. ^: significance at $p < 0.05$ compared to mineralized scaffold. **: significant at $p < 0.05$ compared to all groups. N.S.: no significance between indicated groups.

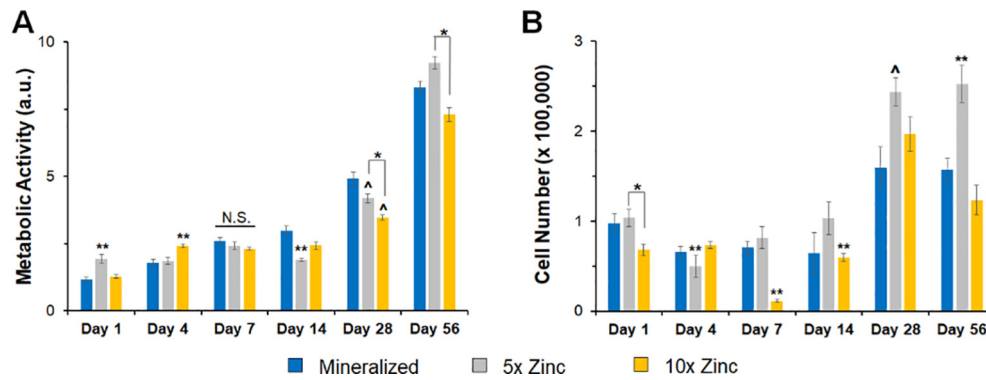


Fig. 5. Long-term cell metabolic activity and cell number. (A) Metabolic activity of pASCs showing the 5× Zinc variant has the highest cell activity at Day 56. (B) Overall number of attached pASCs showing the 5× Zinc variant has the highest cell number starting at Day 7, becoming significant by Day 28. *: significance at $p < 0.05$ for indicated groups. ^: significance at $p < 0.05$ compared to mineralized scaffold. **: significant at $p < 0.05$ compared to all groups. N.S.: no significance between indicated groups.

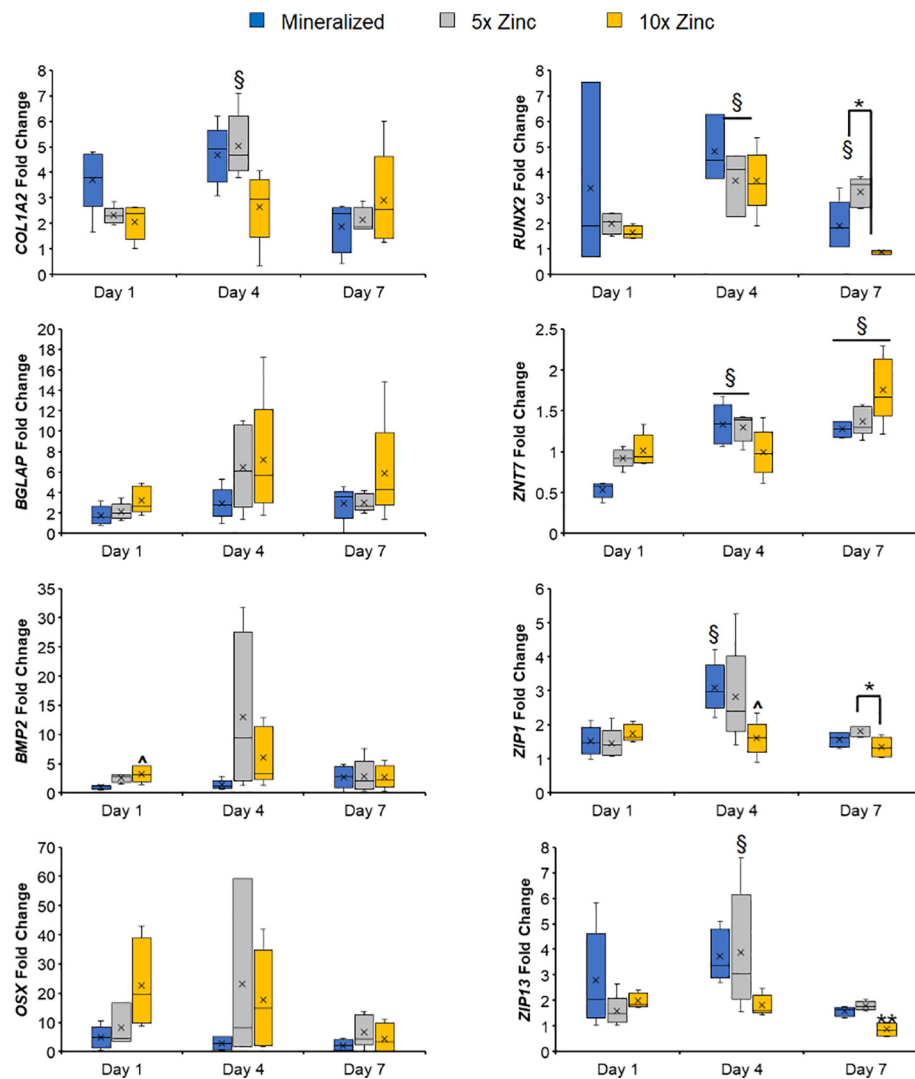


Fig. 6. Gene expression profiles for osteogenesis and zinc transporters. All scaffolds display upregulated genes related to osteogenesis – COL1A2, BGLAP, BMP2, OSX, and RUNX2 – and more dynamic expression profiles of zinc transporters – ZNT7, ZIP1, and ZIP13. See Supplemental Fig. 2 for long-term gene expression profiles. *: significance at $p < 0.05$ for indicated groups. ^: significance at $p < 0.05$ compared to mineralized scaffold. **: significant at $p < 0.05$ compared to all groups. N.S.: no significance between indicated groups. #: significant ($p < 0.05$) decrease compared to Day 1 within experimental group. \$: significant ($p < 0.05$) increase compared to Day 1 within experimental group.

Day 7). pASCs in zinc functionalized scaffolds also exhibited trends towards increased expression of the osteoblast-specific transcription factor OSTERIX (*OSX*; 5× Zinc: Days 4, 7; 10× Zinc: Days 1, 4, 7), the bone protein osteocalcin (*BGLAP*; 5× Zinc: Day 4; 10× Zinc: Days 1, 4, 7), and *BMP2* (5× Zinc: Days 1, 4; 10× Zinc: Day 4).

Interestingly, zinc functionalized scaffolds did not have increased expression of zinc transporters *ZNT7*, *ZIP1*, and *ZIP13* compared to the mineralized scaffolds, except *ZNT7* expression at Day 1 (Fig. 6). Additionally, there is an interesting trend in which *ZNT7* expression increases until Day 7 in all scaffold variants and then decreases to Day 56 while *ZIP13* expression decreases until Day 7 in all scaffold variants and then increases to Day 56 (Supp. Fig. 3).

3.6. Zinc-functionalized scaffolds support new mineral deposition

In addition to metabolic health and gene expression, we evaluated the extent of mineral formation in pASC seeded scaffolds over the course of the 56-day experiment via micro-CT, comparing new mineral formation to unseeded scaffold controls (Fig. 7). Zinc functionalized scaffold controls had more and larger particles than mineralized scaffold controls at Day 0 (Fig. 7A–C). All scaffold had more particles at Day 28 and 56 compared to Day 0 controls (except for 5× Day 28 unseeded group) (Fig. 7B). We observed significantly more particles present in all scaffolds at Day 56 vs. to 28,

showing mineral deposition over time. Further, 5× and 10× Zinc scaffolds contained more particles than unseeded controls by Day 56, though the particles were significantly smaller compared to Day 0 controls (Fig. 7C).

4. Discussion

This work builds on our recent development of a calcium phosphate mineralized collagen scaffold for craniomaxillofacial bone repair applications. Previously we have reported control over mineral weight percent (0–80%) and phase, pore size (100–250 μm), mechanical stiffness, and rate at which a portion of the calcium phosphate mineral is eluted from the scaffold as an osteogenic signal [14,27,32,44,45]. We identified a 40 wt% calcium phosphate variant that promotes human mesenchymal stem cell osteogenic differentiation in the absence of osteogenic supplements (BMP-2, osteogenic media) [14,44,46,47] as well as a mechanism for scaffold induced osteogenesis: activation of canonical (Smad1/5/8) [15] and Smad-independent (ERK1/2, Akt, p38 MAPK) BMP receptor signaling pathways [48]. While the mineralized collagen scaffold shows promising results in both *in vitro* osteogenesis and *in vivo* regeneration studies [17,18], the inclusion of bioactive factors to further enhance osteogenesis and matrix production may be particularly important for large, geometrically complex craniomaxillofacial defects. Recently, studies begun to explore the use of mineral

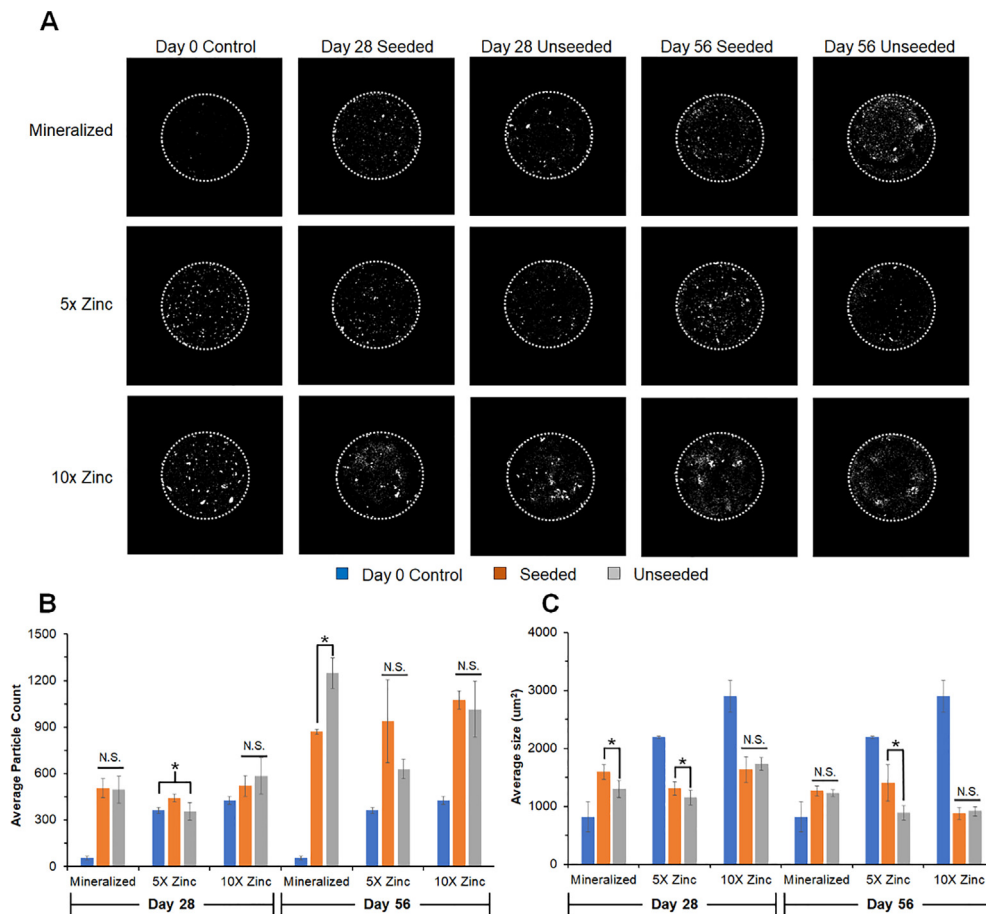


Fig. 7. Micro-CT analysis of mineral remodeling over long-term *in vitro* culture. (A) Representative images (middle slice from each image stack) of mineralized, 1× Zinc, and 5× Zinc scaffolds along with unseeded controls. Diameter of scaffold is 6 mm (dotted white line). (B) Average particle count representative of mineral nodules apparent within the scaffold. Overall, number of nodules increased from Day 28 to Day 56. All groups had significantly more particles than the unseeded Day 0 controls by Day 56. (C) Average particle size within scaffolds, showing mineralized scaffolds display larger particles compared to their unseeded Day 0 controls while the zinc functionalized scaffolds display significantly smaller particles compared to their unseeded Day 0 controls. *: significance at $p < 0.05$ for indicated groups. N.S.: no significance between indicated groups.

additives such as strontium and zinc to aid regenerative therapies for musculoskeletal injuries [49]. And with recent efforts examining the use of soluble zinc to aid osteogenesis [23,50–52], including enhancing osteogenic differentiation of pASCs [25], here we examine incorporation of zinc ions into the mineral phase of a mineralized collagen scaffold and its influence on scaffold biophysical properties and pASC activity.

With the addition of zinc sulfate into the mineralized scaffolds, we did not observe a loss of the open pore network of the scaffold (Fig. 1A) nor significant differences in mean pore size in the 1× and 5× Zinc variants (Table 2). However, we observed significant differences in the mineral microstructure, with zinc functionalized scaffolds presenting elongated crystal structures while traditional mineralized scaffolds present flattened, plate-like crystal structures commonly associated with brushite (Fig. 1B). These findings inspire ongoing efforts to interrogate changes in cell interactions with elongated brushite crystallites, particularly their role in macrophage-scaffold interactions where material anisotropy may be instructive [53]. Interestingly, X-ray diffraction (XRD) analysis confirmed the mineral content of all scaffold variants was brushite (Fig. 2A–B). Unit cell analysis confirmed these findings, showing no change in brushite unit cell characteristics with increasing concentrations of zinc in the scaffold (Fig. 2C). Zinc modified scaffolds (5× and 10× Zinc groups) also exhibited increased overall modulus (Fig. 2F); while modulus is reported for non-hydrated scaffolds we have previously shown that while hydration alters the absolute values of reported moduli it does not alter relative differences in moduli between groups [32,38]. These data suggest the inclusion of sufficient zinc content (5x, 10x) can significantly enhance scaffold elastic modulus, although the role of the added zinc is unclear (overall increase in scaffold relative density, targeted effect of shifts in calcium phosphate morphology) and subject to future characterization. While the modulus of all scaffold variants is sub-optimal for *in vivo* use, separate efforts by our group have described incorporating polymeric support frames into the scaffold to form a collagen-polymer composite with increased compressive strength [28,54] and radial conformal fitting capabilities [54] that contribute to improved craniofacial bone repair in a Yorkshire pig mandible defect model [17].

We quantified the weight percent of zinc, calcium, and phosphorous incorporated into the freeze-dried scaffold structure (Fig. 2D–E). We observed no relationship between incorporated zinc and the brushite weight percent (Fig. 2E), suggesting the inclusion of zinc ions does not alter the amount of brushite precipitated into the scaffolds. Thus, observed differences in XRD peak intensity are likely a result of preferred crystal orientation, not brushite concentration. Plate-like and needle-like crystals result in different peak intensities due to differences in shape and direction of crystal growth. Together, these data suggest that the addition of zinc into our precursor suspension alters *how* brushite is precipitating within the scaffold. These morphological changes we see are consistent with work done by Madsen and Pederson, in which they tested the effects of metal ions on brushite formation [55,56]. In this work they found that zinc was the only ion that caused aggregates of brushite to form, and these aggregates look like the brushite crystal morphology present in our freeze-dried zinc functionalized scaffolds. From our ICP-MS analysis, we observed approximately fifty percent of zinc added to the collagen suspension (across all zinc concentrations) was incorporated into the final scaffold (Fig. 2D). Furthermore, we observed zinc release curves from the mineralized collagen scaffolds that showed dose-dependence (Fig. 3). Interestingly, we created zinc scaffold variants with the goal of matching ion concentrations near the 0.04 mM concentration recently identified by Bertels et al. to promote pASC osteogenesis in 2D tissue culture [25]. We calculated the amount of zinc included into our scaffolds based on previous efforts that

characterize release of calcium and phosphate ions from the mineralized collagen scaffold [14]. While the observed zinc release in this experiment was below this target, we still observed an influence of zinc on enhanced pASC metabolic activity and cell number, setting the stage for future efforts to identify an optimal range for zinc inclusion into three-dimensional scaffolds for bone repair.

A critical finding from this work is that zinc incorporation does not have negative consequences on porcine adipose derived stem cell (pASC) activity. pASCs have previously been shown to have strong osteoinductive properties [39,57–60], making them intriguing targets for craniofacial bone repair applications. pASCs were able to attach and spread on all scaffold variants (Fig. 4A), with no significant differences with the addition of zinc ions. Further, we observed no short-term negative consequences in terms of altered metabolic activity or cell number due to the incorporation of zinc at any tested dose (Fig. 4B). Due to the low concentration of released zinc, largely similar short-term cell response compared to the mineralized scaffold, and the less robust elongated zinc deposits in the 1× zinc variant (Supp. Fig. 1), we chose to remove this group from the extended (8-week) cell activity experiment. Over an elongated culture period, the 5× zinc scaffold group displayed the highest metabolic activity and cell number by Day 56. The 5× Zinc variants had the highest metabolic activity as early as Day 7, with significant differences occurring by Day 28 (Fig. 5). High cell number and increased metabolic activity are important for clinical applications, because we want to see cell proliferation quickly in our defect area in addition to high cell metabolic activity.

Analysis of gene expression profiles across the extended culture reveal a signature that zinc modified scaffolds support pASC osteogenesis. Genes important for bone remodeling and osteogenesis (i.e. all genes besides the zinc transporters – ZNT7, ZIP1, and ZIP13) were upregulated at all timepoints, indicating addition of zinc does not negatively impacting the cells ability to deposit collagen matrix and early osteoblast differentiation [61–63]. Examining zinc transporters, we observed that as ZNT7 expression decreased after Day 7, ZIP13 expression increased, consistent with previous finding that ZnTs decrease cytosolic zinc concentrations while ZIPs increase cytosolic zinc concentrations [64]. Higher expression of ZIP1 and ZIP13 in mineralized and 5× zinc scaffolds suggests cells are trying to transport zinc into their cytosol. With the highest ZnT7 in 10× and 5× Zinc variants, cells are likely decreasing their cytosolic zinc content at earlier timepoints as compared to cells within conventional mineralized scaffolds. These finding motivate future study of a larger selection of zinc transporters via both PCR and Western Blot which would provide more information about the zinc transport in our zinc functionalized scaffolds. While recent work using pASCs shows the osteogenic benefit of zinc ions motivated this study to examine the behavior of pASCs on zinc-functionalized scaffolds, the relative difficulty of finding porcine PCR primers and Western Blot antibodies motivate future studies to examine the role of zinc-functionalized scaffolds using human mesenchymal stromal cells and examining the broad array of identified zinc transporters in human cells [64].

Analysis of mineral deposits via microcomputed tomography (micro-CT) showed increasing number of mineral deposits for all scaffold variants from Day 28 to 56. However, the sizes of particles shifted substantially, with increasing particle size for mineralized scaffolds but decreasing in zinc functionalized scaffolds (relative to Day 0 control). Drivers for changes in particle size include cell mediated deposition, but also shifts due to the release of mineral from the scaffold. Previous studies of mineralized scaffolds showed significant release of Ca and P ions from the scaffolds [14]. Given the particle size of zinc functionalized scaffolds was significantly different (needle-like deposits vs. conventional Brushite plates), the challenge of comparing overall changes in mineral particle size

as a function of time (and mineral release) is difficult. While this work focuses on the development and thorough characterization of a novel class of zinc functionalized mineralized collagen scaffolds for craniofacial bone regeneration application, an important future stage of this work is in depth *in vivo* analysis of bone repair. We have recently completed initial evaluation of bone regeneration induced via the mineralized collagen scaffolds using both a rabbit calvarial defect [18] and a porcine mandible defect model [17]. In these models the mineralized collagen scaffold promoted robust bone regeneration, and we identified a role for the scaffold in both promoting osteogenic differentiation and bone synthesis but also acting to inhibit osteoclast activity [65,66]. The enhanced cell activity identified within zinc-functionalized mineralized collagen scaffolds suggest that an essential next step for these materials is functional assessment via these robust preclinical models.

5. Conclusions

A key challenge for biomaterials designed for craniomaxillofacial bone regeneration is the capacity to promote robust osteoinduction, biomaterial driven cell recruitment, osteogenic differentiation, and new matrix biosynthesis. Here we report modification of a mineralized collagen scaffold under development for craniomaxillofacial bone repair via inclusion of zinc ions as an osteogenic supplement. We report the effects of zinc supplementation on scaffold biophysical and compositional properties as well as the results of *in vitro* cell activity experiments. Notably, zinc-functionalized scaffolds retain the brushite phase of calcium phosphate mineral, but displayed altered mineral microstructure, increased elastic moduli, and with *in vitro* testing reveal the zinc-functionalized scaffold can enhanced the overall number and cell metabolic activity of pASCs seeded within the scaffold. Together, these findings suggest the zinc-functionalized mineralized collagen scaffold provides a biomaterial platform to investigate *in vitro* the role of zinc inclusion on osteogenic signal transduction via calcium and zinc ion transporters as well as to examine new bone formation via a series of established porcine and rabbit craniofacial bone defect models.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.actbio.2019.05.031>.

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