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Research Article

Exogenous lysyl oxidase-like 2 and perfusion culture induce collagen crosslink formation in osteogenic grafts[†]

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Abbreviations

LOXL2	Lysyl oxidase-like 2
MSCs	mesenchymal stem cells
PYD	pyridinoline
DPD	deoxypyridinoline
ECM	extracellular matrix
HA-PLG	hydroxyapatite-poly(lactide-co-glycolide)
HL	hydroxylysine

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ABSTRACT

Lysyl oxidase (LOX)-mediated collagen crosslinking can regulate osteoblastic phenotype and enhance mechanical properties of tissues, both areas of interest in bone tissue engineering. The objective of this study was to investigate the effect of lysyl oxidase-like 2 (LOXL2) on osteogenic differentiation of mesenchymal stem cells (MSCs) cultured in perfusion bioreactors, enzymatic collagen crosslink formation in the extracellular matrix (ECM), and mechanical properties of engineered bone grafts. We administered exogenous LOXL2 to MSCs seeded in composite scaffolds under perfusion culture for up to 28 days. Constructs treated with LOXL2 appeared brown in color and possessed greater DNA content and osteogenic potential measured by a 2-fold increase in bone sialoprotein gene expression. Collagen expression of LOXL2-treated scaffolds was lower than untreated controls. Functional outputs such as calcium deposition, osteocalcin expression, and compressive modulus were unaffected by LOXL2 supplementation. Excitingly, LOXL2-treated constructs contained 1.8- and 1.4-times more pyridinoline (PYD) crosslinks per mole of collagen and per wet weight, respectively, than untreated constructs. Despite these increases, compressive moduli of LOXL2-treated constructs were similar to untreated constructs over the 28-day culture duration. This is the first report of LOXL2 application to engineered, three-dimensional bony constructs. The results suggest a potentially new strategy for engineering osteogenic grafts with a mature ECM by modulating crosslink formation.

INTRODUCTION

The extracellular matrix (ECM) is composed of a tissue-specific repository of proteins, proteoglycans, and sequestered growth factors [1]. Biomaterial-based approaches to bone tissue engineering seek to recapitulate characteristics of the ECM for delivery of bone-forming cells such as mesenchymal stem cells (MSCs) [2, 3]. MSCs are a heterogeneous mixture of progenitor cells with multilineage potential including differentiation to bone-forming osteoblasts when given the appropriate signals. Among other components, collagen plays a significant role in the bone ECM, providing a structural framework for mineralization and integrin-binding sites for directing cell function [3-5]. Post-translational modifications of collagen leading to enzymatic crosslink formation are a natural part of ECM maturation that may affect bone healing. Crosslink formation is mediated by lysyl oxidase (LOX) and its isoforms, LOX-like proteins (LOXL1-4) [6, 7]. LOX deficiency is associated with osteoporosis and weak bones [8, 9]. LOX inhibition results in osteolathyrism and impairs bone and cartilage formation and function both *in vitro* and *in vivo* [10]. Conversely, increases in crosslink formation correlated with improved mechanical properties of the callus developed in a rat model of fracture repair [11]. Treatment of MC3T3-E1 murine preosteoblasts with beta-aminopropionitrile (β -APN), a well-known specific and irreversible inhibitor of LOX activity [12] that targets the active site of LOX and its isoforms, leads to diminished osteoblastic differentiation and crosslink formation in monolayer culture [13, 14]. Collectively, these data suggest that proper enzymatic crosslink formation is essential for osteoblastic differentiation and increases in crosslink formation may provide an avenue to enhance formation of osteogenic grafts. To the best of our knowledge, the relationship between LOX-mediated collagen crosslinks and human MSC osteogenic response has not been reported in 3D culture systems.

LOX is a copper-dependent enzyme produced intracellularly and secreted into the extracellular space where it facilitates pyridinoline (PYD) and deoxypyridinoline (DPD) crosslink formation [7, 15]. Copper-activated LOX is necessary for the critical first step in crosslink formation, where telopeptide lysine and hydroxylysine (HL) residues on collagen fibrils are converted to their aldehyde forms. While lysine is a common component of cell culture media, HL and CuSO_4 must be added to culture media to

promote LOX function [15]. Endogenous LOX upregulation, stimulated through hypoxia or HL and CuSO₄ supplementation, and exogenous LOXL2 supplementation correlate with enhanced compressive and tensile stiffness of native and engineered neocartilage [15, 16]. Overexpression of LOX by genetically modified smooth muscle cells led to increased tensile strength and elastic modulus in engineered vascular constructs [17]. LOXL2 was defined as a critical signaling mediator for chondrogenic differentiation in ATDC5 cells *in vitro* and was also upregulated in chondrocytes during the chondrogenic phase of fracture healing. However, both LOX and LOXL2 were detected in osteoblasts during early fracture healing, suggesting that each may play a role in osteogenic differentiation [18]. Despite reports of the effect of LOX and its paralogues on enhancing mechanical properties and generating functional cartilage and vascular constructs, literature regarding the effect of LOX in bone is limited to LOX-inhibition studies. As increasing mechanical properties of engineered grafts is a key target for bone tissue engineering and in light of similar successes using exogenous LOXL2, we investigated the effect of LOXL2 supplementation during the formation of three-dimensional engineered bony constructs.

Bioreactor culture of three-dimensional osteogenic constructs can improve nutrient availability, facilitate homogenous cell seeding, and enhance osteogenic differentiation and ECM deposition [19-21]. We recently demonstrated that MSCs on composite scaffolds exhibited superior osteogenic differentiation *in vitro* when maintained under perfusion culture for at least 14 days, and these constructs resulted in more bone formation *in vivo* compared to constructs cultured for shorter durations [21]. In this study, we sought to build upon our previous work with perfusion culture by supplementing culture media with LOXL2 to create a mature osteogenic graft. As LOXL2 activity was visible in osteoblasts during fracture healing [18], we hypothesized that LOXL2 treatment of MSC-seeded constructs cultured under continuous perfusion would provide a novel strategy to further enhance ECM maturity and mechanical properties of these grafts. We seeded MSCs in hydroxyapatite-poly(lactide-co-glycolide) (HA-PLG) scaffolds and maintained them in direct perfusion bioreactors for up to 28 days in order to answer the following questions: Does LOXL2 treatment 1) induce enzymatic crosslink formation; 2) enhance the

osteogenic differentiation potential of MSCs cultured under perfusion; and 3) improve the mechanical properties of engineered constructs?

MATERIALS AND METHODS

Scaffold preparation

Composite scaffolds were fabricated using a gas foaming/particulate leaching method as described previously [21-23]. Briefly, microspheres composed of PLG (85:15, DLG 7E; Lakeshore Biomaterials, Birmingham, AL) were prepared using a double-emulsion process and lyophilized to form a free-flowing powder. 9.2 mg of lyophilized microspheres were combined with 23.1 mg of HA crystals (particle size 100 nm, Berkeley Advanced Biomaterials, Berkeley, CA), and 175.6 mg of NaCl particles (300-500 μ m) to yield a 2.5:1:19 mass ratio of ceramic:polymer:salt. The powdered mixture was then compressed for 1 min into solid disks (final dimensions: 8 mm in diameter and 2 mm in height) using a Carver Press (Carver, Inc., Wabash, IN). Compressed disks were exposed to high pressure CO₂ (5.5 MPa) for at least 16 hrs followed by rapid pressure release to cause polymer fusion. NaCl particles from the scaffolds were then leached in distilled H₂O for 24 hrs. Scaffolds were sterilized by 70% ethanol under gentle vacuum, followed by two rinses in sterile phosphate buffered saline (PBS) and then dried in a sterile biosafety cabinet.

Cell culture

Human bone marrow-derived MSCs (Lonza, Walkersville, MD) were expanded without further characterization in growth medium (GM) consisting of minimum essential alpha medium (α -MEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin/streptomycin (Gemini Bio-Products, Sacramento, CA). Cells were cultured under standard conditions (37°C, 21% O₂, 5% CO₂) and utilized at passage 5. For all experiments, cells were initially seeded in GM and then exposed to osteogenic medium (OM: GM supplemented with 10

mM β -glycerophosphate, 50 μ g/mL ascorbate-2-phosphate, and 100 nM dexamethasone, all from Sigma Aldrich, St. Louis, MO).

Hydroxylysine and copper sulfate treatment

Cells were plated in GM at 30,000 cells/cm² in 12-well plates and allowed to adhere for 1 day. Thereafter, monolayer cultures were exposed to OM supplemented with varying levels of HL and CuSO₄ (both from Sigma Aldrich) for up to 21 days to determine potential dose-dependent effects on cell function with these supplements prior to any LOXL2 supplementation studies. HL was added at 0.0146, 0.146, or 1.46 mg/mL, while CuSO₄ was added at 0.00016, 0.0016, or 0.016 mg/mL. Following a full-factorial design with two factors of three levels each, we treated MSCs with nine different combinations of supplements. MSCs in OM served as the control group.

Detection of LOX activity

LOX activity in MSCs in monolayer culture was determined using a LOX activity kit (Abcam, Cambridge, MA). Media samples were collected from well plates and analyzed following the manufacturer's instructions. The resulting fluorescence was read on a microplate reader (excitation/emission= 540/590 nm) and recombinant LOXL2 (R&D Systems, Minneapolis, MN) was used as a standard.

Seeding MSCs on HA-PLG constructs in bioreactors and stimulation with LOXL2

Cells were seeded in bioreactors as described [21, 24]. Briefly, scaffolds were installed in U-CUP flow perfusion bioreactors (Cellec Biotek, Basel, Switzerland) and 10 mL of GM was injected through the bottom port into the bioreactor. Scaffolds were soaked in medium for 30 min before seeding. MSCs (1.2×10^6 in 2 mL GM) were injected *via* the top port into the bioreactor. Up to 10 individual bioreactors were then connected to each syringe pump (Harvard Apparatus, Holliston, MA) to maintain media at a superficial velocity of 3 mL/min for 15-18 hrs. GM was replaced with OM after seeding, and constructs

were maintained for up to 28 days in media supplemented with 0.146 mg/mL HL, 0.0016 mg/mL CuSO₄, and varying concentrations of LOXL2 (0.0015 ng/mL, 0.015 ng/mL, and 0.15 ng/mL; Signal Chem, Richmond, Canada). Regardless of study duration, media was supplemented with CuSO₄/HL/LOXL2 from days 7 to 21 of culture. Constructs received supplements every 3-4 days when media was changed.

Analysis of DNA content, osteogenic differentiation, and construct mechanical properties

Scaffolds were retrieved from bioreactors and washed twice with PBS. A 5 mm biopsy punch (Integra Miltex, York, PA) was used to harvest a disk that was first used for compressive testing and then subsequently for qPCR analysis. The compressive moduli of composite scaffolds were determined using an Instron 5800 Series Testing System (Instron, Norwood, MA). Scaffolds were compressed at a constant deformation rate of 1 mm/min, and compressive modulus was calculated using the linear portion of the stress-strain curve. Samples were processed for qPCR analysis thereafter as described [25]. Samples were collected in TRIzol reagent (Invitrogen) following the manufacturer's protocol. Total RNA was isolated using an RNEasy micro kit (Qiagen, Valencia, CA). RNA was reverse transcribed with the QuantiTect Reverse Transcription kit (Qiagen). qPCR was performed using a QuantiFast Probe PCR kit (Qiagen) on a CFX96 Real-Time System (Bio-Rad, Hercules, CA). Primers and probes for *RPL13* (HS00204173_m1), *COL1A1* (HS00164004_M1), and *IBSP* (HS00173720_M1) were purchased from Applied Biosystems (Foster City, CA). Amplification conditions were 95°C for 3 min, followed by 40 cycles at 95°C for 3 sec and 60°C for 30 sec. Quantitative PCR results were normalized to RPL13 transcript levels to yield ΔC_t , and fold change in expression relative to the housekeeping gene was calculated using $2^{-\Delta C_t}$. The remainder of the scaffold was collected in 250 μ L of passive lysis buffer (Promega, Madison, WI), frozen at -20°C, thawed, sonicated, centrifuged at 5000 rpm for 10 min to pellet the cell debris, and the supernatant was collected for DNA analysis. The supernatant was analyzed for DNA content using the Quant-iT PicoGreen dDNA Assay Kit (Invitrogen) following manufacturer's instructions. The remaining pellet was resuspended in 0.9 N H₂SO₄ and incubated overnight at 37°C. Calcium deposition was quantified by reacting with *o*-cresolphthalein complexone as previously described [26, 27]. The

calcium content of an unused composite scaffold was subtracted from that of cultured composite scaffolds to determine the amount of calcium deposited by MSCs.

To visualize cell distribution and markers of osteogenic differentiation within constructs, samples were washed twice in PBS and fixed in 10% buffered formalin acetate (Fisher Scientific, Fair Lawn, NJ) for 24 hrs at 4°C. Samples were then washed twice in PBS to remove residual formalin acetate and preserved in 70% ethanol at 4°C until further processing. Prior to cryosectioning, samples were soaked in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) overnight to allow OCT to perfuse through the entire scaffold. Constructs were then placed in fresh OCT and frozen on dry ice. 5 µm sections were cut on a Leica CM1850 Cryostat (Leica Microsystems, Bannockburn, IL) and mounted onto microscope slides for analysis. Sections were stained using hematoxylin and eosin (H&E) following standard protocols and immunostained using an anti-osteocalcin (OCN) antibody (1:200, AB13420, Abcam).

Detection and quantification of collagen crosslinks in engineered mineralized tissues

Constructs were washed twice in PBS and tissue wet weight was recorded. Samples were then lyophilized overnight prior to recording dry weight. Samples were decalcified with Calci-Clear Rapid (National Diagnostics, Atlanta, GA) at room temperature for 24 hrs. Decalcified samples were hydrolyzed in 500 µL of 6N HCl at 110°C for 24 hrs. Following hydrolysis, samples were dried using a vacuum concentrator and split into two equal fractions for a collagen content assay and PYD and DPD crosslink analysis. Samples used for HPLC were resuspended in a solution of 10 nmol pyroxydine per mL of water. Samples were then diluted five times with a solution of 10% v/v acetonitrile and 0.5% v/v heptafluorobutyric acid (HFBA) in water to prepare for reverse phase chromatography. For the assay, 50 µL of each sample was injected into the reverse phase column and eluted over 37 min using the following solvent profile: Solvent A (24% v/v methanol and 0.13% v/v HFBA) from 0-17 min, Solvent B (75% v/v acetonitrile and 0.1% HFBA) from 17-27 min, and Solvent A again from 27-37 min. A PYD/DPD calibrator (Quidel, San Diego, CA) was prepared as a standard and analyzed during each run to permit quantification of pyridinoline content in each sample.

Quantification of collagen content

Collagen content in engineered constructs was determined using a hydroxyproline assay as described previously [28]. Briefly, the remaining portion of hydrolysate from HPLC sample preparation was first reacted with 150 μ L Chloramine T (Sigma Aldrich) and incubated at room temperature for 20 min. 150 μ L aldehyde-perchloric acid solution (150 μ L) was then added to each sample and incubated at 60°C for 15 min. Following incubation, tubes were left to cool for 10 min and sample absorbance was read on a microplate reader. Hydroxyproline was converted to collagen mass assuming that collagen contains 13.7% hydroxyproline [29].

Statistical analysis

All data are presented as means $\pm$ standard deviation of the mean. Statistical analysis was performed using unpaired t-tests, one-way ANOVA, and two-way ANOVA with Tukey's multiple comparison *post-hoc* test, where appropriate. All statistical analysis was performed in Prism 6 software (GraphPad, San Diego, CA), and *p* values less than 0.05 were considered statistically significant. Significance is denoted by alphabetical letterings; groups with no significance are linked by the same letters, while groups with significance do not share the same letters.

RESULTS

HL and CuSO₄ influence collagen deposition and LOX activity

In the absence of exogenous LOX supplementation, we examined the effect of HL and CuSO₄ supplementation on collagen deposition and endogenous LOX activity of MSCs in monolayer culture at 14 and 21 days to determine necessary concentrations for use in future studies. At 14 days, we observed similar quantities of collagen deposition following MSC stimulation with all levels of HL and CuSO₄ (**Fig. 1A**). Importantly, high HL/high CuSO₄ yielded lower collagen content than all groups within the medium CuSO₄-treated groups. Collagen content in the high HL/low CuSO₄ group was also significantly lower

than low HL/med CuSO₄ group. At 21 days, all groups had similar or higher collagen content than the control group (**Fig. 1B**). Collagen deposition was comparable for low and medium HL groups across all CuSO₄ groups. Collagen secreted by MSCs stimulated by medium HL levels was enhanced compared to high HL within the low and high CuSO₄ groups.

All treated groups exhibited similar or higher endogenous LOX activity than OM at 14 days. In particular, medium and high HL treatments within the high CuSO₄ group exhibited significantly higher activity than all treatments within the low and medium CuSO₄ groups (**Fig. 1C**). LOX activity was similar in all low and medium CuSO₄ groups. After 21 days in culture, all CuSO₄-treated groups except for low HL- and high HL/low CuSO₄ had higher LOX activity than the OM control (**Fig. 1D**). LOX activity was reduced in all groups after 21 days compared to 14 days. With a goal of using fewer additives, we supplemented osteogenic media with medium levels of HL and CuSO₄ (denoted as OM*) for future studies. This concentration of supplements did not induce detectable differences in proliferation or metabolic activity when measuring DNA content or using an alamarBlue assay, respectively (*data not shown*).

LOXL2 treatment influences MSC proliferation after 21 days of culture

Constructs were treated low, medium, or high levels of LOXL2 corresponding to 0.0015, 0.015, and 0.15 ng/mL, respectively. Constructs exposed to medium and high levels of LOXL2 had a brownish appearance compared to the pink color of OM- and low LOXL2-treated constructs (**Fig. 2B**). We observed homogeneous cell distribution throughout scaffolds of all groups following H&E staining (**Fig. 2D**). DNA content increased with increasing LOXL2 concentration, with medium and high LOXL2 groups exhibiting significantly higher DNA content than the OM control (**Fig. 2C**). We then evaluated the effect of LOXL2 supplementation on the osteogenic response of MSCs *via* gene expression, calcium quantification, and immunohistochemistry. Bone sialoprotein (*IBSP*) gene expression, an intermediate-to-late marker of osteogenic differentiation, was increased in medium and high LOXL2-treated constructs relative to OM control and low LOXL2 groups (**Fig. 3A**). *COL1A1* expression exhibited the opposite trend, with

decreasing collagen expression as a function of increasing LOXL2 treatment (**Fig. 3B**). Both medium and high LOXL2-treated groups had significantly lower *COL1A1* expression than low LOXL2 constructs, but only high LOXL2 was significantly lower than the OM control.

Functional properties of engineered constructs are not affected by LOXL2

Functional properties of engineered constructs were not altered by LOXL2 treatment. Similar levels of total calcium were detected in constructs within all groups of this study (**Fig. 3C**). All LOXL2-treated constructs possessed similar compressive moduli to each other and the control group (**Fig. 3D**). Immunohistochemical evaluation for osteocalcin (OCN) corroborated calcium quantification results. No apparent qualitative differences were observed in OCN expression as a result of LOXL2 supplementation (**Fig. 3E**).

Extended culture duration sustains MSC osteogenic differentiation

In order to allow time for LOXL2-mediated immature crosslinks to form mature enzymatic crosslinks, we cultured constructs for an additional 7 days after the last LOXL2 supplementation and evaluated osteogenic differentiation of MSCs at 28 days (**Fig. 4A**). We treated constructs with medium-level LOXL2 only, since low LOXL2 treatment did not enhance osteogenic differentiation relative to controls and high LOXL2 did not impart significant advantages over medium LOXL2. Similar to the 21-day study, we observed brown discoloration of constructs due to LOXL2 treatment (**Fig. 4B**). We did not observe significant differences in DNA content of treated constructs compared to OM controls following extended culture (**Fig. 4C**), and H&E stains revealed comparable cell distribution between OM and LOXL2-treated scaffolds (**Fig. 4D**). Similar to the 21-day study, we observed increased osteogenic gene expression of MSCs at 28 days due to LOXL2 supplementation. *IBSP* expression was significantly greater in LOXL2-treated constructs (**Fig. 5A**). Again, constructs cultured for 28 days exhibited lower *COL1A1* expression relative to OM controls (**Fig. 5B**). Even with 28 days of culture, we did not observe increases in calcium content of engineered constructs due to LOXL2 treatment (**Fig. 5C**). Compressive moduli of treated and

control samples were not statistically different (**Fig. 5D**), and OCN expression was comparable in both groups (*data not shown*).

Exogenous LOXL2 supplementation leads to pyridinoline crosslink formation

HPLC analysis of constructs cultured for 28 days under perfusion revealed that LOXL2 supplementation significantly increased PYD crosslinks. Compared to untreated samples, we detected 1.4-fold more total PYD, 1.4-fold more PYD/wet weight, and 1.8-fold more PYD/mol of collagen (**Fig. 5E-G**). We did not detect measurable levels of DPD resulting from LOXL2 treatment (*data not shown*).

DISCUSSION

We investigated the effect of LOXL2 treatment on the osteogenic potential of MSCs cultured on engineered constructs under perfusion, mechanical properties of engineered bony tissues, and formation of enzymatic crosslinks. LOXL2 supplementation enhanced *IBSP* gene expression in MSCs, while DNA content was increased with LOXL2 in a dose-dependent manner. Functional properties of LOXL2-treated constructs, such as calcium deposition and compressive moduli, were similar to those of controls. LOXL2 supplementation led to a significant increase in PYD crosslink formation over controls at 28 days. These data demonstrate that exogenous LOXL2 application and perfusion culture may be a novel strategy for inducing enzymatic collagen crosslinks to generate osteogenic constructs with a mature ECM.

Enzymatic collagen crosslink formation in bone is mediated by LOX and its paralogues (LOXL1-LOXL4) [6]. While several studies have characterized expression of LOX and its isoforms in MC3T3-E1 cells [30, 31], few studies have reported LOX expression in MSCs. To our knowledge, no reports exist that directly measure LOX and LOXL expression in human MSCs. Fernandes *et al.* reported that LOX inhibition *via* β -APN did not affect MSC differentiation; however, this study did not measure LOX or LOXL2 expression [12]. Moreover, β -APN targets the active site of LOX and its isoforms, necessitating further study to determine if a single lysyl oxidase or paralogue is involved in osteogenic differentiation. We measured expression of LOX and its isoforms in human MSCs cultured in monolayer for 14 and 21

days and found that the most prominent LOX and LOXL-genes expressed in human MSCs are LOX, LOXL1, and LOXL2 (*data not shown*). Both LOX and LOXL2 were detected *via* immunohistochemistry in osteoblasts during early fracture healing, suggesting that each may play a role in osteogenic differentiation[18]. As LOXL2 was successfully used to enhance the mechanical properties of engineered neocartilage[15], a shared goal of this study, we supplemented media with LOXL2 given similar levels of gene expression at 21 days. Given the data from these studies and the importance of LOXL2 in chondrogenic differentiation[18], future work should investigate the effects of LOX and its other isoforms on enzymatic crosslink formation and MSC differentiation.

Copper is an essential co-factor for the activity of the LOX family of enzymes [32]. Since copper is not a common component of cell culture media, endogenous LOX secreted by cells or exogenously supplemented LOX remain in their inactive form during culture [15]. Moreover, the incorporation of both lysine and HL into collagen fibrils is essential for LOX-mediated crosslink formation. While lysine is a common ingredient in media, HL must be exogenously supplemented. Makris *et al.* demonstrated that supplementing culture medium with 0.146 mg/mL HL and 0.0016 mg/mL CuSO₄ synergistically increased PYD formation, tensile properties, and aggregate modulus of engineered neocartilage [15]. Therefore, we treated MSCs in monolayer with varying levels of both supplements and measured collagen content and LOX activity to ensure that these supplements were not detrimental to either output. From these studies, we determined that medium levels of both supplements (0.146 mg/mL HL, 0.0016 mg/mL CuSO₄) were suitable for further studies.

Perfusion culture of MSCs seeded in biomaterial scaffolds provides nutrients for cell survival, enhances extracellular matrix deposition, and increases osteogenic cell differentiation. We recently reported that constructs maintained in perfusion culture for at least 14 days resulted in the greatest expression of osteoblastic markers *in vitro* and ectopic bone formation when implanted *in vivo* [21]. The goal of this study was to determine if LOXL2 supplementation and bioreactor culture could synergistically improve ECM maturity and create an enhanced osteogenic graft. The effect of LOX-mediated crosslinking on osteogenic differentiation has been studied with murine osteoblasts in monolayer culture [14, 31].

Furthermore, the capacity of LOX and its isoforms to improve mechanical properties of engineered neocartilage or vascular tissues has also been demonstrated in static culture [16, 17, 33]. However, continuous perfusion culture markedly increases ECM deposition by MSCs during osteogenic differentiation [21, 34, 35], providing an abundance of collagen substrate for LOX. Therefore, we applied exogenous LOXL2 to MSCs cultured in HA-PLG scaffolds under perfusion. Constructs treated with medium and high levels of LOXL2 exhibited a brown appearance compared to the pinkish OM controls. Histological evaluation *via* H&E staining revealed no differences between treated and control scaffolds for either study. LOXL2 treatment increased DNA content of constructs in a dose-dependent manner at 21 days. Though LOX and its isoforms are rarely investigated for their effect on cell proliferation, Pischon *et al.* demonstrated that knocking out the LOX gene in primary calvarial osteoblasts significantly decreased DNA synthesis [31]. Furthermore, HL supplementation of culture media increased the number of cells per construct in engineered neocartilage [15]. Our data provide further evidence that HL and/or LOXL2 may play a stimulatory role in proliferation of MSCs. However, we did not observe these differences in the 28-day study in which the LOXL2 supplement cocktail was supplied until day 21 of culture. These results could be attributed to contact inhibition and reduced proliferative capacity of MSCs with increasing osteogenic differentiation, which is present in LOXL2-treated constructs relative to MSCs in untreated constructs [36].

Next, we investigated the effect of LOXL2 supplementation on the osteogenic potential of MSCs. *IBSP*, an intermediate-to-late marker for osteogenic differentiation, was significantly increased in medium and high level-treated LOXL2 constructs, suggesting that exogenous LOXL2 and mechanical stimulation may synergistically enhance osteogenic gene expression of MSCs. LOX-deficient primary calvarial osteoblasts had reduced *IBSP* expression and mineralization during osteogenic differentiation in monolayer culture [31]. Others have demonstrated that LOX inhibition by β -APN can downregulate osteocalcin mRNA expression by 75% [14]. In another study, β -APN treatment of MC3T3-E1 cells impaired osteogenic differentiation by downregulating alkaline phosphatase (ALP) activity and calcium deposition, yet human MSCs were unaffected by reduced collagen crosslinking in the same study [13].

Herein, we demonstrate for the first time that mechanically stimulated MSCs cultured in continuous perfusion bioreactors respond to LOXL2 with enhanced *IBSP* gene expression. Currently, there is no consensus on the effect of LOX on collagen expression during osteogenic differentiation. LOX knockout of murine calvarial primary osteoblasts led to a decrease in *COL1A1* expression [31], while β -APN inhibition of LOX in MC3T3-E1 cultures increased *COL1A1* expression by 140% [14]. In this study, we observed increased *IBSP* expression in human MSCs in 3D perfusion culture, together with decreased *COL1A1* expression, due to LOXL2 treatment at both 21 and 28 days. Taken together, our data demonstrate that exogenous LOXL2 administration to MSCs under perfusion initiates a cascade of events in the osteogenic program that require further examination.

Exogenous LOXL2 supplementation is a promising strategy to improve the functional properties of engineered constructs through the formation of enzymatic collagen crosslinks. Despite increases in osteogenic gene expression and DNA content, we did not observe changes in osteocalcin secretion, a late marker of differentiation, calcium deposition, or compressive modulus. The mechanical properties of bone reflect the inherent material properties of its constituents and the way in which they are arranged and interact. LOX-mediated collagen crosslinks affect the tensile strength and post-yield properties of bone [7]. Although pre-yield properties such as elastic modulus predominantly depend on the mineral phase, collagen crosslinking can influence these as well [7]. Cartilage implants are commonly evaluated using both tensile and compressive testing. Makris *et al.* demonstrated that increased PYD crosslinks correlated with increased compressive and tensile stiffness in engineered cartilage, which is primarily composed of collagen [15, 16]. While the effect of collagen crosslinks may be more easily observed in tension, the physiological role of bone is to withstand compressive stress. Therefore, we chose to measure compressive properties of our constructs. In these studies, MSCs were seeded on clinically relevant, macroporous HA-PLG composite scaffolds, which are significantly stiffer than engineered cartilage tissues. Therefore, small changes in compressive modulus due to LOXL2-mediated crosslinking may be masked in stiff biomaterials such as the ones used in this study. Although we did not detect changes in compressive modulus through compressive testing, treated constructs appeared crunchy,

indicating that LOXL2 may affect the post-yield properties such as toughness. Further studies are needed to investigate the effect of enzymatic crosslinks on tensile stiffness, strength, and toughness of engineered bony constructs.

LOX and its isoforms mediate the formation of several mature enzymatic collagen crosslinks in bone including PYD and DPD [7, 32]. In this study, we detected significant increases in total PYD crosslinks, PYD/wet weight, and PYD/mol of collagen (but not DPD) due to LOXL2 supplementation of culture medium. While total PYD provides a measure for total crosslinks formed in the engineered construct, PYD/wet weight allows us to compare the fraction of crosslinks in our constructs compared to other tissues treated with LOXL2. Moreover, PYD/mol of collagen provides insight into the degree of crosslinking in the collagen present in constructs and enables comparison with native tissues. PYD/wet weight was 1.4-fold that of controls, which is lower than the improvements in crosslink formation reported for exogenous LOXL2 supplementation of engineered neocartilage [16]. This difference may be attributed to the availability of a continuous matrix of collagen fibrils in neocartilage vs. collagen spatially separated by pores in this study. Moreover, as cartilage is predominantly composed of collagen type II and bone contains primarily collagen type I with the presence of collagen type II, there may be differences in the function of crosslinks formed between collagen subtypes. One might also speculate that this moderate increase in collagen crosslinking could be due to an insufficient amount of collagen substrate. This hypothesis is further corroborated by reduced *COL1A1* expression observed due to LOXL2 treatment. Optimizing the timing and duration of LOXL2 administration or increasing the concentration of ascorbate-2-phosphate, a stimulus for matrix deposition, may facilitate enhanced collagen deposition prior to collagen crosslinking. In terms of PYD/mole of collagen, we achieved values higher than the ones reported for native bone [7], indicating that the major limitation in our study was not the amount of available LOXL2, but rather, the scarcity of substrate in these engineered constructs. Strategies that augment collagen deposition prior to LOXL2 administration could result in a denser ECM, which upon crosslinking, may have more drastic effects on osteogenic differentiation and mechanical properties.

This study provides evidence for LOXL2 treatment as a means to regulate enzymatic collagen crosslink formation within MSC-laden engineered bony constructs. While LOXL2 supplementation led to increases in PYD crosslinks, DNA content, and gene expression of osteogenic markers of differentiation, we did not observe increases in functional outputs of differentiation or compressive modulus of engineered constructs. This work introduces exciting new avenues to the field of bone tissue engineering for improving the functional properties of osteogenic grafts.

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CONFLICT OF INTEREST STATEMENT

The authors declare no commercial or financial conflict of interest.

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FIGURE LEGENDS

Figure 1. Full-factorial study to determine concentrations of HL and CuSO₄ required for LOX-mediated crosslinking. Effect of three levels of HL and CuSO₄ on collagen deposition in the absence of exogenous LOX at 14 **(A)** and 21 days **(B)**. LOXL activity at 14 **(C)** and 21 days **(D)**. For **(A)-(D)**, one-way ANOVA and Tukey's *post hoc* tests were performed to determine statistical significance, which is denoted by alphabetical letterings; groups with no significance are linked by the same letter, while groups with significance do not share a letter (n=4).

Figure 2. Effect of exogenous LOXL2 application on morphology and DNA content of constructs cultured for 21 days. **(A)** Schematic representing the LOXL2 supplementation regime. **(B)** Gross morphology of constructs at 21 days. **(C)** DNA content of constructs at 21 days. **(D)** Representative H&E images taken at 10x magnification (scale bar represents 200 μ m) to visualize cellularity of constructs with insets taken at 4x magnification (scale bar represents 500 μ m). Black is indicative of scaffold and some cells are denoted by black arrows. For **(C)**, a one-way ANOVA and Tukey's *post hoc* test was performed to determine statistical significance, which is denoted by alphabetical letterings; groups with no significance are linked by the same letter, while groups with significance do not share a letter (n=4).

Figure 3. Effect of exogenous LOXL2 application on osteogenic differentiation and functional outputs for constructs in perfusion culture for 21 days. **(A)** *IBSP* and **(B)** *COL1A1* gene expression. **(C)** Calcium quantification and **(D)** compressive moduli of constructs. **(E)** Representative immunohistochemistry of constructs stained for osteocalcin (OCN). Black is indicative of scaffold (S); brown is indicative of OCN (scale bar represents 500 μ m). For **(A)-(D)**, one-way ANOVA and Tukey's *post-hoc* tests were performed to determine statistical significance, which is denoted by alphabetical letterings; groups with no significance are linked by the same letter, while groups with significance do not share a letter (n=5 for gene expression; n=3-4 for calcium and compressive modulus).

Figure 4. Effect of extended culture time on morphology and DNA content. (A) Schematic representing the LOXL2 supplementation regime for the 28-day study. **(B)** Gross morphology of constructs. **(C)** DNA content of constructs at 28 days (n=4). **(D)** Representative H&E images taken at 10x magnification (scale bar represents 200 μ m) to visualize cellularity of constructs and insets taken at 4x magnification (scale bar represents 500 μ m). Black is indicative of scaffold and some cells are denoted by black arrows. For **(C)**, a lack of statistical significance was determined using an unpaired t-test.

Figure 5. Effect of extended culture time on osteogenic differentiation and functional outputs. (A) *IBSP* and **(B)** *COL1A1* expression at 28 days. **(C)** Calcium quantification and **(D)** compressive moduli of constructs at 28 days. **(E)** Total PYD content at 28 days. **(F)** PYD content normalized to wet weight at 28 days. **(G)** PYD content normalized to collagen content at 28 days. For **(A)-(G)**, unpaired t-tests were performed to determine statistical significance, which is denoted by an asterisk; groups with significance are indicated with an asterisk symbol * $p < 0.05$ and ** $p < 0.01$ (n=4 for gene expression; n=3 for calcium; n=5 for compressive modulus, n=5 for PYD content).

FIGURE 1

FIGURE 2

FIGURE 4

FIGURE 5