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A bioactive material with dual integrin-targeting ligands regulates specific endogenous cell adhesion and promotes vascularized bone regeneration in adult and fetal bone defects

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ABSTRACT

Significant progress has been made in designing bone materials capable of directing endogenous cells to promote vascularized bone regeneration. However, current strategies lack regulation of the specific endogenous cell populations for vascularized bone regeneration, thus leading to adverse tissue formation and decreased regenerative efficiency. Here, we engineered a biomaterial to regulate endogenous cell adhesion and promote vascularized bone regeneration. The biomaterial works by presenting two synthetic ligands, LLP2A and LXW7, explicitly targeting integrins $\alpha4\beta1$ and $\alpha\nu\beta3$, respectively, expressed on the surfaces of the cells related to bone formation and vascularization, such as mesenchymal stem cells (MSCs), osteoblasts, endothelial progenitor cells (EPCs), and endothelial cells (ECs). *In vitro*, the LLP2A/LXW7 modified biomaterial improved the adhesion of MSCs, osteoblasts, EPCs, and ECs via integrin $\alpha4\beta1$ and $\alpha\nu\beta3$, respectively. In an adult rat calvarial bone defect model, the LLP2A/LXW7 modified biomaterial enhanced bone formation and vascularization by synergistically regulating endogenous cells with osteogenic and angiogenic potentials, such as DLX5⁺ cells, osteocalcin⁺ cells, CD34⁺/CD45⁻ cells and CD31⁺ cells. In a fetal sheep spinal bone defect model, the LLP2A/LXW7 modified biomaterial augmented bone formation and vascularization without any adverse effects. This innovative biomaterial offers an off-the-shelf, easy-to-use, and biologically safe product suitable for vascularized bone regeneration in both fetal and adult disease environments.

1. Introduction

Ideal biomaterials for successful regeneration of bone defects should achieve bone regeneration and functional vascularization simultaneously [1,2]. Currently, due to the high risk of infection, time intensity, and high-cost consumption, the cell-seeding tissue engineering biomaterials are gradually being replaced by the cell-free biomaterial that can direct endogenous cells for vascularized bone formation [3]. Remarkably, the osteo-inductive agent bone morphogenetic protein-2 (BMP-2) loaded biomaterials have been widely used for bone regeneration. However, with the increasing clinical use of BMP-2, the side effects caused by the high dose of BMP-2 have emerged, such as postoperative inflammation and abnormal ectopic bone formation [4–6]. Therefore, the clinical use of BMP-2 remains debatable, especially

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in fetal and pediatric clinical applications [7-9].

During the process of bone regeneration, integrins play a critical role in anchoring cells to the surrounding extracellular matrix (ECM) [10, 11], mediating cell migration to their destined positions [12], and regulating multiple intracellular signaling pathways [13-16]. Hence, integrins expressed on cells are promising targets to promote the adhesion of desired endogenous cells on the biomaterial, thus effectively achieving new bone formation and neovascularization. Integrins $\alpha 4\beta 1$ and $\alpha\nu\beta3$ have been identified to play the crucial roles in mediating the cell processes of bone formation and vascularization [17]. Specifically, integrin $\alpha 4\beta 1$ is highly expressed on mesenchymal stem cells (MSCs) and plays multiple roles, such as MSC homing to the defect site, promoting osteoblast differentiation and bone formation, and increasing bone mass [17]. Osteoblasts also have been shown to express integrins $\alpha 4\beta$ 1 and $\alpha v\beta 3$, which are the primary adhesion molecules for osteoblast binding to the matrix [18,19]. Integrin $\alpha 4\beta 1$ is also highly expressed on early-stage endothelial progenitor cells (EPCs) and regulates EPC retention and mobilization [20,21]. Integrin $\alpha v\beta 3$ is expressed on both endothelial cells (ECs) and late-stage EPCs and is critical in mediating EC adhesion, migration, survival, and late blood vessel organization [22–24]. An inflammatory reaction is the tissue's first response to bone injury, which activates growth factors and cytokines to drive different types of endogenous cells to the injury sites [25]. In addition, some cells may hinder the regeneration process, such as the pro-inflammatory M1-type macrophages inhibiting osteogenesis [26]; however, it is known that neither integrin $\alpha 4\beta 1$ nor integrin $\alpha v\beta 3$ is expressed on M1-type macrophages [27]. Therefore, integrins $\alpha 4\beta 1$ and $\alpha v\beta 3$ hold great promise for promoting bone formation and vascularization by mediating adhesion and migration of the relevant endogenous cells to the bone defect area.

By using One-bead one-compound (OBOC) combinatorial technology, an ultra-high-throughput chemical library synthesis and screening method [28], we identified LLP2A and LXW7 as two high-affinity, high-specificity binding ligands that target integrins $\alpha 4\beta 1$ and $\alpha v\beta 3,$ respectively [29,30]. We demonstrated that LLP2A had a strong binding affinity, via integrin $\alpha 4\beta 1$, to MSCs derived from different tissue sources and significantly improves MSC adhesion, survival, and spreading, as well as facilitates related biological signals on biomaterial scaffolds [31]. Our collaborators have used LLP2A as the MSC targeting molecule and demonstrated that LLP2A can direct MSCs to bone, augment bone formation, and increase bone mass in a murine model [32]. Additionally, we identified that LXW7 possesses a strong and specific binding affinity to EPCs/ECs via integrin $\alpha v\beta 3$ and improves EC proliferation, survival, and related biological signals [33,34]. Using a rat carotid artery model, we further demonstrated that LXW7 significantly improves endothelialization by promoting the recruitment and migration of endogenous EPCs and ECs to the biomaterial scaffold [23]. Hence, in this study, we proposed for the first time to combine the use of LLP2A and LXW7 to construct a cell-free bioactive material for vascularized bone regeneration. The concept of using two unique synthetic ligands with diverse functions to guide the endogenous cells to achieve vascularized bone formation is novel. The use of integrin-regulating biomaterials to guide the stem cell function in the developing fetal environment has never been done in the past. In addition, here we proposed to design a new bone material with these two safe and stable integrin ligands to overcome the drawbacks of the current tissue engineered bone scaffolds in the clinical settings, such as the poor engraftment with cell-seeded bone scaffolds and the safety issues with growth/morphogenetic factor-loaded bone scaffolds, especially in fetal and pediatric clinical applications. Thus, in this study, we used two different animal models, one rat calvarial bone defect model, an adult bone defect model undergoing intramembranous ossification, and one fetal sheep spinal bone defect model, a fetal bone development model undergoing endochondral ossification, to evaluate the function of the LLP2A/LXW7 modified collagen scaffold in promoting vascularized bone formation.

Collagen is the major structural protein of the ECM, accounting for

up to 90% of the bone matrix [35]. Furthermore, we investigated a high-affinity collagen-binding peptide, SILY, derived from platelet membrane receptors, that binds to the D-spacing in collagen [36-38]. We also established an approach to immobilize the ligands onto collagen-based scaffolds using the SILY peptide as a linker, and demonstrated that the ligands still maintained their functions in vitro and in vivo [34]. To meet the physiological environment of vascularized bone development, in this study, we developed a bone ECM mimicking biomaterial by conjugating LLP2A and LXW7 to the collagen-based scaffold via the SILY-collagen conjugation approach. We hypothesized the implanted LLP2A/LXW7 modified collagen-based biomaterial could specifically regulate the adhesion of endogenous cells via integrins $\alpha 4\beta 1$ and $\alpha v\beta 3$ at the bone defects and promote bone formation and vascularization in both adult and fetal vascular bone developmental environments (Fig. 1). In vitro, the LLP2A/LXW7 modified collagen-based biomaterial significantly improved the adhesion of MSCs, osteoblasts, EPCs, and ECs via integrins $\alpha 4\beta 1$ or $\alpha v\beta 3$, respectively. In an adult rat calvarial bone defect model, the LLP2A/LXW7 modified biomaterial significantly improved the recruitment and adhesion of endogenous cells with osteogenic and angiogenic potentials, such as DLX5⁺ cells, osteocalcin⁺ cells, CD34⁺/CD45⁻ cells and CD31⁺ cells, at the bone defect area, as well as promoted bone formation and vascularization. In a fetal sheep spinal bone defect model, the LLP2A/LXW7 modified biomaterial significantly augmented bone formation and vascularization without any adverse effects.

2. Materials and methods

2.1. Cell culture

Human bone marrow-derived MSCs (BMSCs) were purchased from ATCC (PCS-500-012) and expanded in high-glucose Dulbecco's Modified Eagle Medium (DMEM, SH30243.01, HyClone) containing 10% fetal bovine serum (FBS, SH30071.03, HyClone). BMSCs between P3 and P5 were used for all experiments. Human osteoblasts were purchased from ATCC (CRL-11372) and expanded in Human Osteoblast Growth Medium (417–500, Sigma). Osteoblasts between P3 and P5 were used for all experiments. Human umbilical vein endothelial cells (UVECs) were purchased from ATCC (PCS-100-013) and expanded in Endothelial Cell Growth Medium-2 (EGM-2, CC-3162, Lonza). UVECs between P3 and P5 were used for all experiments. Human endothelial colony forming cells (ECFCs) were isolated from human umbilical cord blood as described in our previous studies [23,33,34]. ECFCs were expanded and cultured in EGM-2. ECFCs between P2 and P4 were used for all experiments.

2.2. Preparation and characterization of the integrin ligands modified bone biomaterials

LLP2A, LXW7 and SILY (Fig. 2a) were synthesized as described in our previous studies [31,33,34]. Here, we synthesized (LLP2A)₂-SILY and (LXW7)₂-SILY through three steps: 1) standard solid phase peptide synthesis (SPPS) of SILY-2N3, 2) SPPS synthesis of LLP2A-DBCO or LXW7-DBCO, 3) 1 eq. of SILY-2N3 conjugating with 2 eq. of LLP2A-DBCO or 2 eq. of LXW7-DBCO, respectively via copper-free click chemistry (Fig. 2b). Detailed synthesis was described in (Supplementary Figs. 1 and 2). For the preparation and characterization of the LLP2A/LXW7 modified collagen-based biomaterials, FDA-approved clinical-grade tailorable collagen-based scaffolds (Infuse, 7510050, Medtronic) (Supplementary Fig. 3) were soaked in (LLP2A)₂-SI-LY/(LXW7)2-SILY solution mixed with same molar amount of (LLP2A)2-SILY and (LXW7)2-SILY at the different amounts of (LLP2A)₂-SILY/(LXW7)₂-SILY/collagen (nmol/mg), such as 0, 0.1, 0.5, 1, 2.5, 5 or 10 nmol/mg, in 48-well plates and incubated at 37 °C for 1 h, then 100 µL solution was collected for high-performance liquid chromatography (HPLC) analysis to quantify the amount of unbound free

defect model.



Fig. 1. Schematic of study design. The bone biomaterial designed in this study was constructed by conjugating two integrin-base ligands, LLP2A and LXW7, specifically targeting integrins $\alpha 4\beta 1$ and $\alpha \nu \beta 3$, respectively, to the collagen-based scaffold via SILY, a high-affinity collagen binding peptide. The LLP2A/LXW7 modified biomaterial improved accumulation of endogenous cell with osteogenic and angiogenic potentials, such as MSCs, osteoblasts, EPCs and ECs, at the bone defect area, and promoted bone formation and vascularization without any adverse effects in the adult rat calvarial bone defect model and the fetal sheep spinal bone

(LLP2A)₂-SILY and (LXW7)₂-SILY that were remained in the solution. Briefly, HPLC analysis was performed on the Agilent HPLC system equipped with a Waters Xterra R MS column (5 mm, C18, 150 \times 4.6 mm). A linear gradient was run from 100% solution A (water/0.1% trifluoroacetic acid) to 100% solution B (acetonitrile/0.1% trifluoroacetic acid) within 20 min with a flow rate at 1.0 mL/min. The UV detection wavelength was 214 nm [34]. To further confirm the conjugation of (LLP2A)₂-SILY and (LXW7)₂-SILY on collagen scaffold directly, we synthesized TAMRA-(LLP2A)2-SILY and Cy5.5-(LXW7)2-SILY. The scaffolds were soaked in the solution mixed with the same molar amount of TAMRA-(LLP2A)2-SILY and Cy5.5-(LXW7)2-SILY at 5 nmol/mg of TAMRA-(LLP2A)₂-SILY/Cy5.5-(LXW7)₂-SILY/collagen (nmol/mg), in 48-well plates and incubated at 37 °C for 1 h, then the scaffolds were washed with DPBS for 5 times. Images were captured using the Zeiss Observer Z1 microscope. For the tensile strength test, the dried samples were tested under tension at a rate of 5%/min on a mechanical testing apparatus (Instron) with a 1000 N load cell until failure. Ultimate tensile strength (UTS) was calculated from the load vs extension data based on initial sample geometries. UTS was determined as the maximum stress of each sample's stress-strain curve.

2.3. Cell adhesion study

The 0.125 cm^3 (0.5 cm \times 0.5 cm \times 0.5 cm) untreated collagen scaffolds and the collagen scaffolds modified by LLP2A only, LXW7 only, or LLP2A/LXW7 were placed in 35 mm tissue culture dishes. The biomaterials were incubated with BMSCs, osteoblasts, ECFCs, or UVECs in the relevant media, respectively, at a density of 1×10^6 cells/cm³ at 37 °C. For the integrin blocking experiments, the integrin $\alpha 4\beta 1$ expressed on cells was blocked using the anti- $\alpha 4$ antibody (AB202969, Abcam) and anti- $\beta 1$ antibody (AB183666, Abcam), and the integrin $\alpha v\beta 3$ expressed on the cells was blocked using the anti- $\alpha v\beta 3$ antibody (AB7166, Abcam) as the description in our previous studies [31,33]. For the cell adhesion test, after 0.5 h of incubation, the media was aspirated, and unattached cells were washed off with Dulbecco's

phosphate-buffered saline (DPBS, 14190, Gibco) for three times. The cell-seeded scaffolds were placed in 500 µL of passive lysis buffer (PLB, E1910, Promega). Following a freeze-thaw cycle, the lysate was sonicated (10 s on ice) and separated from the scaffold material via centrifugation (10,000 rpm for 10 min at 4 °C). The total DNA of the adhered cells was characterized by using the Quant-iT PicoGreen dsDNA kit (P11496, Invitrogen). Real-Time Quantitative RT-PCR (RT-qPCR) was performed to determine the adhesion-related gene expression. In detail, total RNA was extracted from cells using RNeasy Plus Mini Kit (74034, Qiagen). DEPC treated water, dNTP mix, random hexamer, DTT, RNaseOUT, FS buffer, and superscript II (all from Invitrogen) were used for cDNA synthesis. The PCR conditions for all genes were as follows: 48 °C for 30 min and then 95 $^\circ C$ for 10 min followed by 40 cycles of 95 $^\circ C$ for 30 s, 58 °C for 30 s, and 72 °C for 30 s, last followed by one cycle of 95 °C for 1 min, 58 °C for 30 s, and 95 °C for 30 s. Results are based on cycle threshold (Ct) values. We calculated differences between the Ct values for experimental and reference (GAPDH) genes and graphed the results as the ratio of each RNA to the calibrated sample. Primers used for gene amplification are shown in Supplementary Table 1. The MTS assay was performed according to the manufacturer's instructions (G9241, Promega) to determine cell viability. The Scanning Electron Microscope (SEM, Quattro, Thermo Fisher Scientific) was used to evaluate cell morphology.

2.4. Chondrogenic, osteogenic and angiogenic capabilities of the LLP2A/ LXW7 modified collagen biomaterial

For the chondrogenic and osteogenic assay, the BMSCs were seeded on the 0.125 cm³ (0.5 cm \times 0.5 cm \times 0.5 cm) untreated collagen scaffolds as well as the collagen scaffolds modified with LLP2A and LXW7 at a density of 1 \times 10⁶ cells/cm³ and then were cultured in chondrogenic (A1007101, Gibco) or osteogenic (A1007201, Gibco) differentiation media respectively for 2 weeks. The expression of chondrogenic genes (SOX9 and COL2A1) and osteogenic genes (RUNX2 and COL1A1) in the MSCs were evaluated by using RT-qPCR described in 2.3. For the



Fig. 2. Construction and characterization of the LLP2A/LXW7 modified biomaterial. a, Chemical structures of SILY, LXW7 and LLP2A. b, Chemical synthesis process of (LLP2A)₂-SILY and (LXW7)₂-SILY. Two equivalent units of LLP2A-DBCO or LXW7-DBCO were conjugated to one equivalent unit of SILY-2N₃ via 'click chemistry' to synthesize (LLP2A)₂-SILY and (LXW7)₂-SILY, respectively. c, HPLC results showed that the (LLP2A)₂-SILY and (LXW7)₂-SILY had been conjugated onto the collagen scaffold successfully. d, the images showing the TAMRA-(LLP2A)₂-SILY (red) and Cy5.5-(LXW7)₂-SILY (green) were distributed on the collagen scaffold uniformly. Scale bar = 100 µm.

angiogenic assay, the ECFCs were seeded on the 0.125 cm³ (0.5 cm \times 0.5 cm \times 0.5 cm) untreated collagen scaffolds and the collagen scaffolds modified with LLP2A and LXW7 at a density of 1 \times 10⁶ cells/cm³ and culture in EGM-2 for 72 h. The expression of angiogenic genes (CD144 and vWF) in the ECFCs was determined by using RT-qPCR described in 2.3. Primers used for gene amplification are shown in Supplementary Table 1.

2.5. Calvarial bone defect model in rat

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California, Davis. Tenweek-old male Sprague-Dawley rats were purchased from the Charles River animal facility. A sample number of 6 rats per treatment group (total 4 treatment groups) per time point (2 weeks and 12 weeks) were utilized in this study. This sample number was determined using GraphPad StatMate 2.01 software to allow for the determination of the statistical significance of 50% variations in experimental values (vascular density, bone mineral density, bone volume fraction) between conditions. This calculation was performed using typical values of standard deviation (approximately 35%) for these experiments based on previously published studies (alpha = 0.05, power level = 80%). As described in our previous studies [39,40], the rats underwent bilateral calvarial osteotomies. The rats were anesthetized (3.0%) and maintained (1.5%) under an isoflurane/O₂ mixture delivered through a nose cone at 6 L/min. A mid-longitudinal 15-mm skin incision was made on the dorsal surface of the cranium. The periosteum was completely cleared from the surface of the cranial bone by scraping. A trephine bur (04-9482-01, ACE Surgical Supply Co., Inc.) was used to create one circular 3.5-mm-diameter defect in the rat cranium on each side of the sagittal suture, and the full thickness (~1.5 mm) of the cranial bone was removed to create a critical-size calvarial bone defect. Collagen scaffolds, collagen scaffolds modified LLP2A only, collagen scaffolds modified LXW7 only, and collagen scaffolds modified LLP2A and LXW7 were generated with a final diameter of 3.5 mm and thickness of 1.5 mm using a 3.5-mm biopsy punch (MT3333, Integra® Miltex®) and immediately placed directly into the osteotomy sites after the defect creation, then the skin was closed using continuous sutures (Prolene monofilament 5-0, Ethicon Inc.). The skin wound was lastly disinfected with betadine, and the rats were returned to cages and allowed to recover from anesthesia. Animals were given analgesia (buprenorphine; 0.05 mg/kg) via subcutaneous injection at the time of anesthesia and for 48 h post-recovery (two injections over 24 h period). Skin sutures were removed within ten days post-surgery, and the longest duration for implants was 3 months.

2.6. Fetal spinal bone defect model in sheep

All procedures were approved by the Institutional Animal Care and Use Committee at the University of California, Davis. Pregnant Dorper mix sheep were purchased from Vanlandingham Farms Inc. Fourteen sheep fetuses obtained from ten ewes were used in this study. Three sheep fetuses were used for the untreated group, four sheep fetuses were used for the collagen scaffold group, four sheep fetuses were used for the collagen scaffold modified with LLP2A and LXW7 group, and three sheep fetuses were used for the normal group (no defect group). Ewes at GA 100 were anesthetized with 2-6 mg/kg Propofol plus 2-5 mg/kg Ketamine, intubated and maintained (3-1%) under an isoflurane/O2 mixture. A laparotomy was performed to expose the gravid uterus and a hysterotomy was made to expose the fetal back. A mid-longitudinal 2-3 cm skin incision was made on the fetal back, and the underlying paraspinal muscles were transected to expose the L4-L6 vertebrae. Next, 2 level (half L4, whole L5 and half L6) lamina was removed using bone rongeurs to expose the spinal cord. Care was taken not to damage the spinal cord or enter the intrathecal space. Next, the biomaterial (1.5 cm imes 0.5 cm imes 0.2 cm) was placed on top of the exposed spinal cord inside

the laminectomy site. The muscles on each side of the spine were then sutured together using absorbable Vicryl 4-0 (Ethicon Inc.), which held the sponge in place at the defect location. Lastly, the fetal skin was closed using absorbable Vicryl 4-0 (Ethicon Inc.). Uterine fluid contents were replaced with 37 °C DPBS, and a dose of Penicillin (1 million units) and Gentamicin (100 mg) was added to the amniotic fluid. The hysterotomy was closed with absorbable Vicryl 2-0 (Ethicon Inc.), and the laparotomy was closed with absorbable PDS 0-0 (Ethicon Inc.) A maximum of 2 fetuses were operated on in each ewe. At GA146, the fetuses underwent survival cesarean section and were maintained for up to 24 h postnatally. Motor function assessments using the validated sheep locomotor rating scale (SLR) [41] were performed at 4 h and 24 h of life. Sheep were then euthanized with sodium pentobarbital and perfused intracardially with 1 L of PBS followed by 1.6 L of 10% formalin. The lumbar spinal column of the sheep was dissected for histological analysis.

2.7. Laser-Doppler perfusion imaging (LDPI) and quantification

Upon anesthesia of the rats, the blood perfusion was measured at 2 weeks and 12 weeks after surgery using a PeriScan PIM 3 laser Doppler blood perfusion imager (Perimed). The hair covering the surgical site was removed before scanning, and the calvariae were cleaned using alcohol wipes immediately before data acquisition. Perfusion measurements were obtained from a circular region of interest superimposed over the defect.

2.8. Micro-CT scan imaging and quantification

For the rat calvarial bone defect model, the calvariae were collected and fixed in 4% paraformaldehyde for 1 day at 4 °C. Samples were washed twice in deionized water to remove residual paraformaldehyde and preserved in 70% ethanol at 4 °C until further processing. Qualitative and quantitative 3D analyses of the explants at 2 weeks and 12 weeks were conducted using micro-CT. Explants were imaged (70 kV peak, 114 µA, 300-ms integration time, average of three images) using a high-resolution micro-CT specimen scanner (mCT 35, Scanco Medical) by the Veterinary Orthopedic Research Laboratory at the University of California, Davis. Contiguous slices of 2048 pixels by 2048 pixels were imaged with 15-µm resolution and slice thickness (voxels). Serial tomograms were reconstructed from raw data of 1000 projections per 180° using a cone beam filtered back projection algorithm. The tomograms were calibrated to 0.0, 99.6, 200.0, 401.0, and 800.3 mg HA cm^{-3} concentrations of HA, so that gray values of the images were converted to units of density in milligrams of HA per cubic centimeter. The entire defect was analyzed by selecting a 3.5-mm-diameter region of interest extending through the bone thickness. The material in the reconstructed images was partitioned by a threshold of 256–3000 mg HA cm^{-3} to discriminate between mineralized and unmineralized tissue. On 3D images of the specimen, bone volume (mm³) at the defect site was measured.

For the fetal sheep spinal bone defect model, the relevant spinal columns were collected and fixed in 4% paraformaldehyde for 3 days at 4 °C. Samples were washed twice in deionized water to remove residual paraformaldehyde and preserved in 70% ethanol at 4 °C until further processing. Qualitative and quantitative 3D analyses of explants were conducted using micro-CT. Explants were imaged (70 kV peak, 114 μ A, 300-ms integration time, average of three images) using a high-resolution micro-CT specimen scanner (mCT 35, Scanco Medical) by the Veterinary Orthopedic Research Laboratory at the University of California, Davis. The material in the reconstructed images was partitioned by a threshold of 220–3000 mg HA cm⁻³ to discriminate between mineralized and unmineralized tissue. After thresholding, the image noise was reduced using a low-pass Gaussian filter ($\sigma = 0.8$, support = 1). The volume of the newly formed bone was determined.

2.9. Histology

For the rat calvarial bone defect model, after micro-CT analysis, explants were demineralized in Calci-Clear (50-899-90140, National Diagnostics) for 7 days, washed twice in water, dehydrated, OCTembedded, and sectioned at 8-µm thickness. The 2-week explants were used to evaluate the distribution and behavior of the endogenous stem cells. The tissue was incubated overnight with the relevant primary antibodies against DLX5 (1:100, AB109737, Abcam), osteocalcin (1:100, AB13420, Abcam), CD31 (1:100, AB182981, Abcam), CD34 (1:100, AB81289, Abcam) and CD45 (1:100, AB33923, Abcam) at 4 °C. After being washed with DPBS 3 times, the tissue was incubated with the relevant secondary antibodies (1:500; Life Technologies) for 1 h at room temperature, and then nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, D1306, Thermo Fisher Scientific). After being washed with DPBS 3 times, the images were captured using the Zeiss Observer Z1 microscope. Quantification of the cell numbers was performed using the Image J software (NIH, USA). To visualize tissue formation and morphology of the 12-week explants, sections were stained with H&E and Alican Blue according to the manufacturer's instructions (AB150662, Abcam), and the images were captured using the Kevence BZ-X800 microscope. The sections were stained with anti-osteocalcin antibody (1:100, AB13420, Abcam) to evaluate and further confirm the osteoblast activity as the description above. The images were captured using the Zeiss Observer Z1 microscope. Osteoblast surface to bone surface (Ob.S/BS) and osteoblast number per bone perimeter (Ob. N/B.Pm) of bone static histomorphometric analyses were performed using the Image J software. Bone histomorphometric parameters were calculated and expressed according to the standardized nomenclature for bone histomorphometry. The sections were stained with anti-CD31 antibody (1:100, AB182981, Abcam) to further evaluate the vascularization as described above. The images were captured using the Zeiss Observer Z1 microscope. The blood vessel density was quantified by using the Image J software.

For the fetal sheep spinal bone defect model, after micro-CT analysis, explants were demineralized in Calci-Clear for 2 weeks, washed twice in water, dehydrated, OCT-embedded, and sectioned at 8- μ m thickness. The H&E staining was performed for the sections to visualize tissue formation and morphology of the explants and quantify the blood vessel density, and the images were captured using the Keyence BZ-X800 microscope. Quantification was performed using the Image J software. For all the quantification by using Image J software, six fields were imaged on each slice, and 3 slices were chosen from each animal.

2.10. Statistical analysis

Graphs and statistical tests were performed using Prism. For multiple-sample comparison, analysis of variance (ANOVA) was used to evaluate whether a significant difference existed between groups with different treatments, and a multiple comparison procedure Holm's *t*-test was used for post-analysis. The n numbers shown in the manuscript indicated the results obtained from the number of different experiments or animals, and 3 replicates were performed within the same experiment or animal. A p-value less than 0.05 indicates a significant difference between samples in comparison.

3. Results

3.1. Construction and characterization of LLP2A/LXW7 modified biomaterials

We previously demonstrated that the (ligand)₂-SILY compound (two ligands linked with one SILY) significantly improved specific cell binding compared to the ligand-(SILY)₂ compound (one ligand linked with two SILYs) due to the higher density of cell binding sites [34]. Hence, in this study, we synthesized (LLP2A)₂-SILY and (LXW7)₂-SILY (Fig. 2a and

b and Supplementary Figs. 1 and 2) and constructed the engineered integrin-specific bone biomaterial by conjugating the two integrin-based ligands LLP2A and LXW7 onto the collagen scaffold via the SILY-collagen binding approach. To evaluate the conjugation efficiency of (LLP2A)2-SILY and (LXW7)2-SILY on collagen scaffold, 2 h after the collagen scaffold was soaked in the solution of different amounts of (LLP2A)₂-SILY/(LXW7)₂-SILY (nmol/mg), HPLC was used to measure the amounts of unbound free (LLP2A)₂-SILY and (LXW7)₂-SILY remained in the solution. The residual (LLP2A)2-SILY or (LXW7)2-SILY compounds were not detectable until the respective molar amounts of the (LLP2A)2-SILY or (LXW7)2-SILY compounds used to modify the collagen-based scaffold were up to 5 nmol/mg, the ligand compound corresponding peaks increased as the molar amounts increased, and the saturation amount of the ligands for the modification was between 2.5 and 5 nmol/mg of the scaffold (Fig. 2c). To further confirm the conjugation of (LLP2A)₂-SILY and (LXW7)₂-SILY on collagen scaffold directly, we synthesized TAMRA-(LLP2A)2-SILY and Cy5.5-(LXW7)2-SILY, and we conjugated them onto the collagen scaffold under 5 nmol/mg. The results showed both the TAMRA-(LLP2A)2-SILY (red) and Cy5.5-(LXW7)₂-SILY (green) were distributed on the collagen scaffold uniformly (Fig. 2d). These results demonstrated that LLP2A and LXW7 had been successfully conjugated onto the collagen-based scaffold. To evaluate the effect of the LLP2A/LXW7 conjugation on the mechanical properties of the collagen scaffold, the tensile strength of the biomaterials was measured using tensile testing. The results showed no significant difference between the untreated collagen scaffolds, LLP2A modified scaffolds, LXW7 modified scaffolds, and LLP2A/LXW7 modified scaffolds (Supplementary Fig. 4), which demonstrates the LLP2A/LXW7 conjugation does not change the mechanical properties of the collagen scaffold.

3.2. The LLP2A/LXW7 modified biomaterial improves adhesion of the cells related to bone formation and vascularization via integrins $\alpha 4\beta 1$ and $\alpha v\beta 3$

Cell adhesion and regulation are fundamental for new tissue formation [42]. Integrins play essential roles in the cell adhesion process via their interactions with the correlated ligands located on the surrounding ECM [43,44]. We evaluated the adhesion of human BMSCs, osteoblasts, ECFCs, and UVECs related to bone formation and vascularization on the LLP2A/LXW7 modified biomaterials. The cell adhesion on the LLP2A/LXW7 modified biomaterials was evaluated by quantifying the total DNA. Compared to untreated collagen-based biomaterial, biomaterial modified with LLP2A improved adhesion of BMSCs, osteoblasts, and ECFCs but not UVECs, while the biomaterial modified with LXW7 improved adhesion of osteoblasts, ECFCs, and UVECs but not BMSCs (Fig. 3a). This indicates that increasing the density of integrin binding ligands on the biomaterial is crucial for promoting the adhesion of cells expressed the corresponding integrins. Moreover, compared to the untreated collagen-based biomaterial, the biomaterial modified with LLP2A/LXW7 improved the adhesion of all these four types of cells (Fig. 3a), which demonstrates that LLP2A and LXW7 possess a synergistic effect on the adhesion of the cells related to bone formation and vascularization. These results suggest that the LLP2A/LXW7 modified biomaterials hold the promise of effectively promoting new bone formation and neovascularization simultaneously. These data also support the notion that complementary signaling is achievable by presenting multiple ligand molecules.

To further confirm the improved adhesion of BMSCs, osteoblasts, ECFCs and UVECs on the LLP2A/LXW7 modified biomaterials via integrins $\alpha 4\beta 1$ and $\alpha v\beta 3$, we performed the blocking experiment using anti-integrin $\alpha 4\beta 1$ and anti-integrin $\alpha v\beta 3$ antibodies. After the integrin $\alpha 4\beta 1$ expressed on the cells was blocked, the LLP2A modified biomaterial no longer improved the adhesion of BMSCs, osteoblasts, and ECFCs (Fig. 3b), which indicates the LLP2A modified biomaterial improved the adhesion of BMSCs, osteoblasts and ECFCs via integrin $\alpha 4\beta 1$. After the



Fig. 3. Adhesion of human BMSCs, osteoblasts, ECFCs and UVECs on the LLP2A/LXW7 modified biomaterials *in vitro*. a, Quantitation of total DNA of human BMSCs, osteoblasts, ECFCs and UVECs adhered biomaterials modified with different integrin binding ligands. b, Quantitation of total DNA of human BMSCs, osteoblasts, ECFCs and UVECs adhered on biomaterials modified with different integrin binding ligands after integrin $\alpha 4\beta 1$ expressed on the cells was blocked. c, Quantitation of total DNA of human BMSCs, osteoblasts, ECFCs and UVECs adhered on biomaterials modified with different integrin $\alpha 4\beta 1$ expressed on the cells was blocked. c, Quantitation of total DNA of human BMSCs, osteoblasts, ECFCs and UVECs adhered on biomaterials modified with different integrin binding ligands after integrin binding ligands after integrin $\alpha \gamma\beta 3$ expressed on the cells was blocked. d, Quantitation of total DNA of human BMSCs, osteoblasts, ECFCs and UVECs adhered on biomaterials modified with different integrin binding ligands after integrins $\alpha 4\beta 1$ and $\alpha \gamma\beta 3$ expressed on the cells were blocked. Data were expressed as mean \pm standard deviation: **p < 0.01, ***p < 0.001 (n = 6).

integrin $\alpha\nu\beta3$ expressed on the cells was blocked, the LXW7 modified biomaterial no longer improved the adhesion of osteoblasts, ECFCs and UVECs (Fig. 3c), which indicates the LXW7 modified biomaterial improved the adhesion of osteoblasts, ECFCs and UVECs via integrin $\alpha\nu\beta3$. After both the integrin $\alpha4\beta1$ and integrin $\alpha\nu\beta3$ expressed on the

cells were blocked, the LLP2A/LXW7 modified biomaterial no longer improved the adhesion of BMSCs, osteoblasts, ECFCs and UVECs (Fig. 3d), which indicates the LLP2A/LXW7 modified biomaterial synergistically improved the adhesion of BMSCs, osteoblasts, ECFCs and UVECs via integrins $\alpha 4\beta 1$ and $\alpha v\beta 3$.

Based on these results, we further evaluated the adhesion-related gene expression by using RT-qPCR, such as RhoA, which has been demonstrated to be directly involved in cell adhesion [45]. Compared to untreated collagen-based biomaterial, biomaterial modified with LLP2A improved RhoA expression in BMSCs, osteoblasts, and ECFCs but not in UVECs, while the biomaterial modified with LXW7 improved RhoA expression in osteoblasts, ECFCs, and UVECs but not in BMSCs (Supplementary Fig. 5). Moreover, compared to the untreated collagen-based biomaterial, the biomaterial modified with LLP2A/LXW7 improved the RhoA expression in all these four types of cells (Supplementary Fig. 5). The results were consistent with the cell adhesion results. To further confirm the conditions or functions of the adhered cells on the biomaterials, we evaluated the viability and morphology of the cells adhered to the biomaterials. The MTS assay showed consistent results with the cell adhesion results, indicating the viability of the cells adhered to the biomaterials is good (Supplementary Fig. 6). The SEM results showed regular cell morphology of the cells adhered to the biomaterials (Supplementary Fig. 7).

In addition to the role in mediating cell adhesion, the integrin family is also known to participate in the modulation of cell differentiation [46]. We further evaluated the effects of the LLP2A/LXW7 modified biomaterials on promoting the chondrogenic, osteogenic and angiogenic potentials to gain further insights. The RT-qPCR results showed no significant difference in expression of the relevant genes, such as chondrogenic genes (SOX9 and COL2A1) and osteogenic genes (RUNX2 and COL1A1) in BMSCs and angiogenic genes (CD144 and vWF) in ECFCs, between the cells cultured on the untreated collagen scaffolds and the collagen scaffolds modified with LLP2A/LXW7 (Supplementary Fig. 8).

3.3. The LLP2A/LXW7 modified biomaterial improves the accumulation of endogenous cells related to vascularized bone formation at the defect area in a rat calvarial bone defect model

Based on the in vitro results, we further evaluated the capabilities of the LLP2A/LXW7 modified biomaterials for improving the accumulation of the endogenous cells with osteogenic and angiogenic potentials in a rat calvarial bone defect model. Two weeks after biomaterial implantation, evaluation of bone formation by micro-CT revealed that LLP2A only, LXW7 only, and the LLP2A/LXW7 modified collagen biomaterials significantly enhanced bone formation compared to untreated collagen biomaterials (Fig. 4a and b). There was no significant difference in the mineral density of the newly formed bone in all groups (Fig. 4c). LDPI showed that LLP2A only, LXW7 only, and LLP2A/LXW7 modified collagen biomaterials did not extensively improve blood perfusion compared to untreated collagen biomaterials (Fig. 4d and e). The immunohistochemical analysis was performed to identify the cells that were adhered to the biomaterials at the defect area at 2 weeks after biomaterial implantation. Staining with anti-distal-less homeobox 5 (DLX5) antibody, a marker expressed on the cells with osteogenic potential [47,48], showed that LLP2A only and LLP2A/LXW7 modified collagen biomaterials significantly improved the accumulation of endogenous DLX5-expressing cells compared to the untreated collagen biomaterial (Fig. 4f and g). Staining with anti-osteocalcin antibody, another marker expressed on osteogenically differentiating progenitor cells [49,50], showed that LLP2A alone, LXW7 only, and LLP2A/LXW7 modified collagen biomaterials all significantly improved the accumulation of endogenous osteocalcin-expressing cells compared to the untreated collagen biomaterials (Fig. 4f and g). Staining with anti-CD34 and anti-CD45 antibodies showed that LLP2A only and LLP2A/LXW7 modified collagen biomaterials significantly improved the accumulation of endogenous CD34⁺/CD45⁻ EPCs compared to the untreated collagen biomaterials (Fig. 4f and g). Staining with anti-CD31, expressed on ECs and late-stage EPCs [51], showed that LXW7 only and LLP2A/LXW7 modified collagen biomaterials significantly improved the accumulation of endogenous ECs and late-stage EPCs compared to the untreated collagen biomaterials (Fig. 4f and g). Overall, these results demonstrated

that the LLP2A/LXW7 modified collagen biomaterial significantly promotes the accumulation of endogenous cells with osteogenic and angiogenic potentials at the bone defect area, thus indicating the LLP2A/LXW7 modified collagen biomaterial holds promise to promote bone formation and vascularization.

3.4. The LLP2A/LXW7 modified biomaterial promotes bone formation and vascularization in the long-term rat calvarial bone defect model

To evaluate the capabilities of the integrin-specific biomaterials for bone formation and vascularization, we implanted the integrin-specific biomaterials into the adult rat calvarial bone defect model, an intramembranous ossification model. Twelve weeks after biomaterial implantation, the micro-CT results showed that LLP2A only, LXW7 only, and LLP2A/LXW7 modified collagen biomaterials significantly enhanced bone formation compared to untreated collagen biomaterials (Fig. 5a and b). The LXW7 only modified collagen biomaterial showed more new bone formation than the LLP2A only modified collagen biomaterial (Fig. 5a and b), possibly because LXW7 specifically improved the accumulation of endogenous osteoblasts that directly contribute to new bone formation [52]. In contrast, LLP2A improved the accumulation of endogenous MSCs undergoing osteogenic differentiation to osteoblasts, which needs a longer time for new bone formation [53]. The LLP2A/LXW7 modified collagen biomaterials induced the greatest new bone formation (Fig. 5a and b), probably because the presence of both LLP2A and LXW7 synergistically engaged both integrins $\alpha 4\beta 1$ and $\alpha v\beta 3$ expressed on different types of cells at different stages during the new bone formation process [54]. There was no significant difference in the mineral density of the newly formed bone in all groups (Fig. 5c), which indicated the biomaterials modified with different integrin-based ligands could promote uniform new bone formation. The LDPI results showed that LLP2A only, LXW7 only, and LLP2A/LXW7 modified collagen biomaterials significantly promoted blood perfusion (Fig. 5d and e). H&E staining revealed consistent results with the micro-CT results, and the LLP2A/LXW7 collagen modified biomaterial showed the most robust bone regeneration among all the groups (Fig. 5f). Immunohistochemical analysis was performed to further confirm the new bone formation and vascularization. Osteocalcin is a biomarker of osteoblasts that is crucial to bone formation [55]. Staining with anti-osteocalcin antibody revealed that osteocalcin expression was upregulated in LLP2A only, LXW7 only, and LLP2A/LXW7 modified collagen biomaterial groups compared to the untreated collagen biomaterial group, and the highest expression in the LLP2A/LXW7 modified collagen biomaterial group (Fig. 5g). The ratio of Ob.S/BS and Ob.N/B.Pm was higher in the LLP2A only, LXW7 only, and LLP2A/LXW7 modified collagen biomaterial groups compared to the untreated collagen biomaterial group. The ratio was also highest in the LLP2A/LXW7 modified collagen biomaterial group (Fig. 5 h, i). These data suggest that LLP2A only, LXW7 only, and LLP2A/LXW7 modified collagen biomaterials all enhanced osteoblast activity compared to the untreated collagen biomaterial, but the LLP2A/LXW7 modified collagen biomaterial showed the best capability [55-57]. Staining with anti-CD31 antibody revealed LLP2A only, LXW7 only, and LLP2A/LXW7 modified collagen biomaterials all significantly enhanced the density of the blood vessels compared to the untreated collagen biomaterial, and the LLP2A/LXW7 modified collagen biomaterial showed the best capability (Fig. 5j and k), which was consistent with the LDPI results. Overall, results demonstrate that these integrin-specific biomaterials possess the capability to improve bone formation and vascularization, and the synergy of the two ligands could further improve the capability for bone formation and vascularization.

3.5. The LLP2A/LXW7 modified biomaterial promotes bone formation and vascularization in the fetal sheep spinal bone defect model

Based on the data shown in the rat calvarial bone defect model, the



Fig. 4. Accumulation of endogenous cells with osteogenic and angiogenic potentials at the defect area at 2 weeks after biomaterial implantation in the rat calvarial bone defect model. a, Representative micro-CT images of defects denoted by a red circle. Scale bar = 1 mm. b-c, Quantitation of new bone formation (b) and bone mineral density (c) of the micro-CT results. d, Representative LDPI images of the blood perfusion at the defect area denoted by a red circle. Scale bar = 1 mm e, Raw perfusion values of LDPI results. f, Immunohistochemical analysis of endogenous cell accumulation regulated by the integrin ligands modified biomaterials at the defect area. Scale bars = 50 μ m g, Quantitation of the population of different types of endogenous cells at the defect area. Data were expressed as mean \pm standard deviation: *p < 0.05, **p < 0.01, ***p < 0.001 (n = 6).



(caption on next page)

Fig. 5. Bone formation and vascularization at 12 weeks after biomaterial implantation in the rat calvarial bone defect model. a, Representative micro-CT images of defects denoted by a red circle. Scale bar = 1 mm. b-c, Quantitation of new bone formation (b) and bone mineral density (c) of the micro-CT results. d, Representative LDPI images of the blood perfusion at the defect area denoted by a red circle. Scale bar = 1 mm e, Raw perfusion values of LDPI results. f, H&E staining of the tissue and matrix formation in the different groups. Blue dotted line indicates the defect area. Scale bar = 1 mm g, Immunohistochemical staining of osteolast surface/bone surface ratios (h) and osteolast number/bone perimeter ratios (i) in different groups. j, Immunohistochemical staining of CD31 in different groups. Scale bar = 100 μ m k, Quantitation of blood vessel density in different groups. Data were expressed as mean \pm standard deviation: *p < 0.05, **p < 0.01 (n = 6).

collagen scaffolds presenting both LLP2A and LXW7 possessed the best capacity for bone formation and vascularization. We evaluated the safety and capacity of LLP2A/LXW7 modified collagen biomaterial in promoting bone formation and vascularization in the fetal sheep spinal bone defect model, an endochondral ossification model [58]. Briefly, Dorper sheep at a gestational age of 100 days (GA100) underwent a laparotomy to expose the gravid uterus and a hysterotomy to expose the fetal back. The back muscle layers were incised to expose the spinal bone. A mid-longitudinal 2-3 cm skin incision was made on the fetal back, and the underlying paraspinal muscles were transected to expose the L4-L6 vertebrae. Then 2 level (half L4, whole L5 and half L6) lamina was removed using bone rongeurs to expose the spinal cord, and the biomaterial was implanted at the defect. The defects could be visualized during surgery by exposure of the spinal cord (Fig. 6a) and after surgery via X-Ray imaging, in which they appear as a groove in the spine in the cross-sectional view (Fig. 6b).

At GA146, the fetuses underwent survival cesarean section, and the motor function assessments were performed for the baby sheep at 4 h and 24 h of life using our established, validated sheep locomotor rating (SLR) scale [41,59]. The results showed that all the baby sheep had a great SLR scale of 15 at 4 h and 24 h of life (Supplementary Fig. 9), demonstrating that the integrin-specific biomaterial transplantation did not have any adverse effects on the motor function of the baby sheep. The micro-CT results showed the LLP2A/LXW7 modified collagen biomaterial treatment significantly improved new bone formation compared to the untreated collagen biomaterial treatment and the defect without any treatment (Fig. 6c and d). There was no significant difference in the mineral density of newly formed bone for all groups (Fig. 6e). H&E staining revealed that the new bone formed in the LLP2A/LXW7 modified collagen biomaterial group achieved more coverage of the bone defect. However, there was low osteogenesis in both the untreated and untreated collagen biomaterial groups (Fig. 6f), which was consistent with the micro-CT results. The H&E staining also revealed no adverse events, such as abnormal tissue growth and tumorigenesis at the defect area and surrounding tissue. Alican Blue staining showed varying degrees of cartilage formation in the untreated, collagen, and collagen + LLP2A + LXW7 groups. More cartilage formation was shown in the collagen group, and more bone formation in the collagen + LLP2A + LXW7 group, which indicates the healing occurred through endochondral ossification, and the LLP2A/LXW7 modified collagen biomaterial promoted the process of endochondral bone formation compared to the collagen biomaterial and untreated group. Remarkably, the structure of the cartilage and bone formation was consistent with the normal group (Fig. 6g). Moreover, H&E staining showed significant enhancement of blood vessel density in the LLP2A/LXW7 modified collagen biomaterial group, compared to the untreated group and the untreated collagen biomaterial group (Fig. 6h and i), and there was no significant difference between the LLP2A/LXW7 modified collagen biomaterial group and the normal group. These results demonstrate that the LLP2A/LXW7 modified collagen biomaterial can improve new bone formation and neovascularization in the fetal developmental environment, and it will be a safe and efficient approach for the treatment of fetal bone defects.

4. Discussion

MSCs are precursors of chondrocytes and osteoblasts, and once the osteoblastic progenitors are directed to the bone defect area, they

synthesize a range of proteins that will further enhance the adhesion and maturation of the osteoblasts [32,53,60]. Also, the MSC-derived secretome possess strong capabilities in regulating EPCs and ECs for promoting vascularization [61, 62]. Hence, mobilization of the endogenous cells with osteogenic and angiogenic potentials, such as MSCs, osteoblasts, EPCs and ECs, to the bone defect area is crucial for bone formation and vascularization [63, 64]. In addition, integrins and their ligands play key roles during this mobilization process [65]. In this study, we developed a biomaterial modified with two synthetic high-affinity ligands, LLP2A and LXW7, against integrins $\alpha 4\beta 1$ and $\alpha v\beta 3$, respectively, for promoting vascular bone regeneration by recruiting and directing the endogenous cells with osteogenic and angiogenic potentials, such as MSCs, osteoblasts, EPCs and ECs. As the results show in Fig. 3, scaffolds modified with LLP2A promoted the adhesion of BMSCs, osteoblasts and ECFCs, but not UVECs, because BMSCs, osteoblasts and ECFCs express integrin $\alpha 4\beta 1$, but UVECs do not express $\alpha 4\beta 1$. Scaffolds modified with LXW7 promoted the adhesion of osteoblasts, ECFCs and UVECs, but not MSCs, because osteoblasts, ECFCs and UVECs express integrin $\alpha v\beta 3$, but MSCs do not express avp3. Remarkably, scaffolds modified with LLP2A and LXW7 promoted the adhesion of all the BMSCs, osteoblasts, ECFCs and UVECs synergistically. Both the osteoblasts and their progenitor MSCs are important for bone formation, and both ECs and their progenitor EPCs are important for vascularization. Hence, in this study, we propose to design the scaffolds modified with LLP2A and LXW7 together to improve the adhesion of as many different types of cells related to bone formation and vascularization as possible to further enhance the efficiency of the vascularized bone regeneration. The results shown in Fig. 4, compared to the unmodified biomaterial, LLP2A modified biomaterial, and LXW7 modified biomaterial, the LLP2A/LXW7 modified biomaterial synergistically improved accumulation of endogenous cells with osteogenic and angiogenic potentials, such as DLX5⁺, osteocalcin⁺, CD34⁺/CD45⁻ and CD31⁺ cells, at the bone defect area and promoted bone formation and vascularization in the rat calvarial bone defect model. These results from the in vivo studies were consistent with the in vitro results.

Compared to the RGD peptide, a non-specific integrin-based ligand recognized by most types of integrins and widely used to modify bone biomaterials [66,67], these two integrin-based ligands, LLP2A and LXW7, both have high binding specificity to integrin $\alpha 4\beta 1$ and integrin $\alpha v\beta$ 3, respectively. Previously, we explicitly compared LXW7 against the conventional RGD peptide [68] and confirmed that LXW7 had a stronger binding affinity and higher binding specificity to integrin $\alpha v\beta 3$ [33]. These data indicate that the LLP2A/LXW7 biomaterial we developed in this study holds promise for providing the specific adhesion sites, improving binding specificity for the endogenous cells related to bone formation and vascularization, thereby increasing the regenerative efficiency of bone formation and vascularization, and avoiding abnormal tissue formation. In addition, the cell-free bioactive bone material modified with two safe and stable integrin-based ligands, LLP2A and LXW7, holds promise for overcoming the drawbacks of the functional osteoinductive agent BMP-2 in clinical applications, such as ectopic bone growth and cancer [69,70]. Currently, serious safety concerns rapidly arise in patients treated with BMP-2 products, especially in pediatric clinical applications and fetal developmental environments [71]. Fetal bone defects and diseases significantly affect and hold lifelong influence on children and their families [72]. For example, spina bifida (SB), a bone and neural tube defect, is the most common congenital cause of lifelong paralysis in the United States, and approximately four

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Fig. 6. Bone formation and vascularization after biomaterial implantation in the fetal sheep spinal bone defect model. a, Process of biomaterial implantation. Scale bars = 1 cm. b, X-Ray images showing the spinal bone defect. Scale bar = 1 cm. c. Representative micro-CT images of defects. The red circle indicates the defect area. The red arrows indicate the newly formed bone. Scale bars = 5 mm. d-e, Quantitation of new bone formation (d) and bone mineral density (e) from the micro-CT results. f, Cross-sectional H&E staining of the spinal bone formation at the defect area. Scale bars = 5 mm h, Cross-sectional H&E staining of the spinal bone formation at the defect area. Scale bars = 5 mm h, Cross-sectional H&E staining of the blood vessel formation at the defect area. The blue arrows indicate the blood vessels. Scale bar = 200 μ m i, Quantitation of blood vessel density at the defect area. Data are expressed as mean \pm standard deviation: *p < 0.5, **p < 0.01 (n = 3 for the untreated group and normal group, n = 4 for the collagen group and the collagen + LLP2A + LXW7 group).

children are born with this congenital defect daily [73]. Fetal bone tissue engineering is a promising approach to promote bone formation in the fetal environment [74]. However, the developing fetal environment is unique, and there are several limitations to applying conventional tissue engineering principles and approaches to treat bone defects in the developing fetus. Particularly, no growth factors or morphogenetic factors are allowed to be used in the developing fetuses due to potential adverse effects and safety concerns [8]. Engineered biomaterial scaffolds that interact with specific integrins represent a biologically safe treatment strategy to modulate the cellular and extracellular factors and guide new tissue formation. In this study, we developed a fetal sheep spinal bone defect model and evaluated the LLP2A/LXW7 modified biomaterial in the fetal developmental environment. We demonstrated that the LLP2A/LXW7 modified biomaterial promoted new bone formation and neovascularization in the short-term fetal developmental process; moreover, it did not cause any abnormal tissue formation in the fetal developmental environment and did not affect the normal functions of newborn sheep. Hence, the LLP2A/LXW7 biomaterial could be a promising biomaterial with vascularized bone regenerative potential and higher safety in clinical treatment for adult and fetal bone defects. In this study, we proposed to investigate the LLP2A/LXW7 modified biomaterial in the fetal developmental environment; therefore, we only evaluated the bone formation, vascularization, and safety issues of the sheep at birth, a total of 46 days from biomaterial implantation to evaluation. The vascularized bone development after birth may need to be monitored at later stages in future studies.

Bone defects that occur in different diseases undergo different bone developmental modes, such as intramembranous ossification or endochondral ossification [75]. In this study, we demonstrated that the LLP2A/LXW7 modified biomaterial promoted the bone formation and vascularization in both the rat calvarial bone defect model, undergoing intramembranous ossification, and the fetal sheep spinal bone defect model, undergoing endochondral ossification, which indicates the LLP2A/LXW7 modified biomaterial possesses the potential to treat the bone defects undergoing different bone developmental modes.

Collagen is the main component of the native bone matrix [35], and the integrin binding sites on the matrix support the cell-matrix interactions critical for tissue regeneration [76]. In this study, we used collagen-based material for the structural scaffold of the LLP2A/LXW7 biomaterial, and the two integrin-based ligands LLP2A and LXW7 were immobilized onto the collagen-based scaffold to increase the binding sites for the desired endogenous cells. The high simulation of the native bone matrix will enable the LLP2A/LXW7 modified biomaterial to better regulate cell-matrix interactions and direct the behaviors of endogenous cells for tissue regeneration. In addition, the LLP2A/LXW7 modified biomaterial is a collagen-based biomaterial with high structural flexibility, so the LLP2A/LXW7 modified biomaterial could be used in bone defects with different shapes easily.

5. Conclusion

The high-affinity and high-specificity integrin-targeting ligands LLP2A and LXW7 identified from OBOC technology are first used to construct a vascularized bone biomaterial. The LLP2A/LXW7 modified biomaterial accurately improved the accumulation of endogenous cells with osteogenic and angiogenic potentials at the bone defect area and promoted bone formation and vascularization without any adverse effects in the adult rat calvarial bone defect model and the fetal sheep spinal bone defect model. This new biomaterial holds promise to be a safe, easy-to-use, off-the-shelf bone product for a wide range of clinical applications for the treatment of bone defects. This study offers a concept and strategy for fabricating biomaterials with precise regulation of the specific endogenous cells for tissue regeneration.

Ethics approval and consent to participate

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California, Davis. Written informed consent was obtained from all participants.

CRediT authorship contribution statement

Dake Hao: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Writing – original draft. Ruiwu Liu: Data curation, Software. Tomas Gonzalez Fernandez: Formal analysis. Christopher Pivetti: Methodology. Jordan Elizabeth Jackson: Methodology. Edwin Samuel Kulubya: Methodology. Hong-Jiang Jiang: Writing – review & editing. Hai-Yang Ju: Writing – review & editing. Wen-Liang Liu: Writing – review & editing. Alyssa Panitch: Writing – review & editing. Kit S. Lam: Methodology, Writing – review & editing. J. Kent Leach: Formal analysis, Methodology, Writing – review & editing. Diana L. Farmer: Writing – review & editing. Aijun Wang: Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2022.05.027.

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