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RESEARCH ARTICLE

In-situ engineering of native extracellular matrix to improve vascularization and tissue regeneration at the ischemic injury site

Dake Hao[1,2] · Lu Lu[1] · Hengyue Song[1,2] · Juan-Maria Lopez[1,2] · Ruiwu Liu[3] · Ping Zhou[4] · Jan Nolta[4] · Diana L. Farmer[1,2] · Eric Kurzrock[5] · Alyssa Panitch[6] · Kit S. Lam[3] · Aijun Wang[1,2,7]

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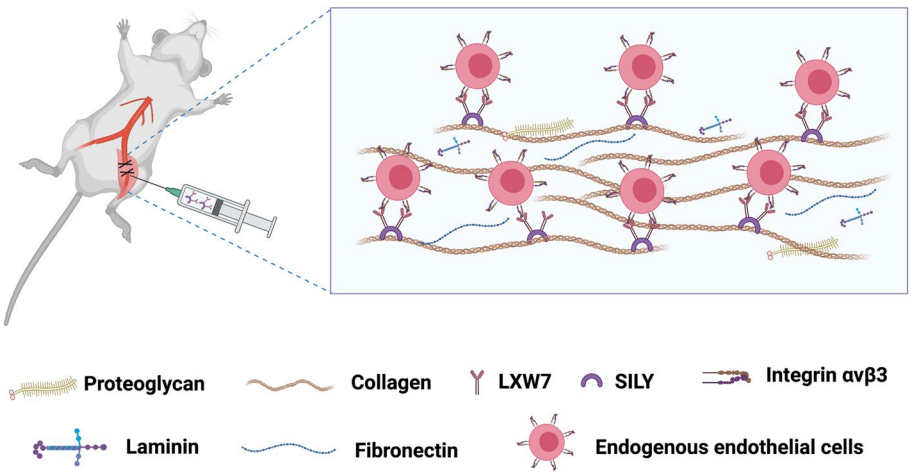
Abstract

Ischemic injury causes dynamic damage to the native extracellular matrix (ECM), which plays a key role in tissue homeostasis and regeneration by providing structural support, facilitating force transmission, and transducing key signals to cells. The main approach aimed at repairing injury to ischemic tissues is restoration of vascular function. Due to their potential to form capillary niches, endothelial cells (ECs) are of greatest interest for vascular regeneration. Integrin binding to ECM is crucial for cell anchorage to the surrounding matrix, spreading, migration, and further activation of intracellular signaling pathways. In this study, we proposed to establish an *in-situ* engineering strategy to remodel the ECM at the ischemic site to guide EC endogenous binding and establish effective EC/ECM interactions to promote revascularization. We designed and constructed a dual-function molecule $(LXW7)_2$-SILY, which is comprised of two functional domains: the first one (LXW7) binds to integrin αvβ3 expressed on ECs, and the second one (SILY) binds to collagen. In vitro, we confirmed $(LXW7)_2$-SILY improved EC adhesion and survival. After in situ injection, $(LXW7)_2$-SILY showed stable retention at the injured area and promoted revascularization, blood perfusion, and tissue regeneration in a mouse hindlimb ischemia model.

Graphical Abstract



Dake Hao and Lu Lu the authors contributed equally to this work.

Extended author information available on the last page of the article

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Highlights

- A dual-function peptide developed for in-situ engineering native extracellular matrix.
- The dual-function peptide specifically anchors endogenous endothelial cells to extracellular matrix.
- The dual-function peptide promotes vascularized tissue regeneration.

Keywords Extracellular matrix · Integrin · Cell-binding sites · Revascularization · Ischemic injury

Introduction

Ischemic vascular diseases (IVDs) are a leading health concern and the principal cause of morbidity and mortality throughout the world. IVD is a local manifestation of atherosclerosis and results from the blockage or narrowing of myocardial and non-myocardial arteries [1]. Generally, IVDs mainly include coronary heart disease (CHD) [2], carotid artery disease (CAD) [3] and peripheral arterial disease (PAD) [4, 5]. End organ ischemia is the main pathological feature of IVDs. Impaired blood circulation deprives the organs and tissues of oxygen and nutrient supply, which leads to pathological conditions such as heart attack, stroke, or limb ischemia [6, 7]. Surgical and endovascular interventions are the common therapies for the restoration of blood supply to the ischemic tissue, but they only delay the progression of ischemia and do not induce revascularization [8]. Most ischemic patients have recurrent symptoms and periprocedural complications [9]. Hence, sufficient revascularization has been identified as a key challenge for successful ischemic injury repair [10, 11].

Extracellular matrix (ECM) is a three-dimensional extracellular network and an important regulator of cell function in tissue regeneration [12–14]. The ECM not only provides structural support for tissue integrity and stability, but also regulates vascular cell growth, migration, differentiation, cellular signal transduction and responses [15–18]. In normal conditions, the ECM is a well-organized dynamic structure that controls tissue homeostasis and regeneration processes through continuous remodeling [19, 20]. However, under ischemic injury conditions, the balance of ECM structure and function is dynamically damaged. Dysregulated ECM is directly associated with the pathogenesis of ischemic vascular injury and even exacerbates disease progression [21, 22]. Therefore, ECM remodeling plays a crucial role for promoting vascularization in preventing and treating IVDs [10, 19, 23, 24].

ECM is composed of various components, mainly including collagens, proteoglycans, elastin, fibronectin, and laminins, which provide cell binding sites for facilitating signal transduction during tissue regeneration [25–27]. Integrins, a family of heterodimeric transmembrane receptors expressed on the cell surface, mediate cell-ECM adhesion through interactions with the ligands in the ECM [28, 29]. Integrin αvβ3 is highly expressed on the surface of endothelial cells (ECs), which contributes to EC adhesion and vascularization [30–33]. Previously, we used One-Bead One-Compound (OBOC) combinatorial technology and identified a ligand, LXW7, which has specific and high binding affinity to ECs via binding integrin αvβ3 [30]. Our previous studies demonstrated that ECM-mimicking scaffolds modified with LXW7 can prominently enhance endogenous EC recruitment and promote vascularization [34, 35]. Additionally, LXW7-functionalized collagen-based scaffolds showed the ability to improve EC adhesion, survival, and revascularization in the ischemic-mimicking environment [36–38]. Thus, LXW7 is an ideal ligand to increase the number of integrin binding sites in the ECM and regulate endogenous EC functions for improving vascularization. Moreover, collagen is the main component of ECM, and we have identified a strong and specific collagen-binding peptide SILY and used it to modify collagen-based scaffolds [15, 37, 39–41]. In this study, we designed and constructed a dual-function compound, which is comprised of two functional domains: LXW7 binds to integrin αvβ3 expressed on ECs and SILY binds to collagen, to enhance the revascularization potential of the ECM by increasing the αvβ3 integrin-mediated EC binding sites within the ECM at the ischemic injury area and stimulate the endogenous ECs to promote vascularization for ischemic tissue repair (Fig. 1).

Results

Collagen surface treated with $(LXW7)_2$-SILY improved EC attachment

The dual-function molecule $(LXW7)_2$-SILY was synthesized by conjugating the integrin ligand LXW7 to the collagen-binding peptide SILY via click chemistry (Fig. 2A). Electrospray Ionization Mass Spectrometry (ESI–MS) has been performed and confirmed the molecular weight and structure of $(LXW7)_2$-SILY (Fig. 2B), and High-Performance Liquid Chromatography (HPLC) has been performed and demonstrated the high purity of $(LXW7)_2$-SILY (Fig. 2C). Collagen surface treated with $(LXW7)_2$-SILY significantly enhanced EC adhesion compared to the surfaces treated with PBS or LXW7 (Fig. 3A, B), and no significant difference between the PBS group and the LXW7 group, indicating

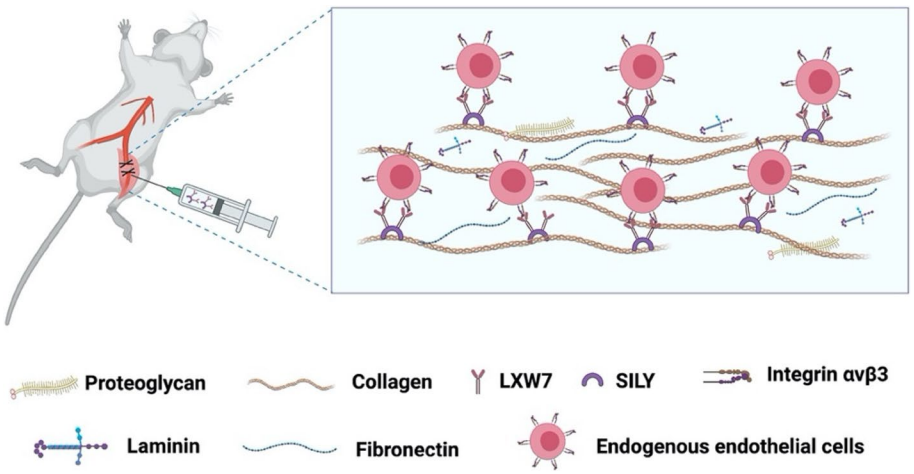

Fig. 1 Schematic diagram of the study design. The dual-function molecule $(LXW7)_2$-SILY was designed to increase the density of integrin-mediated EC binding sites on the injured ECM and to regulate endogenous ECs for improving revascularization in the mouse hindlimb ischemia model

$(LXW7)_2$-SILY treatment could promote EC binding on the collagen surface via providing more specific EC binding sites.

Collagen surface treated with $(LXW7)_2$-SILY inhibited EC apoptosis and promoted EC survival under simulated ischemic environmentin vitro

Collagen surface treated with $(LXW7)_2$-SILY significantly decreased caspase 3 expression in ECs under the simulated ischemic environment in vitro, compared to the PBS and LXW7 groups (Fig. 3C). These results may be caused by the additional EC binding sites provided by $(LXW7)_2$-SILY treatment. Subsequently, the results showed that the $(LXW7)_2$-SILY treated collagen surface significantly improved EC survival under the simulated ischemic environment in vitro (Fig. 3D), indicating the $(LXW7)_2$-SILY treated collagen surface was beneficial for EC survival by increasing the number of specific EC binding sites.

$(LXW7)_2$-SILY promoted blood perfusion, tissue regeneration, and revascularization in the mouse hind limb ischemia model

We evaluated the effects of $(LXW7)_2$-SILY in the mouse ischemic hind limb model. By using Laser Doppler perfusion imager (LDPI), we demonstrated the $(LXW7)_2$-SILY treatment significantly increased the blood perfusion at different time points after injection (Fig. 4A, B), compared to the PBS and LXW7 groups. Also, no significant difference was found between the PBS and LXW7 groups (Fig. 4A, B). In addition, compared to the PBS and LXW7 groups, micro-CT imaging confirmed the $(LXW7)_2$-SILY treatment significantly improved the vessel volume (Fig. 4C, D), indicating the $(LXW7)_2$-SILY possesses the ability to reconstruct the blood vessel structure.

Furthermore, the histologic analysis and immunofluorescence staining were performed to determine the tissue regeneration and revascularization. Compared to the PBS and LXW7 groups, the Hematoxylin and Eosin (H&E) staining results showed the $(LXW7)_2$-SILY treatment significantly reduced centrally located nuclei (Fig. 5A, B), and the Masson Trichrome staining results confirmed $(LXW7)_2$-SILY treatment significantly decreased the collagen deposition (Fig. 5A, C), demonstrating the SILY conjugation improves the regeneration function of LXW7. The CD31 and α-smooth muscle actin (α-SMA) staining were performed to evaluate the formation of capillaries and arterioles respectively, and the results showed $(LXW7)_2$-SILY significantly enhanced the revascularization (Fig. 6A, B, and C), compared to PBS and LXW7 groups. These results further indicated the $(LXW7)_2$-SILY approach designed in this study promotes the regenerative function of LXW7 via including a collagen-binding peptide to immobilize LXW7 on the native ECM.

Discussion

ECM is a non-cellular structure that displays a high number of cell binding sites, which support cell–matrix adhesion and regulates many cell functions [10, 42, 43]. Cell surface receptors transduce signals into cells from the ECM, an action which regulates diverse cellular functions, such as attachment, growth, proliferation, migration, survival, differentiation, and some vital roles in maintaining cell homeostasis for tissue regeneration [44–47]. As such, ECM plays an important role in tissue repair. Hence, current efforts have largely been focused on constructing artificial implantable biomaterial scaffolds/matrices by mimicking the physical properties and biological function of native ECM for tissue repair [48–52]. However, to fully mimic the native ECM

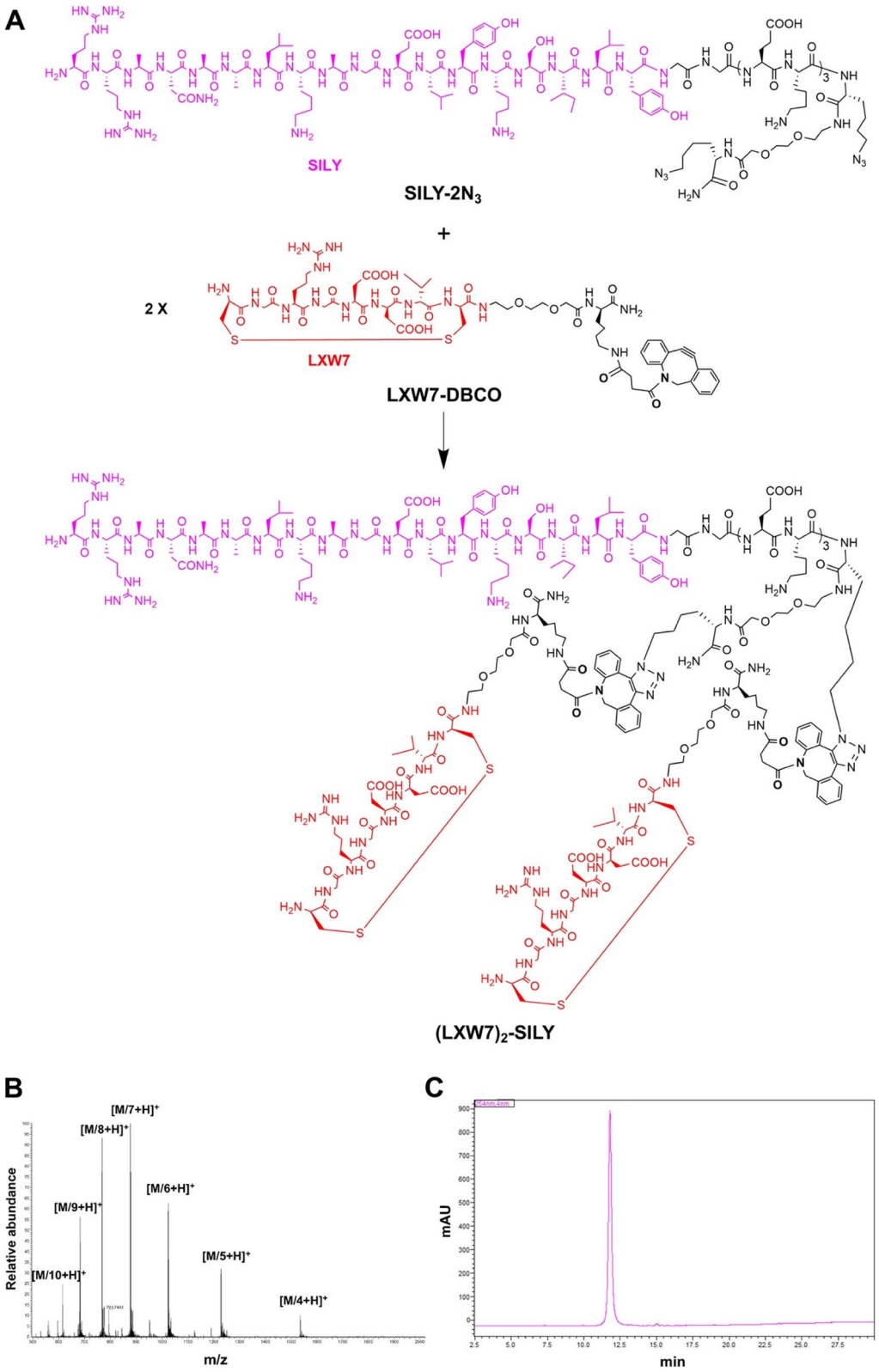

Fig. 2 Synthesis and characterization of $(LXW7)_2$-SILY. **A** The chemical synthesis approach of $(LXW7)_2$-SILY. **B** ESI–MS profile. **C** HPLC profile

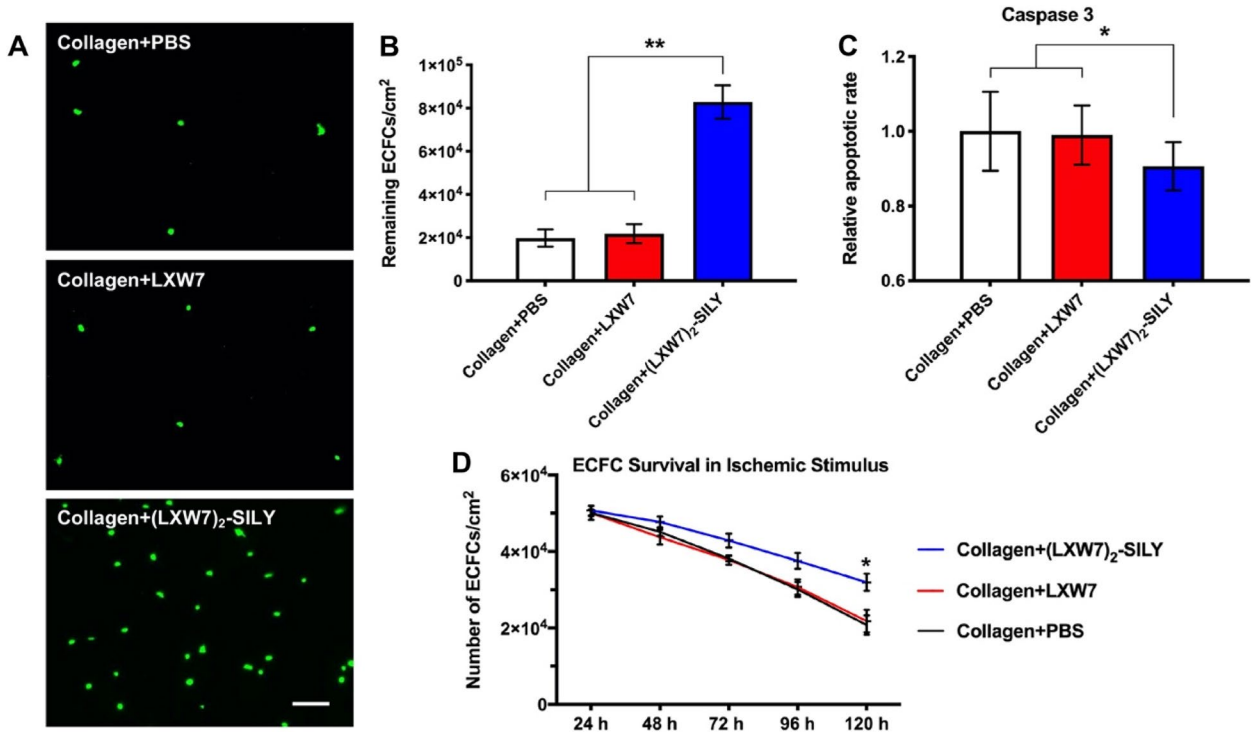

Fig. 3 Attachment, apoptosis, and survival of ECs on collagen surface with $(LXW7)_2$-SILY. **A** Images of ECs attached on collagen surfaces treated with PBS, LXW7, or $(LXW7)_2$-SILY. The attached ECs were stained with Calcein-AM (green). Scale bar = 50 μm. **B** Number of ECs attached on the different treated surfaces. **C** Caspase 3 expression in ECs cultured on different treated surfaces with simulated ischemic environment. **D** Survival of ECs cultured on the different treated surfaces with simulated ischemic environment. Data were expressed as mean ± standard deviation: $*p < 0.05$ ($n = 6$)

is technically challenging because of its dynamic structure and function [46, 53, 54]. Additionally, applying the ECM-mimicking artificial scaffolds often involves surgical procedures which in turn causes more injury to the tissue [26, 43]. Native ECM is the best template for cell binding and tissue regeneration. If we could fully utilize and optimize the physical properties and biological functions of the native ECM in a minimally invasive manner, it could provide an effective approach to modulate the microenvironment and guide tissue repair [55–57]. Therefore, in this study, we attempted to develop an innovative approach to engineer the native ECM in situ, to modulate endogenous EC binding and function to thus improve revascularization for tissue repair.

ECs play critical roles in vascularization and blood perfusion, both of which are crucial processes during ischemic tissue repair. Here, we engineered and remodeled the native ECM to modulate the behaviors of endogenous ECs by increasing the density of αvβ3 integrin ligands to improve vascularization and blood perfusion. The ECM motifs that bind to cell-surface integrins, play a major role in regulating cell adhesion and other cell-ECM interactions [58]. Additionally, enhanced cell-ECM engagement through increased density of integrin-binding motifs has been shown to stimulate key cellular functions [59]. In previous studies we also found that increased density of the αvβ3 integrin binding peptide, LXW7, stimulates angiogenesis through activation of VEGFR2 [30]. Further, in previous studies investigating the application of LXW7, we established a way to immobilize LXW7 on the collagen, using a collagen-binding peptide SILY and showed that the higher density of the integrin-based EC binding sites held greater potential for promoting vascularization. In the previous study [36] and the current study, we characterized $(LXW7)_2$-SILY of varying concentrations using HPLC, indicating $(LXW7)_2$-SILY possesses high solubility. Moreover, based on the in vitro and in vivo results obtained in the previous study [36] and the current study on different cell types and animal models, $(LXW7)_2$-SILY demonstrates strong stability without noticeable toxicity. The muscle fiber size is another important indicator for identifying muscle tissue remodeling. Unfortunately, the processing method of muscle tissue for histological studies used in this study showed the muscle fiber are shrunk, thus, are not good enough for calculating fiber size. To avoid the shrunk of muscle fibers, the frozen isopentane method should be used to freeze muscle tissues for histological analysis in the future studies [60].

Based on the indispensable role of ECM in cell function and tissue regeneration, this study utilized molecular engineering technology to optimize the structure and

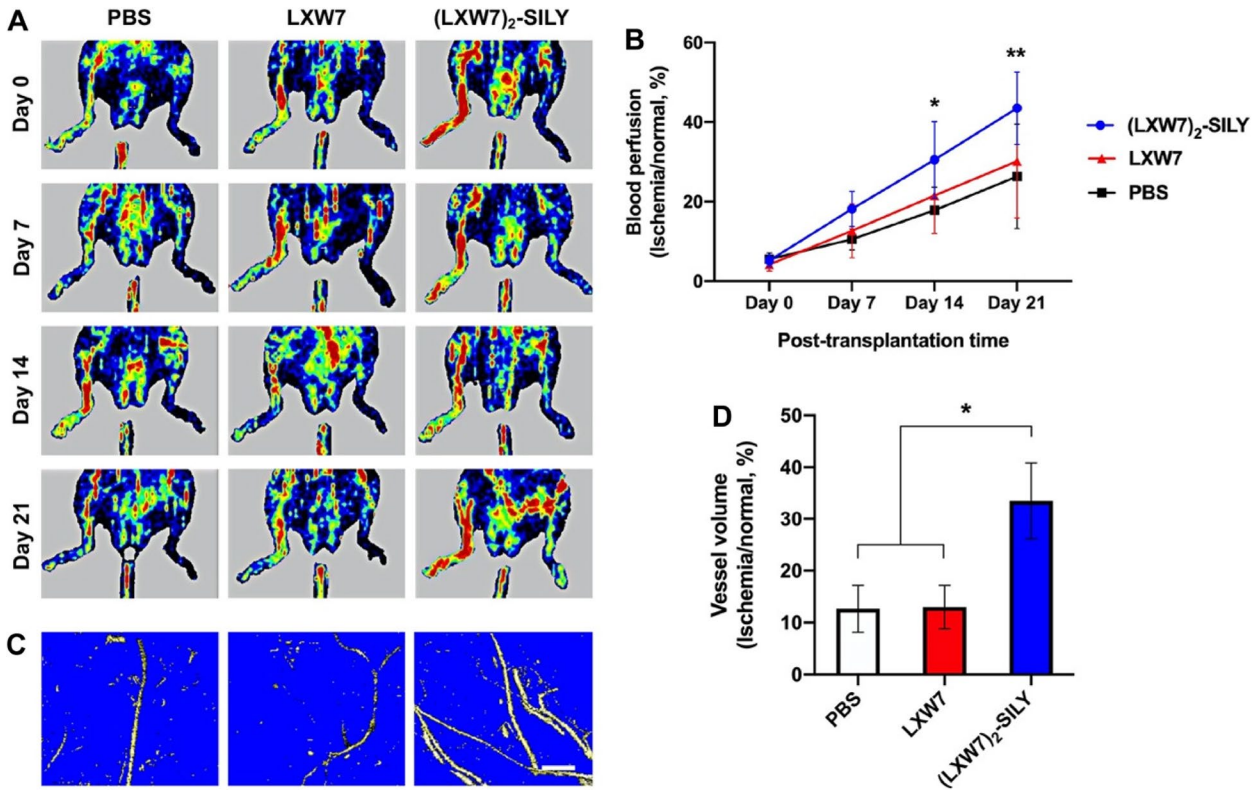

Fig. 4 $(LXW7)_2$-SILY promoted blood perfusion and vascular regeneration in the mouse hind limb ischemia model. **A** LDPI images and (**B**) quantification of blood perfusion. **C** Micro-CT images and (**D**) quantification of regenerated blood vessels. Scale bar = 1 mm. Data are expressed as mean ± standard deviation: $*p < 0.05$, $**p < 0.01$ ($n = 6$)

function of natural ECM, achieving regulation of specific endogenous cell behaviors and tissue regeneration. This study establishes new proof of concept for in situ ECM engineering to modulate the interactions between cells and ECM and provides an innovative approach to engineer and utilize native ECM to guide cell behaviors and promote tissue regeneration.

Conclusion

This study sought to design and construct a dual-function compound $(LXW7)_2$-SILY to engineer the native ECM and promote revascularization for ischemic injury repair. With the abundant expression of collagen across tissues and organs and the significance of vascularization in tissue repair and regeneration, $(LXW7)_2$-SILY technology holds promise across various tissue regeneration and clinical applications. Moreover, $(LXW7)_2$-SILY could also be used in functionalizing collagen-based biomaterials and scaffolds for improved vascularization in treating various diseases and conditions.

Materials and methods

Cells

We used endothelial colony forming cell (ECFC)-derived ECs from our cell banks [30, 34]. ECs were expanded in Endothelial Cell Growth Medium-2 (EGM-2, Lonza).

Synthesis and characterization of $(LXW7)_2$-SILY

Previously, we have demonstrated the dual-function molecule $(LXW7)_2$-SILY held the greatest potential to improve EC functions [36]. We synthesized $(LXW7)_2$-SILY through three steps: 1) standard solid phase peptide synthesis (SPPS) of SILY-$2N_3$, 2) SPPS synthesis of LXW7-DBCO, 3) DBCO-N_3 conjugation by mixing SILY-$2N_3$ with 2 eq. of LXW7-DBCO. Detailed synthesis was described in Fig. 2. $(LXW7)_2$-SILY was characterized by using ESI–MS to confirm the molecule weight and structure and HPLC to validate the purity of the synthesized molecule.

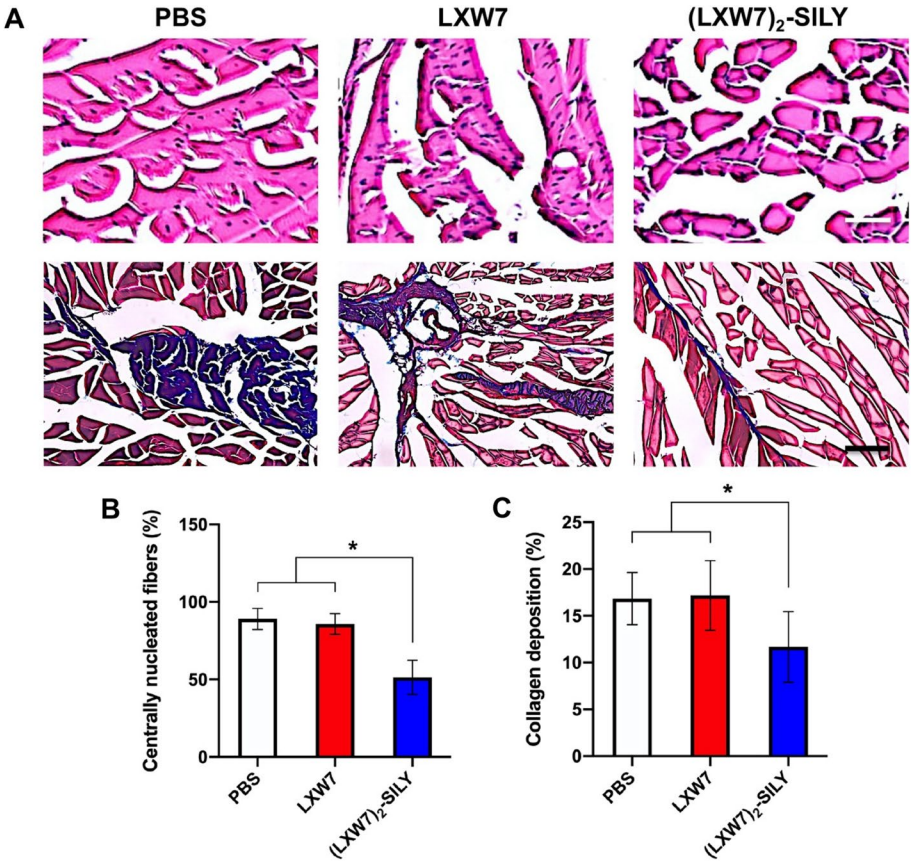

Fig. 5 $(LXW7)_2$-SILY promoted tissue regeneration in the mouse hind limb ischemia model. **A** H&E and Masson Trichrome images of tissue treated with PBS, LXW7, or $(LXW7)_2$-SILY. Scale bar = 20 μm in H&E images, and 100 μm in Masson Trichrome images. $(LXW7)_2$-SILY treatment significantly decreased (**B**) centrally nucleated myofibers and (**C**) collagen deposition in tissue, compare to the PBS and LXW7 groups. Data are expressed as mean ± standard deviation: $*p < 0.05$ ($n = 6$)

EC attachment on $(LXW7)_2$-SILY treated collagen surface

24-well plate cell culture wells were incubated with 500 μL of 100 μg/mL type I collagen (PureCol) at 37℃ for 1 h. After rinsed with PBS (HyClone), the wells were incubated with 500 μL of 40 μM LXW7 or 500 μL of 20 μM $(LXW7)_2$-SILY for 1 h [36]. The wells were treated with 1% BSA (Thermo Fisher Scientific) for 1 h. 5×10^3 ECs were incubated in the wells for 5 min and washed with PBS. The adhered cells were fixed in 10% formalin for 20 min and stained with Calcein AM (Thermo Fisher Scientific). Image was generated using a Carl Zeiss Axio Observer D1 inverted microscope and quantified using the ImageJ software.

EC apoptosis and survival on $(LXW7)_2$-SILY treated collagen surface under simulated ischemic environment

The simulated ischemic environment was as described previously [61, 62]. ECs were seeded in collagen-coated 96-well plates treated with PBS, LXW7, or $(LXW7)_2$-SILY under the simulated ischemic environment. The cells were cultured for 6 h and determined by using a Caspase 3 Assay Kit (Cell Signaling Technology). The cells were cultured for 5 days, and the MTS Assay (Promega) was performed to determine cell survival [63].

Animal study

Female mice (C57BL/6 J, 8-wk-old, Jackson Laboratory) were used in this study. Mice were operated with unilateral hind limb ischemia surgeries under anesthesia as described in our previous study [21]. The experimental groups were designed as: (1) PBS ($n = 6$); (2) LXW7 ($n = 6$); (3) $(LXW7)_2$-SILY ($n = 6$). In line with our previous research [21, 36, 64], we administered 100 μL of 100 μM LXW7 or 100 μL of 50 μM (LXW7)2-SILY via intramuscular injection at four distinct locations surrounding the ischemic region of the hind limb. Blood perfusion was monitored weekly for up to three weeks, using a LDPI system to calculate the perfusion ratio between the ischemic and non-ischemic limbs. Afterward, the mice were euthanized, and a catheter was placed in the left ventricle. The vasculature was flushed with PBS containing 100 U/mL of heparin sodium, followed by perfusion with 4% paraformaldehyde. To enhance contrast, Microfil MV-120 (Flow Tech, Inc.)

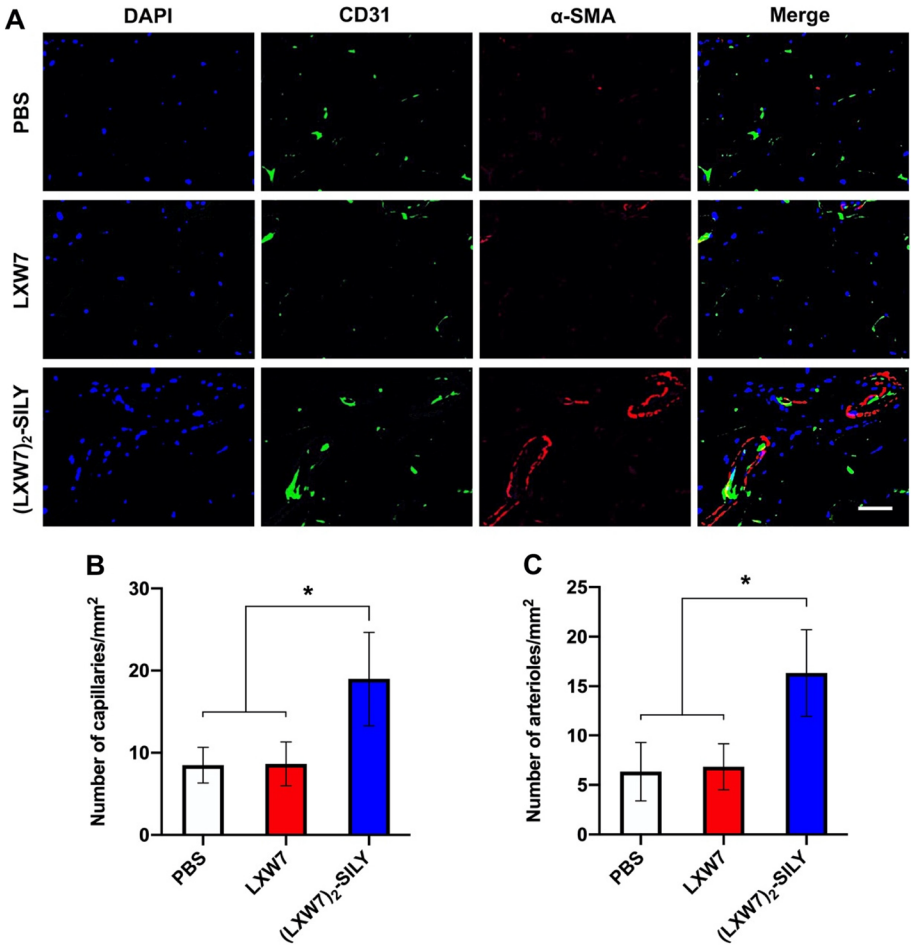

Fig. 6 $(LXW7)_2$-SILY improved revascularization in the mouse hind limb ischemia model. **A** Immunofluorescence staining of CD31 and α-SMA. Scale bar = 50 μm. Quantification of the density of (**B**) capillaries and (**C**) arterioles. Data are expressed as mean ± standard deviation: $*p < 0.05$ ($n = 6$)

was injected into the left ventricle. Finally, the leg muscles were harvested and analyzed using micro-CT imaging.

Histologic analysis and immunofluorescence staining

The animals were euthanized. The hind limb tissue were harvested and immersed in 4% paraformaldehyde at 4 °C for 48 h. 6-μm cryosections were prepared and stained with H&E (Thermo Fisher Scientific), Masson Trichrome (Thermo Fisher Scientific), CD31 antibody (Abcam), or α-SMA antibody (Abcam) according to the manufacturer's instructions respectively. The The images were captured using microscope and analyzed using ImageJ.

Statistical analysis

Prism was used for the statistical analysis. Analysis of variance (ANOVA) was used to evaluate the significant difference between different groups, and the post-analysis was performed using Holm's t test. A value of $p < 0.05$ indicates the significant difference.

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Authors' contributions DH designed and performed the in vitro and in vivo evaluation, performed the animal surgery, wrote the manuscript, and discussed the results. LL and PZ assisted the animal surgery. HS and JML assisted the in vitro evaluation. RL synthesized $(LXW7)_2$-SILY. JN, DF, EK, AP, and KL were involved in the results discussion. AW and DH supervised the study and provided funding support. All authors contributed to the article and approved the submission.

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Data availability The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at University of California, Davis.

Consent for publication Not applicable.

Competing interests AP, KL, and AW are founders in VasoBio Inc, which has a license to the LXW7 peptide. Other authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Authors and Affiliations

Dake Hao[1,2] · Lu Lu[1] · Hengyue Song[1,2] · Juan-Maria Lopez[1,2] · Ruiwu Liu[3] · Ping Zhou[4] · Jan Nolta[4] · Diana L. Farmer[1,2] · Eric Kurzrock[5] · Alyssa Panitch[6] · Kit S. Lam[3] · Aijun Wang[1,2,7]

✉ Dake Hao
dkhao@ucdavis.edu

✉ Aijun Wang
aawang@ucdavis.edu

1 Department of Surgery, University of California Davis, Sacramento, CA 95817, USA

2 Institute for Pediatric Regenerative Medicine, Shriners Hospitals for Children, Sacramento, CA 95817, USA

3 Department of Biochemistry and Molecular Medicine, University of California Davis, Sacramento, CA 95817, USA

4 Stem Cell Program and Gene Therapy Center, Institute for Regenerative Cures, University of California Davis, Sacramento, CA 95817, USA

5 Department of Urologic Surgery, University of California Davis, Sacramento, CA 95817, USA

6 Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA 30332, USA

7 Department of Biomedical Engineering, University of California Davis, Davis, CA 95616, USA