### MATERIALS SCIENCE

### Engineered bridge protein with dual affinity for bone morphogenetic protein-2 and collagen enhances bone regeneration for spinal fusion

Priscilla S. Briquez<sup>1</sup>\*, Hsiu-Ming Tsai<sup>2</sup>, Elyse A. Watkins<sup>1</sup>, Jeffrey A. Hubbell<sup>1,3,4</sup>\*

The revolutionizing efficacy of recombinant human bone morphogenetic protein (rhBMP-2) for clinical spinal fusion is hindered by safety issues associated with the high dose required. However, it continues to be widely used, for example, in InFUSE Bone Graft (Medtronic). Here, we developed a translational protein engineering–based approach to reduce the dose and thereby improve the safety of rhBMP-2 delivered in a collagen sponge, as in InFUSE Bone Graft. We engineered a bridge protein with high affinity for rhBMP-2 and collagen that can be simply added to the product's formulation, demonstrating improved efficacy at low dose of rhBMP-2 in two mouse models of bone regeneration, including a newly developed spinal fusion model. Moreover, the bridge protein can control the retention of rhBMP-2 from endogenous collagenous extracellular matrix of tissue. Our approach may be generalizable to other growth factors and collagen-based materials, for use in many other applications in regenerative medicine.

#### INTRODUCTION

Back pain ranks as the leading cause of disability in the world, with about 50 to 80% of adults experiencing it during their lifetimes. It strongly reduces mobility, quality of life, and ability to work, thus being one of the main causes of absenteeism (1). While most people recover from back pain within a few months following onset, it has been estimated that in the United States, about 20 to 44% of back pain episodes recur within the first year (2-4). While the causes of back pain are often nonspecific and hard to determine, spine trauma, fractures, deformity, instability, spondylolisthesis, herniated disc, and degenerative disc disease are common causes that can be treated by spinal fusion.

Spinal fusion is a common surgical technique that consists of fusing two adjacent vertebrae to eliminate their relative movement that causes the pain. It can be performed using fixation devices, such as plates, screws, and rods, interbody devices that are placed between the two vertebrae to restore spacing and provide support, and/or graft materials and biologics to induce bone growth and seal the adjacent vertebrae together. For example, bone allo- and autografts can be shaped as implants to fit the intervertebral space and promote intervertebral fusion. In the United States, more than 350,000 spinal fusion interventions are performed yearly, a number that is expected to increase in the coming years (5, 6) because of an aging population and the rise of robotic-assisted minimally invasive surgeries for spinal fusions. From an economical perspective, the annual spinal device market in 2020 represented \$1.9 billion and \$6.5 billion in the United States and worldwide, respectively, and is projected to reach more than \$8 billion globally by 2027 (7).

In this market, a product called InFUSE Bone Graft (Medtronic), a premium bone graft substitute to autografts, has been estimated to be used in 100,000 spinal fusion procedures per year in the United Copyright © 2021 The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original U.S. Government Works. Distributed under a Creative Commons Attribution NonCommercial License 4.0 (CC BY-NC).

States (8). InFUSE Bone Graft is composed of an absorbable bovine type I collagen sponge laden with recombinant human bone morphogenetic protein-2 (rhBMP-2), the latter being a very potent growth factor (GF) that induces bone formation at the site of implantation. InFUSE Bone Graft is used in combination with an interbody cage made of titanium or polyether ether ketone. Following its approval for application in anterior lumbar interbody fusion (ALIF) by the U.S. Food and Drug Administration (FDA) in 2002, the revolutionizing efficacy of rhBMP-2 fostered its broad use in more than 40 to 50% of primary ALIF surgeries and in off-label applications, such as posterior lumbar or anterior cervical fusions (9, 10). In 2004 and 2007, rhBMP-2 was further approved for nonunion tibial repair and oral maxillofacial reconstruction, respectively, expanding its clinical use.

Nevertheless, serious safety concerns rapidly arose in patients treated with InFUSE Bone Graft, triggering an important controversy on the product. Adverse effects were life-threatening and included ectopic bone growth, cancer, nerve damage, severe inflammation, and male sterility (9, 11). Consequently, the FDA issued a safety warning on the use of rhBMP-2 for cervical spinal fusion (an off-label use) in 2008 and rejected a product called Amplify (Medtronic) that contains a higher dose of rhBMP-2 in 2011. Very recently, in March 2020, *Becker's Spine Review* announced that Medtronic has voluntarily and momentarily withdrawn InFUSE Bone Graft from the Australian market to limit off-label use, keeping it accessible only via a special request program (12).

While the controversy on InFUSE Bone Graft safety continues, Medtronic has expanded its product line and received a new FDA approval for additional spinal implants containing rhBMP-2 (13). In parallel, the experience of rhBMP-2 use in the clinic has strongly accelerated the research on new biomaterials capable of better controlling the release of rhBMP-2 to reduce therapeutic dosing and, thus, side effects (14). While some of these biomaterials are very effective, modification of the carrier material or rhBMP-2 might lead to long and expensive regulatory procedures, with increased safety requirements due the history of rhBMP-2 (15).

Consequently, in this study, we undertook the challenge of developing a simple and highly translatable approach to efficiently control rhBMP-2 release from InFUSE Bone Graft, without altering

<sup>&</sup>lt;sup>1</sup>Pritzker School of Molecular Engineering, University of Chicago, Chicago, IL 60637, USA. <sup>2</sup>Integrated Small Animal Imaging Research Resources (iSAIRR), Department of Radiology, University of Chicago, Chicago, IL 60637, USA. <sup>3</sup>Committee on Immunology, University of Chicago, Chicago, IL 60637, USA. <sup>4</sup>Committee on Cancer Biology, University of Chicago, Chicago, IL 60637, USA.

<sup>\*</sup>Corresponding author. Email: jhubbell@uchicago.edu (J.A.H.); pbriquez@uchicago.edu (P.S.B.)

either the rhBMP-2 or the collagen sponge. Taking inspiration from the natural extracellular matrix (ECM), which controls the release of GFs via ECM glycoproteins that bind to both collagens and GFs, we engineered "bridge" proteins with dual high affinity for type I collagen and rhBMP-2 to increase rhBMP-2 retention within the collagen sponges (Fig. 1A). To do so, we linked an antibody fragment (Fab) derived from an anti-collagen antibody, here called FabCol, to a GF-binding domain with promiscuous affinity for heparinbinding GFs, including for rhBMP-2. We explored two different GF-binding domains, derived from the ECM proteins fibrinogen (a domain that we call FgHBD) (16) and laminin (a domain that we call LamLG4) (17), and covalently attached them to the FabCol domain via chemical conjugation or by recombinant expression as fusion protein. Since we designed our bridge proteins to be an addin to products such as InFUSE Bone Graft, we assessed their potential in an InFUSE-like formulation very close to the clinical one, containing a bioactive rhBMP-2 produced in Chinese hamster ovary (CHO) cells, which is the same expression system that is used in the clinical drug substance, and a bovine type I collagen sponge manufactured by Integra LifeSciences, which is the same as that used in

InFUSE Bone Graft. In addition, we strictly followed the InFUSE Bone Graft instructions for material preparation, in which rhBMP-2 should be incubated onto the collagen sponge for a minimum of 15 min before implantation. We do note, however, that the rhBMP-2 doses per unit volume that we use in our studies are much lower than that used in the clinical product, providing a very favorable dose reduction opportunity, as presented in detail below.

Among the four bridge proteins designed and evaluated, we found that the FabCol-LamLG4 fusion protein was the most effective in bridging rhBMP-2 to collagen, allowing for increased sequestration of rhBMP-2 in collagen sponges and in the local endogenous collagenous ECM upon release. In a calvarial bone defect model in mice, we showed that adding FabCol-LamLG4 into the InFUSE-like formulation improves bone defect coverage and regenerated bone volume at the very low dose of 50 ng of rhBMP-2 per defect (2  $\mu$ g/ml), at which the InFUSE-like formulation alone was ineffective. We lastly developed an intervertebral defect model in mice, mimicking spinal fusion applications, to confirm the superiority of the FabCol-LamLG4 in promoting intervertebral bone growth. In conclusion, here, we propose a single bridge fusion protein that can be recombinantly produced



### A Illustration of the engineered bispecific bridge proteins for retention of rhBMP-2 into collagen sponge

**Fig. 1. Engineered bridge proteins have dual affinity to collagen I and rhBMP-2. (A)** Schematic representation of a bridge protein with dual specific affinities to collagen I and to rhBMP-2. The bridge proteins are composed of the Fab domain of an anti-collagen I antibody (here, named FabCol) associated with a GF-binding domain with strong affinity to rhBMP-2. (**B**) SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of the engineered bridge proteins. Linkage of the FabCol domain (lane 1) to the GF-binding domains was achieved either by chemical conjugations (conj; lanes 2 and 3) or by expression as fusion proteins (lanes 4 and 5). Two GF-binding domains were evaluated: the heparin-binding domain of fibrinogen (FgHBD) and the LG4 domain of laminin  $\alpha$ 3 (LamLG4). (**C**) Binding of the bridge protein variants to type I bovine collagen. The FabCol domain allows strong binding of all the bridge proteins to collagen I with dissociation constant ( $K_D$ ) around 1 nM (mean ± SD). (**D**) Binding of the bridge protein variants to rhBMP-2. GF-binding sites chemically conjugated or recombinantly fused to the FabCol domain allow significant binding to rhBMP-2, particularly for the FabCol-LamLG4 fusion protein variant, which displayed the highest binding [mean ± SD; one-way analysis of variance (ANOVA) with Tukey's post test, \*\*\* $P \leq 0.001$ ; stars on top of the columns indicate comparisons to FabCol and bovine serum albumin (BSA)].

and added into InFUSE Bone Graft to significantly reduce the required therapeutic dose of rhBMP-2 while preserving efficacy in regenerating bone.

#### RESULTS

## Bridge proteins display dual affinity for bovine type I collagen and rhBMP-2

We started by designing four bispecific bridge proteins that include in their structure a collagen-binding domain (FabCol) linked to a GF-binding domain. FabCol was obtained by grafting the variable regions of an anti-collagen Fab with validated affinity for human type I and II collagens (as published by others in the patent WO2016016269A1) onto human Fab constant regions. As GF-binding domains, we selected the heparin-binding domains of fibrinogen and of laminin  $\alpha$ 3, named FgHBD and LamLG4, respectively, which we previously found and characterized as being promiscuous GF-binding domains, with affinity for several heparin-binding GFs, including BMP-2 (16, 17). Two bridge proteins were engineered by chemical conjugation of FabCol to FgHBD or to a LamLG4-derived peptide and two others by recombinant expression of the fusion proteins FabCol-FgHBD and FabCol-LamLG4 (table S1). To design the fusion protein FabCol-FgHBD, we fused three copies of FgHBD to both the heavy and light chain of FabCol, as FgHBD dimerization is important for its GF binding function. In contrast to the FabCol-LamLG4, the LamLG4 domain was fused solely to the heavy chain of FabCol via its natural laminin  $\alpha$ 3 linker domain. In addition, glycine-serine (GlySer) linkers were used to increase molecular spacing between the collagen- and GF-binding domains. For all four bridge proteins, as expected, we observed an increase in molecular weight as compared to the FabCol without GF-binding domains on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1B). For the chemically conjugated variants, this increase was about 10 to 15 kDa, which suggests the attachment of about three to five peptides per FabCol. As to the fusion proteins, FabCol-FgHBD showed two bands, both lower than the expected 81.0-kDa theoretical size, which could be due to partial degradation or the particular structure of the protein. FabCol-LamLG4, on the other hand, appeared a main single band around the theoretical molecular size of 71.3 kDa, with a few additional faint bands of higher weights, potentially being multimeric forms of the protein. In addition, the presence of FgHBD and LamLG4 onto FabCol was confirmed by the ability of both fusion proteins to bind to heparin affinity chromatography columns during their purification.

We then assessed the binding of the different bridge proteins to bovine type I collagen using an enzyme-linked immunosorbent assay (ELISA)–based assay wherein the collagen was coated on the plate and the bridge proteins were detected via an antihuman Fab antibody. We found that all bridge proteins strongly bound to bovine collagen, with dissociation constant  $K_D$  ranging between 0.7 and 1.12 nM, similar to FabCol only, which has a  $K_D$  of 0.65nM (Fig. 1C). Therefore, the addition of the GF-binding domains to FabCol did not impair its binding capacity to collagen.

Last, we compared the ability of the different bridge proteins to bind to rhBMP-2, using a similar assay in which rhBMP-2 was coated on the plate. We found that all bridge proteins displayed some affinity for rhBMP-2, as compared to FabCol and bovine serum albumin (BSA), the latter being used as a control for nonspecific binding (Fig. 1D). Among the different variants, the fusion protein FabCol-LamLG4 showed a significant superiority in binding rhBMP-2. Notably, the superiority of the FabCol-LamLG4 fusion protein compared to the conjugate is likely due to a lower affinity of the shorter LamLG4-derived peptide used for conjugation as compared to the full LamLG4 domain used in the fusion protein (table S2).

### Bridge proteins enhance retention of rhBMP-2 into collagen sponges

Second, we assessed the bridge function of the proteins that we developed by measuring the binding of rhBMP-2 to collagen in their presence. We sequentially incubated ELISA plates coated with bovine type I collagen with the different bridge proteins and with rhBMP-2 and measured the amount of bound rhBMP-2. In this assay, we found that rhBMP-2 itself binds to collagen to some extent, a natural affinity that was not inhibited by the addition of FabCol (Fig. 2A and fig. S1). Moreover, we observed that all four bridge proteins significantly enhanced the binding of rhBMP-2 to collagen.

We then studied the release of rhBMP-2 in that presence of the bridge proteins from a three-dimensional (3D) bovine type I collagen sponge. To do so, the rhBMP-2 was mixed with the various bridge proteins and directly added onto the sponges. Wetted sponges were incubated for 15 min before being immersed into a release buffer, which was collected and refreshed daily. The amount of rhBMP-2 released was then quantified by ELISA (Fig. 2B). The results showed that collagen sponges alone did not efficiently control the release of rhBMP-2, which released to more than 80% after the first day. Similarly, the addition of the FabCol domain or the GF-binding peptides only was not sufficient to delay rhBMP-2 release. In contrast, the bispecific bridge proteins strongly increased rhBMP-2 retention, particularly the fusion protein variants, which released only 30% of the rhBMP-2 during the first day. The FabCol-LamLG4 fusion protein was the most efficient of the bridge proteins and still retained about 35% of the rhBMP-2 after 1 week. The chemically conjugated variants performed less well than the fusion proteins but were still capable of slowing rhBMP-2 release, especially for FabCol-FgHBDconj.

Furthermore, we performed an anti–BMP-2 immunofluorescence staining on the collagen sponges after 7 days of release to visualize the sequestered rhBMP-2. We observed that rhBMP-2 was detectable in sponges that contained the bridge proteins but not in any other controls (i.e., rhBMP-2 without a bridge protein or rhBMP-2 in the presence of the collagen-binding or the GF-binding domains only) (Fig. 2C). Here, again, the fusion protein variants performed better than the chemically conjugated ones, with the FabCol-LamLG4 fusion protein being impressively superior to the others.

In addition to being more effective, recombinant bridge fusion proteins would present multiple translational advantages as compared to chemically conjugated variants, particularly for production purposes and regulatory approval procedures since the number of GFbinding domains per collagen-binding domain and the subsequent protein design can be precisely controlled by the encoding DNA sequence. Therefore, we decided to focus the next part of our study on the characterization of the FabCol-FgHBD and FabCol-LamLG4 fusion proteins for in vivo applications.

### FabCol-LamLG4 can control the delivery of rhBMP-2 from the endogenous collagenous ECM

Because in vivo experimentation often requires larger doses of rhBMP-2 than the ones studied in vitro, we sought to determine the maximum amount of the bridge proteins that can be efficiently incorporated

rhBMP-2 binding to bovine Α collagen via bridge proteins



B Controlled release of rhBMP-2 from bovine collagen sponges



#### Retention of rhBMP-2 into bovine collagen sponges in the presence of the engineered bridge proteins С



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19

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Fig. 2. Bridge proteins enhance the retention of rhBMP-2 into bovine type I collagen sponges. (A) Binding of rhBMP-2 to bovine type I collagen via the engineered bridge proteins. The presence of any of the bridge proteins significantly enhanced the binding of the rhBMP-2 to collagen I as compared to rhBMP-2 only or in the presence of the FabCol domain only (mean  $\pm$  SD; one-way ANOVA with Tukey's post test, \*\*\*  $P \leq 0.001$ ). (B) Release of rhBMP-2 from collagen sponges in the presence of the engineered bridge proteins over 7 days. The release of rhBMP-2 is slowed by FabCol-LamLG4, FabCol-FgHBD, FabCol-FgHBDconj, and, to a lesser extent, FabCol-LamLG-4conj. In contrast, the FabCol domain or the GF-binding peptides (FgHBDpep and LamLG4pep) only are not sufficient to control rhBMP-2 release (mean ± SD; two-way ANOVA with Tukey's post test, \*P < 0.05; statistics are indicated for the day 1 time point). (C) Immunofluorescence images of the rhBMP-2 retained in the collagen sponges after 7 days and relative quantification of the fluorescence intensity of the immunostaining (scale bar, 100 µm; average of five fields of view per data point; mean ± SD; one-way ANOVA with Tukey's post test, \*\*\* $P \le 0.001$ ).

into the collagen sponges to deliver rhBMP-2. To determine that, we incubated various concentrations of fluorescently labeled Fab-Col domain, ranging from 0.01 to 10  $\mu$ M, in the collagen sponges for 15 min, vigorously washed them, and measured the remaining amount in the sponges via fluorescence. We unexpectedly found that a relatively low amount of FabCol was able to be retained into the collagen sponges (Fig. 3A), with an apparent maximal saturation concentration close to 0.4 µM.

This result implies that upon delivery of the bridge proteins, a substantial amount will be released in the tissues to encounter the endogenous ECM. Consequently, we questioned whether the bridge proteins would attach locally to the endogenous ECM upon release, considering that type I collagen is the most abundant ECM protein in almost all tissues. To evaluate that, we injected 1 µg of the fluorescent FabCol domain intradermally in the back of mice and monitored its presence over time using the IVIS In Vivo Imaging System. As a control for nonspecific tissue retention, we used a similar

fluorescent Fab domain called outer surface protein A (OspA) (from Borrelia burgdorferi) with no known specific affinity for any endogenous mouse proteins. We found that the FabCol domain was strongly retained in the endogenous collagenous ECM as compared to OspA, with about 50% of the initial dose retained after 6 days, and detectable levels observed for more than 10 days upon injection (Fig. 3B).

As a next step, we evaluated the retention of rhBMP-2 into the endogenous ECM in vivo when delivered via the bridge proteins. We intradermally injected 200 ng of rhBMP-2 mixed with the bridge proteins at a 1:10 molar ratio (i.e., with the bridge protein in excess) in the back of mice and quantified the amount of rhBMP-2 sequestered locally in the tissue after 1, 2, 4, and 7 days. We found that the FabCol-LamLG4 bridge protein was capable of controlling the release of rhBMP-2 from the endogenous matrix for 2 to 3 days, in contrast to rhBMP-2 alone, which was not retained in the tissue (Fig. 3C). Unexpectedly, FabCol-FgHBD did not improve the retention of rhBMP-2 within the physiological ECM, although it successfully did from the

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Maximal retention of FabCol Α in collagen sponges

0.5

0.4

0.3

0.2

0 1

0.0

0.01

final concentration in µM)

**Retained FabCol** 

Ε

### B Intradermal retention of FabCol into the endogenous collagenous ECM



1.0

#### In vivo retention of rhBMP-2 in endogenous collagen С



#### D FabCol binding to human collagens



Fig. 3. The FabCol domain binds to endogenous collagens, which permits FabCol-LamLG4 to retain rhBMP-2 into the native endogenous ECM. (A) Saturation concentration of the FabCol domain into collagen sponges (mean ± SD). (B) Binding of fluorescently labeled FabCol domain (1 µg) to endogenous collagen upon intradermal injection. OspA is a non-tissue-binding Fab here used as a negative control (mean  $\pm$  SD; multiple t test with Bonferroni correction, \*P  $\leq$  0.05 and \*\*\*P  $\leq$  0.001). (C) Retention of rhBMP-2 (200 ng) into endogenous collagen upon intradermal injection, when coinjected with the bridge proteins (\*\*P < 0.01). (D) Binding of the FabCol to human collagen I and III (mean ± SD; one-way ANOVA with Tukey's post test at the 100 nM concentration). (E) Schematic representation of the bridge protein variants delivered by carrier collagen sponges into native tissues. A fraction of the bridge proteins strongly binds into the collagen sponge from which it can efficiently deliver rhBMP-2, while the fraction in excess can bind to the surrounding collagenous matrix from which it continues to fulfill its role in controlling rhBMP-2 release. Through its ability to deliver GFs from the native ECM, the bridge protein mimics the role of native ECM glycoproteins and permits in vivo modulation of the matrix composition.

exogenous bovine type I collagen sponge (Figs. 2, B and C, and 3C). This might be due the presence of soluble fibrinogen in native tissues, which could compete with the interaction between FgHBD and rhBMP-2. Fibrinogen naturally contains the FgHBD domain and is present at relatively high levels in the blood, interstitial fluid, and lymph (16, 18).

To understand whether FabCol-LamLG4-mediated delivery of rhBMP-2 from the endogenous collagenous ECM would be possible in human ECM, we further evaluated binding of FabCol to purified

human collagen I and to purified human collagen III, the expression of which commonly increases in wounded tissues. We assessed the binding to collagens using an ELISA-like assay wherein collagencoated plates were incubated with various concentrations of FabCol, further revealed with an anti-Fab antibody. In an attempt to compare the relative binding to collagen I and III, we stopped the ELISA development reactions at the same time. We observed that FabCol significantly binds to both human collagens I and III and that the binding to collagen I was relatively much higher than to collagen A Dose response to rhBMP-2 delivered via bridge proteins in the calvarial defect model in mice



## C µCT scans of the regenerated bone in the calvarial defect model in mice



**Fig. 4. FabCol-LamLG4-mediated delivery of rhBMP-2 promotes bone regeneration in the calvarial defect model in mice.** Unilateral defects (4 mm) were created in mice calvarial bone and treated with collagen sponges laden with rhBMP-2 and/or the bridge proteins or PBS as a negative control. Both defect coverage and volume of regenerated bone were measured every 2 weeks by  $\mu$ CT scans as readouts of regeneration (BV/TV, regenerated bone volume over the total bone volume before defect) (mean ± SEM; \*P ≤ 0.05, \*\*P ≤ 0.01, and \*\*\*P ≤ 0.001; n.s., not significant). (**A**) Dose study of the regenerated bone at week 4 upon treatment with 50, 100, and 150 ng of rhBMP-2 in the presence of the bridge proteins ( $n \ge 6$  at 50 to 100 ng and n = 4 at 150 ng, mixed-effects analysis with Tukey's post test). (**B**) Quantification of defect coverage and regenerated bone volume at week 4 upon delivery of 50 ng of rhBMP-2 in the presence of the bridge proteins ( $n \ge 6$ , one-way ANOVA with Tukey's post test). (**C**) Representative CT scan images of the regenerated bone at week 4 after treatment (red circles, initial defect). (**D**) Increase in bone regeneration between weeks 2 and 4 upon treatment with 50 ng of rhBMP-2 in the presence of the bridge proteins ( $n \ge 6$ , one-way ANOVA with Sidak's post test).

III, assuming a similar coating efficiency of both collagens (Fig. 3D). In addition, we found that both dissociation constants to collagen I and III were in a comparable range of 0.3 to 0.4 nM.

Together, these findings suggest that upon implantation of a collagen sponge containing rhBMP-2 and a bridge protein, a fraction of the bridge proteins would be sequestered into the exogenous sponge and would efficiently deliver rhBMP-2 from there, while another unbound fraction would release from the sponge, attach within the local endogenous ECM, and control the release of the exogenous rhBMP-2 from the native tissue directly. The released fraction of the bridge proteins would modulate the composition of the native ECM by providing additional rhBMP-2 binding sites (Fig. 3E). These bridge proteins, attached to native collagens and providing GF-binding domains, strongly mimic the natural role of ECM glycoproteins, which controls the spatiotemporal release of GFs from the ECM during morphogenetic processes (19, 20).

## FabCol-LamLG4 promotes rhBMP-2-mediated bone regeneration in the calvarial defect model

We started to assess the in vivo efficacy of the bridge proteins in a calvarial defect model in mice. In this model, unilateral bone defects of 4 mm in diameter were surgically created and treated with bovine type I collagen sponges laden with 50, 100, or 150 ng of rhBMP-2 with or without the bridge proteins. Collagen sponges were incubated

B Bone regeneration at week 4 after defect upon delivery of 50 ng of rhBMP-2



D Bone growth over time

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#### A Intervertebral spine defect model in mice





**Fig. 5. FabCol-LamLG4 improves rhBMP-2-mediated bone regeneration in an intervertebral defect model in mice.** (A) Development of the intervertebral defect model. An intervertebral bone defect between the lumbar vertebra L2 and L3 in mice is surgically induced, which results in exposure of the undamaged underlying spinal cord. The defect is then covered with the collagen sponge laden with the different treatments to promote bone formation and L2-L3 vertebral fusion. Sagittal and transversal  $\mu$ CT imaging confirms the presence of the defect (red arrow) after the surgery. (B) Representative  $\mu$ CT images of the bone formed at 4 week upon treatments with 100 ng of rhBMP-2 with or without FabCol-LamLG4 or untreated (visible defects after 4 weeks are circled in red; regenerated bone is circled in white). (C) Quantification of the bone volume formed in the spinal defect upon treatment with 100 ng of rhBMP-2 with or without FabCol-LamLG4 (mean ± SEM;  $n \ge 6$ , one-way ANOVA with Tukey's post test, \*\* $P \le 0.01$  and \*\*\* $P \le 0.01$ ). (D) Proportion of spinal fusions upon treatments with rhBMP-2 ± FabCol-LamLG4 (n = 6 per group; complete fusion, substantial amount of bone bridging the vertebrae L2-L3 with no more visible defect; partial fusions, presence of bone between vertebrae L2-L3 with partially visible defect; no fusion, visible defect with no bone bridging the top of L2-L3). (E) Increase of bone formation between weeks 2 and 4 upon treatments (repeated-measurements two-way ANOVA with Sidak's post test, \* $P \le 0.01$ ).

for 15 min at room temperature before implantation, as indicated in the InFUSE Bone Graft preparation instructions. Here, the InFUSE-like formulation that we used as a clinically relevant comparison is indicated as rhBMP-2 only. As experimental readouts for bone regeneration, we measured the defect coverage and the formed bone volume using micro-computed tomography ( $\mu$ CT) scan imaging. The bone volume/total volume ratio (BV/TV) represents the amount of formed bone over the initial bone volume measured in uninjured skulls. Accordingly, a BV/TV > 100% indicates a thicker regenerated bone than in uninjured skulls.

Overall, we observed that both defect coverage and bone formation increase with the dose of rhBMP-2, which is expected, considering that rhBMP-2 is the bioactive compound that promotes osteogenesis. For all studied doses, we showed that FabCol-LamLG4 strongly enhanced rhBMP-2-mediated bone regeneration both in terms of defect coverage and bone volume as compared to rhBMP-2 only, as evaluated 4 weeks after surgery (Fig. 4A). This effect was statistically significant at the very low dose of 50 ng of rhBMP-2 per defect (delivered in a volume of  $25 \,\mu$ ), at which rhBMP-2 alone had very limited efficacy (Fig. 4, A and B). The addition of FabCol-LamLG4

Briquez et al., Sci. Adv. 2021; 7 : eabh4302 11 June 2021

to rhBMP-2 improved defect coverage from about 28 to 75% and bone volume from 45 to 110%. We further verified that this effect was not due to FabCol-LamLG4 alone in the absence of rhBMP-2. On the other hand, addition of FabCol-FgHBD to rhBMP-2 enhanced bone regeneration as compared to defects treated with phosphatebuffered saline (PBS) (i.e., collagen sponges only) and gave consistent trends toward increased bone regeneration as compared to rhBMP-2 only, although it was significantly less effective than FabCol-LamLG4 (Fig. 4, A and B). Representative µCT scans and histological images of the defect coverages are presented in Fig. 4C and fig. S2, respectively, to highlight the substantial differences between the treatments. Last, since the bridge proteins prolong the retention of rhBMP-2 within the collagen sponges and local ECM, we assessed the increase of bone formation at a delayed time after the treatment. We particularly observed that, between weeks 2 and 4 after treatment, bone regeneration increased in all three groups containing rhBMP-2 but that statistical differences were detected only in the groups with admixture of a bridge protein (Fig. 4D). This supports the hypothesis that the in vivo biological effects of rhBMP-2 are sustained for longer periods in the presence of the bridge proteins. In this model, we observed no further bone growth after 4 weeks (fig. S3).

## FabCol-LamLG4 improves InFUSE-like formulation in a spinal fusion model in mice

With the goal of improving the formulation of InFUSE Bone Graft for spinal fusion applications, we developed an intervertebral defect model in mice to mimic such applications. In this model, we incised the back muscle layers to expose the spinal bone and created surgical bone defects on the dorsal parts of the lumbar vertebra L2-L3, without damaging the underlying spinal cord. The vertebral defects can be visualized during surgery by exposure of the spinal cord and after surgery via  $\mu$ CT imaging, in which they appear as holes in the spine in the cross-sectional views (Fig. 5A).

Upon surgery, animals were treated with an InFUSE-like formulation (i.e., rhBMP-2 in bovine collagen sponges) with or without FabCol-LamLG4. The collagen sponges were preincubated for 15 min with the different treatments before being placed on top of the vertebral defect and maintained in place by suturing back the dorsal muscle layers. In this experiment, we used a very low dose of 100 ng of rhBMP-2 (in 25  $\mu$ l) to evaluate differences in spinal fusion and bone formation by  $\mu$ CT imaging 4 weeks after treatment. Representative images of the newly formed bone in the vertebral defects are illustrated in Fig. 5B and fig. S4A, highlighting spinal fusion between L2-L3 in the group treated with FabCol-LamLG4 + rhBMP-2.

At this dose, we found that rhBMP-2 only was not sufficient to induce significant bone formation as compared to PBS-treated defects, whereas the addition of FabCol-LamLG4 strongly increased the volume of generated bone (Fig. 5C), which resulted in a higher number of lumbar spinal fusions (Fig. 5D). Similar superiority of FabCol-LamLG4 + rhBMP-2 was observed at the higher dose of 500 ng of rhBMP-2, at which rhBMP-2 alone showed some bone generation (fig. S3). Increasing the rhBMP-2 dose also increased the proportion of complete spinal fusions. Last, we observed that a significant volume of bone continued to develop between weeks 2 and 4 after treatment in the presence of the FabCol-LamLG4 but not in the rhBMP-2 only group, consistent with an extended presence of rhBMP-2 at the implantation site when delivered via the bridge protein (Fig. 5E).

### DISCUSSION

While rhBMP-2 is the most potent osteogenic GF approved in the clinic for bone regeneration, its safety profile has raised important concerns, with about 20 to 50% of the patients suffering from side effects, which have included ectopic bone formation, seroma formation, nerve injuries, retrograde ejaculation, and cancer (10, 21). Nevertheless, the clinical product containing rhBMP-2, namely, InFUSE Bone Graft, continues to be widely used as a first-choice solution for autograft replacement in spinal fusion. Accordingly, the improvement of rhBMP-2 safety, in addition to efficacy at lower doses, remains an ongoing objective.

To address this issue, a multitude of new biomaterials have been developed to increase retention of rhBMP-2 at the site of implantation, therefore enhancing on-site therapeutic efficacy while preventing side effects in neighboring and distant sites (14, 15). Strong local retention of rhBMP-2 would permit significant reduction of its therapeutic dosing. Such retention was implemented, for example, by covalent linkage of rhBMP-2 to a carrier material, such as to fibrin hydrogels (22), by noncovalent yet specific interactions to a carrier material, such as to heparan sulfate–supplemented collagen matrices (23) or to heparin microparticles (24), or by biophysical- or

biochemical-based retention, such as through rhBMP-2 encapsulation into polymer microspheres (25). Alternative strategies also explored the in vivo delivery of rhBMP-2–expressing mesenchymal stem cells to achieve sustained release of low doses of rhBMP-2 (15, 26). However, despite proven efficacy in preclinical models, clinical translation of these approaches might be challenging because of the use of multicomponent materials and materials with complex formulation procedures, the incorporation of living cells and associated challenges to control their long-term fates, or the sourcing of these materials. In addition, the translation of complex materials is commonly hampered by long and expensive regulatory procedures (15).

Taking that into account, we here aimed to provide a simple single-component proteinaceous material that can be added to the already-approved clinical products such as InFUSE Bone Graft to localize GF dose to the scaffold and neighboring tissue. Using protein engineering, we created a recombinant bispecific bridge protein, FabCol-LamLG4, made by the fusion of an anti-collagen antibody Fab domain to the GF-binding domain LG4 of laminin  $\alpha 3$  (17). While laminin had been demonstrated to naturally interact with rhBMP-2 (17), we here highlighted that this interaction occurs at the LG4 domain of laminin more particularly. We then found that, by displaying dual high affinity to rhBMP-2 and to type I collagen, FabCol-LamLG4 efficiently controlled the release of rhBMP-2 from collagen sponges and significantly enhanced rhBMP-2-mediated osteogenesis in two different models of bone regeneration in vivo, including in a newly developed intervertebral defect model of spinal fusion in mice. FabCol-LamLG4 was the most effective of the four bridge proteins evaluated, the others being made either by the fusion of FabCol to the fibrinogen-derived GF-binding domain FgHBD (16) or by chemical conjugation of FabCol to the GF-binding domains.

We demonstrated that FabCol-LamLG4 improved bone regeneration at very low doses of rhBMP-2. In the calvarial bone defect model, 50 ng of rhBMP-2 in the implant site gave significant regeneration, while the commonly reported therapeutic doses for rhBMP-2 in similar defect sizes in mice or rats range between 0.5 and 5 µg (22, 27-29) or are around 200 ng when combined with other GFs (30). In our intervertebral defect model, 100 ng of rhBMP-2 delivered via FabCol-LamLG4 was sufficient to allow spinal fusions in mice, although a slight increase in dose might further improve the rate of complete fusion, here being about 50%. As a comparison, the dose reported to observe fusion in a similar intertransverse lumbar fusion model in mice was about 10 times higher (31). As to the retention kinetics of rhBMP-2 within collagen in the presence of FabCol-LamLG4, we observed release over a week from the exogenous collagen sponge in vitro and over 4 days in vivo in the endogenous matrix upon intradermal injection. However, we highlighted that bone significantly continued to grow between weeks 2 and 4 in vivo in both of the studied bone defect models, which suggested that low levels of rhBMP-2 remained sequestered for longer times (32) or that the cellular differentiation and tissue ossification processes triggered by rhBMP-2 continue beyond actual exposure (33). While complex materials have been developed to control the release kinetics of rhB-MP-2 over a few days to a few weeks (32), the optimal release kinetics for rhBMP-2 is not clearly established and depends on the studied model and defect size. Very slow release kinetics of rhBMP-2 can also result in a lack of exposure to the recipient cells and poor osteogenesis (34).

Despite the successful validation of our strategy in mice, the current study presents some limitations. Although it is expected that a reduction in rhBMP-2 therapeutic dosing will reduce its side effects, further dose optimization and toxicology evaluation would be necessary to establish the safety of FabCol-LamLG4 combined with rhBMP-2 in larger animal models, with bone defects sized to human needs. In these models, mechanical assessment of the newly formed bone would be valuable to confirm its ability to restore the support and locomotor functions of the repaired bone defect. Last, the ease of translation of FabCol-LamLG4 is difficult to anticipate and depends on its classification as a biological drug or as a combination product with InFUSE Bone Graft. Although proteins are generally not classified as medical devices, it is interesting to note here that FabCol-LamLG4 does not have a bioactive domain per se and that its primary action is limited to the delivery of another molecule, that being the GF drug. In addition, it seems reasonable to expect FabCol-LamLG4 to be safe, considering that it is based on a human antibody scaffold and contains only human endogenous sequences from the laminin  $\alpha$ 3 LG4 domain separated by GlySer linkers to avoid the formation of immunogenic epitopes.

Furthermore, in this study, we confirmed that the FabCol domain efficiently bound to type I collagens from multiple species, particularly bovine, murine, and human, because of their high homology. In the clinic, collagen-based biomaterials, including decellularized matrices, are widely used in tissue regeneration applications and are often derived from human, bovine, or porcine origins (*35*). Therefore, it is likely that the FabCol-LamLG4 protein could be used in combination with a variety of other commercially available collagen products to efficiently deliver GFs. Moreover, although our present study focused on the delivery of BMP-2, we previously characterized that the LamLG4 domain demonstrates promiscuous affinity to multiple GFs, including angiogenic and neurotrophic factors (*17*), potentially making it highly versatile to several regenerative medicine applications.

Last, because type I collagen is the most abundant ECM protein in almost all tissues of the body, FabCol-LamLG4 can additionally be retained in endogenous ECMs upon in vivo delivery, as demonstrated upon intradermal injection. From the endogenous ECM, FabCol-LamLG4 can efficiently control the release of GFs, here rhBMP-2. While not being observed in the present study, the delivery of rhBMP-2 from the local ECM surrounding the bone defect could possibly lead to heterotopic ossification or inflammation in the adjacent tissues because of increased local concentrations of rhBMP-2. The presence of these side effects would need to be specifically assessed in future safety studies and would likely depend on rhBMP-2 and FabCol-LamLG4 dosing.

By being capable of delivering GFs from the endogenous ECM, FabCol-LamLG4 strongly mimics the natural role of the ECM glycoproteins that decorate collagen to provide GF-binding sites, which are essential to regulate the spatiotemporal release of GFs and guide morphogenetic cell behaviors (20). In this perspective, fusion proteins containing both a matrix-binding domain and a GF-binding site could be used as powerful tools to modulate in vivo the native composition of ECMs and the signaling microenvironment of cells.

### **MATERIALS AND METHODS**

#### Materials

rhBMP-2 was produced in CHO and purchased from R&D Systems (Bio-Techne, Minneapolis, MN, USA). Absorbable bovine type I collagen sponges were purchased from Integra LifeSciences (Plainsboro Township, NJ, USA).

# FabCol-LamLG4 and FabCol-FgHBD fusion protein design, production, and purification

The sequences of the variable regions of FabCol were taken from the patent WO 2016016269A1 (clone C11) and synthesized by GenScript (Piscataway, NJ, USA), before being incorporated into a plasmid containing human Fab constant regions. LamLG4 and FgHBD sequences were synthesized by GenScript. In the FabCol-LamLG4 fusion protein, the LamLG4 domain was placed at the C terminus of the FabCol heavy chain via an eight–amino acid GlySer linker. As to the FabCol-p(FgHBD) fusion protein, three copies of the FgHBD domain were inserted at the C termini of both the light and the heavy chains of the FabCol, with each copy linked to another by an eight–amino acid GlySer linker. Protein sequences are detailed in table S1.

DNA plasmids of FabCol, FabCol-LamLG4, and FabCol-FgHBD were transfected into human embryonic kidney (HEK) 293-F cells using polyethyleneimine-mediated transfection and cultured in suspension for 7 days in FreeStyle 293 medium (Thermo Fisher Scientific, USA). The supernatant was then collected and purified using HiTrap MabSelect column and an Akta Pure M25 fast protein liquid chromatography systems (GE Healthcare Life Sciences, USA) according to the manufacturer's instructions. FabCol-LamLG4 and FabCol-FgHBD recombinant fusion proteins were further purified using HiTrap Heparin HP columns (GE Healthcare). Proteins were then dialyzed in PBS (pH 7.4), sterile-filtered, and stored at -80°C. Protein purity and size were assessed by SDS-PAGE. All protein batches used for in vivo experimentations were tested to be <5 EU/mg of endotoxins, using HEK-blue-mTLR4 assays.

### Chemical conjugation of FgHBD and LamLG4 to FabCol

FgHBD and LamLG4 peptides (>95% pure) were synthesized by GenScript and chemically conjugated to FabCol using sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) cross-linker (Thermo Fisher Scientific). One milligram of FabCol was incubated with 30-fold molar excess of the sulfo-SMCC in PBS at room temperature for 1 hour, after which the excess cross-linker was removed using Zeba Spin desalting columns, 7K MWCO (Thermo Fisher Scientific). The peptides were then added to the FabCol at 30-fold molar excess, and the mixture was incubated for 1 hour at room temperature. Unconjugated peptides were then removed using Amicon 30-kDa centrifugal filters by dilution/concentration cycles in PBS. The removal of unconjugated peptides was then assessed by SDS-PAGE. The conjugates were kept at 4°C for a maximum of 2 weeks before experimentation. Peptide sequences are detailed in table S2.

### Binding assay to bovine and human collagens

ELISA plates were coated overnight with bovine type I collagen (10 µg/ml; PureCol, Advanced BioMatrix, San Diego, CA, USA) or purified human type I or III collagens (3 µg/ml; EMD Millipore, Burlington, MA, USA) at room temperature. The plate was further blocked using 2% BSA for 2 hours at room temperature. Then, the bridge proteins (100 nM or indicated concentrations) were diluted in PBS–0.05% Tween (PBST) + 0.1% BSA and incubated for 1 hour at room temperature. The plate was washed thrice in PBST, and a horseradish peroxidase–conjugated antihuman Fab antibody (1:10,000; Jackson ImmunoResearch) was used to detect bound FabCol variants. The plate was revealed using 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution and stopped with 1 M H<sub>2</sub>SO<sub>4</sub>. Absorbance at 450 nm was read using an Epoch plate reader (BioTek, Winooski, VT, USA) and corrected with the absorbance at 570 nm. Curve fits

and  $K_D$  were computed using Prism (GraphPad Software Inc., San Diego, CA, USA).

### **Binding assay to rhBMP-2**

ELISA plates were coated with 50 nM recombinant rhBMP-2 overnight at room temperature and blocked using 2% BSA for 2 hours at room temperature. The plates were then washed thrice in PBST and incubated with 100 nM bridge proteins or FabCol-diluted PBST + 0.1% BSA for 1 hour at room temperature. Bound bridge proteins were detected via the Fab domain as described above. Specific binding of the bridge proteins to rhBMP-2 was computed by subtracting the absorbance to the one obtained on a plate coated with BSA (i.e., no rhBMP-2), here used as a nonspecific control.

### Binding assay of rhBMP-2 to collagen via the bridge proteins

The procedure was the same as described for the binding to bovine type I collagen, until the probing with the bridge proteins. Then, the plates were washed thrice in PBST, and 50 nM rhBMP-2 was incubated in the wells for 1 hour at room temperature in PBST–0.1% BSA. Plates were washed again, and the bound rhBMP-2 was detected using the anti–BMP-2 detection antibody and the streptavidin–horseradish peroxidase from the human BMP-2 DuoSet ELISA kit (R&D Systems) as instructed by the manufacturer.

### Preparation of the collagen sponges

In all experiments, the bovine type I collagen sponges from Integra were cut using sterile scalpel blades and biopsy punches into 4-mmdiameter and 1.5-mm-thick sponges, under a sterile biological hood.

### **Release of BMP-2 from collagen sponges**

Collagen sponges were loaded with 25  $\mu$ l of PBS containing rhBMP-2 (500 ng/ml) and the bridge proteins (0.5  $\mu$ M; molar ratio, ~1:13) for 15 min at room temperature. Collagen sponges were then placed in 1 ml of release buffer [150 mM NaCl and 20 mM tris (pH7.4) + 0.1% BSA + 1% penicillin-streptomycin] at 37°C. The release buffer was collected and refreshed daily for 7 days and stored at -20°C until analysis. A well that contained only rhBMP-2 served as a 100% released control. The amount of released rhBMP-2 was quantified using the human BMP-2 DuoSet ELISA kit (R&D Systems), according to the manufacturer's instructions.

## rhBMP-2 retention into collagen sponges by immunohistochemistry

The collagen sponges were prepared as in the release assay described above. After 7 days, the sponges were stained for rhBMP-2 using the detection anti–BMP-2 biotinylated antibody from the human BMP-2 DuoSet ELISA kit (R&D Systems), overnight at 4°C. The sponges were then washed thrice for 1 hour in PBS and incubated with streptavidin–Alexa Fluor 647 (AF647; 1:200) for 1 hour at room temperature. The sponges were then washed overnight in PBS at 4°C and imaged with a Leica DMi8 (Leica Biosystems Inc., Wetzlar, Germany). Images were analyzed using Fiji software (ImageJ, National Institutes of Health, USA).

### Saturation concentration of FabCol into collagen sponges

The FabCol protein was first conjugated with AF647 *N*-hydroxysuccinimide ester (succinimidyl ester; Thermo Fisher Scientific) as instructed by the manufacturer, and the unconjugated fluorophores were removed using Zeba Spin desalting columns. Various concentrations of FabCol ranging from 10  $\mu$ M to 2.5 nM were incubated in 25- $\mu$ l collagen sponges at room temperature for 15 min and immediately washed in 50 ml of PBS per sponge for 4 hours under vigorous shaking. A 100% retention standard was made using unwashed collagen sponges incubated with fluorescently labeled FabCol. The sponges were last digested with collagenase D (1 mg/ml) at 37°C, and the fluorescence was read using a plate reader (Cytation 3, BioTek).

### **Animal experimentations**

All animal experimentations were approved by the University of Chicago's Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Mice used in this study were female C57BL/6 of 10 to 12 weeks old at the start of the experiments.

## Intradermal retention of FabCol in endogenous collagenous ECM

Fluorescently labeled FabCol or OspA (a Fab domain that specifically binds to the OspA on *Borrelia burgdorferi*, used here as a collagen nonbinding control) were injected intradermally in the back of mice at a dose of 1  $\mu$ g, with the FabCol and the OspA control being at the contralateral sides. Decrease of fluorescence was measured every 2 days using IVIS Spectrum In Vivo Imaging System (PerkinElmer, Waltham MA, USA) and analyzed with the associated software. Fluorescence measurements were normalized to the respective day 0 time point for each Fab.

## In vivo retention of the rhBMP-2 in endogenous collagen via bridge proteins

rhBMP-2 (200 ng) was mixed with the different bridge proteins at a 1:10 molar ratio in 30 µl of sterile PBS, about 15 to 30 min before injection. Mice were injected intradermally in the back (four injection spots per mouse, multiple groups per mouse) and euthanized 1, 2, 4, and 7 days or immediately (d0) after the injections. Full-thickness skin at injected sites was harvested using a 10-mm biopsy punch and finely cut into small pieces before being transferred into 0.9 ml of T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific) containing collagenase IV (1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) for 1 hour at 37°C for enzymatic tissue digestion. Then, 100 µl of 5 M NaCl containing protease inhibitors was added to the lysate (one tablet of cOmplete Protease Inhibitor Cocktail for 10 ml; Roche, Basel, Switzerland). The lysates were centrifuged at 5000g for 5 min, and the supernatants were collected for further quantification of rhBMP-2 using the human BMP-2 DuoSet ELISA kit (R&D Systems). Data were normalized for each group to the d0 time point.

### Calvarial bone defect model in mice

Mice were injected with analgesia (buprenorphine; 0.1 mg/kg) about 30 min before the surgery. Mice were anesthetized by isoflurane inhalation throughout the duration of the surgery (~20 min), and the absence of deep reflexes was confirmed by toe pinch. The skull was shaved, and the animal was placed on a heating pad. Artificial tears were applied to prevent corneal dryness, and a subcutaneous injection of a nonsteroidal anti-inflammatory drug (meloxicam; 2 mg/kg) was performed. The skull was disinfected using ethanol and betadine alternatively three times in circular movements. The skin was incised at the midline of the skull, and the underlying periosteum was removed using forceps. The craniotomy defect was created

using a 4-mm-diameter trephine (Meisinger USA, Centennial, CO, USA) and a surgical drill (Foredom K.1070, Stoelting, Wood Dale, IL, USA) on the parietal bone of the skull, without damaging the underlying brain. The defect was washed with sterile PBS and sterile surgical gauzes. A collagen sponge preincubated with 20  $\mu$ l of rhBMP-2 (50, 100, or 150 ng) and/or the bridge proteins (1:13 molar ratio) in sterile PBS for 15 min at room temperature was then placed in the defect, after which the skull skin was closed using continuous sutures (Prolene monofilament 5-0; Ethicon Inc., Somerville, NJ, USA). The skin wound was lastly disinfected with betadine, and the mice were monitored until fully awakened. Analgesia was repeated every 12 hours until the mice showed no sign of pain. Sutures were removed after 7 days.

### Intervertebral defect model in mice

Analgesia and anesthesia treatments, as well as the mice preparation procedures, were the same as in the calvarial defect model. The backs of the mice were shaved and disinfected with ethanol/betadine cycles. A 2.5-cm midline incision was performed in the mouse back skin, and the underlying muscles were cut on each side of the vertebrae L2-L3 (~0.5-cm incision) to expose the spine bone. The spine was firmly held, and a bone defect was carefully created using a bone rongeur starting at the spinous process of L3 and extended toward L2 until the spinal cord was exposed at the junction L2-L3. The defect was limited to the dorsal part of the vertebrae, and the spinal cord remained undamaged. A collagen sponge preincubated with rhBMP-2 (100 ng) with or without FabCol-LamLG4 (molar ratio, ~1:13) for 15 min at room temperature was placed into the defect. The muscles on each side of the spine were sutured together using absorbable Vicryl 6-0 (Ethicon Inc.), holding the sponge at the defect location. The back skin was lastly closed using interrupted Prolene 5-0 sutures (Ethicon Inc.), and the wound was disinfected with betadine. The mice were monitored until fully awakened and observed for at least 30 min after surgery to confirm healthy mobility (including standing position on their hind legs). External sutures were removed after 7 days.

### Histology

Upon euthanasia of the mice, the calvarial bone at the defect location was dissected and fixed in 4% paraformaldehyde for 16 hours at 4°C. The bones were washed with water and decalcified for 10 days in 14% ethylenediaminetetraacetic acid (pH 7.0). Decalcified explants were embedded in paraffin, sliced at the defect location (thickness, 5  $\mu$ m), and stained with Masson's trichrome or hematoxylin and eosin. Histology was imaged using a Leica DMi8 microscope (Leica Biosystems Inc.).

### $\mu\text{CT}$ scan imaging and quantification

 $\mu CT$  images of cranial bones were taken on the XCUBE (Molecubes NV., Gent, Belgium) by the Integrated Small Animal Imaging Research Resource at The University of Chicago. Spiral high-resolution  $\mu CT$  acquisitions were performed with an x-ray source of 50 kVp and 440  $\mu A$ . Volumetric CT images were reconstructed by applying the iterative image space reconstruction algorithm in a 400  $\times$  400  $\times$  370 format with an isotropic voxel size of 100  $\mu m$ .

Quantification of the  $\mu$ CT scans was done using Fiji (ImageJ). All images were all processed with the same algorithm, which enhances image brightness with linear transformation, defined 3D regions of interest (ROIs) around the bone defects and thresholds the images to select the "bone" positive voxels within the ROI. For the calvarial model, the coverage was then determined as the percent positive area using a 2D projection in the bone defect plane and the bone volume as the total amount of positive voxels in the 3D ROI. Data were lastly normalized such that untreated defects (i.e., no collagen sponge, no rhBMP-2, and no bridge protein) are at 0% defect coverage or 0% BV/TV and that mice without defect (i.e., wild-type and no surgery) are at 100%.

### Data collection, analysis, and statistics

All experiments comparing rhBMP-2 with rhBMP-2 + bridge proteins were repeated at least twice independently or at multiple doses independently, with at least two different batches of proteins. Representative experiments are shown rather than pooled data. Statistical outliers were detected using Iglewicz and Hoaglin's robust test for multiple outliers (two-sided test) and removed from the analysis. No outliers were removed when the sample size was  $\leq$ 5. Graphs and statistical tests were performed using Prism.

### SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/ content/full/7/24/eabh4302/DC1

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