Thermoresponsive Citrate-Based Graphene Oxide Sca
Mesenchymal Stem Cells Enhances Bone Regeneration from BMP9-Stimulated Adipose-Derived

Chen Zhao,$^1,4$ Zongyue Zeng,$^1,8$ Nader Taheri Qazvini,$^1$ Xinyi Yu,$^1,4$ Ruyi Zhang,$^4,8$ Shujuan Yan,$^4,8$
Yi Shu,$^4,8$ Yunxiao Zhu,$^1,^\wedge$ Chongwen Duan,$^1$ Elliot Bishop,$^1$ Jiayan Lei,$^1,4$ Wenwen Zhang,$^4,8$
Chao Yang,$^4,8$ Ke Wu,$^4,8$ Ying Wu,$^4,\square$ Liping An,$^3,\square$ Shifeng Huang,$^4,8$ Xiaojian Ji,$^4,8$ Cheng Gong,$^\odot$
Chengfu Yuan,$^\triangle$ Linghuan Zhang,$^4,8$ Wei Liu,$^4,8$ Bo Huang,$^4,8$ Yixiao Feng,$^4,8$ Bo Zhang,$^4,8$
Zhengyu Dai,$^\triangle,\square$ Yi Shen,$^\triangle,\wedge$ Xi Wang,$^4,8$ Wenping Luo,$^4,8$ Leonardo Oliveira,$^\square$ Aravind Athiviraham,$^\square$
Michael J. Lee,$^\square$ Jennifer Moriatis Wolf,$^\square$ Guillermo A. Ameer,$^\wedge$ Russell R. Reid,$^\wedge,^\#$^\wedge$
Tong-Chuan He,$^\wedge,^4,8,^\wedge\wedge$ and Wei Huang$^*,4$

$^1$Departments of Orthopedic Surgery, Nephrology, Cardiology, Clinical Laboratory Medicine, and Breast Surgery, The First
Affiliated Hospital of Chongqing Medical University, 1 Youyi Road, Chongqing 400016, China
$^2$Molecular Oncology Laboratory, Department of Orthopaedic Surgery and Rehabilitation Medicine, The University of Chicago
Medical Center, 5841 South Maryland Avenue MC 3079, Chicago, Illinois 60637, United States
$^3$Ministry of Education Key Laboratory of Diagnostic Medicine and School of Laboratory Medicine, The Affiliated Hospitals of
Chongqing Medical University, 1 Medical College Road, Chongqing 400016, China
$^4$Institute for Molecular Engineering, The University of Chicago, 5640 South Ellis Avenue, Chicago, Illinois 60637, United States
$^5$Department of Biomedical Engineering, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208, United States
$^6$Department of Surgery, Laboratory of Craniofacial Biology and Development, Section of Plastic Surgery, The University of Chicago
Medical Center, 5841 South Maryland Avenue MC6035, Chicago, Illinois 60637, United States
$^7$Department of Laboratory Medicine and Clinical Diagnostics, The Affiliated University-Town Hospital of Chongqing Medical
University, 55 Daxuecheng Zhonglu, Chongqing 401331, China
$^\square$Department of Immunology and Microbiology, Beijing University of Chinese Medicine, 11 N. Third Ring Road E., Beijing 100029, China
$^\wedge$Key Laboratory of Orthopaedic Surgery of Gansu Province and the Department of Orthopaedic Surgery, The Second Hospital of
Lanzhou University, 82 Cuiyingmen, Lanzhou 730030, China
$^\odot$Department of General Surgery, Zhongnan Hospital of Wuhan University, 169 Donghu Road, Wuhan 430071, China
$^\wedge$Department of Biochemistry and Molecular Biology, China Three Gorges University School of Medicine, 8 Daxue Road,
Yichang 443002, China
$^\triangle$Department of Orthopaedic Surgery, Chongqing Hospital of Traditional Chinese Medicine, 35 Jianxin East Road,
Chongqing 400021, China
$^\wedge$Department of Orthopaedic Surgery, Xiangya Second Hospital of Central South University, 139 Renmin Road, Changsha 410011, China
$^\wedge$Department of Surgery, Feinberg School of Medicine, Northwestern University, 420 East Superior Street, Chicago, Illinois 60616,
United States
$^\wedge\wedge$Center for Advanced Regenerative Engineering (CARE), 2145 Sheridan Road, Evanston, IL 60208, United States

Supporting Information

**ABSTRACT:** Effective bone tissue engineering is important to over-
come the unmet clinical challenges as more than 1.6 million bone
grafts are done annually in the United States. Successful bone tissue
engineering needs minimally three critical constituents: osteoprogen-
itor cells, osteogenic factors, and osteoinductive/osteoconductive
scaffolds. Osteogenic progenitors are derived from multipotent
mesenchymal stem cells (MSCs), which can be prepared from
numerous tissue sources, including adipose tissue. We previously
showed that BMP9 is the most osteogenic BMP and induces robust
bone formation of immortalized mouse adipose-derived MSCs

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entraped in a citrate-based thermosensitive hydrogel referred to as PPCNg. As graphene and its derivatives emerge as promising biomaterials, here we develop a novel thermosensitive and injectable hybrid material by combining graphene oxide (GO) with PPCNg (designated as GO-P) and characterize its ability to promote bone formation. We demonstrate that the thermosensitive behavior of the hybrid material is maintained while effectively supporting MSC survival and proliferation. Furthermore, GO-P induces early bone-forming marker alkaline phosphatase (ALP) and potentiates BMP9-induced expression of osteogenic regulators and bone markers as well as angiogenic factor VEGF in MSCs. In vivo studies show BMP9-transduced MSCs entrapped in the GO-P scaffold form well-mineralized and highly vascularized trabecular bone. Thus, these results indicate that GO-P hybrid material may function as a new biocompatible, injectable scaffold with osteoinductive and osteoconductive activities for bone regeneration.

KEYWORDS: graphene, graphene oxide, PPCN, thermosensitive, scaffold, mesenchymal stem cells, BMP9, bone tissue engineering

■ INTRODUCTION

Engineering tissues in vitro or in vivo can potentially improve human health by restoring tissue functions that have been compromised by disease or injury. Clinical problems that would benefit from engineered tissues include significant segmental defects, fracture nonunion, and/or medical conditions such as tumor resection and infection sites. In fact, more than 1.6 million bone grafts are done annually in the United States alone. However, successful regeneration of bone tissue requires a multidisciplinary approach involving the integration of materials science, stem cell biology, biomechanical sciences, and translational medicine as bone has complex structure and its function requires well-orchestrated interactions between cells, the extracellular matrix, biomechanical forces, and gene and protein regulatory factors. Thus, the successful regeneration of bone requires minimally three integral components: osteoprogenitor cells, osteogenic factors, and osteoinductive and osteoconductive scaffolds.

Osteogenic progenitors are derived from mesenchymal stem cells (MSCs). MSCs are multipotent progenitor cells that are able to self-renew and undergo differentiation into several cell types such as osteogenic, chondrogenic, and adipogenic lineages. While bone marrow stromal stem cells are one of the best-studied MSCs, multiple types of MSCs have been isolated from various tissues. In particular, adipose tissue has become a favorite source of progenitor cells for tissue regenerative therapies. Adipose-derived mesenchymal stem cells (AD-MSCs) can be isolated from adipose tissue and exhibit osteogenic potential. To effectively utilize AD-MSCs as a staple source of cells to form new bone, we have recently established reversibly immortalized mouse adipose-derived MSC (iMAD) cells, which exhibit the features of multipotent mesenchymal stem cells and respond effectively to BMP9 to induce osteogenic differentiation.

Although numerous growth factors and signaling molecules such as Wnts, IGFs, PDGF, FGFs, and Notch play important roles in regulating osteogenic differentiation, bone morphogenetic proteins (BMPs) are among the most potent osteoinductive factors. Through a systematic profiling of the 14 types of BMPs, we demonstrated that BMP9 (or GDF2) is the most potent bone-forming BMP. We further showed that BMP9 induces effective bone formation by regulating several important target genes and lncRNA H19 and cross-talks with several other critical pathways. Therefore, it is conceivable that the use of BMP9-transduced MSCs should enhance bone regeneration in big osseous defects and fracture nonunion in clinical settings.

Even though MSCs can be easily identified and isolated, and many biological factors for bone regeneration are now available, successful bone tissue engineering for repairing large defects and fracture nonunion is still a huge clinical challenge, largely due to the lack of ideal scaffolds, which provide cell-friendly micro-environment and solicit efficient in-growth of bone tissue at the repair sites. An ideal scaffold can be either osteoconductive, which is supportive for the growth of new bone, and/or osteoinductive, which is able to recruit bone progenitors and stimulate new bone formation. Currently, most of the commonly used scaffolds consist of bioactive glasses, calcium phosphates (in the forms of hydroxypatite and β-tricalcium phosphate), and biopolymers. Most of these materials function only as osteoconductive scaffolds for bone in-growth with or limited osteoinductivity for inducing osteoprogenitor differentiation. Thus, it is imperative to develop new scaffolds that possess osteoinductive and osteoconductive activities for bone tissue engineering.

Graphene consists of a single-layer of carbon atoms and has attracted a broad range of interest in many fields, including chemistry, physics, and materials science. Graphene oxide (GO)-based nanomaterials offer multifaceted biomedical applications. GO is hydrophilic and contains ample reactive chemical functionalities, including hydroxyl, carbonyl, carboxyl, and epoxy groups, enabling its surface to be easily modified with biocompatible polymers and enhance its biofunctionalization with higher biocompatibility and more diverse applications in biomedical fields. In fact, numerous biomolecules, growth factors, and drugs were coated to GO surface and showed promising biomedical utilities. GO-derived hybrids were shown to possess a substantial increase in physical properties such as elastic modulus, tensile strength, thermal stability, and electrical conductivity even at low amount of GO filler, essential for an ideal bone tissue engineering scaffold material. However, the applications of graphene and its derivatives, including GO, in bone tissue engineering are limited. We recently demonstrated that the thermoresponsive macromolecule PPCN when mixed with gelatin, served as a highly biocompatible scaffolding material to deliver BMP9-stimulated MSCs for effective bone formation. In this study, we investigated whether incorporating GO into PPCN, the resultant hybrid materials referred to as GO-P, would enhance its ability to support the formation of new bone. We demonstrate that the addition of GO maintains thermoresponsive behavior of the hybrid material and effectively supports MSC survival and proliferation. Furthermore, GO-P induces early bone forming marker alkaline phosphatase (ALP) and potentiates BMP9-regulated osteogenic markers, as well as the angiogenic factor VEGF in MSCs. We further show that BMP9-transduced MSCs entrapped in the GO-P scaffold form well-mineralized and highly vascularized trabecular bone. Therefore, the addition of GO renders PPCN osteoinductive and

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angiogenic activities while preserving PPCNg’s thermoresponsiveness. Thus, our results indicate that the GO-P hybrid material may be used as a novel injectable scaffold with osteoinductive and osteoconductive activities to support the formation of new vascularized bone.

**MATERIALS AND METHODS**

**Cell Lines and Chemicals.** HEK-293 was purchased from ATCC. 293pTP and RAPA lines were derived from HEK-293 as previously described. Mesenchymal progenitor cells, iMADs (immortalized mouse adipose-derived cells), were previously characterized. These cells were cultured in DMEM with 10% FBS (Invitrogen, Carlsbad, CA, United States) and cultured in 37 °C with 5% CO2. GO (2 mg/mL suspension in H2O) was obtained from Sigma-Aldrich (St. Louis, MO). All other reagents were purchased from ThermoFisher (Waltham, MA) or Sigma-Aldrich.

**Preparation of Adenoviral Vectors.** AdBMP9, AdR-GLuc, and AdGFP were constructed by using the AdEasy technology as described. Specifically, the coding regions of human BMP9 and Gaussia luciferase were PCR amplified and cloned into a shuttle plasmid, followed by the generation of recombinant adenoviral vectors in BJ5183 bacterial cells. Recombinant adenoviruses were generated following the protocol described above. Fifty microliters of culture medium in BJ5183 bacterial cells. Recombinant adenoviruses were generated and amplified in HEK-293, 293pTP, and/or RAPA cells as previously described. Resulting in AdBMP9 and AdR-GLuc, each of which contain GFP and RFP, respectively. An adenoviral vector expressing GFP only (i.e., AdGFP) served as a mock infection control. All adenovirus infections were determined by strain sweep measurements.

**Rheological Property Analysis.** The PPCNg and GO-P hybrid scaffolds were loaded as room temperature saturated with water by using a solvent trap sponge. The dynamic moduli (storage, and loss, respectively) of PPCNg and GO-P were recorded under a fluorescence microscope. Each condition was carried out in triplicate.

**3D Cell Culture of PPCNg and GO-P Hybrid Scaffolds Entrapped with iMAD MSCs.** PPCNg was synthesized as previously described. PPCNg powder was dissolved in PBS (100 μg/mL), sterilized by syringe filtration with 0.22 μm filters, and kept at 4 °C. PPCNg–gelatin (PPCNg) was prepared by diluting PPCNg stock solution at 1:1 ratio with 0.2% gelatin/PBS (i.e., PPCNg final concentration at 50 μg/mL) as described. Graphene oxide–PPCNg (GO–PPCNg) hybrid scaffold was prepared by adding graphene oxide to PPCNg (final concentration at 0.4 μg/mL) and mixing well on ice. Unless indicated otherwise, the composition of the GO–PPCNg hybrid material was set at PPCNg (50 μg/mL):GO(2 μg/mL) = 5:1 for the reported studies.

For assembling the cell–containing scaffolds, subconfluent iMAD cells were transduced with respective adenoviral vectors at MOI (multiplicity of infection) of 50 for 24 h. The infected cells were collected and resuspended in cold PPCNg or GO-P. The cell–polymer mixtures were placed into cell culture plates that were prechilled at 4 °C or directly used for subcutaneous injection of athymic nude mice. For in vivo culturing, each well contained 40 μL of cell–polymer mixed with 2 × 105 cells. The plates were prewarmed at 37 °C 5% CO2 for 20 min and then refilled with 2 mL of the 37 °C prewarmed complete DMEM. The gels were transferred to new plates after 24 h. Fluorescence signals were recorded under a fluorescence microscope. Each condition was carried out in triplicate.

**Rheological Property Analysis.** The rheological properties of the PPCNg alone and GO-P hydrogels, with various ratios of PPCNg (50 μg/mL) vs GO (2 mg/mL) (e.g., at 1:0, 10:1, 1:1 and 1:10), were characterized by a Rheoscope Rheometer (HR2, TA Instruments, United States). A 2” stainless steel cone and plate geometry (diameter 20 mm) with a truncation gap of 59 μm was used. To prevent water loss during measurements, the air around the sample was saturated with water by using a solvent trap filled with a water-soaked sponge. The dynamic moduli (storage, G’ and loss, G″) were measured within the linear viscoelastic region as a function of temperature by oscillatory temperature ramp experiments (0.5 °C/min) at a frequency, f = 1 Hz and deformation, γ = 1%. The linear viscoelastic region was determined by strain sweep measurements.

**Gaussia Luciferase (GLuc) Activity Assay.** The PPCNg alone and GO-P scaffolds containing with AdR-GLuc infected iMADs were constructed as described above. Fifty microliters of culture medium was taken from each well for GLuc activity assay at the indicated time points by using the BioLux Gaussia Luciferase Assay Kit (NEB).

**Preclinical Imaging System (GE Healthcare, Piscataway, NJ, United States).** A 2° filled with 2 mL of the 37 °C prewarmed complete DMEM medium. The culture wells were replenished with the same volume of the 37 °C prewarmed complete DMEM medium.

**Scanning Electron Microscopy (SEM).** The morphology of the PPCNg and GO-P scaffolds was visualized following the quick-freezing deep etch (QFDE) method as previously described. Briefly, the PPCNg and GO–PPCNg were loaded as room temperature quick-freezing deep etch solution directly on the QFDE specimen disks, heated above its LCST until the scaffolds turned solid, and slam frozen in the BioCryo Leica EM HPM100 High-Pressure Freezer. The samples were then dried with the critical point dryer (Leica EM CPD300), loaded onto aluminum SEM pin stub mount using double-sided carbon tape, and imaged with the Nova NanoSEM 230 scanning electron microscope with an accelerating voltage of 5 kV as described. Each assay condition was carried out in triplicate.

**Immunohistochemical (IHC) Staining of VEGF Expression.** IHC was carried out as described. Briefly, the paraffin-embedded tissue sections were deparaffinized, rehydrated, and subjected to IHC staining with a VEGF antibody (mouse, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, United States). Sections were then washed and incubated with donkey antimouse IgG antibody conjugated with HRP (Jackson ImmunoResearch Inc., West Grove, PA, United States) for 30 min at RT. The presence of VEGF was visualized by DAB staining. Stains without the primary antibody were used as negative controls.

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**Hematoxylin & Eosin (H&E) Staining and Trichrome Staining.** After μCT imaging, the retrieved masses were decalcified, paraffin-embedded, and sectioned. The sections were stained as previously described. The use and care of animals was approved by the Institutional Animal Care and Use Committee. All experimental procedures were performed in accordance with the approved guidelines. The subcutaneous ectopic bone formation was carried out as previously reported. Briefly, subconfluent iMADs were infected with AdBMP9 or AdGFP for 36 h, collected and resuspended in 100 μL of PPCNg or GO-P scaffolds on ice, and injected into the flanks of athymic mice subcutaneously (Envigo; n = 5, female, 6-week old; 2 × 106 cells per injection site). At 4 weeks, the animals were sacrificed for harvesting ectopic masses.

**Micro-Computed Tomography (μCT) Analysis.** The retrieved ectopic masses were fixed with 10% PBS-buffered formalin and subjected to μCT imaging using the GE Triumph Trinomality Preclinical Imaging System (GE Healthcare, Piscataway, NJ, United States). Amira 5.3 (Visage Imaging, Inc., San Diego, CA, United States) was used to perform 3D reconstruction and determine volumetric data as described.

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Infecting subconfluent iMADs with AdR-GLuc, which coexpresses Gaussia luciferase (GLuc) and RFP, when the same number of AdR-GLuc-transduced iMADs was mixed with the same volume of PPCNg or GO-P gel, we found that the RFP signal was compatible in both PPCNg and GO-P gels at day 7 (Figure 2A). However, the RFP signal dropped rapidly in PPCNg gel and became undetectable after day 14, whereas high levels of RFP signal were readily detected in GO-P hybrid scaffold up to day 21 (Figure 2A). Quantitative measurements of the GLuc activities in the culture medium yielded a similar trend, and we found that GLuc activities were consistently higher in the GO-P group than that in the PPCNg group after day 7 and became more pronounced after day 15.

To test whether the decreased RFP signal was caused by the decreased numbers of viable cells or by the dilution of AdR-GLuc-mediated transient infection, we added a fresh dose of AdR-GLuc to reinfect the 3D cultured cells and found that more RFP-positive cells reappeared in the GO-P group, while no detectable RFP-positive cells were observed in the PPCNg group (Figure 2C), suggesting that the GO-P hybrid scaffold may provide a superior 3D environment for cell survival and cell proliferation.

Morphological Features of the Entrapped iMADs and SEM Analysis of the Surface Features of the GO-P Scaffold. We also examined the morphological features of the AdBMP9-transduced iMAD cells seeded in the GO-P scaffold.
and found that the entrapped iMAD cells underwent significant morphological changes and adopted elongated cell shapes at as early as day 1 (Figure 3A), becoming more pronounced at days 3 and 5 (Figure 3B and C), compared with those in the PPCNg group. Thus, consistent with the results about the increased viscoelastic properties of PPCNg by GO (Figure 1), these results indicate that the GO-P hybrid scaffold may provide a supportive environment for the proliferation and differentiation of MSCs.

SEM revealed that the GO-P gel had a rougher scaffold surface when compared to that of PPCNg alone (Figure 4A a and b vs c and d). When the AdBMP9-transduced iMADs were seeded in the GO-P hybrid scaffold, the iMAD cells adhered to the surface at day 3 of seeding (Figure 4B, a and b), and significant amounts of minerals were deposited on the surface at day 7 (Figure 4B, c and d).

**GO-P Hybrid Scaffold Has Osteoinductive Activity in Vitro.** To test whether GO-P hybrid scaffold exhibits osteoinductive activity, we infected iMADs with AdBMP9 or AdGFP and mixed the infected cells with the GO-P gel as well as with the control PPCNg gel, which showed robust adenovirus infection in both types of scaffolds (Figure 5A, a and b). BMP9 was shown to induce a robust level of alkaline phosphatase (ALP) activity in the PPCNg group (Figure 5B). However, the GO-P scaffold treated with AdGFP showed a high level of ALP activity, which was potentiated by BMP9 stimulation (Figure 5B). Furthermore, quantitative analysis revealed that GFP-treated iMADs seeded in the GO-P scaffold (GFP + GOP group) induced significantly high levels of ALP activity at day 5 and day 7, compared with that of the GFP-treated PPCNg group (p < 0.05) (Figure 5C). Moreover, BMP9-induced ALP activity was significantly potentiated in the GO-P scaffold when compared with that in the PPCNg gel group (Figure 5C). Thus, these results indicate that the GO-P hybrid scaffold may exhibit both osteoinductive and osteoconductive activities.

**GO-P Hybrid Scaffold Potentiates BMP9-Regulated Expression of Osteogenic Markers and Angiogenic Factor and Augments BMP9-Induced Ectopic Bone Formation.** To assess the effect of GO-P scaffold on BMP9-induced expression of osteogenic regulators/markers, we infected subconfluent iMAD cells with Ad-GFP or Ad-BMP9 and mixed the cells with PPCNg or GO-P. We revealed that BMP9-induced expression of Runx2 was enhanced in GO-P gel at day 3, while the GFP+ GO-P group also exhibited higher Runx2 expression at day 5 (Figure 6A). Similarly, AdBMP9-induced Bsp expression was significantly enhanced in the GO-P group, whereas the AdGFP-treated GO-P group also exhibited Bsp expression higher than that of the PPCNg group at both day 3 and day 5 (Figure 6A). Furthermore, the expression of other osteogenic markers such as Osx, Alp, Ocn, Opn, and Coll1a1 was not only up-regulated by
BMP9 but also enhanced in AdBMP9-treated GO-P group, compared with that in AdBMP9-treated PPCNg group ($p < 0.01$) (Figure 6B). Similarly, a higher expression level of these osteogenic markers was found in the AdGFP-treated GO-P group than that of AdGFP-treated PPCNg group ($p < 0.05$) (Figure 6B), further suggesting that GO-P hybrid scaffold may exhibit osteoinductive activity. Consistent with our early report that BMP9 is able to induce angiogenic factor HIF1α,49 we found that BMP9 was shown to induce the expression of VEGF in the iMADs ($p < 0.05$), which was drastically enhanced in the presence in the GO-P hybrid scaffold (Figure 6B).

Lastly, we determined the in vivo features of the GO-P hybrid scaffold for BMP9-transduced MSCs in ectopic bone formation in athymic nude mice. We found that under the same conditions, the BMP9-transduced iMADs mixed with the GO-P scaffold yielded slightly larger bony masses than that in the PPCNg group ($p < 0.05$) (Figure 7A, a and b). No detectable bone masses were retrieved from AdGFP-transduced iMAD groups. H&E staining analysis indicated that the bony masses retrieved from the GO-P group exhibit significantly more mature and denser trabecular bone than that from the PPCNg group (Figure 7B, a), which was also confirmed by trichrome staining (Figure 7B, b). A quantitative analysis revealed that the average trabecular bone area was larger in the GO-P group than that in the PPCNg group ($p < 0.01$) (Figure 7C). Furthermore, the histological evaluation indicated that the bony masses from the GO-P group were highly vascularized. We performed immunohistochemical staining to detect VEGF expression in the retrieved bony masses, and significantly higher numbers of VEGF-positive cells were observed in the bony masses retrieved from the GO-P group than that from the PPCNg group (Figure 7B, c), which is consistent with the qPCR results about marked expression of VEGF induced by BMP9 and GO-P (Figure 6B).

**DISCUSSION**

Optimized biocompatible scaffolds are essential to generate healthy bone. Strategies to regenerate bone include the use of cells, signaling molecules, and scaffolds.2 We recently demonstrated that a biodegradable thermosensitive PPCNg can function as a scaffolding delivery carrier of BMP9-transduced MSCs for the formation of well-mineralized and highly vascularized trabecular bone-like structures in a mouse model,24,71 suggesting that the PPCN-gelatin may function as a new biodegradable and injectable scaffolding material for stem cell-based bone regenerative tissue engineering.

It is conceivable that biocompatible scaffolds that can sustain cell migration, attachment, and proliferation in bone tissue engineering applications may be easily constructed with hybrid systems containing more than two types of materials. Here, we investigated the impact of adding GO to PPCNg with the goal of enhancing osteoinductive and osteoconductive properties. As a 2D crystal with one-atom thickness, graphene has become one of the hottest topics in materials science and nanotechnology.62 More recently, graphene-based nanosheets have been considered as a fine nanofiller for the fabrication of hybrid scaffolds.55,56,61,63−65,68,69 We recently assembled gelatin-derived
graphene/laponite nanosheets using a cost-effective synthesis procedure. We demonstrated that the GL scaffold is bio-compatible for supporting long-term proliferation of MSCs. The carbonized GL-powder hybrids were shown to effectively enhance BMP9-induced osteoblastic differentiation of MSCs in vitro and promote BMP9-induced bone formation in vivo, indicating that the addition of carbonized GL hybrids to BMP9-stimulated MSCs can lead to more effective bone formation in vivo. Nonetheless, the nanosheet or powder nature of the GL-hybrid scaffold possesses fabrication challenges as biomaterials for stem cell-based tissue engineering. The GO-P hybrid scaffold may provide an ideal osteogenic and angiogenic microenvironment for BMP9-transduced MSC-based bone tissue engineering. Here, we took advantage of the favorable physical properties of GO and sought to develop a hybrid scaffold by incorporating GO into the thermoresponsive PPCNg hydrogel. We showed that the addition of GO changes the viscoelastic properties of the PPCNg. The GO-P hybrid material is osteoinductive in vitro and significantly enhances the formation of more mature, better mineralized, and highly vascularized trabecular bone in vivo, indicating that the GO-P hybrid scaffold may provide a progenitor cell-friendly and osteogenic and angiogenic microenvironment for new bone formation. Furthermore, it is conceivable that the thermoresponsive property renders the GO-P flexible utility for being used as an injectable material to deliver MSC-containing constructs for bone regeneration. Taken together, our findings suggest that the GO-P hybrid material may function as a novel biocompatible and injectable scaffold with osteoinductive and osteoconductive activities to support the formation of new vascularized bone. Nonetheless, our studies have several limitations. First, the GO-P scaffold should be further tested for osteogenic efficiency in segmental defect and/or fracture healing animal models. Second, the mix ratios between GO and PPCNg should be further assessed in vivo to maximize the osteoinductive and osteoconductive activities of GO-P scaffold. Third, the long-term biocompatibility and biosafety of GO-P scaffold materials should be extensively analyzed in vivo. Lastly, the exact mechanism through which GO itself induces osteogenic differentiation in MSCs needs to be elucidated. Therefore,
Figure 5. Osteoinductive and osteoconductive activities of the GO-P hybrid scaffold in vitro. (A) AdGFP or AdBMP9-infected iMADs were mixed with PPCNg (a) or GO-P (b) and examined at 48 h after infection under bright field (BF) or GFP fluorescence microscope (GFP). Representative images are shown. (B and C) ALP activity analysis. AdGFP or AdBMP9-transduced iMADs were mixed with PPCNg or GO-P and seeded in 24-well plates. ALP staining was carried out on day 5 (B), while quantitative ALP assay was conducted at 3, 5, and 7 days after infection (C). All assays were done in triplicate. * $p < 0.05$ and ** $p < 0.01$ when compared to respective GFP groups.

Figure 6. GO-P hybrid scaffold potentiates BMP9-induced expression of osteogenic regulators/markers and angiogenic regulator VEGF. Subconfluent iMAD cells were infected with Ad-GFP or Ad-BMP9 and mixed with PPCNg or GO-P. Total RNA was isolated at day 3 and day 5 and subjected to TqPCR analysis using gene-specific primers for mouse Runx2 and Bsp (A) and bone markers including Osx, Alp, Ocn, Opn, Col1a1, and angiogenic regulator VEGF (day 5 only) (B). All assays were done in triplicate. * $p < 0.05$ and ** $p < 0.01$ when compared to respective GFP groups.
future directions should focus on overcoming these limitations so that the GO-P hybrid material can be translated into possible preclinical and clinical bone tissue engineering applications.

**CONCLUSION**

In our search for highly biocompatible, osteoinductive and osteoconductive scaffolds, we investigated whether incorporating GO into PPCNg would enhance its ability to support the formation of new bone. We demonstrated that the addition of GO maintains thermoresponsive behavior of the hybrid material and effectively supported MSC survival and proliferation. Furthermore, GO-P induced ALP and potentiates BMP9-regulated expression of osteogenic regulators and bone forming markers as well as the angiogenic factor VEGF in MSCs. We further showed that BMP9-transduced MSCs entrapped in the GO-P scaffold form well-mineralized and highly vascularized trabecular bone in vivo. Thus, our findings demonstrate that the GO-P hybrid material may be used as a novel biocompatible and injectable scaffold with osteoinductive and osteoconductive activities to support the formation of new vascularized bone.

**ASSOCIATED CONTENT**

*Supporting Information*

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.8b00179.
Table S1: list of qPCR primers; Figure S1: Effect of PPCN:GO compositions on the temperature evolution of the viscoelastic properties (PDF)

■ AUTHOR INFORMATION

Corresponding Authors
*Tel.: (773) 702-7169; Fax: (773) 834-4598; E-mail: tche@uchicago.edu.
*Tel./Fax: (86)23- 89011212; E-mail: huangwei68@263.net.

O R C I D

Tong-Chuan He: 0000-0001-7721-3934

Author Contributions
T.C.H., W.H., C.Z., R.R.R., G.A.A., J.M.W., M.J.L., A.A., and L.O. conceived and designed the study. C.Z., Z.T.Q., X.Y., R.Z., and S. Yan performed most experiments and collected data. Y. Shu, Y.Z., C.D., E.B., J.L., W.Z., C. Yang, K.W., Y.W., L.A., S.H., X.J., C.G., C. Yuan, L.Z., W. Liu, B.H., Y.F., B.Z., Z.D., Y. Shen, X.W., and W. Luo provided essential experimental materials (such as adenovirus construction and high titer amplification), and assisted in SEM sample preparation and analysis, histological preparations, immune-staining, and qPCR data analysis and interpretations. T.C.H., C.Z., Z.T.Q., W.H., C.Z., R.R.R., G.A.A., J.M.W., M.J.L., A.A., and L.O. drafted the manuscript. All authors reviewed and approved the manuscript.

Notes
The authors declare no competing financial interest.

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