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Research progress of gene therapy combined with tissue engineering to promote bone regeneration

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Xiangyu Chu,[1,2] Yuan Xiong,[3] Li Lu,[1,2] Yiqing Wang,[4] Jing Wang,[5] Ruiyin Zeng,[1,2] Liangcong Hu,[1,2] Chenchen Yan,[1,2] Zhiming Zhao,[6] Sien Lin,[7,a)] Bobin Mi,[1,2,a)] and Guohui Liu[1,2,a)]

AFFILIATIONS

[1]Department of Orthopedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Avenue, Wuhan 430022, China
[2]Hubei Province Key Laboratory of Oral and Maxillofacial Development and Regeneration, Wuhan 430022, China
[3]Department of Orthopedics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Wuhan 430030, China
[4]Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing 100730, China
[5]Department of Nuclear Medicine and PET, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China
[6]Department of Orthopedics, Suizhou Hospital, Hubei University of Medicine, Suizhou 441300, China
[7]Department of Orthopaedics & Traumatology, Stem Cells and Regenerative Medicine Laboratory, Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Hong Kong, China

[a)]**Authors to whom correspondence should be addressed:** sienlin@cuhk.edu.hk. **Tel.:** 027-85726541; mibobin@hust.edu.cn; and liuguohui@hust.edu.cn

ABSTRACT

Gene therapy has emerged as a highly promising strategy for the clinical treatment of large segmental bone defects and non-union fractures, which is a common clinical need. Meanwhile, many preclinical data have demonstrated that gene and cell therapies combined with optimal scaffold biomaterials could be used to solve these tough issues. Bone tissue engineering, an interdisciplinary field combining cells, biomaterials, and molecules with stimulatory capability, provides promising alternatives to enhance bone regeneration. To deliver and localize growth factors and associated intracellular signaling components into the defect site, gene therapy strategies combined with bioengineering could achieve a uniform distribution and sustained release to ensure mesenchymal stem cell osteogenesis. In this review, we will describe the process and cell molecular changes during normal fracture healing, followed by the advantages and disadvantages of various gene therapy vectors combined with bone tissue engineering. The growth factors and other bioactive peptides in bone regeneration will be particularly discussed. Finally, gene-activated biomaterials for bone regeneration will be illustrated through a description of characteristics and synthetic methods.

I. INTRODUCTION

Bone repair and regeneration process involves a complex and well-regulated series of physiological events, which recapitulates aspects of different cell types in combination with several signaling pathways.[1,2] However, large segmental bone defect (SBD) increases the risk of non-union. In the United States, it is estimated that 10%–15% of long bone fractures that occur annually experience delayed healing or non-union due to large SBD.[3] Auto-grafting can be an effective treatment for non-union fractures. However, tissue availability and the second surgery to harvest bone and donor site complications necessitates the further exploration of new strategies to treat these clinical problems. Therefore, bone tissue engineering, which aims to accelerate functional bone regeneration by combining mesenchymal stem cells (MSCs) and signaling molecules within biomaterial scaffolds,[4,5] is developing rapidly. Human MSCs (hMSCs), with the reliable ability to differentiate into distinct lineages including osteoblasts, chondrocytes, and adipocytes, are widely used. In addition, the bone morphogenetic protein (BMP) family members have been extensively investigated in bone tissue engineering.[6,7] However, the difficult maintenance of therapeutic concentrations at wound sites and the rapid diffusion into the

bloodstream restrict their applications. Therefore, it is of paramount importance to construct a safe and efficient delivery system to achieve a sustained and long-term osteogenic effect on hMSCs, which will not only reduce the large dose of growth factor but also promote faster and more uniform bone formation. Currently, promotion of osteogenesis to treat large SBD, delayed unions, and non-unions remains a crucial clinical challenge.[8]

There are a number of treatment strategies available to encourage bone regeneration process, including autologous bone graft, allograft implantation, distraction osteogenesis, and induced membrane technique,[9] which have been commonly used either singly or in combination for complex clinical situations. Most of these methods exhibit relatively optimistic results for bone regeneration. Nevertheless, there are several disadvantages and limitations, including associated complications, issues of immunogenicity, and time-consuming treatment procedures. Additionally, large SBD caused by trauma, infection, tumor resection, and skeletal abnormalities[10,11] make the treatment more difficult, although the overall incidence is relatively low. Restoration of bone conductivity, osteoinducibility, and osteogenesis by tissue engineering constructs could increase bone formation. Scaffolds with composite bioactive factors, which can achieve highly oriented repair with bone induction ability, are often used in the research of bone regeneration.[12] Therefore, gene and cell-based bone tissue engineering provide a potential alternative approach in the reconstruction of bone tissue. Bone tissue engineering and gene/cell-based therapies, specifically in treating delayed fracture healing, non-unions, and large SBD, have made remarkable progress in recent years.[13] In this review, we provide an overview of *in vivo* and *ex vivo* studies combining gene therapy associated with bone tissue engineering for bone regeneration. A thorough understanding of gene therapy vectors and gene delivery-based biomaterials may allow for better incorporation of these potential applications in bone repair and regeneration.

II. HISTOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF FRACTURE HEALING

A. Normal fracture healing process

Due to the strong bone regeneration ability, fracture can be completely healed after a good reduction in 3–4 months generally. The proliferation of osteoblasts and the generation of new bone is the basis of fracture healing.[14] The fracture will fully heal after hematoma formation, fibrous and bony callus formation, and callus remodeling, returning the bone to normal structure and function (Fig. 1).

B. Cellular and molecular changes during fracture healing

Fracture hematoma formation is the foremost important stage of fracture healing. Within a few hours, blood coagulation and local necrotic tissue cause inflammatory response. Macrophages, platelets, polymorphonuclear leukocytes, and lymphocytes are observed in the fracture area.[15,16] These inflammatory cells could release inflammatory cytokines such as tumor-necrosis factor-α (TNF-α), platelet-derived growth factor (PDGF), interleukin-1 (IL-1), and interleukin-6 (IL-6).[17] The inflammatory response typically peaks within 24 h and ends after 7 days. Hematoma gradually becomes organized, forming granulation tissue and then evolving into connective tissue to form fibrous connections. Then, callus formation is driven by growth factors, chondrocytes, fibroblasts, and mechanical stimulation at the fracture site. Proliferation and differentiation typically peaks on 7–10 days and completes within 2–3 weeks after fracture. The final stage of fracture healing is the transformation of irregular woven bone into structured lamellar bone, which is regulated by a coordinated relationship between osteoblasts and osteoclasts. The changes in the main cells involved in the process of fracture healing have been summarized as shown in Fig. 2.

III. BENEFICIAL ASPECTS OF GENE THERAPY FOR THE TREATMENT OF BONE REGENERATION

With the continuous development of transgenic technology and stem cell technology, gene therapy has shown incomparable advantages and broad application prospects in the field of bone repair and regeneration.[18] These advantages include: (1) local and targeted release of gene products; (2) enhanced local treatment effect, while reducing systemic side effects; and (3) the ability to transfer and regulate multiple genes separately, with proteins synthesized from endogenous sources having stronger biological activity compared to exogenous recombinant proteins. Gene therapy overcomes the limitation of protein delivery and is considered as one of the most promising methods to maintain the effective therapeutic concentration of local growth factors in bone defects. However, how to reduce the side effects of gene therapy is still the main clinical concern.

Gene therapy to achieve bone tissue regeneration does not involve changing genetic material and replacing non-functional genes, just achieving the desired goal of bone regeneration. At present, *in vivo* (direct) and *ex vivo* (transduced cell-mediated) are two main gene transfer methods, providing a promising treatment for bone regeneration.[19] As show in Fig. 3, vectors carrying the gene of interest are directly injected or integrated into a biomaterial scaffold and implanted into the injury site. They are also used to genetically modify host cells, which is then seeded into a suitable scaffold or directly implanted into the injury site. *Ex vivo* transgenes do not inject virus particles or DNA complexes directly into the body, which makes target cells technically highly efficient in cell transduction. The commonly used adenovirus (AdV) vectors are relatively safe for *ex vivo* transgene. Meanwhile, cell acquisition, infection, transduction, and implantation into appropriate anatomical sites can be combined with artificial bone materials according to actual needs. However, the disadvantages include complicated operation, expensive cost, and time-consuming application. The *in vivo* method is relatively simple and low cost, which still needs to improve the transfection efficiency and targeting and reduce the risk of immune response.

The combined application of gene therapy and tissue engineering methods, which is called gene-activated materials (GAMs), has great potential for improvement of bone regeneration. The GAM approach implies that provisional template will be enriched with vectors carrying therapeutic genes, which was used to directly transfect recipient's cells and stimulate local secretion of encoded proteins for new bone formation in the defect area. Currently, GAM has shown its potential in clinical and pre-clinical works. However, transfection efficiency, which is highly dependent on the healthy resident cell population, remains a serious barrier to clinical translation.[20] Additionally, the technique of GAM positioning at the defect area depends on the properties of the materials and treated pathology. For example, fixation of gene-activated bone substitute within the bone grafting area was achieved via soft tissue suturing in a clinical study.[21] At present, there are a

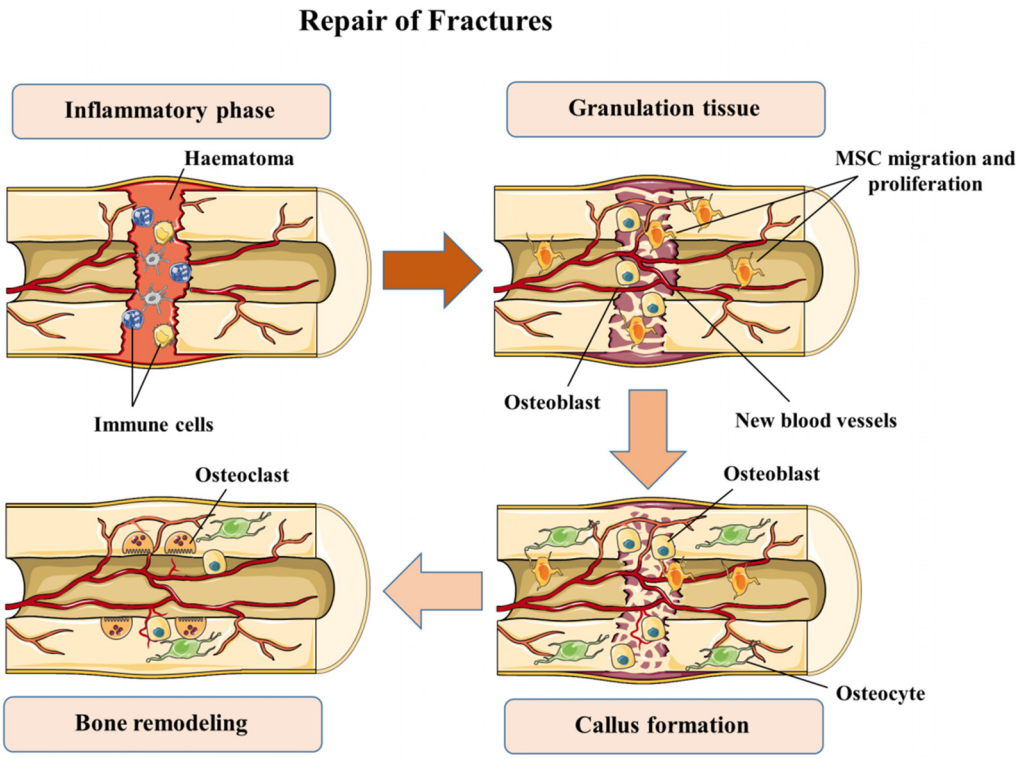

FIG. 1. Normal fracture healing process. Fracture healing process can be divided into inflammatory phase, granulation tissue, callus formation, and bone remodeling stages.

variety of methods for delivering GAMs, primarily including viral and non-viral delivery vectors. However, the use of different gene vectors may be limited by the comprehensive cost consideration. It is still difficult to develop reasonable vectors and put them into large-scale preclinical trials. With the development of gene vectors and novel scaffold materials, the clinical use of gene activating media needs to be further optimized.

IV. GENE THERAPY VECTORS IN BONE REGENERATION

Gene therapy refers to delivery of foreign genes to tissues of interest using viral or non-viral vectors. Many non-viral vectors have been used in gene therapy, including lipid-based system, polymer-based system, and inorganic nanoparticle (NP) system.[22] Nevertheless, major obstacles including transfection efficiency and spatiotemporal release of molecules, as well as the interplay of multiple biological factors still hinder their translational potential. Based on current studies, non-viral gene vectors are more feasible for translation due to their non-immunogenicity and safety that could be promising in clinical applications.[23–25] Non-viral gene delivery strategies typically combine physical techniques (electroporation or sonoporation) to trigger transient cell membrane permeability and chemical techniques (liposomal- or polymer-based transfection strategies) to enable reduced genetic material degradation and more environmentally friendly cell uptake. When compared to viral transduction, non-viral gene delivery methods had lower DNA transfection efficiencies, which led to the development of multiple methods such as GAMs and NPs for increasing transfection efficiency.[26,27]

Viral gene therapies for bone regeneration have been commonly used, including AdV, adeno-associated virus (AAV), retrovirus, and lentivirus.[20] Compared with non-viral gene vectors, viral gene vectors have been proven to be relatively effective gene transfer vectors and are widely used in cell transformation. However, viral vectors have initially prompted questions about their immunological safety, possible worries regarding off-target consequences and associated toxicity, especially when systemic viral vectors are used.[28] The development of safer vectors have improved due to advancements in virology and gene therapy. Simultaneously, local gene therapy procedures could mitigate the potential dangers of systemic system. The safety of gene therapy techniques for bone healing in clinical applications needs to be further improved. As shown in Fig. 4, we described the most recent viral and non-viral vectors used and their characteristics.

A. Viral vectors

1. Adenovirus (AdV)

AdV is currently the most commonly used vector in clinical trials, with large gene capacity and high transfection efficiency. The genes carried by AdV do not integrate into the host cell genome and are independently expressed outside the host genome, which can achieve high abundance and instantaneous expression of target genes.[29] The advantages of AdV as gene delivery vector mainly include: (1) clear biological function and easy genome operation; (2) low virulence or

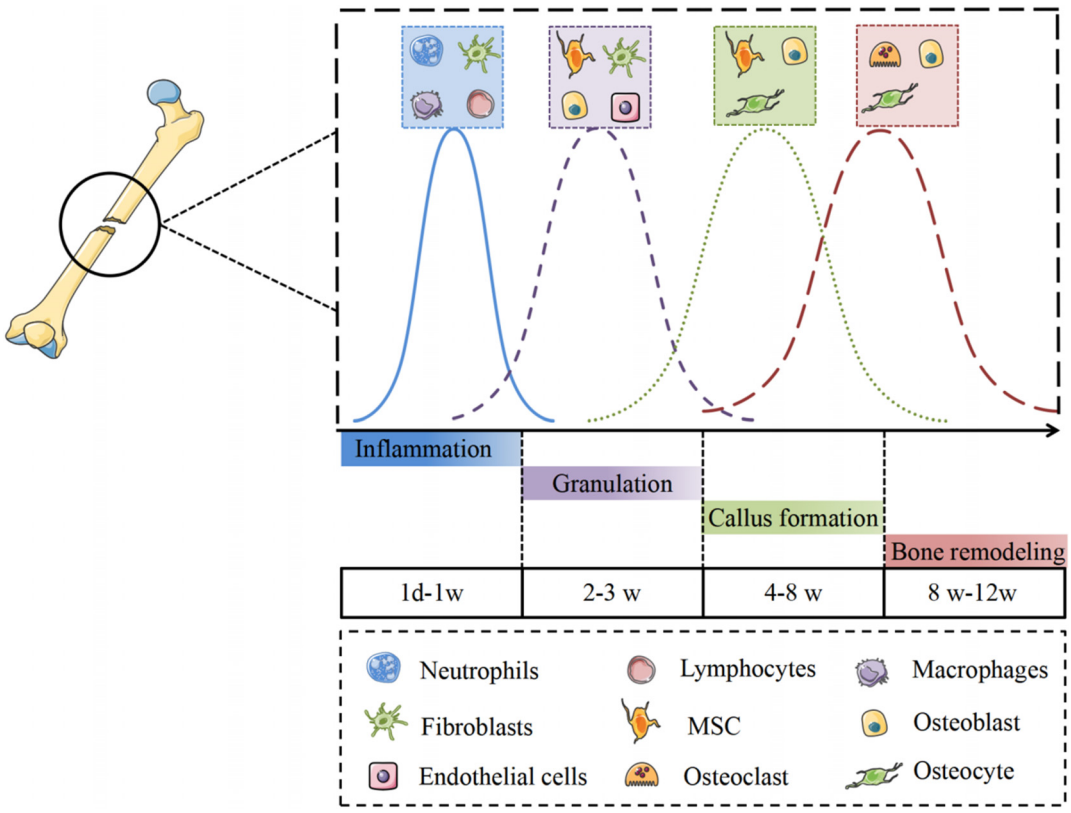

FIG. 2. Cellular changes during fracture healing. The healthy fracture healing process occurs in four sequential and overlapping stages, involving different cell groups secreting a variety of cytokines.

non-virulence in human body and high transduction efficiency for both dividing and non-dividing cells; (3) controllable production cost and high yield; and (4) low risk of insertion mutation. However, AdV also has some disadvantages as a gene therapy vector: (1) short-term gene expression lasting for a week before gradually declining and disappearing and (2) high immune prototypes and potential for severe toxic reactions. As shown in Fig. 5, Sharma *et al.* used AdV-mediated vascular endothelial growth factor A (VEGFA) combined with BMP-2 to express in bone MSCs (BMSCs). Then, the edited BMSCs were implanted into the recently developed scaffold, which promoted the expression of bone markers and bone formation.[30] Takanche *et al.* applied AdV-loaded c-myb (Ad/c-myb) and gelatin-modified biodegradable membrane to injured tibial bones in rats, and the results showed increased osteogenic molecules, bone volume, bone density, and new bone formation.[31]

2. Adeno-associated virus (AAV)

AAV vectors are widely used as gene vectors due to their non-pathogenicity, long-term expression, and ability to infect a variety of cells.[32] The advantages of AAV as a gene delivery vector mainly include the following: (1) high safety, low immunogenicity, and non-pathogenicity; (2) AAV does not integrate into host genome to avoid carcinogenic risk; (3) AAV genome combines with histones to form a structure similar to chromosomes, which is very stable and will not be degraded and can be expressed stably for a long time; and (4) tissue targeting. Different serum types of AAV have different tissue tropism and can target different tissues. However, AAV can cause serious side effects when administered systematically at high doses. In addition, the amount of exogenous target genes accommodated by AAV vectors is generally less than 4.7 kb, which limits its application. In the recent development of clinical trials, AAV vectors have been used more frequently by researchers.[33] For instance, Chen *et al.* recently developed circStag1-loaded AAV construct that could promote new bone formation and prevent bone loss in ovariectomized rats.[34] Sun and colleagues have practiced recombination AAV (rAAV)-mediated *in vivo* gene transfer of BMP-2 as a treatment option to promote bone regeneration.[35] Through this approach, efficient BMP-2 were produced in the hydrogel scaffold and released at a therapeutic level to achieve sustained MSCs osteogenesis for bone regeneration. As shown in Fig. 6, Won-Taek Oh *et al.* designed an AAV9 capsid with the bone-targeting peptide motifs to bind an allograft bone or hydroxyapatite (HA)-based scaffold. Notably, rAAV9 could attach to the HA scaffold, effectively transduce MSCs, and eventually generate skeletal organoids with high bone formation activity, which could be used in the repair of large SBD.[36]

3. Lentivirus

Lentivirus belongs to the retrovirus family, which could continuously express target proteins by integrating target genes into host

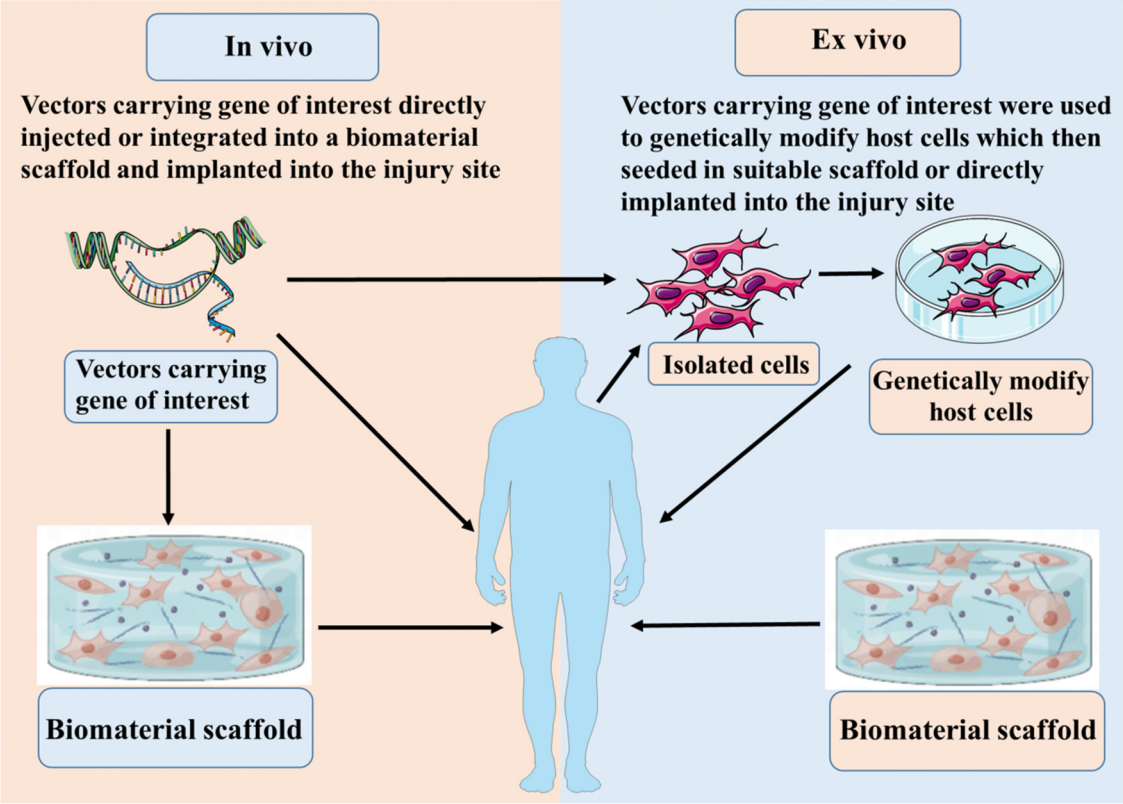

FIG. 3. *In vivo* and *ex vivo* are two main gene delivery methods. *In vivo* indicates that vectors carrying gene of interest are directly injected or integrated into a biomaterial scaffold and implanted into the injury site. *Ex vivo* indicates that vectors carrying the gene of interest are used to genetically modify host cells, which is then seeded into a suitable scaffold or directly implanted into the injury site.

chromosomes.[37] Lentiviral vector-mediated gene therapy technology has the following advantages: (1) high infection efficiency on both dividing and non-dividing cells; (2) osteogenic growth factors with high biological activity can be released locally, efficiently, continuously, and controllably; and (3) the ability to sustain and stably express foreign genes, increase the expression of regulated target proteins, and accommodate large fragments (8–10 kb) of exogenous target genes. However, since the natural host of human immunodeficiency virus type 1 (HIV-1) lentivirus vector, its biological safety is of particular concern. Owing to the infection efficiency and long-term stability in dividing and non-dividing cells,[38] lentiviral gene vehicle becomes more popular in *ex vivo* gene transfer. Meanwhile, researchers prefer the design of reporter gene to monitor and assess transduction efficiency. For example, a recent study evaluated the efficiency of lentivirus as a BMP-2 gene delivery system to maintain stable expression of BMP-2 in human BMSCs (hBMSCs).[39] In this study, Lin and colleagues reported a novel procedure, involving the use of hBMSCs that had been transduced with a lenti-BMP-2 construct and gelatin-based scaffolds, which were synthesized by visible light-based projection stereolithography (VL-PSL) technology, for bone regeneration. Using the computer-aided design (CAD)-derived 3-dimension (3D) structure, hBMSCs were seeded uniformly within the scaffold and expressed BMP-2 gene to stimulate differentiation toward the osteogenic lineage without the exogenous BMP-2 protein. Also, efficient bone formation *in vivo* were observed in severe combined immunodeficiency (SCID) mice, which were given an intramuscular implantation, suggesting the feasibility and self-driven osteogenic capability of this new procedure[40] [Fig. 7(a)]. Additionally, Sun *et al.* induced rapid bone formation after surgery by using BMSCs transfected with BMP-7 gene in a demineralization and acellular allograft bone-collagen biphasic scaffold.[41] Rowland *et al.* used chondrogenic matrix (CDM) scaffold-mediated lentiviral gene delivery of IL-1 receptor antagonists, chondrogenic transforming growth factor-3 (TGF-β3), or osteoblastic BMP-2 to MSCs. CDM hemispheres were found to support robust bone and cartilage tissue formation even in the presence of IL-1. These structures provide a microphysiological *in vitro* articular organoid model with a site-specific, tunable, and inducible protein delivery system [Fig. 7(b)].[42] Meanwhile, researchers have used lentiviral vectors to mediate circular RNA (circRNA) and microRNA (miRNA) transmission. For example, Yu *et al.* overexpressed circ_0003204 in human adipose-derived stem cells (hASCs) through lentiviral vectors. Methacryloylated gelatin (GelMA) was then used to deliver gene-edited hASCs with 3D porous and interconnected structures to treat bone defects in mice.[43] Xiong *et al.* designed a new porous poly-L-lactic acid (PLLA) and polyhedral oligomeric silsesquioxane (POSS) scaffold combined with BMSCs for tissue engineering and transfected

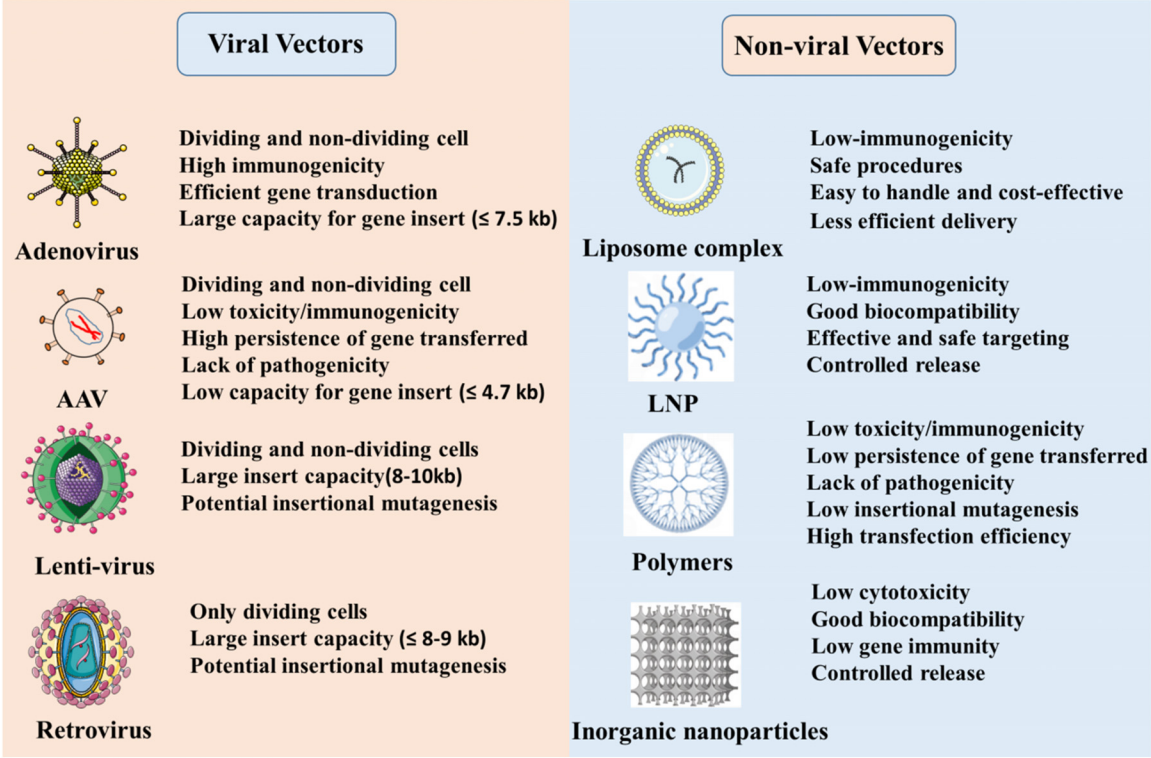

FIG. 4. Viral and non-viral vector types used in GAMs. Viral gene therapies for bone regeneration, including AdV, AAV, retrovirus, and lentivirus are described. The most recently used non-viral vectors, including lipid-based system, polymer-based system, and other systems, have been evaluated.

BMSCs with lentivirus. It was confirmed that miR-19b-3p could promote osteogenic differentiation of BMSCs by inhibiting the expression of SMAD ubiquitylation regulatory factor-1 (Smurf1).[44]

4. Retrovirus

Retrovirus is one of the earliest vectors used in gene therapy. The retroviral vector has the following characteristics: (1) The retrovirus can randomly integrate the target gene into the host genome and stably express the target gene for a long time. (2) Due to the random integration characteristic of the retrovirus, it is easy to interfere with the normal expression of host genes, which has potential tumorigenic risk. (3) Retrovirus has high transduction efficiency to dividing cells, but low expression efficiency in stem cells. Therefore, the retroviral vector is mainly used to deliver cells *ex vivo* and is not suitable for treating non-fatal diseases.

B. Non-viral vectors

1. Lipid-based systems

Liposome is a kind of spherical structure of bilayer lipid molecules. The ordered phospholipid molecular bilayer forms a closed vesicle, and the hydrophilic cavity structure is formed inside. Liposome can be used to deliver drugs or genetic materials into cells, taking advantage of their ability to fuse with cell membranes. Lipid NPs (LNPs) are a special type of NPs different from liposomes. LNPs exist in the interior through the electrostatic complexation of cationic phospholipids and negatively charged nucleic acid substances, forming a multilayer core dispersed between lipid layers. Liposomes have played an important role in the field of drug delivery, involving numerous indications, and LNPs are currently used for delivery of nucleic acid substances.[45] Compared with viral vectors, LNPs have the following advantages: (1) LNPs do not have pre-existing immunogenicity and have fewer interference from natural immune mechanisms; (2) they have less chance of contamination and risk of random integration into the genome; (3) easy control of raw materials and mature process development technology; and (4) controllable quality standard and clear development path. At the same time, in order to further break the bottleneck, the following aspects need to be optimized: (1) optimize the relationship between distribution in the body and efficacy/toxicity; (2) expand the application range of drug and gene delivery; and (3) optimize the effective delivery of the vector to target cells and reduce the ineffective delivery to non-target cells.

A number of lipid-based non-viral vectors have been investigated for MSCs transfection. For example, Garcia-Garcia *et al.* recently described that using LNPs to induce Smurf1 gene silence could promote osteogenic differentiation of MSCs.[46] Imran Vhora *et al.* have also shown that novel ionizable lipid nucleic acid NPs can be successfully used for whole-body BMP-9 gene delivery, inducing BMSCs osteogenesis and improving bone regeneration in rats after oophorectomies.[47] Mi-RNAs can also be delivered via lipid-based vectors. For instance, Hu *et al.* generated hybrid NPs by fusion of C-X-C

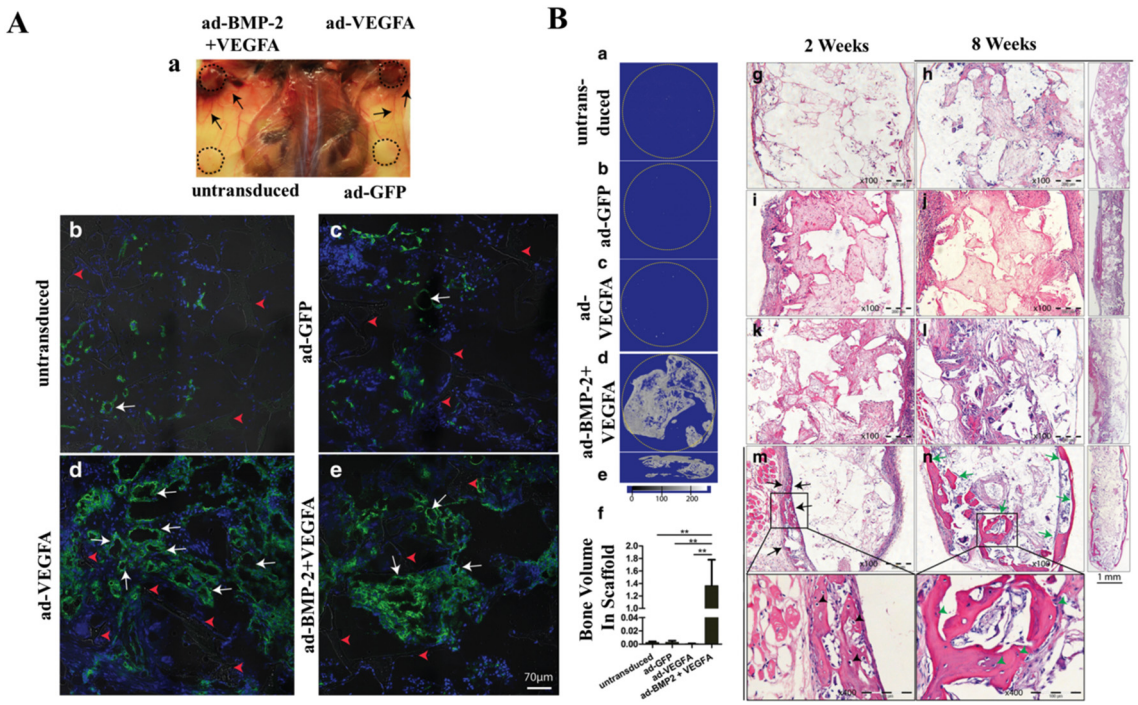

FIG. 5. AdV-mediated gene therapy for bone regeneration. (a) AdV-mediated expression of VEGFA alone and in combination with BMP-2 was associated with enhanced angiogenesis in scaffold explants. (b) Combined delivery of BMP-2 and VEGFA induced ectopic bone formation in scaffold explants in a mouse model. (a) and (b) Adapted with permission from Sharma *et al.*, Stem Cell Res. Ther. **9**(1), 23 (2018). Copyright 2018 Author(s), licensed under a Creative Commons Attribution (CC BY) license.[30]

motif chemokine receptor 4 (CXCR4)-positive exosomes with liposomes carrying antagomir-188. Hybrid NPs specifically aggregate in bone marrow and release antagomir-188 to promote osteogenic differentiation of BMSCs, thereby reversing age-related trabecular bone loss and reducing cortical bone porosity in mice [Fig. 8(a)].[48]

Exosomes, which have received much attention in the field of biomedicine in recent years, can also achieve targeted therapy.[49,50] For example, Xiong *et al.* found that miRNA-5106 was overexpressed in exosomes of M2-type macrophages. Osteogenic differentiation of BMSCs could be induced by directly targeting the Salt-inducible kinase 2 and 3 (SIK2 and SIK3) genes.[51] In addition, Li *et al.* used liposome gene delivery technology to engineer MSCs, promoting exosomes secreted by MSCs to have more significant osteogenic effects. The result provides a new idea for promoting bone regeneration through genetic engineering of exosomes [Fig. 8(b)].[52]

2. Polymer-based systems

The polymer materials used for gene carriers in bone repair can be divided into natural polymers and synthetic polymers. Natural polymers mainly include proteins, peptides and chitosan. Synthetic polymers include polyethylenimide (PEI), dendritic macromolecules, and polyphosphoesters.[53] These polymer structures are more stable than those of a lipid complex. Polymer-mediated gene therapy technology has the following advantages: (1) compared with liposome vectors, polymer gene vectors have stronger transfection ability; (2) polymers protect DNA from degradation; (3) wow immunogenicity; and (4) targeting and biocompatibility can be increased through molecular design. However, the polymer carrier also has some limitations: (1) The DNA release rate of the refractory polymer is slow, which prolongs the treatment cycle and (2) polymers are easy to be excluded by plasma and difficult to be applied *in vivo*. Currently, a number of polymer-based systems have been investigated for bone regeneration. For example, Yanagihara and colleagues have demonstrated that transplanting runt-related transcription factor 2 (Runx2)-transfected MSC spheroids, which were coated with a temperature-responsive polymer, into bone defect on rat femurs could enhance bone regeneration.[54] Liang *et al.* recently developed a non-viral gene vector, which was obtained by coating BMP-2 plasmids and PEI on microvesicles (MVs) of BMSCs. The gene-activated demineralized bone matrix (DBM) scaffold showed significant bone formation and angiogenesis ability.[25,55]

3. Inorganic nanoparticle-based systems

Inorganic NPs play a role in the treatment of diseases by transporting drugs or biomolecules into organisms through cell membranes. Inorganic NPs used in gene transport include calcium phosphate, silica, and gold NPs.[56,57] Various other inorganic materials, such as calcium carbonate, iron oxide, carbon nanotubes, and graphene, are also widely utilized for this purpose.[58,59] Calcium phosphate particle is the earliest used particle, which has the advantages of biocompatibility, biodegradability, easy to be absorbed, and high binding affinity. However, the nano-calcium phosphate crystals grow larger over time, reducing their storage capacity. Silica particles are used for nucleic acid delivery due to their good biocompatibility and adjustability.[60] In serum-containing media, reduced delivery efficiency is a major

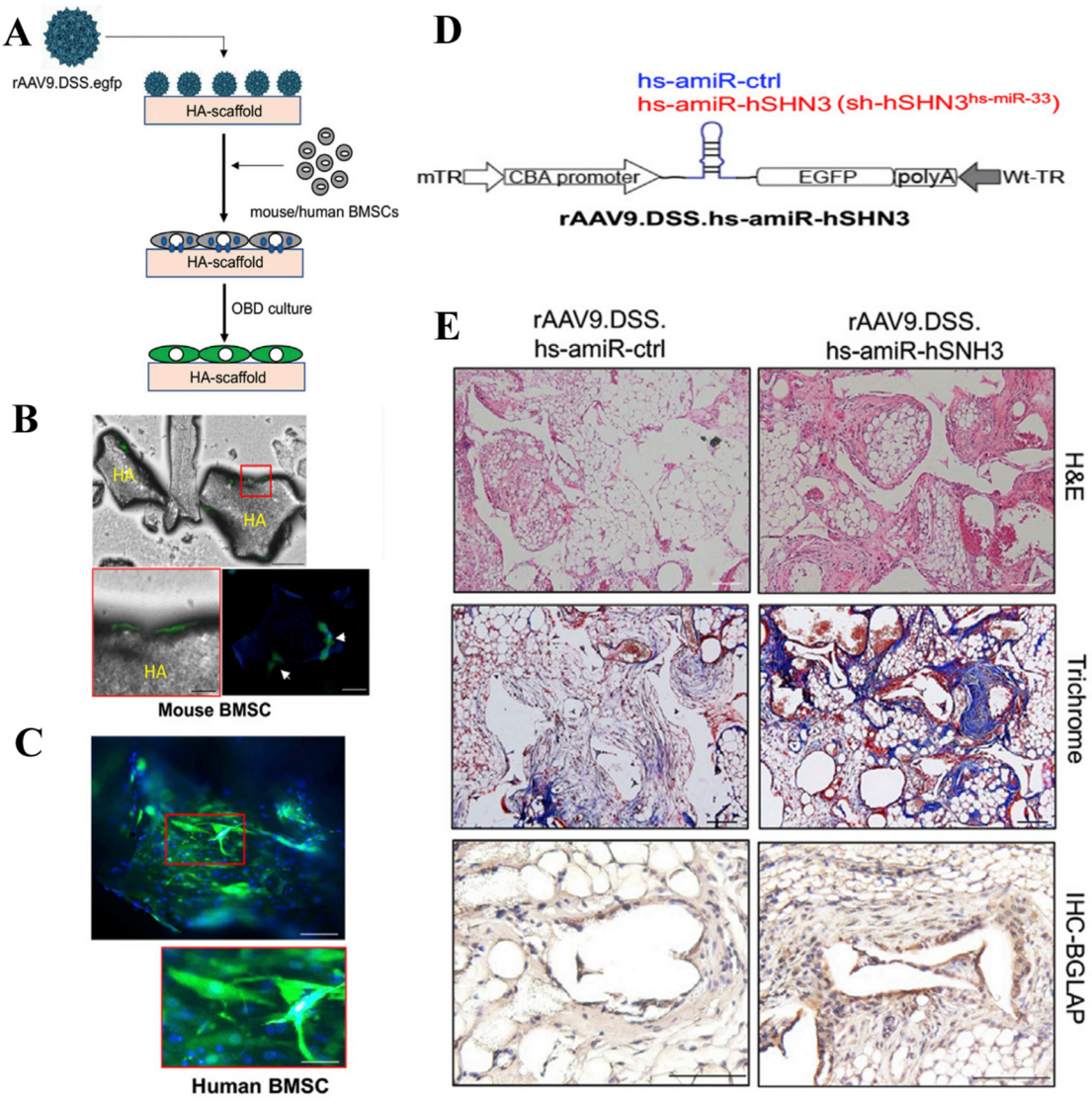

FIG. 6. AAV-mediated gene therapy for bone regeneration. (a) Diagram of the study and treatment methods. (b) Mouse or (c) human BMSCs were seeded on the scaffold. (d) Diagram of the construct. (e) Longitudinal sections of the scaffold were stained for H&E and trichrome and immuno-stained for bone gamma-carboxyglutamic acid protein (BGLAP). (a)–(e) Adapted with permission from Won *et al.*, Mol. Ther. **31**(2), 435–453 (2023). Copyright 2023 Author(s), licensed under a Creative Commons Attribution (CC BY) license.[36]

limiting factor due to interactions between serum proteins. In recent years, gold NPs have been paid increasing attention by researchers.[61] In conclusion, inorganic NPs have the following characteristics: (1) easy preparation and infinite surface characterization and inertia; (2) DNA modified gold surface can use photothermal effect to achieve cell transgene and control gene release; and (3) *ex vivo* transfection efficiency is high, and toxicity is low. However, high chemical stability makes inorganic NPs difficult to dissolve and accumulate in cells, which is not beneficial to cell growth.

Inorganic NPs have been widely used in bone regeneration. For instance, Xing and colleagues successfully coupled multifunctional targeting small interfering RNA (siRNA) onto the surface of gold NPs. Then, they coated the above-modified gold NPs on the surface of titanium implants layer-by-layer (LbL) technology and achieved multilevel nanostructure coating. The results confirmed that the nanolayer could effectively interfere with the expression of cathepsin K in periimplant osteoclast cells, thereby synergically promoting angiogenesis and bone regeneration [Fig. 9(a)].[62] Additionally, Yu *et al.* designed a bioactive nanofibrous scaffold, which was fabricated by co-spinning poly ε-caprolactone (PCL), elastomeric poly citrates-siloxane (PCS), and bioactive osteogenic miRNA nanocomplexes, realizing multiple functional effects of photoluminescence, nanostructure, elasticity, silicon-based active component, and long-term miRNA release. The results demonstrated that this material can effectively promote

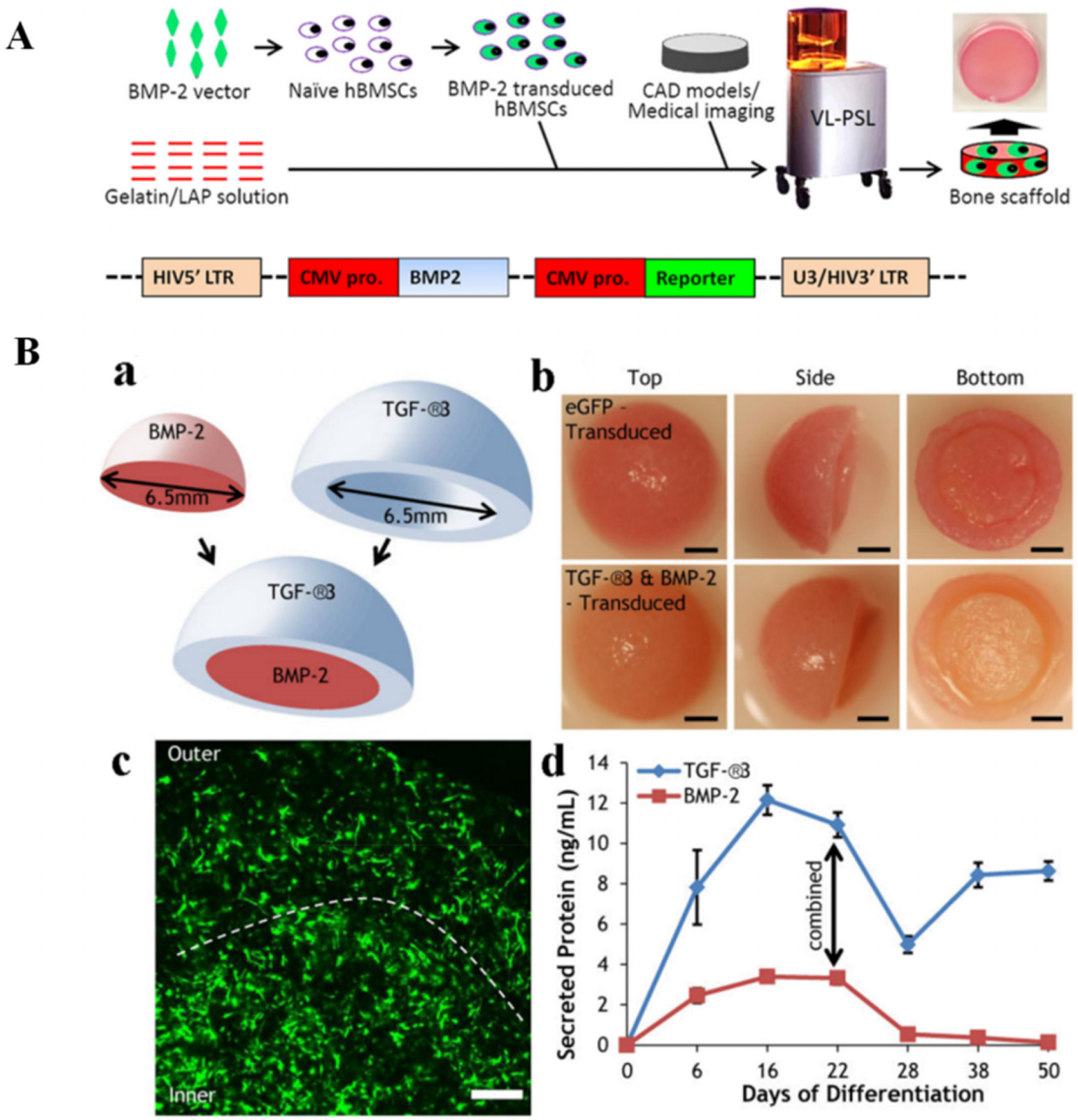

FIG. 7. Lentivirus-mediated gene therapy for bone regeneration. (a) Fabrication scheme of bone scaffold with simultaneous incorporation of lenti-BMP-2 vector-transduced hBMSCs. Adapted with permission from Lin *et al.*, Stem Cell Res. Ther. **10**(1), 254 (2019). Copyright 2019 Authors(s), licensed under a Creative Commons Attribution (CC BY) license.[40] (b) Illustration of chondrogenic outer hemispherical shell with immobilized TGF-β3 lentivirus and osteogenic inner hemisphere core with immobilized BMP-2 lentivirus. Reproduced with permission from Rowland *et al.*, Biomaterials **177**, 161–175 (2018). Copyright 2018 Elsevier.[42]

osteogenic differentiation of stem cells. The *in vivo* bone defect model further verified the ability of the elastic silicon material system to promote rapid bone tissue repair and reconstruction.[63] Huang *et al.* designed a noninvasive and intelligent monitoring carbon nanotube scaffold, which could make up the easy deactivation shortfall of BMP-2 by sustainably enhancing MSCs osteogenic differentiation and bone formation [Fig. 9(b)].[64] At the same time, the same team reported novel bioactive glass nanoclusters (BGNCs) with very large pore sizes (10–30 nm), which can effectively deliver miRNAs to the treatment area to accelerate bone regeneration in critical size bone defects.[65]

In conclusion, compared with viral vectors, non-viral vectors have lower cytotoxicity, immunogenicity, and mutagenicity, which attract more researchers to explore, thus promoting the development of gene therapy. Over the past decade, the trend of using non-viral vectors for gene therapy has been increasing significantly. In recent years, many non-viral vectors have been studied, such as polymers, lipids, inorganic particles, or combinations of different types. These successes offer hope for the discovery of more effective and safe non-viral vector gene therapy products for bone regeneration in the future.

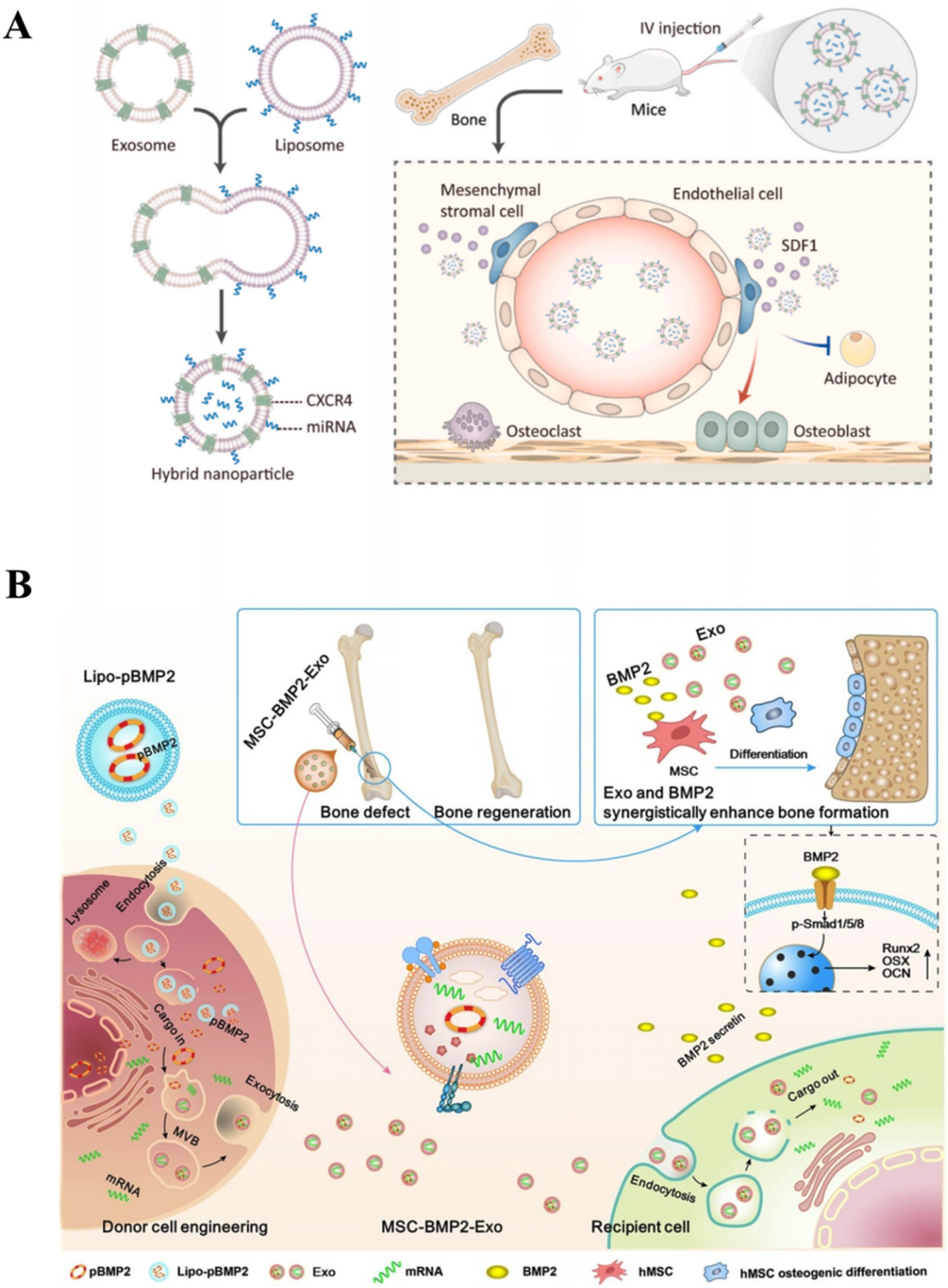

FIG. 8. Lipid-based systems-mediated gene therapy for bone regeneration. (a) Schematic illustration of exosome-guided miRNA blocking. Adapted with permission from Hu *et al.*, Bioact. Mater. **6**(9), 2905–2913 (2021). Copyright 2021 Author(s), licensed under a Creative Commons Attribution (CC BY) license.[48] (b) Schematic illustration of exosomes derived from BMP2 genetically engineered MSCs promote bone regeneration. Adapted with permission from Li *et al.*, J. Nanobiotechnol. **20**(1), 135 (2022). Copyright 2022 Author(s), licensed under a Creative Commons Attribution (CC BY) license.[52]

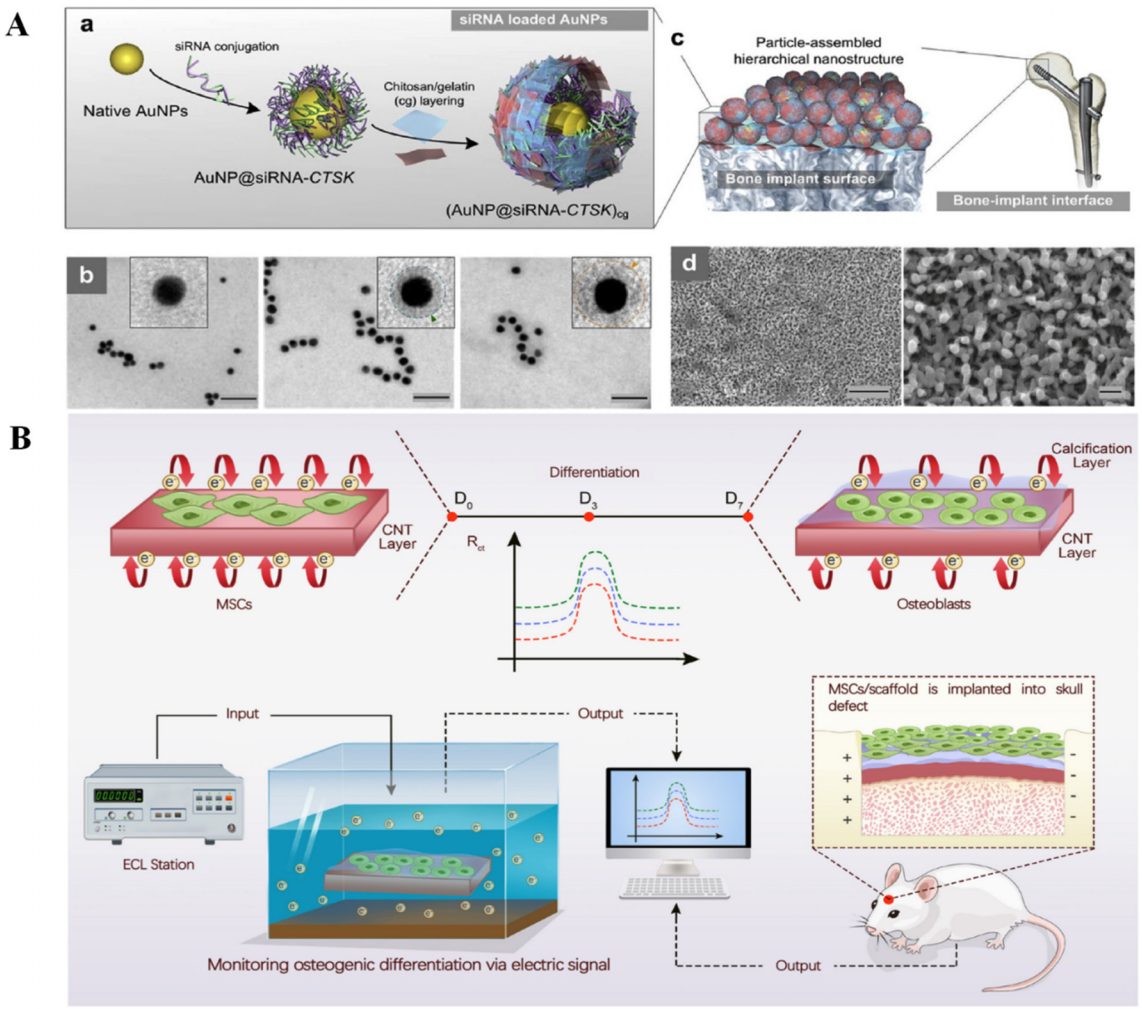

FIG. 9. Inorganic NP-based system-mediated gene therapy for bone regeneration. (a) Particle-based hierarchical nanostructured implant coatings. Reproduced with permission from Xing *et al.*, Biomaterials **235**, 119784 (2020). Copyright 2020 Elsevier.[62] (b) Schematic diagram of carbon nanotubes scaffold preparation and function to recover bone tissue defect. Adapted with permission from Huang *et al.*, Bioact. Mater. **19**, 499–510 (2022). Copyright 2022 Author(s), licensed under a Creative Commons Attribution (CC BY) license.[64]

V. MSCS, GROWTH FACTORS, AND OTHER BIOACTIVE PEPTIDES IN BONE REGENERATION

Scientific consensus indicates that the MSCs reside in many adult tissues, including bone marrow, adipose tissue, muscle, and connective tissue and play a role in tissue repair.[66,67] MSCs are ideal seed cells in the treatment of a variety of refractory diseases in orthopedics. Although the mechanisms of differentiation and mobilization of MSCs are highly complex, the multipotent properties make them an appropriate choice for clinical applications. At present, there are more than 800 medical trails on human beings associated with MSCs as therapy.[68] Especially, the combination of MSCs and various scaffolds is a hot spot in the current research, and it has also made some progress in the treatment of bone regeneration.[5,69] MSCs have also become the most commonly used cells in regenerative medicine due to their ease of isolation and *in vitro* expansion. Although the efficacy and safety still require further investigation, promising findings on bone regeneration of MSCs have been extensively described and utilized in clinical settings.[70,71] However, there are still many difficulties in the study of MSCs, such as the specific surface markers of MSCs, the molecular mechanism of MSC differentiation, the influence of different scaffold materials on MSCs proliferation and differentiation and the specific mechanism, and the physical and chemical properties of the prepared scaffold materials with requirements of treatment. At the same time, many studies are still *in vitro* or at the animal experimental stage. It is believed that with the deepening of research, the application of MSCs in the treatment of bone regeneration will make breakthrough progress.

The stem cells used in clinical treatment mainly include: autologous bone marrow and its bone marrow aspirate concentrate (BMAC), bone marrow nucleated cells (BMNCs), BMSCs, umbilical

cord mesenchymal stem cells (UCMSCs), adipose-derived mesenchymal stem cells (ADMSCs), and peripheral blood stem cells (PBSCs).[72–74] The efficacy and safety of BMNCs and BMSCs have been clinically verified, while there are relatively few studies related to other types of cells.[75] Allogeneic sources including UMSCs and BMSCs are relatively few. At present, there are two main approaches to stem cell therapy, including direct injection of stem cells at the site of injury and composite transplantation of stem cells and scaffolds. However, the clinical repair of large SBD is still a major challenge in the field of orthopedics. We are looking forward to the development of stem cell therapy and clinical application of ideal 3D printed implants with bioactive substances.

Meanwhile, it should be noted that the inclusion of growth factors approved by food and drug administration (FDA),[76] such as BMP-2, which is naturally present in fracture healing[77,78] and has been found to encourage bone regeneration by helping induce differentiation and increase proliferation of osteoblast.[79] However, there are some challenges in the successful application of BMP-2 protein to promote bone regeneration *in vivo*. First, bone regeneration requires long-term active growth factors under temporal and spatial control, resulting in sustained cellular stimulation. Furthermore, it is difficult to maintain therapeutic concentration of BMP-2 at defect sites due to its short half-time and rapid diffusion by the bloodstream, as demonstrated by *in vivo* experiments.[80] Finally, it may be necessary to deliver multiple growth factors including VEGF[81] and TGF-β[82] simultaneously. The combined delivery has the potential to induce higher bone formation than single BMP-2 delivery due to the complex mechanisms of growth factor and cellular crosstalk. Different reports have shown that combined delivery of growth factors results in synergistic effects that enhance bone formation, but it is more complicated.[83] Currently, two major strategies have been developed to optimize the spatiotemporal delivery of BMP-2.[84,85] The first consists in trapping BMP-2 protein in delivery vehicles and functionalizing the surface of these materials. The second major strategy consists in introducing BMP-2 gene into cells through an *ex vivo* gene transfer approach, which has been demonstrated to be more efficient in maintaining the high concentration and bioactivity of BMP-2 protein expressed by engineered MSCs. Additionally, Yongsun Kim and colleagues transduced BMP-7 gene expressing lentivirus particles into ADMSCs to achieve a stable BMP-7 production and bone regeneration in critical-sized bone defects.[86] Meanwhile, BMP-9 has been reported promising results in robust osteogenic cellular differentiation from a bone regeneration perspective.[87–89] In summary, these experiments demonstrated the great potential of BMP family to support bone regeneration.

In addition, other recombinant growth factors have been also extensively used to promote bone regeneration, including insulin-like growth factor-1 (IGF-1), fibroblast growth factor-2 (FGF-2), TGF-β, VEGF, and PDGF.[90,91] Those growth factors were applied individually, sequentially, or simultaneously. For example, Gugjoo and colleagues indicated that the combined application of laminin scaffolds containing MSCs with IGF-1 and TGF-β could enhance significant cartilage formation and subchondral bone formation.[92] Bioencapsulated IGF-1, which used codon-optimized pro-IGF-1 with e-peptide to facilitate oral delivery, was gavaged to femoral fractured diabetic mice by Park and colleagues.[93] The novel delivery system was demonstrated to promote significant increase in bone volume, density, and area. Therefore, the use of this system should have promising applications for clinical treatment of large SBD and non-union fractures.

VI. GENE-ACTIVATED BIOMATERIAL (GAM) AND MANUFACTURING TECHNOLOGY

Biomaterial scaffolds are increasingly being used in regenerative medicine to promote cellular attachment and provide superior mechanical and physical properties.[94] Commonly, small molecular drugs and growth factors are incorporated into these scaffolds to encourage bone regeneration. While the delivery of bioactive therapeutics on scaffolds-based constructs and its application in *in situ* delivery is prospected, it remains hindered by the sustained and long-term osteogenic doses of proteins required. As a result, GAMs, which combined gene therapy and tissue engineering based on the use of vectors that are capable of efficiently transfecting host cells, are the main trends and characteristics of biomaterial research in bone regeneration. As show in Fig. 10, GAM scaffold have been widely used to accelerate bone regeneration. In order to promote more complete tissue repair, cells should be uniformly incorporated within the scaffolds, rather than seeded on the scaffold surface, which is usually inefficient and incomplete. For bone regeneration, scaffolds that maintain biological activity and deliver growth factor genes to transduced hBMSCs into animal models are critical factors. The most widely used carrier materials are hydrogels,[95,96] as they intrinsically exhibit many of the features of native extracellular matrix (ECM) structures.[97] For instance, methacrylated gelatin, a protein-based hydrogel, which are polymeric 3D networks, is enriched with several advantages for cell entrapment purpose, such as providing appropriate cellular microenvironment, promoting efficient exchange of nutrients with the extracellular milieu, and modulating cell behavior.[98] In addition, some injectable hydrogels can be delivered via minimally invasive surgery to reduce patient discomfort, long recovery process, and healthcare costs.[35,99] Tang and colleagues developed an injectable and crosslinkable gelatin microribbon hydrogel to induce *in vivo* bone regeneration.[100] However, there are some limitations of these different types of hydrogels. The mechanical properties of the photocrosslinked gelatin scaffold are relatively weak at the beginning to approach the optimal conditions needed for bone regeneration. Furthermore, clinical problem of substantial bone defects are mostly heterogeneous and usually accompanied by soft tissue defects and additional injuries.[101] Therefore, the necessity of the application of medical imagining, such as computed tomography (CT) and magnetic resonance imaging (MRI), guided for anatomically specific bone tissue printing is presented. In future studies, more sophisticated biomaterials that fulfill requirements including optimal mechanical properties, osteoinductivity, and support of revascularization should be examined to circumvent current shortcomings. As shown in Fig. 11, gene-activated MSCs suitable biomaterials combined with novel printing technology could be applied for clinical treatment of bone defects and fractures,[102] including cranial bone defect,[103,104] mandible bone defect,[105,106] and cartilage and osteochondral defect.[107,108]

For example, Lin and colleagues used an advanced visible light-based projection stereolithography (VL-PSL) technology to fabricate hydrogel scaffolds for stem-cell-based gene delivery in their study.[40] Microporous scaffolds with a pore size of 150 μm were produced via 3D printing. Furthermore, gelatin hydrogels absorb and retain large quantities of water, favoring MSCs proliferation, adhesion, and infiltration. The strong green fluorescent protein (GFP) fluorescent signal within the construct was observed, which demonstrated the high

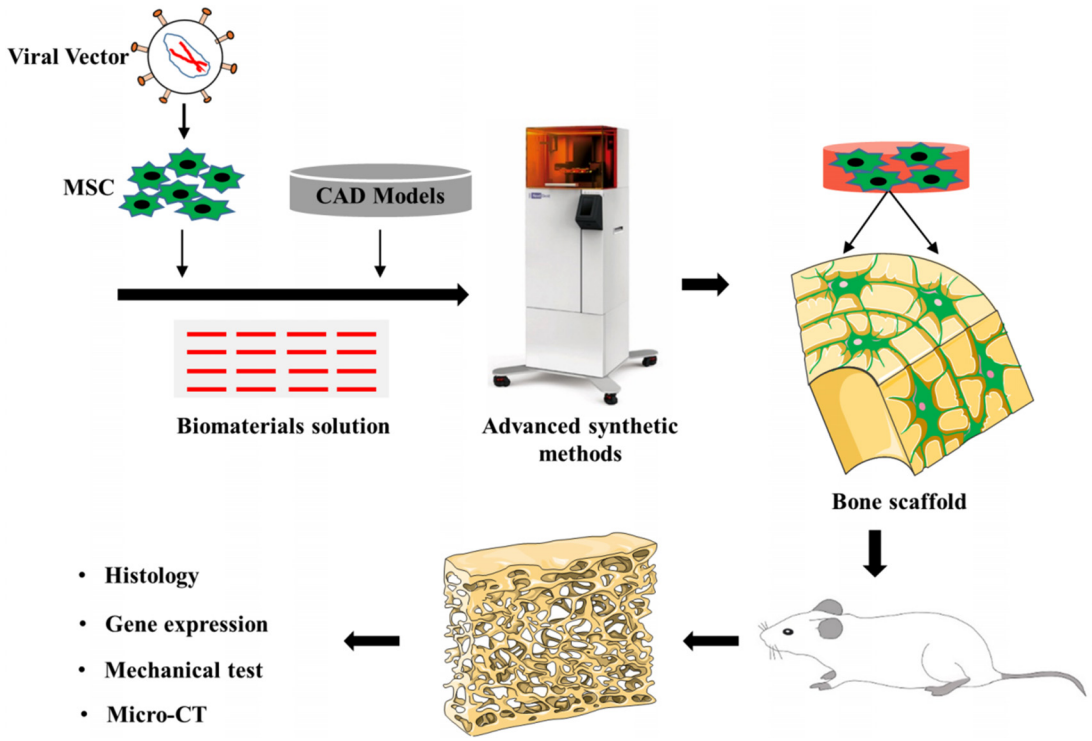

FIG. 10. GAM scaffold accelerates bone regeneration. Technology combines biomaterials, MSC-based tissue engineering, and CAD model as a whole. Medical imaging can be used as the template for 3D printing. A single-step fabrication method for gene-engineered MSC-seeded scaffold could induce enhanced osteogenesis and enhance regeneration in animal models.

transfection efficiency by the lentivirus delivery system. In addition, to maintain cell viability after encapsulation and avoid generating DNA breaks, hydrogels and visible light were thus taken into consideration. Together, these innovative aspects give this technique its high practicability and flexibility. The bone volume of the gene group was higher than that of the protein group, although somewhat reduced at 28 days. The density of the constructs increased gradually as expected. In further examination, cylindrical shape of the constructs was maintained, and irregular ossification was not observed outside, demonstrating that most ossification process took place within the scaffolds. These characterization results demonstrate the strong practicability of the VL-PSL technology combined with 3D printing to fabricate bone scaffolds and simultaneously incorporate BMP-2 transduced hBMSCs within the scaffolds. In the future, medical imaging guided VL-PSL will be effectively used in the bio-medical field, particularly in bone repair and regeneration.

Additionally, calcium phosphate scaffolds (CPS), which were characterized by mechanical properties and biodegradability, lead to significant developments in bone regeneration when combined with MSCs and polymeric materials.[109–112] The novel methods utilizing magnetic field and nano-scaffolds with stem cells are being developed to promote osteogenic differentiation and bone regeneration.[113–115] At the same time, to accurately imitate architectures of native tissue or to completely match the injured defects, 3D models or clinical images were employed as a template for scaffold fabrication.[116–118] More recently, 4-dimension (4D) printing, which combined programmable biomaterials, living cells, and bioactive factors provides a promising technology for clinical treatment of complex structure formation and functional maturation.[119–121] The 4D printing means that the shape or properties of an object can change itself after it has been created. This ability to change its structure over time or in response to an external field is very consistent with the characteristics of biomedical materials.

VII. CONCLUSION AND PERSPECTIVE

Gene therapy delivers therapeutic genes to tissues of interest using viral and non-viral vectors through an *in vivo* or *ex vivo* gene transfer approach, providing a promising treatment for bone regeneration. This strategy has promising applications for clinical treatment of large SBD and non-union fractures. In this review, we highlighted the most recent viral and non-viral vectors used for gene therapy. Many non-viral vectors, including lipid-based system, polymer-based system, and other systems, have been evaluated for bone gene therapy. However, transfection efficiency still remains in the usage of this vector type. In order to overcome present limitations, viral gene therapies, including AdV, AAV, retrovirus, and lentivirus, have been commonly used for bone regeneration. Furthermore, the growth factors and other bioactive peptides in bone regeneration have been particularly discussed in this review. Sustained release of these bioactive factors from optimal scaffold is becoming more efficient and show potential therapeutic effect on clinically relevant animal models. Finally, GAMs and advanced synthetic methods have been discussed. On the pathway to clinical use, the combination of these approaches, including suitable

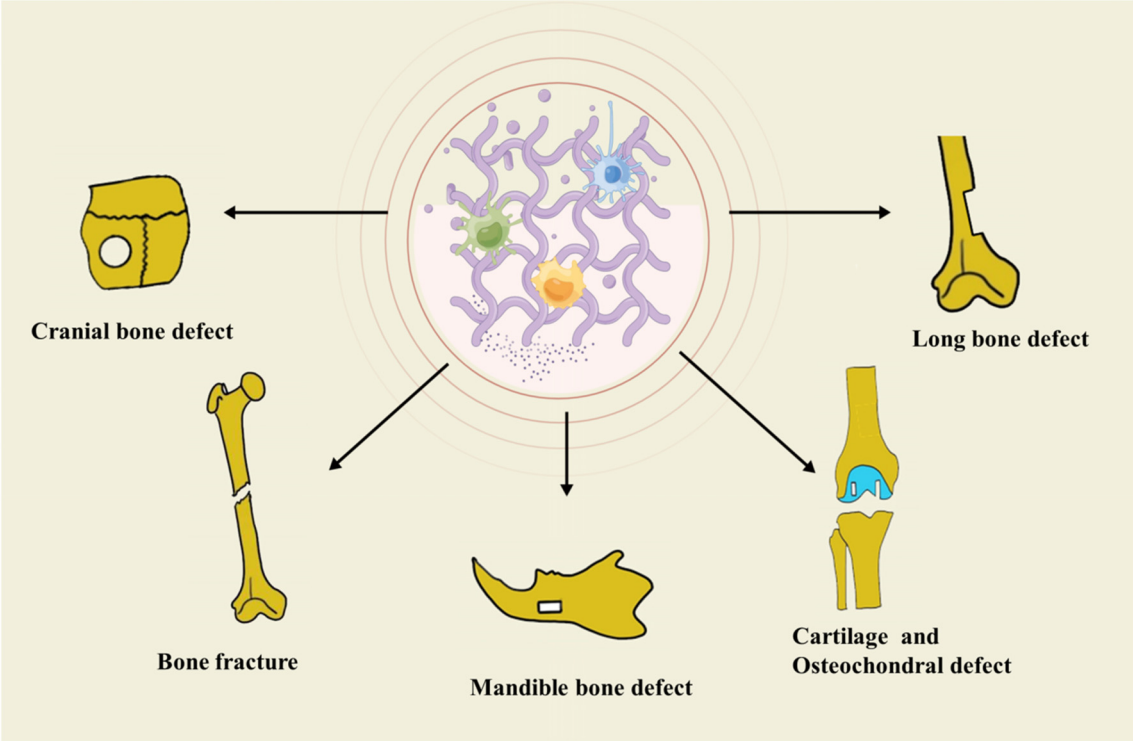

FIG. 11. Applications of GAMs scaffold in bone regeneration. GAMs could be applied for clinical treatment of bone defects and fractures including cranial bone defect, mandible bone defect, cartilage, and osteochondral defect.

cell types, appropriate engineered scaffolds that resemble native bone, and advanced synthetic methods will be essential to achieve optimal bone regeneration.

The number of preclinical and clinical studies related to stem cell therapy and gene therapy has shown exponential growth. However, the promotion of clinical stem cell therapy still confronts many challenges and lacks standards. In order to solve the effectiveness and safety problems of clinical application, several standards are needed to standardize and lead clinical trials, including the source, dose, injection times, and adjuvant optimization of stem cells.[122] First, the number and quality of stem cells from individuals who suffer from different diseases are heterogeneous. The source of autologous stem cells and the selection of clinical patients will help to optimize the therapeutic effect. Second, the types of diseases are complex and diverse, making it challenging to develop a reasonable and feasible individualized treatment plan, including the acquisition and implantation method of stem cells, whether stem cells are expanded, and factors such as implantation dose, times, and time window. Finally, it is important to ensure the bio-safety of stem cells after injection and regulate the proliferation and differentiation of stem cells *in vivo*.[123] Preconditioning of stem cells has showed excellent potential to improve outcomes and facilitate clinical implementation.[124,125] With the advancement of cell reprogramming and gene editing technology, adult stem cells and pluripotent stem cells induced by somatic cells will further boost the application of stem cell therapy in the field of bone repair.

There is much work to be done. The innovative aspects for gene therapy approaches for bone tissue engineering should include the followings: (1) technology that integrates synthetic methods, stem cell-based tissue engineering, and gene therapy as a whole. New bone formation will be robustly augmented through enhancing osteogenesis of MSCs beneficial from gene engineering; (2) using medical imaging including CT and MRI as the template, which enables personalized medicine for the repair of bone defect with effective structure matching and integration; (3) a single-step fabrication method for gene-engineered MSC-seeded scaffold, which results in enhanced osteogenesis and enhance bone regeneration using reagents that are non-toxic, which is FDA-approved and readily available, without complex starting materials; (4) the continuous development of modern medicine should be carried out gradually for precise treatment. For example, the treatment of osteonecrosis of the femoral head is not limited to total hip arthroplasty surgery at the end stage of the disease. It has gradually become a consensus to choose a more targeted treatment based on disease stage classification and individual conditions; and (5) individualized tissue-engineered bone, which offers optimal histocompatibility, osteogenesis induction, and bone healing. It provides a promising technology for clinical applications in bone regeneration.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Ethics Approval

Ethics approval is not required.

Author Contributions

Xiangyu Chu, Yuan Xiong, and Li Lu contributed equally to this work.

Xiangyu Chu: Conceptualization (lead); Funding acquisition (equal); Writing – original draft (lead). **Yuan Xiong:** Conceptualization (equal); Software (lead); Visualization (lead); Writing – original draft (equal). **Li Lu:** Software (equal); Visualization (equal); Writing – original draft (equal). **Yiqing Wang:** Conceptualization (equal); Methodology (equal); Software (equal); Visualization (equal). **Jing Wang:** Writing – review & editing (equal). **Ruiyin Zeng:** Conceptualization (equal); Project administration (equal); Software (equal); Visualization (equal). **Liangcong Hu:** Conceptualization (equal); Formal analysis (equal); Investigation (equal); Software (equal). **Chenchen Yan:** Conceptualization (equal); Investigation (equal); Methodology (equal); Project administration (equal); Software (equal). **Zhiming Zhao:** Conceptualization (equal); Methodology (equal); Software (equal). **Sien Lin:** Supervision (equal); Writing – review & editing (equal). **Bobin Mi:** Conceptualization (equal); Funding acquisition (equal); Supervision (equal); Writing – review & editing (equal). **Guohui Liu:** Conceptualization (equal); Funding acquisition (equal); Supervision (equal); Writing – review & editing (equal).

DATA AVAILABILITY

The data that support the findings of this study are available within the article.

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