Messenger RNA therapy in bone and joint diseases: Rationale, delivery systems, and applications

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ABSTRACT

Bone and joint diseases, including bone fractures, osteoarthritis, and bone tumors, pose significant health challenges due to their debilitating effects on the musculoskeletal system. Conservative therapy and surgical treatment do not always achieve satisfactory outcomes in orthopedics, especially for degenerative bone and joint diseases. Messenger RNA (mRNA) therapy refers to the production of functional proteins and peptides by introducing mRNA into the body. The success of mRNA vaccines during the COVID-19 pandemic highlights the unique advantages of mRNA therapy, including biocompatibility, avoidance of genomic integration, and flexible, sustained delivery. These features make mRNA therapy a versatile therapeutic modality for the treatment of orthopedic diseases. In this review, we first provide an overview of the latest advances in mRNA therapy. We introduce structural modifications of mRNA and advanced gene-editing technologies, including modifications to nucleosides, mRNA domains, and codon sequences. We then discuss the development of mRNA delivery systems, such as nanomaterials, biomimetic carriers, and hydrogels, which enhance mRNA stability, reduce immunogenicity, and improve targeted delivery. This review also explores the application of mRNA therapy in orthopedic diseases, with a particular focus on its utilization in treating bone tumors and degenerative disorders. Despite promising developments, several challenges remain, including optimizing delivery efficiency, prolonging protein expression, and addressing tissue-specific barriers. Accordingly, the current limitations and future directions of mRNA therapy in orthopedic applications are emphasized. In conclusion, mRNA therapy holds great promise and may open new avenues for the treatment of orthopedic diseases and related fields.

Keywords:
Messenger RNA; Bone and joint diseases; mRNA modifications; Delivery system; mRNA therapy

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1. Introduction

Messenger RNA (mRNA) is a single-stranded ribonucleic acid that carries the encoded information for protein synthesis, which is then transcribed and processed into functional proteins. mRNA therapy involves delivering synthetic sequences into cells to produce certain therapeutic peptides or proteins capable of replacing, modulating, or repairing dysfunctional endogenous proteins,[1] thereby achieving therapeutic effects. Recent advances in RNA biology, bioinformatics, separation science, and nanotechnology have enabled breakthroughs in mRNA stability and delivery efficiency.[2,3] Compared to other molecular therapies, mRNA therapy offers several advantages, including rapid onset, good durability, and low immunogenicity. As a result, mRNA therapy has garnered increasing attention from researchers in clinical research and applications. Various mRNA vaccine platforms targeting infectious diseases and cancer have shown encouraging results in human trials.

Bone and joint diseases, including fractures, osteoporosis, osteoarthritis (OA), bone tumors, and bone infections, often lead to chronic pain and mobility impairment. The chronic, degenerative, incurable, and disabling nature of these orthopedic conditions severely diminishes patients' quality of life and imposes substantial socioeconomic burdens. Moreover, population aging is expected to significantly increase the incidence of orthopedic diseases. Although surgery remains the primary treatment of bone and joint diseases, it carries high intraoperative and post-operative risks. To address these challenges, emerging alternative treatments such as stem cell therapy show great potential for bone tissue regeneration and arthritis therapy due to the multidirectional differentiation and anti-inflammatory abilities of stem cells. However, the mechanisms underlying these therapeutic effects are still unclear, and their long-term safety, as well as research and clinical efficacy, have not been fully validated.[4,5] In addition, DNA therapy has also been explored in the context of orthopedic diseases, such as intra-articular injections of DNA encoding transforming growth factor beta (TGF-β)/SRY-box transcription factor 9 for OA and human tumor necrosis factor-immunoglobulin Fc fusion genes[6] or interleukin (IL) 1 receptor antagonist[7] for rheumatoid arthritis. However, low gene transfer efficiency, poor replicability, and dose-dependent complications limit the clinical application of DNA therapies.[8,9] Fatal complications have also been reported in DNA therapy.[10]

Compared with these emerging treatment approaches, the success of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) mRNA vaccines has confirmed the safety and efficacy of mRNA therapy.[11] Unlike DNA-based strategies, mRNA therapy avoids the risk of genomic risks while enabling precise and transient regulation of gene expression.[12] Current pre-clinical studies have demonstrated enhanced specificity in targeting pathological mechanisms in bone and joint diseases, making mRNA therapy a pathogenesis-driven and customizable tool for personalized orthopedic interventions. Therefore, this review provides a comprehensive overview of mRNA-based therapy, including strategies for mRNA structure design and the development of mRNA delivery systems. Applications of mRNA therapy in the treatment of orthopedic diseases are evaluated, and the current challenges and future prospects in this field are discussed.

2. Overview of mRNA therapy

Distinct from other molecular approaches, mRNA exerts its therapeutic effects through cytoplasmic ribosome-mediated translation, thereby circumventing nuclear entry and the associated risks of genomic integration/mutation. This mechanism enhances transfection efficiency and accelerates therapeutic onset compared to DNA-based strategies. Moreover, mRNA enables sustained protein production, surpassing the transient activity of conventional peptide/protein drugs. Although the half-life of mRNA is relatively short, the metabolic byproducts are all naturally occurring substances that do not lead to the accumulation of foreign materials in the body. The low toxicity and immunogenicity of mRNA minimize immune reaction risks during treatment.[13-15]

Since the early 1990s, when scientists at the University of Wisconsin first reported that *in vitro*-synthesized mRNA could achieve protein expression when directly injected into animals, mRNA therapy has undergone significant development. The Nobel Prize in Physiology or Medicine in 2023 was awarded to Katalin Karikó and Drew Weissman for their work on nucleotide structure modifications in mRNA open reading frames (ORFs), recognizing their significant contributions to the development of mRNA vaccines against SARS-CoV-2.

In recent years, mRNA-based therapies have attracted growing attention in both basic research and clinical applications. For example, mRNA can encode tumor antigens to activate the immune system and target cancer cells.[16] In infectious diseases, mRNA vaccines can encode pathogen antigens to induce an immune response.[17] Notably, direct injection of mRNA molecules can be absorbed by antigen-presenting cells (APCs), subsequently inducing immune response, which is a crucial mechanism in the prevention and treatment of infectious diseases. In the treatment of genetic disorders, mRNA therapy can deliver therapeutic mRNA *in situ* to supplement missing or functionally abnormal proteins, efficiently targeting the root cause of disease.[18] For cancer treatments, mRNA vaccines encoding tumor-associated antigens (TAAs) have demonstrated safety and efficacy in clinical trials.[19] TAAs are generally expressed at low levels in normal tissues and overexpressed in tumors; however, they typically show weak tumor specificity, strong central tolerance, and low immunogenicity,[20] necessitating mRNA engineering strategies to overcome immune tolerance. mRNA is first delivered to APCs to produce TAAs or new antigens. The major histocompatibility complex on the surface of APCs presents these antigens to T lymphocytes, activating clusters of differentiation (CD) 8^+ and $CD4^+$ T cells, which then kill tumor cells.[21,22] This process achieves targeted tumor cell elimination and enhances anti-tumor immunity, showing effectiveness in eliminating micrometastases.[23] Beyond conventional vaccination, mRNA vaccines can also be used to transfect and purify patient-derived cells *in vitro*, which are then reinfused back into the patient. For example, mRNA-encoding TAAs can be used to transfect patient-derived dendritic cells (DCs), allowing expression of the antigens on DCs and activation of antigen-specific T cells within the body.[24]

Compared with DNA, mRNA vaccines can more stably and efficiently transfect non-dividing cells, such as APCs represented by DCs, resulting in the effective elimination of melanoma,[25-27] gastrointestinal tumors,[28-30] and nasopharyngeal

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carcinoma.[31] In addition, compared to traditional vaccines, mRNA vaccines have faster design and production timelines, do not require assembly into complete proteins, and offer high adjustability and adaptability.[32] They do not contain active pathogens or cells, thereby eliminating the risk of infection and significantly improving safety while maintaining efficacy. mRNA vaccines exhibit robust clinical translation potential via multiple administration routes, including intravenous, intratumoral, intradermal, and intramuscular, with numerous candidates progressing to Phase II clinical trials.[33]

Beyond antigen delivery, mRNA therapy also modulates the tumor microenvironment through immunoregulatory cytokine expression.[34] This is mainly achieved by expressing interferons and ILs (e.g., IL-2, IL-7, IL-12, IL-15[19]) that enhance tumor cell killing or by expressing inhibitory cytokines[35] (e.g., IL-10, IL-22) to protect against inflammatory damage. Unlike the traditional systemic administration of cytokine products, which is prone to off-target effects and dose-limiting toxicity,[36] mRNA-mediated cytokine therapy offers a feasible optimization strategy. For instance, studies have shown that delivering mRNA encoding tumor suppressor proteins such as phosphatase and tensin homolog (PTEN) and tumor protein p53 can modulate the immune microenvironment, enhance immunotherapy efficacy, and reverse resistance to monoclonal antibodies, resulting in potent anti-tumor effects.[37-41] Another study demonstrated that delivering IL-12 mRNA via lipid nanoparticles (LNPs) significantly promoted a shift in the tumor microenvironment toward a T helper type 1 immune response, conducive to anti-tumor activity.[42] When combined with inhibitory IL-18 decoy-resistant variant 18[43] and the inhibitory anti-inflammatory factor IL-27 from the same family,[44] this approach can synergistically enhance anti-tumor effects while avoiding excessive activation toxicity typically associated with IL-12 alone. IL-23, a member of the IL-12 family with similar functions, can be combined with IL-36γ from the IL-1 family and the T-cell co-stimulatory factor tumor necrosis factor superfamily member 4 (OX40L) to form a triplet mRNA mixture that effectively overcomes tumor microenvironment-associated resistance.[45] Other immune-stimulatory cytokines, such as interferon (IFN)-α and IFN-γ, also play significant roles in tumor therapy.[46,47] Furthermore, mRNA vaccines can be designed based on a patient's specific tumor mutation sites, offering a personalized treatment approach. This personalized method is similar to chimeric antigen receptor (CAR) T-cell therapy but offers greater flexibility and broader potential applicability.

Recent advances highlight chemically modified mRNA (modRNA) as a transient, effective, and dose-controllable expression platform with low immunogenicity.[48] In addition, modRNA constructs are synthetically derived, allowing for rapid, large-scale production, making them suitable for use in disease models and pre-clinical research.[49,50] For example, Kaur *et al.*[13] developed 7G-modRNA, a composite platform integrating cardiac reprogramming genes (GATA binding protein 4 *[Gata4]*, myocyte-specific enhancer factor 2C [*Mef2c*], T-box transcription factor 5 [*Tbx5]*, heart and neural crest derivatives expressed 2 [*Hand2]*) with reprogramming auxiliary genes (dominant-negative [DN]-*Tgfβ*, DN-*Wnt8a*, and acid ceramidase). This platform was delivered to non-cardiomyocytes (CMs), inducing stem cell-like effects and reprogramming scar cells (i.e., non-CMs) into functional CMs, thereby improving heart function without inducing pathological hypertrophy. These examples demonstrate the significant clinical potential of mRNA therapy in various diseases (**Table 1**).

3. Modifications of mRNA

Unmodified mRNA entering the body is easily degraded by various nucleases or activates innate immune cells by stimulating Toll-like receptors (TLRs), particularly TLR3, TLR7, and TLR8. Therefore, in mRNA delivery, inappropriate activation of the innate immune system can lead to insufficient target gene expression, significantly reducing therapeutic effects. To address this, sequence optimization and structural modification of the mRNA structure, through the alteration of one or two nucleotides during *in vitro* transcription, can enable mRNA to evade detection by the host cell's innate immune response. These modifications enhance mRNA stability, reduce immunogenicity, and do not alter the expression of the target protein.[58,59]

Recent advances in high-throughput RNA sequencing technology[60] have elucidated how mRNA modifications and mRNA nucleotide sequences regulate mRNA stability. Several RNA modifications, including N6-methyladenosine (m^6A), N6,2'-O-dimethyladenosine (m^6Am), pseudouridine (Ψ), 5-methylcytidine (m^5C), N1-methyladenosine (m^1A), and N1-methylpseudouridine ($m^1Ψ$), have been shown to regulate various molecular processes of mRNA, thereby affecting numerous cellular and biological processes.[61] Moreover, when naturally occurring modified nucleosides, such as Ψ, m^5C, m^6A, 5-methyluridine (m^5U), or 2-thiouridine (s^2U), are incorporated into transcripts, most TLRs are no longer activated, thereby lowering immunogenicity.

In addition to nucleoside modifications, structural alterations across various components of mRNA also significantly enhance its overall stability. Changes in each specific mRNA segment can markedly influence translation efficiency, functional properties, and immunogenicity. Therefore, localized structural modifications are critically important for the advancement of mRNA therapy. Eukaryotic mRNA typically consists of five key structures: the 5' cap, 5' untranslated region (UTR), coding sequence, also known as the ORF, 3' UTR, and the poly(adenine [A]) tail. Modifying these regions can improve mRNA expression stability and biocompatibility within the cytoplasm, thereby helping to overcome physicochemical barriers (**Figure 1**).

3.1. Modifications of nucleosides

3.1.1. N6-methyladenosine

Among all mRNA modification approaches, m^6A is the most common RNA modification in eukaryotes. The methyltransferase complex composed of methyltransferase-like (METTL) 3, METTL14, Wilms' tumor 1-associating protein, and vir-like m6A methyltransferase associated (VIRMA or KIAA1429) transfers the methyl group to the N-6 position of the adenosine base (**Figure 2**).[62] This modification can be removed

Table 1. Examples of mRNA therapy applications in various diseases

Disease application	Cargos	Delivery vectors	Delivery site	Translation substance	Therapeutic effect	References
Breast cancer, lymphoma, and lung cancer	Fβ[2] mRNA	-	Intratumor	IFN-β, TGF-β	Promotes the infiltration of tumor-specific T cells	51
Cervical cancer	E7-TriMix mRNA	-	Tumor nest	CD40L, CD70, caTLR4	Promoting the migration of $CD8^+$T lymphocytes into tumor nest	52
Advanced melanoma	TriMixDC-MEL mRNA	-	-	CD40L, CD70, caTLR4	Protecting from a non-salvageable melanoma recurrence following the resection of macrometastases	53
Transplant operation	TGF-β3/IL-10 modRNA	ADSCs	Scar tissue	TGF-β3	Reducing skin fibrosis and scar formation	54
Ischemic stroke	Self-replicating HO1-mRNA	DA-PEI	Ischemic brain	HO1	Catalyzing heme degradation and reducing obstruction	55
Myocardial damage	7G-modRNA	Non-cardiomyocyte	Heart after injury	GATA4, MEF2C, TBX5, and HAND2; DN-TGF-B, DN-WNT8A, and AC	Reprogramming cells into cardiomyocytes and promoting cardiac regeneration	13
Variegate porphyria	hPBGD mRNA	LNPs	Systemic administration	PBGD	Increasing hepatic PBGD activity and normalizing ALA and PBG accumulation	56
Athetosis	IRES-Il-10 mRNA	Exosome	Inflamed macrophages	IL-10	Presenting anti-inflammatory and anti-atherosclerotic effects	57

Notes: Fβ[2]: Fusion protein consisting of IFN-β and the ectodomain of the TGF-β receptor II; TriMix: A combination of CD40L, CD70, and a caTLR4.
Abbreviations: AC: acid ceramidase; ADSC: Adipose-derived stem cells; ALA: δ-aminolevulinic acid; caTLR4: Constitutively activated TLR4; CD: Cluster of differentiation; CD40L: CD40 ligand; DA-PEI: Deoxycholic acid-conjugated polyethylenimine; DN: Dominant-negative; E7: HPV16-E7 oncoprotein; Gata4: GATA binding protein 4; Hand2: Heart and neural crest derivatives expressed 2; HO1: Heme oxygenase-1; IFN: Interferon; IL-10: Interleukin; IRES: Internal ribosome entry site; LNPs: Lipid nanoparticles; Mef2c: Myocyte-specific enhancer factor 2C; modRNA: Chemically modified mRNA; mRNA: Messenger RNA; PBG: Porphobilinogen; PBGD: Hepatic porphyrinogen deaminase; Tb×5: T-box transcription factor 5; TGF: Transforming growth factor.

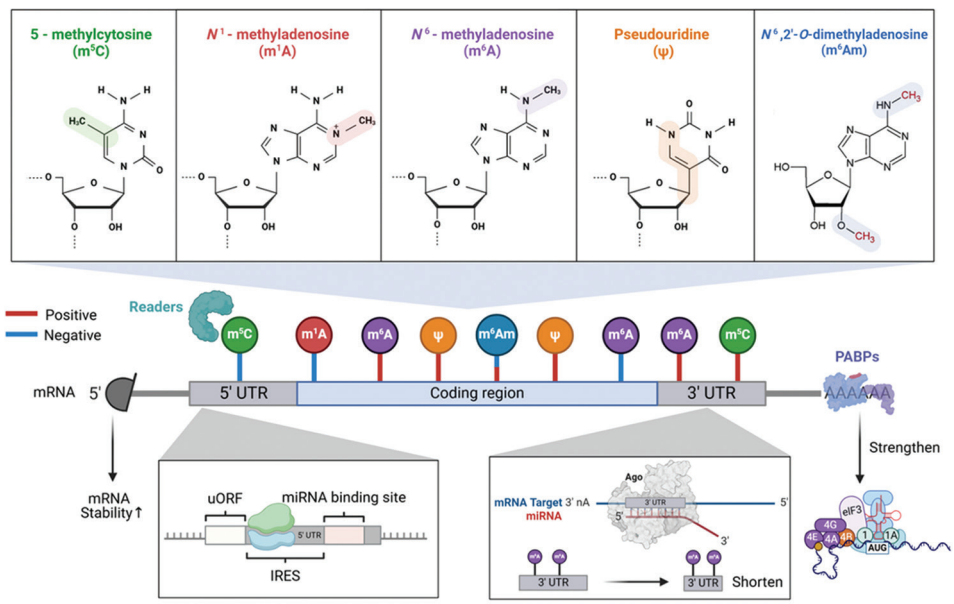

Figure 1. Schematic illustration of various RNA modifications affecting mRNA stability. The top portion of the diagram displays the chemical structures of five RNA modifications discussed in this review, with distinct fluorescent colors highlighting the modified chemical groups. The lower portion illustrates various localized modifications, including those to the 5' cap, 5' UTR, 3' UTR, and the poly(A) tail. Line colors represent their respective impacts on translation. Modifications to the 5' cap influence the structural stability of mRNA. Both the 5' UTR and 3' UTRs contain miRNA binding sites, which interact to regulate mRNA expression. The poly(A) tail binds with PABPs, enhancing translation and increasing mRNA expression levels.
Abbreviation: IRES: Internal ribosome entry site; miRNA: MicroRNA; mRNA: Messenger RNA; PABP: Poly (A)-binding protein; uORF: Upstream open reading frame; UTR: Untranslated region.

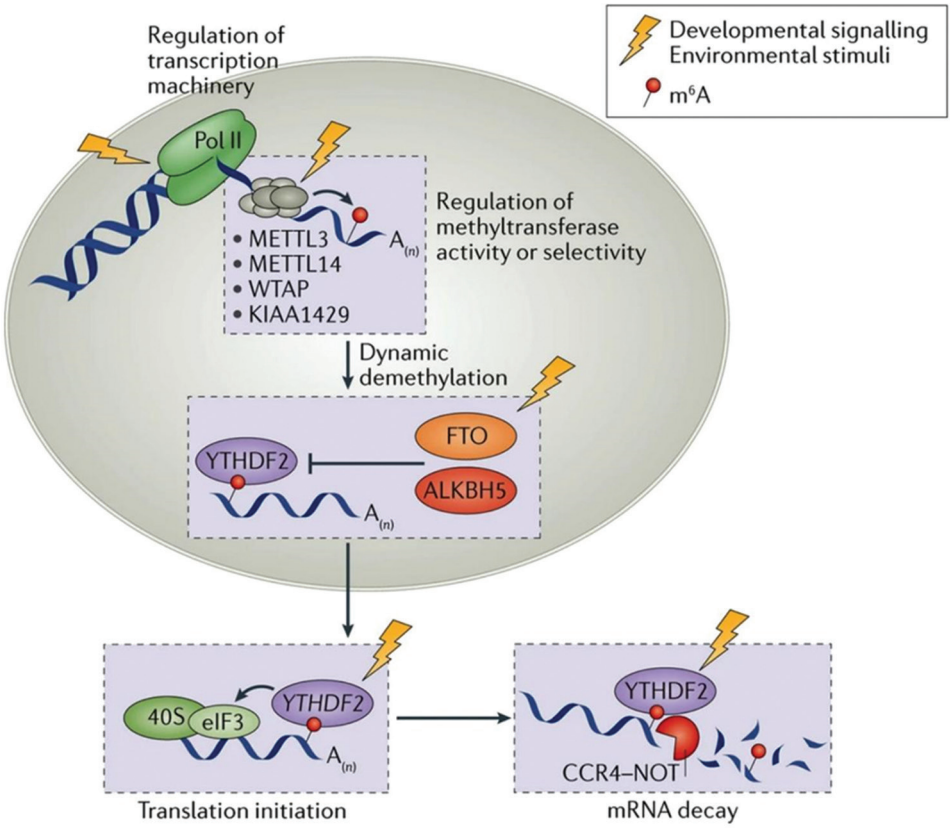

Figure 2. Multiple regulatory mechanisms of m^{6}A modification in gene expression**.** The activities of writers, readers, and erasers may be linked to mRNA expression signaling pathways and to internal or external stimuli. This is a dynamic process that reflects developmental and environmental signals. m^{6}A modification not only influences the initiation of mRNA translation but also accelerates mRNA degradation through recognition by YTHDF2, enabling rapid regulation of gene expression within cells. Reproduced with permission from Zhao *et al.*[70] Copyright © 2016 Springer Nature Limited.

Abbreviations: ALKBH5: α-ketoglutarate-dependent dioxygenase AlkB homolog 5; CCR4-NOT: Carbon catabolite repression 4—negative on TATA-less; eIF3: Eukaryotic initiation factor 3; FTO: Fat mass and obesity-associated protein; METTL: Methyltransferase like; mRNA: Messenger RNA; m^{6}A: N6-methyladenosine; Pol II: DNA polymerase II; WTAP: Wilms' tumor 1-associating protein; YTHDF: YT521-B homology domain family; 40S: Eukaryotic small ribosomal subunit.

by demethylases and is therefore reversible. The function of m^{6}A is diverse across different cell types and biological processes.[63] It regulates various molecular processes, including pre-mRNA maturation, mRNA nuclear processing and export, mRNA transcription, translation, and decay. Molecular events occurring through m^{6}A modification are directed by various reader proteins that recognize m^{6}A, such as YT521-B homology domain family (YTHDF) proteins. m^{6}A modification regulates mRNA splicing, translation, and degradation by binding to different YTHDF proteins.[64,65] Among these, YTHDF2 is the most representative m^{6}A reader protein involved in the degradation of mRNA-containing m^{6}A. When mRNA containing m^{6}A is recognized by YTHDF2, rapid degradation of mRNA is initiated through accelerated deadenylation or decapping pathways.[66,67] Moreover, the dysregulation of m^{6}A modification is directly associated with the development of tumors, indicating that research on mRNA modifications may provide new insights for disease treatment.[68,69]

3.1.2. N6,2'-O-dimethyladenosine

N6,2'-O-dimethyladenosine is structurally similar to m^{6}A. When the first transcribed nucleotide in mRNA near the 5' 7-methylguanosine (m^{7}G) cap structure is adenosine, and the adenosine at the 2'-OH position is methylated to form 2'-O-methyladenosine (Am), further methylation at the N6 position of Am generates m^{6}Am. Recent studies have shown that mRNA initiated with m^{6}Am is more abundant and has a longer half-life.[71] m^{6}Am has been proven to be resistant to the mRNA decapping enzyme DCP2,[72] thus enhancing transcript stability. Moreover, mRNAs initiated with m^{6}Am are also more resistant to microRNA (miRNA)-mediated mRNA degradation.

3.1.3. Pseudouridine

Pseudouridine is generated by the C–C glycosidic isomerization of uridine and exhibits structural and biochemical differences from uridine. The presence of Ψ affects mRNA secondary structure and protein-coding potential. Ψ increases the rigidity of the phosphodiester backbone and strengthens base pairing between Ψ and adenine, indicating that Ψ may directly or indirectly affect mRNA stability.[61] The first position of translation termination codons (UAA, UGA, and UAG) and nonsense codons contains a uridine residue. By artificially converting the uridine in nonsense codons to Ψ,

that is, targeted pseudouridylation, it can become a missense codon. Ψ in termination codons leads to strong nonsense suppression,[73] effectively inhibiting translation termination. In addition, nonsense-mediated mRNA decay recognizes and removes defective mRNA before the production of truncated polypeptides, preventing the accumulation of truncated protein products that could harm cells. Thess *et al.*[74] further demonstrated that completely replacing UTP with Ψ enhances mRNA stability while maintaining protein expression levels.

3.1.4. 5-methylcytidine

5-methylcytidine in the transcript is generated by NOP2/Sun RNA methyltransferase 2, which catalyzes the deposition of a methyl group at the 5' position of cytosine. The presence of m^5C has also been found to promote mRNA transport, indicating that different modifications play diverse functions in the mRNA life cycle.[64]

3.1.5. N1-methyladenosine

N1-methyladenosine is a previously known modification that regulates the structure and stability of transfer RNA (tRNA) and ribosomal RNA. The function of m^1A in mRNA is not yet fully understood. It inhibits translation in the coding sequence region of mitochondrial and nuclear mRNA, while it is associated with increased translation in the 5' UTR.[75] Studies[76,77] have identified the presence of m^1A in eukaryotic cell mRNAs. The positive charge associated with this modification may enhance its biological impact by strengthening RNA–protein interactions or altering RNA secondary structures.

3.1.6. N1-methylpseudouridine

In addition, one study[78] demonstrated that $m^1\Psi$ used for therapeutics does not change translation efficiency. However, it subtly regulates the fidelity of amino acid incorporation in a codon position- and tRNA-dependent manner, both *in vitro* and in human cells. Currently, many mRNA transcripts used in COVID-19 mRNA vaccines have replaced all uridine nucleosides with $m^1\Psi$.[79] Incorporating $m^1\Psi$ limits the cellular innate immune response, thereby avoiding immune reactions and cytotoxicity caused by the introduction of mRNA into cells. This significantly stabilizes mRNA transcripts, enhances translation capacity, and ultimately increases protein synthesis.[80,81] Krienke *et al.*[82] systemically delivered $m^1\Psi$ mRNA encoding disease-related autoantigens to treat several mouse models of multiple sclerosis using nanoparticles , resulting in antigen presentation on splenic $CD11c^+$ APCs without co-stimulatory signals. Hence, these benefits of $m^1\Psi$ modification make mRNA an excellent tool for *in vivo* expression of therapeutic proteins, vaccination, and gene replacement.

3.2. Modifications of mRNA domains

3.2.1. Modifications of 5'UTRs

Codon optimization in the UTR and sequence manipulation have been shown to increase protein expression.[83] Elements on both sides of the 5' and 3' UTR have a profound impact on mRNA structure and function. The 5' UTR is a non-translated sequence upstream of the mRNA coding sequence and serves as the binding site for ribosomes to initiate translation. It is a major determinant of translation efficiency. Therefore, optimizing the 5' UTR sequence to enhance stability and improve translation efficiency is a viable strategy. Ryczek *et al.*[84] summarized that the 5' UTR contains many regulatory elements, including upstream ORFs (uORFs), internal ribosome entry sites, miRNA binding sites, and structural components involved in mRNA stability regulation. Among these, uORFs are key components that control the translation of the main ORF. For example, under stress conditions, the expression of the gene activating transcription factor 4 is not regulated by the rest of the sequence but is instead subjected to regulation by the uORF present in the 5' UTR. This mechanism is called leaky scanning, in which ribosomes bypass the uORF, do not translate the upstream protein, and, upon termination of uORF translation, upregulate main ORF translation through enhanced reinitiation.

Moreover, regulatory mechanisms involving the 5' UTR are not limited to its internal regulatory elements. The mTOR signaling pathway has been shown to affect the translation of mRNAs with structured 5' UTRs, indicating that external signal transduction also influences translation efficiency.[85] Further research revealed that YB-1, a 5' UTR-binding protein, plays a role in translation regulation through the mTOR pathway.[86] This interaction emphasizes the complexity of translation control mediated by the 5' UTR.

The function of UTRs may vary depending on the cell type. Hence, UTR sequences can be adjusted and optimized according to different target cells, followed by customization and screening to achieve the desired level of protein expression. However, cis-regulatory elements, RNA-binding proteins, and other RNAs can create unpredictable combinatorial effects on UTR functionality. As a result, quantitative models based on deep learning have emerged that can predict translation efficiency and mRNA stability based on the 5' UTR sequence.[87-89] Combined with genetic algorithms, these models optimize ribosome loading at specific levels, thereby providing suitable sequences for optimal protein expression.[88]

The aforementioned $m^1\Psi$ modification also affects the translation efficiency of the 5' UTR.[90] Tang *et al.*[91] developed a model for the $m^1\Psi$-modified 5' UTR and created a new machine learning tool called Smart5UTR, which uses a deep generative model to identify optimal nucleotide combinations and high-quality $m^1\Psi$–5' UTR pairs. By designing a COVID-19 mRNA vaccine using Smart5UTR, this vaccine induced a stronger immune response against the SARS-CoV-2 Delta and Omicron variants, significantly outperforming vaccines that used high-expression endogenous gene 5' UTRs.

3.2.2. Modifications of 3'UTRs

Similar to the 5' UTR, some regulatory elements of mRNA are located within the 3' UTR. The 3' UTR directs the transcription product to specific cellular regions, influencing mRNA localization, stability, and translation.[92] Sandberg *et al.*[93] pointed out that rapidly proliferating cells exhibit shorter 3' UTR sequences, resulting in fewer miRNA binding sites.[94] With fewer miRNAs binding to the UTR regions,

the repression of mRNA expression is reduced, leading to increased protein translation efficiency. In neuronal cells, the 3' UTR contains specific miRNA target sites that limit mRNA expression in neurons.[94] Therefore, the function of the 3' UTR may vary depending on the cell type. To personalize the 3' UTR, one main approach is to modify this sequence through alternative polyadenylation. Yue *et al.*[95] demonstrated that the VIRMA protein mediates preferential m^6A methylation in the 3' UTR, which is associated with alternative polyadenylation and influences mRNA stability and localization.[95] This modification can change the length of the 3' UTR, thereby affecting mRNA degradation sensitivity and its interaction with regulatory proteins.

3.2.3. Modifications of 5' cap

The 5' cap in mRNA contributes to transcript stability, pre-mRNA splicing, polyadenylation, mRNA export, and translation initiation. The 3' poly(A) tail primarily promotes nuclear export, translation initiation, and recycling. It also enhances mRNA stability through binding with poly(A)-binding proteins (PABPs). The 5' cap of human mRNA comprises an inverted m^7G connected to the first transcribed nucleotide by a unique 5' – 5' triphosphate bond. Subsequent 2'-O-methylation of the first two nucleotides by cap methyltransferases generates the mature Cap-1 and Cap-2 structures. The cap guides transcript processing and translation selection through interactions with cap-binding proteins and contributes to the overall efficiency of transcript processing, translation, and stability.[96] In addition, it protects mRNA from degradation by 5' – 3' exoribonucleases.[97] Recent studies[98,99] have highlighted the importance of cap modifications in enhancing mRNA stability and translation. For instance, a properly methylated cap structure interacts with cap-binding proteins such as eukaryotic translation initiation factor (eIF) 4E, which are crucial for the assembly of the translation initiation complex.

mRNAs with a 5' cap are selectively recognized by Quaking protein (QKI).[100] QKI7 interacts with the stress granule core protein (Ras-GTPase-activating protein binding protein 1) through its C-terminus, transporting internally m^7G-modified transcripts to stress granules to regulate mRNA stability and translation under stress conditions. Moreover, QKIs may also play a role in mRNA metabolism and cellular drug resistance. Approaches to manipulate mRNA capping have emerged, including the use of photocaged cap analogs that allow for spatial and temporal control over mRNA translation. These analogs can be activated by light to release functional cap structures, providing a powerful tool for investigating the dynamics of mRNA translation in live cells.[101,102] Moreover, uncapped mRNAs can be re-capped in the cytoplasm, contributing to mRNA stability and translational regulation under certain conditions.[103] Furthermore, studies[104] have identified that 5' methylation plays a critical role in distinguishing self from non-self (e.g., viral) mRNA, allowing innate immune proteins to differentiate endogenous transcripts from unmethylated foreign RNA. Therefore, proper capping can enhance the acceptance of mRNA by the host immune system and reduce immunogenicity.

3.2.4. Modifications of the poly(A) tail

The poly(A) tail plays a critical regulatory role in both mRNA translation and stability. It consists of a long string of adenosine nucleotides at the 3' end of eukaryotic mRNA, helping to maintain the translational state and stability of mRNA. Under normal circumstances, mRNA lifespan is tracked and determined by the dynamics of tail length.[105] The rate of deadenylation determines the stability of most mRNAs: the longer the poly(A) tail, the slower the deadenylation process, and thus the more stable the mRNA.[106,107] The poly(A) sequence is usually flanked by spacer elements, which separate the sequence without affecting the translation efficiency or half-life of *in vitro* transcribed mRNA.[108] Poly(A) tail length undergoes dynamic regulation during various biological processes. Liu *et al.*[109] emphasized a global trend of reduced poly(A) tail length during the transition from oocyte to embryo, which is crucial for regulating maternal gene expression without transcription. Lee *et al.*[110] further supported this dynamic regulation concept by identifying a wave of extended global mRNA deadenylation occurring during the oocyte-to-embryo transition, laying the foundation for translational regulation.

In cap-dependent translation, the rate-limiting step is the assembly of the translation initiation complex centered around the m^7G cap by eukaryotic translation initiation factors (eIF4E and eIF4G). Studies have demonstrated that the translation initiation complex is further stabilized through the interaction of eIF4G with PABPs,[111,112] and this molecular synergy enhances translational efficiency. PABPs play a dual role where they initiate translation by binding with eIFs and extend mRNA stability by sequestering the poly(A) tail to prevent deadenylation. Furthermore, PABPs can act as allosteric regulators, with their binding to poly(A) RNA influenced by other proteins, such as poly(A) binding protein interacting protein 2 (PAIP2). PAIP2 competes with eIF4G for binding to PABPs, thereby inhibiting translation.[113] In addition, branched chemical modifications of the poly(A) tail can enhance mRNA translatability. Aditham *et al.*[114] introduced specific sites of exonuclease-resistant modifications into the poly(A) tail, effectively increasing mRNA stability and protein production. They also polymerized[115] the poly(A) tail using a branched topology, giving each poly(A) tail extensive exonuclease resistance. This architectural innovation better preserves mRNA translational capacity, thus enabling sustained protein production *in vivo*.

3.3. Modifications of codon sequences

Codon sequences can be adjusted to optimize reading efficiency in the ORF region.[116] Compared to naturally occurring mRNA sequences, researchers often reduce the use of rare tRNAs in the ORF to improve translation efficiency. However, some rare codons are crucial for maintaining the proper three-dimensional structure of proteins, and arbitrary substitution may disrupt protein folding and decrease translation yield.[117] This warrants further mechanistic investigation.

Another feasible approach is to increase the guanine and cytosine (GC) content. Uridine can be recognized by TLR7/8, triggering innate immune activation, which leads to mRNA degradation

and poses potential risks. Increasing GC content reduces the total number of uridine bases in the mRNA transcript, thereby enhancing transcript stability[118] and protecting mRNA from ribonuclease-mediated degradation, ultimately improving efficiency.[119] Courel *et al.*[120] demonstrated that GC-rich transcripts exhibit higher translation efficiency at the 5' end compared to adenine–uridine-rich transcripts. Therefore, sequence optimization, including nucleotide modifications, substitutions, and increased GC content within mRNA, can enhance translation efficiency, attenuate immunogenicity, and improve the stability of mRNA expression.

3.4. Gene editing technologies

Gene editing technologies, including Cre recombinase, transposases, zinc-finger nucleases, transcription activator-like effector nucleases, and the widely applied clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) system,[121] play a critical role in biomedical research, gene-drug development, and gene therapy. These systems can enter target cells through mRNA-encoded components to achieve therapeutic gene editing. The CRISPR-Cas9 system, a naturally occurring defense mechanism in prokaryotes, has become the most efficient gene editing technology currently in use due to its usability, simplicity, and outstanding efficiency. mRNA-based CRISPR methods are considered safer because they reduce the risk of genomic integration, have a limited duration of action, and thus limit the possibility of off-target DNA editing.[122] CRISPR-Cas9 delivery via mRNA platforms requires the co-delivery of Cas9 mRNA and single-guide RNA (sgRNA). After entering the cell, the Cas9 mRNA is translated by ribosomes into the functional endonuclease, which subsequently assembles with the sgRNA to form an active ribonucleoprotein complex. Abbasi *et al.*[123] demonstrated that PEGylated polyplex micelles can co-deliver Cas9 mRNA and sgRNA in a single material. These polyplex micelles induced efficient genome editing in neurons, astrocytes, and microglia following direct injection into the cerebral cortices of mice.

Unlike other mRNA therapies, mRNA-based gene editing can produce the target enzyme directly to enable genomic editing, thereby overcoming mRNA instability and ensuring robust expression. However, DNA integration poses risks, including chromosomal deletions, translocations, and insertions, which can disrupt normal gene expression and potentially lead to tumor formation.[124]

4. Delivery system of mRNA therapy

Modifications of mRNA nucleosides and internal structural elements effectively attenuate immunogenicity while improving overall stability, thereby increasing intracellular protein expression. However, successful mRNA expression requires that the modified constructs be delivered to target cells in a stable and intact form. Therefore, the design and preparation of delivery systems are crucial for ensuring the effective and targeted transport of mRNA, particularly in overcoming cellular and environmental barriers. Physicochemical barriers refer to the series of obstacles encountered from outside the body to the interior of cells. These include the protective barriers of skin and mucous membranes; impediments formed by various cells, enzymes, and molecules in the internal environment; the cell membrane that must be crossed to reach target cells; and the endosomal membrane that must be breached once inside the cell. Although naked mRNA can induce immune effects in the body, it is prone to degradation by nucleases during transport and is only suitable for *in situ* injection.[47] Carrier design and structural improvement are the two main areas of research in developing effective mRNA delivery systems. The first approach involves designing carriers that facilitate mRNA transport to the target site, protect it from degradation by tissue nucleases, and assist its entry into target cells. The second approach focuses on improving the mRNA structure itself to reduce degradation by cytoplasmic nucleases, thereby enhancing its persistence, expression stability, and, ultimately, protein production, which leads to improved therapeutic outcomes.

In recent years, viral and non-viral carrier delivery systems have developed rapidly.[18] However, viral vectors have not been widely applied in clinical therapy due to unresolved issues such as potential genomic integration, immunogenicity (especially with repeated doses), high production costs, risks of secondary carcinogenesis, and limited packaging capacity.[125] In contrast, non-viral vectors, including LNPs, biomimetic carriers, and engineered polymeric materials, have received widespread attention due to their superior biocompatibility, high encapsulation efficiency, and ability to facilitate endocytosis via the cell membrane (**Figure 3** and **Table 2**).

4.1. Nanoparticles

4.1.1. LNPs

LNPs are considered the most important carriers for non-viral RNA delivery.[126,127] Structurally, LNPs integrate cholesterol, ionizable lipids, polyethylene glycol (PEG)-modified phospholipids, and auxiliary lipids, with the ability to carry mRNA internally. Cholesterol intersperses among phospholipid molecules to reduce membrane permeability and minimize mRNA leakage. Moreover, cholesterol regulates phospholipid membrane fluidity and phospholipid phase transitions, preventing LNP oxidation and enhancing resistance to external environmental changes. Cationic LNPs are stable complexes formed between synthetic cationic lipids and anionic nucleic acids.[33] In relatively acidic environments, ionizable cationic phospholipids carry a positive charge, forming electrostatic complexation with negatively charged mRNA molecules,[128] thereby facilitating mRNA encapsulation and release. In addition, PEGylated lipids are commonly modified to improve the biocompatibility and circulation time of LNPs.[129,130] Beyond protecting the payload, LNPs actively facilitate cellular uptake and endosomal escape for cytoplasmic delivery.[131]

LNPs can deliver mRNA through various routes, such as intravenous injection or inhalation. In most cases, LNPs encapsulate mRNA encoding missing enzyme sequences. Following cellular internalization, mRNA undergoes ribosomal translation to produce therapeutic proteins,[132] restoring metabolic function and alleviating pathological substrate

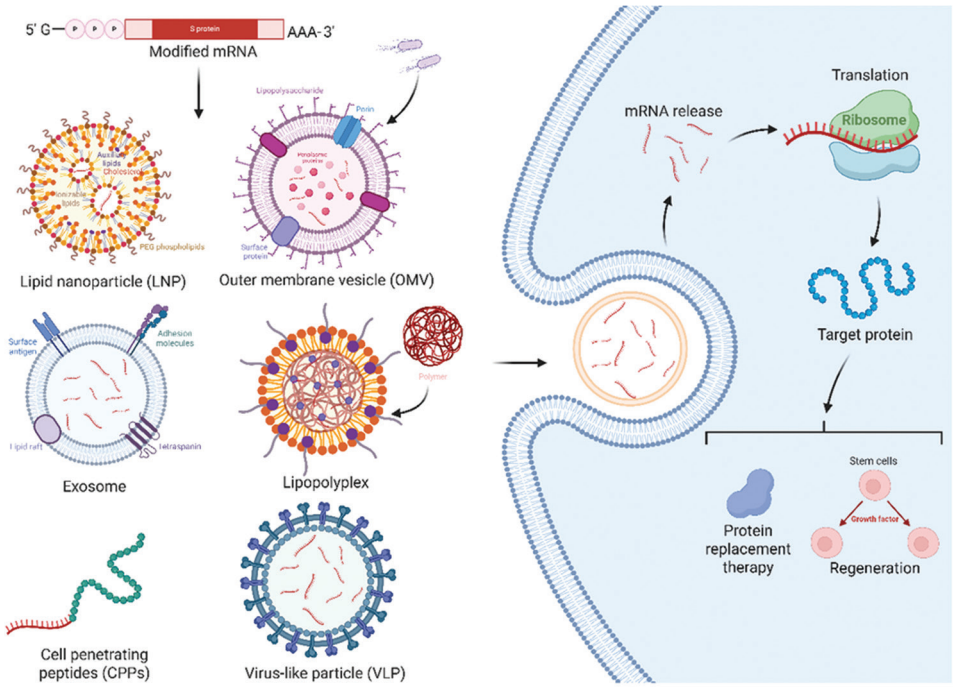

Figure 3. Overview of messenger RNA (mRNA) therapy delivery vector systems. *In vitro*-modified mRNA is loaded into lipid nanoparticles, biomimetic carriers (such as bacterial outer membrane vesicles and exosomes), polymeric materials, peptides, or virus-like particles. These vectors enter target cells through endocytosis, where the therapeutic mRNA is released and translated into the target protein to exert its therapeutic effect.

accumulation in the blood, thereby slowing down disease progression. Examples include treating phenylketonuria with phenylalanine hydroxylase mRNA,[133] glycogen storage disease with glucose-6-phosphatase mRNA,[134,135] Fabry disease with α-galactosidase A mRNA, and α-1-antitrypsin deficiency with α-1-antitrypsin mRNA.[136,137] Moreover, the development of biodegradable LNPs has addressed concerns regarding the long-term stability and toxicity of these delivery systems, making them more suitable for clinical applications.[138]

However, as LNPs tend to preferentially accumulate in the liver, extrahepatic delivery of mRNA remains challenging. To date, research has been devoted to developing new strategies for organ-specific delivery based on LNPs to fully realize the potential of gene therapy.[139] To enhance extrahepatic targeting specificity, antibody-conjugated LNP systems have been engineered to mediate mRNA delivery, providing efficient vascular immune targeting for target organs other than the mouse liver. At the same time, these systems also use modified nucleosides to reduce innate immune activation, increasing mRNA translation levels and further enhancing therapeutic effects.[140] Very recently, Xue *et al.*[141] developed a siloxane-incorporated LNP that enhances not only the cellular endocytosis of mRNA-LNPs but also their endosomal escape ability, thereby improving the efficiency of mRNA delivery. In addition, modifying the structure of siloxane can create various organ-selective mRNAs, and even minor structural changes in siloxane-incorporated lipidoids can significantly alter organ tropism. This system successfully delivered fibroblast growth factor-2 (FGF-2) mRNA and CRISPR-Cas9-based gene editor to modify the disease state. Moreover, Xue *et al.*[142] developed a series of bisphosphonate (BP)-conjugated ionizable lipid-like nanomaterials (BP-LNPs). BP exhibits strong chelation with calcium ions in hydroxyapatite, enabling rapid adsorption and high affinity to the bone surface. Following intravenous administration of BP-LNPs encapsulating bone morphogenetic protein (*Bmp*) 2 mRNA, *Bmp2* expression in the bone microenvironment was significantly elevated, and the effective secretion of BMP-2 protein demonstrates the great potential of mRNA-LNP therapeutics for bone defect healing. These findings highlight the bone-targeting capability of BP-LNPs.

4.1.2. Polymeric nanoparticles

Polymers encompass natural polymers such as chitosan, alginate, and hyaluronic acid, as well as synthetic polymers such as polyamidoamine, poly(lactic-co-glycolic acid) (PLGA), and poly(ethyleneimine) (PEI). Compared to LNPs, polymeric systems exhibit lower transfection efficiency and potential cytotoxicity; however, they offer long-term storage capabilities, even in the form of lyophilized powder.[143] The tunable chemical functionality of polymers also makes it possible to achieve higher *in vivo* stability and cellular uptake rates through chemical modifications and to optimize their distribution and targeting in the body by modulating structure and surface charge.[144] Ligand conjugation to polymeric nanoparticles mediates receptor-specific endocytosis, enhancing the accumulation, uptake, and release of target mRNA in recipient cells. Some ligands can even improve intracellular delivery following cellular uptake by facilitating endosomal escape, promoting successful translation of mRNA. In addition, tumor cells and tumor microenvironments often overexpress specific antigens,[145] which can serve as target receptors for ligand-based nanocarrier design.

Table 2. Examples of different delivery systems for messenger RNA (mRNA) therapy

Vectors	Cargos	Curative effect	References
LNP	PAH mRNA	Treating phenylketonuria	133
LNP	G6Pase-α mRNA	Treating glycogen storage disease	134,135
LNP	A1AT mRNA	Treating α-1-antitrypsin deficiency	136,137
Lung-targeted SiLNP	FGF-2 mRNA	Increasing vascular repair in a viral infection lung damage model	141
SiLNP	Cas9 mRNA and sgRNA	Enabling robust gene editing	141
DA-PEI2k	HO1-mRNA	Reducing the infarct size	149
PVES	eGFP mRNA	Serving as a vaccine delivery platform and inducing potent antibody response	148
Polyplexes	eGFP mRNA	Delivering mRNA targeting pulmonary microvascular endothelial cells	150
PLGA NPs	CFTR modRNA	Restoring critical lung function parameters	151
hPBAE polyplexes	Luciferase IVT-mRNA	Enabling nebulized lung delivery and producing sufficient protein	152
PLGA/PEI NPs	GFP IVT-mRNA	Targeting moDCs and enabling efficient delivery	156
OMVs	OMV-L7Ae mRNA	Delivering mRNA into cells via endocytosis of OMVs	163
Engineered exosome	Catalase mRNA	Reducing neurotoxicity and neuroinflammation	183
VLP	SpCas9 mRNA and sgRNA transcripts	Realizing efficient, dose-controlled, and non-toxic delivery of CRISPR/Cas9 RNAs into target cells	164
VLP	SpCas9 mRNA and HELP	Blocking HSV-1 replication and inhibiting the occurrence of herpetic stromal keratitis	165
PLA-PEG-PLA hydrogel	AMELX modRNA	Realizing almost complete regeneration of rat periodontal defects *in vivo*	168
Hybrid hydrogels	hGLuc mRNA	Prolonging mRNA delivery for 21 days *in vitro* through chitosan-alginate hydrogels	169
DNA nano-hydrogel	mRNA	Controlling mRNA intelligent release	170
LNPs-hydrogel	Tumor antigen-encoding mRNA	Realizing effective cancer immunotherapy	171
MXene hydrogel	PDGF and FGF-7 and VEGF triplet mRNA	Promoting tissue regeneration and collagen deposition	172
CPP-RALA	Antigen-encoding mRNA	Eliciting cytotoxic immunity	175
ADSCs	IGF-1 modRNA	Repairing corneal damage and maintaining stemness	177
hADSCs	VEGF modRNA	Enhancing cell proliferation and angiogenesis in fat grafts	178
DNA origami	mRNA	Improving gene expression of luciferase mRNA in the lung	189
RNA origami	*Smad4* mRNA	Inhibiting tumor growth in colorectal cancer models	190

Abbreviations: ADSCs: Adipose-derived mesenchymal stem cells; AMELX: Amelogenin; A1AT: Alpha-1-antitrypsin; Cas9: CRISPR associated protein 9; CFTR: Cystic Fibrosis transmembrane conductance regulator; CPP-RALA: Cell-penetrating peptides–arginine-alanine-leucine-alanine; CRISPR: Clustered regularly interspaced short palindromic repeats; DA-PEI2k: Deoxycholic acid-conjugated PEI; eGFP: Enhanced green fluorescent protein; FGF: Fibroblast growth factor; GFP: Green fluorescent protein; G6Pase-α: Glucose-6-phosphatase alpha; HELP: HSV-1-erasing lentiviral particle; hGLuc: Humanized Gaussia luciferase; HO1: Heme oxygenase-1; HSV-1: Herpes simplex 1; IGF-1: Insulin-like growth factor-1; IVT: In vitro-transcribed; LNP: Lipid nanoparticle; moDCs: Modified dendritic cells; modRNA: Modified mRNA; (MXene: Metal carbide/nitride; NPs: Nanoparticles; OMV: Outer membrane vesicle; PAH: phenylalanine hydroxylase; PBAE: Poly(β-amino ester); PDGF: platelet-derived growth factor; PEG: Polyethylene glycol; PEI: Poly (ethyleneimine); PLA: Poly (lactic acid); PLGA: Poly (lactic-co-glycolic acid); PVES: Hydrophobic Vitamin E succinate-modified water-soluble PEI copolymer; sgRNA: Single-guide RNA; SiLNP: Siloxane-incorporated LNP; *Smad4*: SMAD family member 4; SpCas9: *Streptococcus pyogenes* Cas9; VEGF: vascular endothelial growth factor; VLP: Virus-like particle.

Among polymeric nanoparticles, PEI is one of the most widely used cationic polymers for nucleic acid delivery, capable of forming complexes with mRNA to protect it and facilitate its entry into cells.[146] The strong cationic property of PEI promotes interactions between positively charged nanoparticles and negatively charged cell membranes, enhancing the release of encapsulated biomolecules and endosomal escape.[147] Moreover, its toxicity can be reduced by adjusting its molecular weight and structure. For instance, the development of low-molecular-weight PEI significantly reduces its toxicity. Ren *et al.*[148] designed a novel self-assembled polymeric micelle based on a hydrophobic Vitamin E succinate-modified water-soluble PEI copolymer (PVES). PVES micelles and mRNA can form more stable nanoparticles through electrostatic interactions, effectively encapsulating and protecting mRNA from degradation, with no significant cytotoxicity observed in experiments. A subsequent study demonstrated[149] that deoxycholic acid-conjugated PEI successfully delivered heme oxygenase-1 mRNA to the brain for the treatment of ischemic stroke, effectively reducing infarct size, with higher mRNA transfection rates than other carrier systems and relatively lower cytotoxicity compared to lipofectamine delivery systems. Furthermore, Dunn *et al.*[150] functionalized PEI with biological fatty acids and carboxylate-terminated PEG to construct polyplexes, exhibiting high specificity for pulmonary microvascular endothelial cells and successfully delivering enhanced green fluorescent protein mRNA while maintaining high targeting.

PLGA is a United States Food and Drug Administration (FDA)-approved polyester widely used for drug delivery. Due to the hydrolytic characteristic of its ester bonds, PLGA produces non-cytotoxic degradation products, has a small volume, and exhibits strong stability. Haque *et al.*[151] delivered cystic fibrosis

transmembrane conductance regulator (CFTR) modRNA using biodegradable chitosan-coated PLGA nanoparticles in nanoparticles, successfully restoring pulmonary function in CFTR-deficient mice, although the accumulation of human CFTR modRNA in lung cells was lower than that achieved by intravenous injection under equivalent conditions. In addition, poly(β-amino ester) (PBAE) is a biocompatible and biodegradable polymer with relatively simple synthesis.[146] One study[152] designed hyperbranched PBAE to deliver luciferase mRNA to the lung epithelium via nebulization, producing sufficient protein in the lungs and providing comprehensive coverage of both the upper and lower airways compared to tracheal administration.

4.1.3. Lipid-polymer hybrid nanoparticles (LP-NPs)

LP-NPs typically comprise a polymeric core for payload encapsulation, a lipid shell, and an outer lipid-PEG layer.[153] LP-NPs benefit from the structural versatility of lipid membranes and the broad tunability of polymers. The lipid component's hydrophobicity allows it to interact with and encapsulate mRNA within its core, protecting the mRNA payload from degradation during storage and systemic transportation. Integrating polymers into the core lipid bilayer enables controlled surface modifications, enhancing flexibility in terms of chemical functionality without affecting the core characteristics of the lipid/mRNA system. Overall, LP-NPs possess long-term stability and high transfection efficiency,[154] featuring defined release kinetics, high cellular uptake rates, and adjustable organ-targeting properties.[155] For instance, a study[156] used PLGA/PEI nanoparticles to deliver *in vitro* transcribed mRNA encoding a green fluorescent protein and targeted it to human monocyte-derived DCs, observing the effective expression of the green fluorescent protein. In addition, such as polymeric nanoparticles, LP-NPs can also reduce toxicity by modifying polymer components, and adjusting the polymer-to-lipid ratio of the nanoformulation can achieve controlled release.

4.2. Biomimetic carriers

4.2.1. Outer membrane vesicles (OMVs)

Biomimetic carriers, or biomembrane-camouflaged nanoparticles, refer to the technique of encapsulating mRNA within cellular membranes or similar membrane structures and delivering it into the cytoplasm through membrane fusion. This method mimics the infection mechanism of viruses, using membrane-coated nanoparticles to achieve intracellular delivery of mRNA.[157,158] Membrane-encapsulated nanoparticles preserve mRNA integrity while enabling cell-specific delivery.[159] However, the system cannot fully evade the body's immune system during the delivery process, which can hinder its efficiency.[160] Various types of biomimetic carriers, including cellular membrane vesicles, bacterial OMVs, and extracellular vesicles (such as exosomes), have now been developed and are being explored as novel platforms for mRNA delivery.

OMVs are nanoscale spherical structures naturally released from the outer membrane of Gram-negative bacteria and play a crucial role in various biological processes, including cell–cell communication, pathogenesis, and immune modulation. OMVs are composed of a lipid bilayer containing proteins, lipopolysaccharides, and other biomolecules that can affect host interactions.[161,162] Li *et al.*[163] used *Escherichia coli*-derived OMVs as an mRNA carrier. They integrated L7Ae (an RNA binding protein) and listeriolysin O (a lysosomal escape protein) onto the OMV surface (OMV-LL) through electroporation. OMV-LL can rapidly adsorb mRNA antigens (OMV-LL-mRNA), successfully delivering them to DCs in mice and significantly inhibiting the progression of melanoma. This method is rapidly prepared, low-cost, and has good immunogenicity, showing promising therapeutic efficacy.

4.2.2. Virus-like particles (VLPs)

VLPs encompass the major structural proteins required for virus capsid assembly but do not package viral genomic components. Utilizing the principle of specific recognition of phage capsid proteins by mRNA stem-loop structures, this new VLP-mRNA delivery method is created through viral engineering techniques. On the one hand, VLP-mRNA leverages the viral shell, granting it high transfection efficiency. On the other hand, based on the transient nature of mRNA itself, gene editing therapies become safer and more controllable. For instance, one research[164] indicated that VLP-mRNA has significant advantages in delivering Cas9 mRNA. Compared to traditional viral vector systems, the VLP-mRNA system can rapidly release Cas9 mRNA within cells and express the Cas9 protein in a short timeframe, with the presence of the protein lasting only 72 h. The brief expression duration helps reduce the risk of off-target effects, allowing cells to quickly return to a normal state after editing, thus minimizing potential impacts on non-target genes. Based on this, Yin *et al.*[165] engineered two guide RNAs targeting HSV-1 to enable Cas9 to directly cleave the HSV-1 genome, causing its degradation. To enhance the safety of gene editing, they employed VLP-mRNA to deliver *Streptococcus pyogenes* Cas9 mRNA and viral gene-targeting sgRNAs, successfully curing herpetic stromal keratitis in mice.

4.3. Hydrogels

Hydrogels are materials based on hydrophilic polymers, characterized by high water content and physical properties similar to those of natural extracellular matrices.[166] Currently, injectable and biodegradable hydrogels are widely used for *in situ* drug delivery and sustained release due to their adjustable physical properties, controllable degradability, and minimally invasive administration. Considering the polyanionic character of mRNA, gels composed of PEI, chitosan, polyamidoamine, PBAEs, and poly L-lysine have been shown to confer advantages for mRNA delivery.[167] Pan *et al.*[168] combined hydrogels with liposomal delivery systems and found that poly(lactic acid) (PLA)-PEG-PLA hydrogels inhibited the degradation of modRNA and enabled sustained release. In a rat model, they observed nearly complete regeneration of periodontal defects. Steinle *et al.*[169] complexed humanized Gaussia luciferase mRNA with alginate, chitosan, or chitosan-alginate hybrid hydrogels, demonstrating that hydrogels loaded with mRNA, administered via minimally invasive local injection could sustain delivery of the cargo into cells over several weeks. Fu *et al.*[170] developed DNA nanohydrogels

that self-assemble into nanoballs, promoting cellular uptake and thereby improving the delivery efficiency of mRNA. They also incorporated pH-responsive i-motif structures into the nanohydrogel, achieving a controlled release of mRNA. Furthermore, a dynamically cross-linked hyaluronic acid hydrogel[171] can be encapsulated around the LNP delivery system to restrict LNP migration and fusion, enhancing the stability of the LNP-hydrogel system. mRNA-LNPs can be released upon a phase transition of hyaluronic acid. Very recently, Wang *et al.*[172] delivered a novel triplet mRNA formulation for diabetic wound healing using transition metal carbide/nitride (MXene) hydrogel microneedles, leveraging the photothermal conversion property of MXene to achieve controlled release.

4.4. Peptide carriers

Polypeptides are natural polymers formed by multiple amino acids linked by phosphodiester bonds. Specifically, cell-penetrating peptide (CPP)-based carriers represent emerging mRNA vectors capable of traversing the plasma membrane to deliver therapeutic cargo into cells. The chemically modified residues within their structure can enhance endosomal escape and reduce degradation by endosomal proteases, making CPP-based delivery systems more resistant to degradation compared to other drug delivery systems.[173] Characterized by positive charges or amphiphilicity, CPPs can form nanostructures with negatively charged nucleic acids, facilitating the transmembrane transport of mRNA into cells. These features of CPP-based systems also enhance their cellular uptake, intracellular distribution, and expression of mRNA.[174] Udhayakumar *et al.*[175] developed an mRNA vaccine using an amphiphilic CPP-arginine-alanine-leucine-alanine (CPP-RALA), composed of positively charged arginine and hydrophobic alanine and leucine residues. After intradermal injection of model antigens into mice, CPP-RALA successfully elicited potent cellular immunity and efficiently cured the disease. However, polypeptides exhibit relatively low biological stability, and thus, the long-term delivery efficacy in the body remains a key challenge for peptide carriers.

4.5. Cellular delivery systems

Delivering mRNA using living cellular carriers holds significant importance in gene therapy and vaccine development. This delivery technique not only protects mRNA from degradation but also allows for targeted delivery to specific tissues, ensuring the safety of mRNA therapies without involving genome integration, thus avoiding the risks associated with gene therapy. Multipotent mesenchymal stem cells (MSCs), which possess self-renewal and differentiation properties, include adipose-derived mesenchymal stem cells (ADSCs) and bone marrow-derived mesenchymal stem cells (BMSCs).[176] For example, one study[177] utilized insulin-like growth factor-1 (IGF-1) modRNA to engineer and transfect ADSCs to treat corneal burns in mice, which effectively promoted the restoration of corneal morphology and function while maintaining stemness, offering broader effects than IGF-1 protein eye drops.[177] Similarly, Yu *et al.*[178] obtained human ADSCs from liposuction and transfected them with modified vascular endothelial growth factor (modVEGF). They found that modVEGF-engineered ADSCs performed better than unmodified ADSCs in terms of cell survival rate and therapeutic protein production, ensuring the long-term survival and angiogenesis of the graft *in vivo*. BMSCs are often combined with BMP-2 to treat bone injury-related diseases, inducing bone healing and regeneration.[179]

4.6. Emerging technologies

4.6.1. Engineered exosomes

Exosomes, a type of extracellular vesicle secreted by cells, have emerged as promising mRNA carriers owing to their excellent biocompatibility, low immunogenicity, small size, ability to cross physiological barriers, and cell-specific targeting capabilities. Engineered exosomes are developed by structurally, compositionally, or functionally modifying natural exosomes through genetic engineering, chemical modification, physical fusion, or drug-loading technologies. These engineered exosomes retain their inherent low immunogenicity and ability to evade immune clearance while gaining enhanced targeting specificity and payload capacity. Therapeutic mRNA encapsulation can be achieved through endogenous engineering of parental cells or exogenous cargo-loading approaches. In endogenous loading,[180] donor cells are engineered with specific sequences and transfection techniques to ensure the secreted exosomes naturally carry the desired RNA. Exogenous loading involves purifying exosomes from various sources and loading RNA into them using electroporation, chemical transfection reagents, or hybrid technologies such as exosome-liposome fusion or cell nanoporation.[181,182] Kojima *et al.*[183] developed an implantable exosome-producing cell system that delivered catalase mRNA to the brain, mitigating neurotoxicity and neuroinflammation in Parkinson's disease models. Wan *et al.*[184] electroporated Cas9 ribonucleoprotein (a protein complexed with sgRNA) into exosomes derived from hepatic stellate cells, utilizing immortalized cell lines to enhance production efficiency. This engineered exosome system boosted therapeutic efficacy in murine models of acute liver injury, chronic hepatic fibrosis, and hepatocellular carcinoma.

In addition, exosomes can be chemically modified, or donor cells can be genetically engineered to enable exosomes to target specific cells or tissues. Research has demonstrated that expressing ligands for specific receptors, such as low-density lipoprotein receptors, on exosome surface proteins enhances central nervous system targeting capabilities.[185] Moreover, the amino groups on exosome proteins can be easily modified with alkynes, and alkylated exosome proteins can be conjugated with tumor-targeting peptides, such as neuropilin-1-targeted peptide (RGE), through copper-catalyzed azide-alkyne cycloaddition.[186] After intravenous injection, RGE-conjugated exosomes can cross the blood–brain barrier and home to brain tumor regions.

4.6.2. Nucleic acid nanomaterials

DNA origami, a programmable nanotechnology based on molecular self-assembly principles, enables the precise folding of a long single-stranded DNA scaffold into predefined two-dimensional/three-dimensional nanostructures through

sequence-specific base-pairing interactions with hundreds of short-staple strands.[187,188] DNA origami exhibits exceptional biocompatibility, tissue penetrability, and biostability, but its application is limited by rapid systemic clearance via hepatic and renal pathways. To address this, Liu *et al.*[189] encapsulated DNA origami within LNPs functionalized with selective organ-targeting molecules, which significantly prolonged the *in vivo* circulation half-life. By introducing ultraviolet-crosslinked thymine dimers to reinforce structural rigidity and modulating the LNP surface charge distribution via selective organ-targeting molecules, they achieved co-encapsulation of mRNA and DNA origami within a single LNP (mRNA/origami-LNPs). This strategy extended circulation half-life and enabled lung-specific mRNA delivery.

RNA origami, an extension of DNA origami technology, leverages the conformational flexibility of single-stranded RNA and the presence of ribose 2'-hydroxyl groups to form dynamic secondary structures (e.g., hairpins). These features preserve nanoscale stability while allowing full exposure of mRNA active regions to maintain translational activity. Hu *et al.*[190] developed a lantern-shaped flexible RNA origami, which serves dual roles as both genetic drug cargo and origami scaffold. Using SMAD family member 4 (*Smad4*) mRNA as the scaffold, key positions were fixed by two cyclic RNA staples modified with arginylglycylaspartic acid-targeting peptides, compressing the mRNA into nanoparticles (diameter ≤90.8 nm) while retaining most single-stranded regions. Compared to traditional DNA origami, which has rigid structures requiring complete double-strand formation, this design enables rapid mRNA release and efficient translation after cellular internalization. Importantly, no significant cytotoxicity or inflammatory responses were observed in both *in vitro* and *in vivo* experiments. Tumor growth was significantly inhibited in colorectal cancer models.

5. Applications of mRNA therapy in orthopedic diseases

5.1. Bone fracture and osteoporosis

Bone fracture is the break or crack of a bone, typically caused by direct or indirect external forces acting on the bone due to trauma. In addition to trauma-induced fractures, osteoporosis (an age- and estrogen-related systemic bone metabolic disorder affecting individuals worldwide) increases bone fragility and fracture risk. Regardless of the type of fracture, bone regeneration and healing are complex processes.[191,192] At the molecular level, numerous mediators and cellular components drive the initiation and progression of bone repair. Current clinical methods include the use of various bone grafts (autografts, allografts, bone graft substitutes), distraction osteogenesis and bone transport, growth factors, and cell therapies. Although these methods offer therapeutic benefits, they have limitations related to safety and cost-effectiveness. Similarly, osteoporosis management relies on anabolic agents and anti-resorptive agents.[193] However, it is important to note that both types of drugs have serious side effects due to their impact on the bone remodeling cycle.

Protein replacement therapy primarily involves the artificial introduction of therapeutic proteins to compensate for missing or defective proteins. BMPs, members of the TGF-β superfamily, are critical osteoinductive factors. Specifically, BMP-2 plays a role in maintaining the dynamic balance of newly formed bone tissue[194] and in regulating the transcription of osteogenesis-related genes. BMP-9 potently induces the differentiation of osteoid,[195] stimulating the expression of late osteoclast markers while enhancing trabecular bone volume and collagen matrix deposition. Recombinant human BMP-2 (rhBMP-2) and rhBMP-7 were approved for use in the United States and Europe, but due to concerns over inefficient protein delivery, dosage costs,[196] and safety issues, this therapeutic strategy has faced strict clinical restrictions and has even been withdrawn from the market in some cases.[197]

Local gene delivery offers a promising alternative method.[198] *In vitro* transcribed mRNA can encode any protein sequence and, upon infusion, enables transient protein expression within cells, correcting physiological functions without altering the genome. Regarding BMP gene delivery, pre-clinical studies have made significant progress in transferring genetic material into the genome of target cells. Through endogenous cellular transcription mechanisms, a sustained release of bioactive mediators can be achieved within bone lesions, promoting bone healing. modRNA not only retains transcriptional function but also significantly improves stability and reduces immunogenicity.[199] Compared to DNA-based gene therapies, using inexpensive and safe biomaterials embedded with BMP-2 coding RNA results in modRNA-treated MSCs exhibiting higher expression levels of BMP-2, alkaline phosphatase (ALP), and osteocalcin (OCN), along with significantly increased calcium deposition *in vitro*.[199] Murine bone defects treated with modRNA exhibited a significantly higher proportion of mineralized bone volume relative to total tissue volume (BV/TV) and increased connectivity of regenerated bone compared to controls, with mature mineralized bone extensively bridging the defect. Another study[200] also demonstrated the efficacy of a lipid complex formulation containing modRNA encoding human BMP-2, applied to fibrin gel matrices in non-critical femoral defects in rats, in stimulating bone regeneration. De La Vega *et al.*[201] compared modRNA and recombinant protein treatments in a rat critical-sized femoral osteotomy model, analyzing mechanical strength, transcriptomics, and side effects on other organs. They were the first to confirm that modRNA can heal large, critical-sized, segmental long bone defects more effectively than recombinant proteins.

In addition, hydrogel-encapsulated engineered exosomes rich in *BMP-2* mRNA achieved sustained release,[202] enabling more efficient and safe bone regeneration. By leveraging the inherent homing effect, high circulation stability, biocompatibility, low immunogenicity, low toxicity, and effective molecular signaling of exosomes, mRNA was delivered into recipient cells, significantly increasing the expression of key osteogenic genes such as *Alp*, osteopontin, and type I collagen (*Col1a*), as well as enhancing mineralization levels in the treated cells. Notably, *in vivo* experiments showed that by week 8 post-surgery, the new lamellar bone had formed, almost bridging the defect area. This combination effectively increased the sustained release of exosomes, promoting osteogenesis in severe bone defects.

Another formulation[203] used therapeutic small extracellular vesicles endogenously loaded with human *BMP-2* mRNA and delivered through a PEGylated poly(glycerol sebacate acrylate) hydrogel. Measurements of BV/TV, vascular volume, bone mineral density, and trabecular thickness confirmed that *BMP-2* mRNA was effective in promoting bone regeneration in rat femoral critical-sized defects (**Figure 4**). Moreover, Wang *et al.*[204] demonstrated that dual delivery of unmodified BMP-2 mRNA with non-structural protein-1 mRNA as a translation enhancer could increase BMP-2 production by 8.5-fold within 24 h, reaching 10.5-fold by 48 h compared to *BMP2* mRNA monotherapy in murine pluripotent stem cells. This strategy improved osteogenic gene expression, upregulating Runt-related transcription factor (*Runx*) 2 and activating the expression of bone matrix proteins in the middle-to-late stage, including ALP, osteopontin, and OCN. Thus, the non-structural protein-1 co-delivery platform served as an effective alternative to modRNA.

Similarly, the *in vitro* bioactivity of BMP-9 encoded by modRNA was evaluated by measuring various osteogenic markers in BMSCs. *In vivo* experiments involved collecting bone samples for micro-CT and histological evaluation, where higher levels of ALP and OCN were also observed, and BV/TV was significantly increased. Comparative analysis[205] demonstrated that BMSCs transfected with BMP-9 modRNA exhibited higher ALP expression, greater calcification, and increased calcium production. Compared to the BMP-2 modRNA treatment group, the collagen scaffold loaded with BMP-9 modRNA resulted in twice the bone connectivity density in the regenerated bone after implantation in a rat cranial defect model. This indicates that BMP-9 modRNA loaded into a collagen matrix holds potential for critical-sized defect regeneration and exhibits significant clinical potential for bone repair.

5.2. Bone tumor

Bone tumors are neoplasms that occur in the skeletal system and its accessory tissues, with the majority being benign. Primary malignant bone tumors have a low incidence rate, accounting for <0.2% of all tumor cases,[206] but they are highly

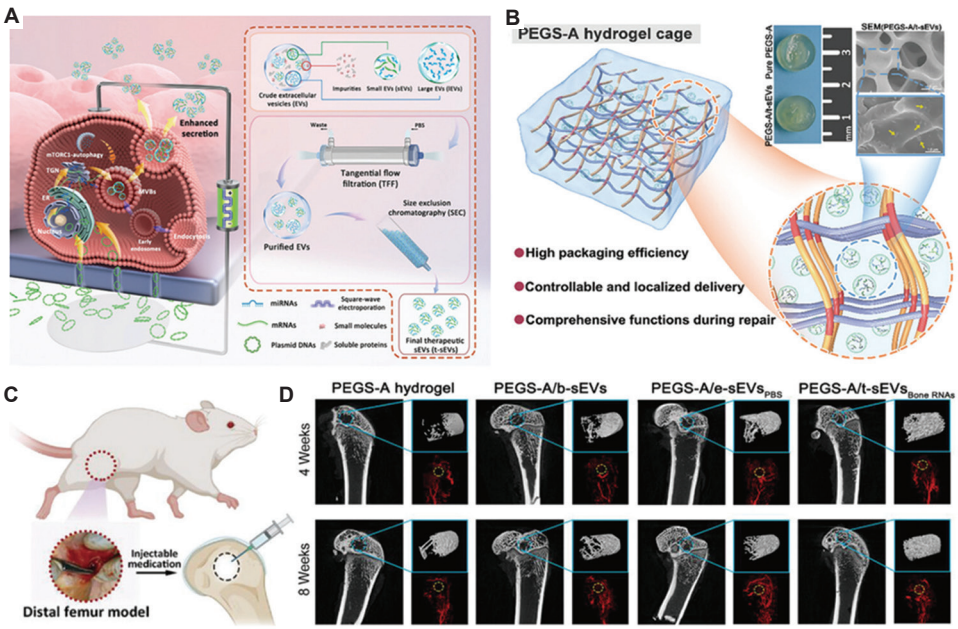

Figure 4. Exosomes carrying BMP-2 mRNA-loaded t-sEVs combined with a hydrogel for bone repair. (A) Schematic diagram of the large-scale production of t-sEVs using trace-etched membrane nano-electroporation (TM-nanoEP). Transfected plasmid DNA is transcribed into mRNA, which is stored within intraluminal vesicles of multivesicular bodies (MVBs). TM-nanoEP induces endosome formation and activates mTORC1-autophagy activity, thereby enhancing the secretion of tEVs. The subsequent purification stage employs tangential flow filtration and size-exclusion chromatography to isolate small EVs (sEVs) with optimized therapeutic agents from crude EVs. (B) Schematic diagram of the custom-designed PEGS-A hydrogel scaffold, encapsulating RNA-rich t-sEVs. The mesh structure not only ensures high encapsulation efficiency but also controls the localized release of exosomes, exhibiting potent bone regeneration capabilities. (C) Schematic of therapeutic injection in rats with femoral defects. At the distal femur, the hydrogel and exosomes are injected into the bone defect area. (D) Micro-CT images from mice with reconstructed femoral defects. The PEGs-A/t-sEV group showed the best outcomes, demonstrating more comprehensive bone repair and new bone formation, confirming the robust *in vivo* regenerative efficacy of the therapeutic hydrogel. Reproduced with permission from Ma *et al.*[203] Copyright © 2023 Authors.

Abbreviations: BMP: Bone morphogenetic protein; ER: Endoplasmic reticulum; miRNA: MicroRNA; mRNA: Messenger RNA; mTORC1: Mammalian target of rapamycin complex 1; PBS: Phosphate-buffered saline; PEGS-A: PEGylated poly(glycerol sebacate acrylate); SEM: Scanning electron microscope; TGN: Trans-Golgi network; t-sEV: Therapeutic small extracellular vesicles.

invasive and significantly impact patients' quality of life. The most common primary malignant bone tumors in adults are chondrosarcoma, followed by osteosarcoma (OS), chordoma, and Ewing's sarcoma. Notably, in adolescents and children, the incidence of OS and Ewing's sarcoma is much higher than that of other bone cancers. Unfortunately, current treatments[207-209] for bone tumors are very limited. Even when combined with chemotherapy and radiotherapy, survival rates for OS and other bone tumor patients after surgical resection remain low, and the tumor recurrence rates are still high.[210]

mRNA therapy has the potential for broad application in bone tumors. Miao *et al.*[211] found that the total m^{6}A level and the expression level of the METTL3 in human OS tissue are increased. Silencing METTL3 suppressed the proliferation, migration, and invasion ability of OS cells. This mechanism may involve reduced ATPase family AAA domain containing 2 m^{6}A levels and decreased lymphoid enhancer factor-1 RNA expression, which in turn suppresses Wnt/β-catenin signaling activity.[212] These findings indicate that METTL3/m^{6}A methylation may be a potential therapeutic target for OS.

Anti-tumor-specific immune responses are closely related to T lymphocytes in the human body.[213] Numerous studies have reported that a significant reason for the immunosuppression observed in solid tumors such as OS and Ewing's sarcoma is the lack of T cell infiltration,[214] suggesting that tumor treatment can target tumor cells through T cells to induce apoptosis. Lehner *et al.*[215] showed that the chimeric natural killer (NK) group 2D receptor, expressed via lentiviral transduction or mRNA transfection, can redirect T cells to the Ewing's sarcoma family of tumors, effectively mediating Ewing's sarcoma family of tumor cell death triggered by activated T cells, with mRNA transfection being safer compared to lentiviral transduction. In addition, the use of autologous DCs transfected with allogeneic OS mRNA antigens can also induce specific anti-tumor effects.[216,217]

For bone metastatic tumors, studies have shown that mRNA therapy is also effective. For example, one study[218] developed mRNA-engineered MSCs that simultaneously express P-selectin glycoprotein ligand-1/homing factor, cytidine deaminase, and osteoprotegerin, and targeted them to mouse models of breast cancer bone metastasis, showing low toxicity and high therapeutic efficacy (**Figure 5**). Recently, Lam *et al.*[219] engineered a novel CAR-NK cell therapy targeting the ephrin type-A receptor-2 (EphA2) antigen, which is highly expressed in various pediatric sarcomas. The EphA2-CAR mRNA, co-modified with m$^1\Psi$ and adenosine-5'-(α-thio)-triphosphate (ATPαS), was transiently delivered into NK cells using the MaxCyte STx electroporation system. The incorporation of 10% ATPαS into enhanced green fluorescent protein m$^1\Psi$ mRNA enhanced target specificity toward

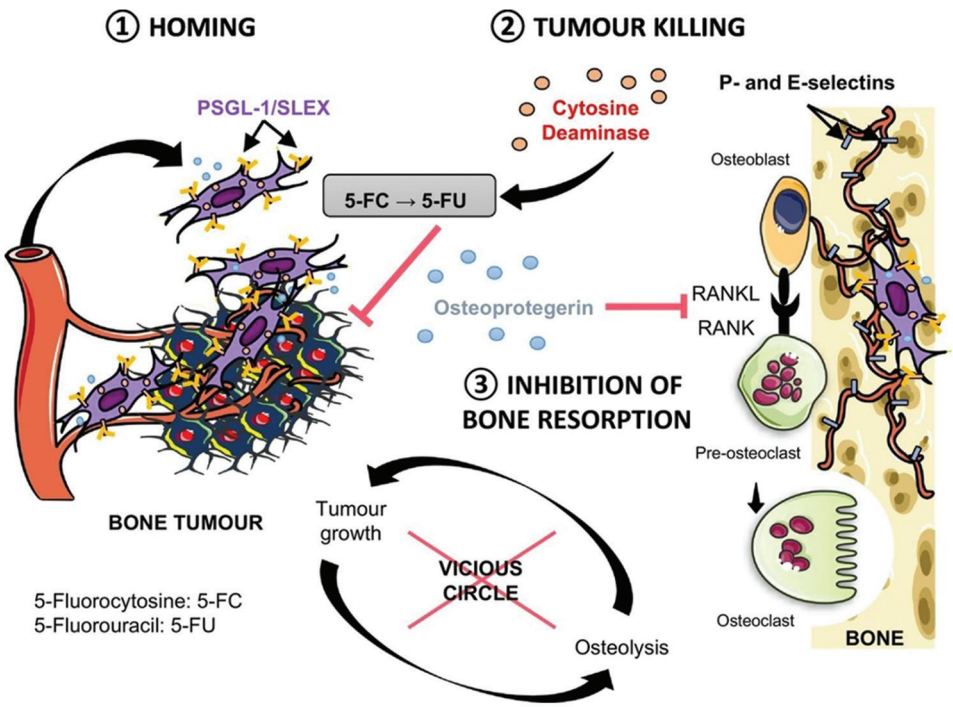

Figure 5. Bone metastasis targeted therapy using messenger RNA (mRNA)-engineered mesenchymal stem cells (MSCs). This strategy employs mRNA-engineered MSCs to deliver multiple factors that target bone metastases via three pathways: (1) P-selectin and E-selectin are typically expressed on the surface of endothelial cells, and their binding with PSGL-1/Sialyl-Lewis x glycan structure (SLEX) enables the therapeutic cells to target bone tumor tissue. (2) Therapeutic cells or vectors engineered with the cytosine deaminase gene convert inactive 5-FC into the toxic anticancer drug 5-FU within the tumor microenvironment, directly targeting and killing tumor cells. (3) Blockade of the RANKL-RANK axis reduces bone resorption, further interrupting the vicious cycle induced by bone tumors. Reproduced with permission from Segaliny *et al.*[218]
Abbreviations: PSGL-1: P-selectin glycoprotein ligand-1; RANK: Receptor activator of nuclear factor kappa B; RANKL: Receptor activator of nuclear factor kappa-B ligand.

EphA2 antigens in sarcoma cells, improving mRNA stability and prolonging CAR protein expression for more than 168 h without inducing detectable immunogenicity in primary NK cells. EphA2-specific CAR-NK cells exhibited superior cytotoxicity against sarcoma cell lines *in vitro* and improved antitumor activity in mouse models of rhabdomyosarcoma and OS. Furthermore, one clinical trial (NCT05660408) is currently investigating the feasibility, safety, and immunogenicity of the RNA-lipid particle vaccine in patients with recurrent lung cancer or inoperable OS. However, this trial is still ongoing, and the vaccine's efficacy remains unclear.

5.3. OA

OA is a chronic degenerative joint disease characterized by the breakdown of articular cartilage, subchondral sclerosis, and osteophyte formation. Progressive cartilage degeneration is a key pathological change in the onset of OA, including apoptosis of chondrocytes and alterations in the extracellular matrix (ECM) components synthesized by chondrocytes.[220] Various factors, such as mechanical injury, cellular senescence, and upregulation of soluble proteases, collectively contribute to OA pathogenesis. Increased levels of inflammatory cytokines, proteases, and reactive oxygen species accelerate the degeneration of the cartilage ECM.[221] There is no specific treatment for OA; thus, non-steroidal anti-inflammatory drugs are usually used to control joint pain. Ultimately, OA patients often undergo joint replacement, facing challenges such as infection and prosthesis wear.

Given the multifactorial pathogenesis of OA, mRNA therapy can focus on modulating key processes involved in cartilage degradation and joint inflammation, such as enhancing chondrocyte synthetic activity, inhibiting catabolic pathways driving cartilage degeneration, or targeting pro-inflammatory cytokines that mediate cartilage destruction. Moreover, mRNA therapy encoding growth factors, such as IGF-1 or TGF-β, can stimulate cartilage regeneration by promoting ECM protein synthesis and enhancing chondrocyte survival. For example, Aini *et al.*[222] developed an mRNA delivery system based on PEG-polyamino acid block copolymer nanomicelles to deliver *Runx1* mRNA into a mouse knee OA model. They observed a significant reduction in the expression of key pro-inflammatory molecules such as IL-1β and a marked increase in the expression and proliferation of anabolic markers in chondrocytes. Another study[223] employed polyamino acid nanomicelles to deliver IL-1 receptor antagonist mRNA via intra-articular injection in a rat temporomandibular joint OA model. This method effectively inhibited the expression of various inflammatory cytokines, offering high safety and prolonged pain relief. Wu *et al.*[224] isolated ADSCs and used liposomes to transfect plasmids containing *Igf1* mRNA, injecting them into the joint cavity of rats with OA. They found that modifying ADSCs with mRNA technology enhanced their ability to secrete IGF-1, significantly improving cartilage damage in rats, reducing inflammation in the joint cavity, and improving joint function, showing a stronger therapeutic effect compared to unmodified ADSCs (**Figure 6**).

The FGF signaling pathways play critical roles in articular cartilage development and homeostasis. Recently, Kong *et al.*[225] developed an LNP-based mRNA delivery system in which *Fgf18* mRNA was encapsulated into LNPs following sequential modifications: uridine-to-m$^1\Psi$ substitution, 5' Cap1 capping, poly(A) tail elongation, and UTR optimization. These modifications increased the FGF18 protein translation efficiency by 1,000-fold. *In vivo* studies revealed a fivefold greater cartilage penetration depth with LNPs compared to recombinant FGF18 protein alone, sustaining intra-articular expression in knee joints for six days with good biocompatibility and low immunogenicity. Mechanistically, *Fgf18* mRNA protected chondrocytes by activating the forkhead box O3-autophagy axis, leading to significant downregulation of the senescence markers p16 and p21. Hence, this *Fgf18* mRNA therapy significantly delayed OA progression in both destabilization of the medial meniscus-induced OA and aging-induced OA models (**Figure 6**).

5.4. Vertebral disc degenerative disease

The intervertebral disc (IVD) is composed of a highly hydrated gelatinous nucleus pulposus at the center, surrounded by lamellar collagen rings known as the annulus fibrosus, along with cartilaginous and bony endplates.[226] The tightly wound annulus fibrosus maintains high osmotic pressure within the nucleus pulposus, allowing the IVD to resist compressive loads. Type II collagen fibers and elastin constitute the ECM components of the IVD, maintaining its homeostasis. Under certain conditions, such as genetic predisposition, aging, mechanical stress, or injury, this homeostasis can be altered, initiating a cascade of IVD degeneration.[227] An imbalance between anabolic and catabolic processes leads to the downregulation of ECM synthesis in the IVD, compromising structural integrity. Reduced production of proteoglycans and type II collagen within the nucleus pulposus leads to dehydration,[228] fibrosis, and fissure formation. This subsequently leads to a loss of hydration and disc height, triggering further IVD degeneration and clinical symptoms.

Currently, treatments for IVD-related symptoms primarily include physical therapy, surgical interventions, and pharmacological management, all of which have limited effectiveness in halting the progression of IVD degeneration. Procedures such as discectomy, arthroplasty, spinal fusion, total disc replacement, and partial nucleotomy can alleviate patient pain but do not restore full IVD function.[229] Moreover, surgical approaches are highly invasive and may lead to loss of mechanical properties, adjacent segment degeneration, and uncertain long-term outcomes. As a result, gene therapy has emerged as a promising alternative. Chang *et al.*[230] developed self-assembling polyplex nanomicelles to deliver mRNA encoding the cartilage-anabolic factor Runx1 to a rat model of disc degeneration. This treatment increased disc hydration, enhanced type II collagen expression, reduced disc space narrowing, stimulated ECM secretion in the damaged disc, and promoted tissue regeneration at the lesion site (**Figure 7**). Similarly, Lin *et al.*[231] applied *Runx1* mRNA via polyplex nanomicelles to a rat tail IVD degeneration model, observing preserved disc height and hydration content, along with the prevention of disc tissue fibrosis.

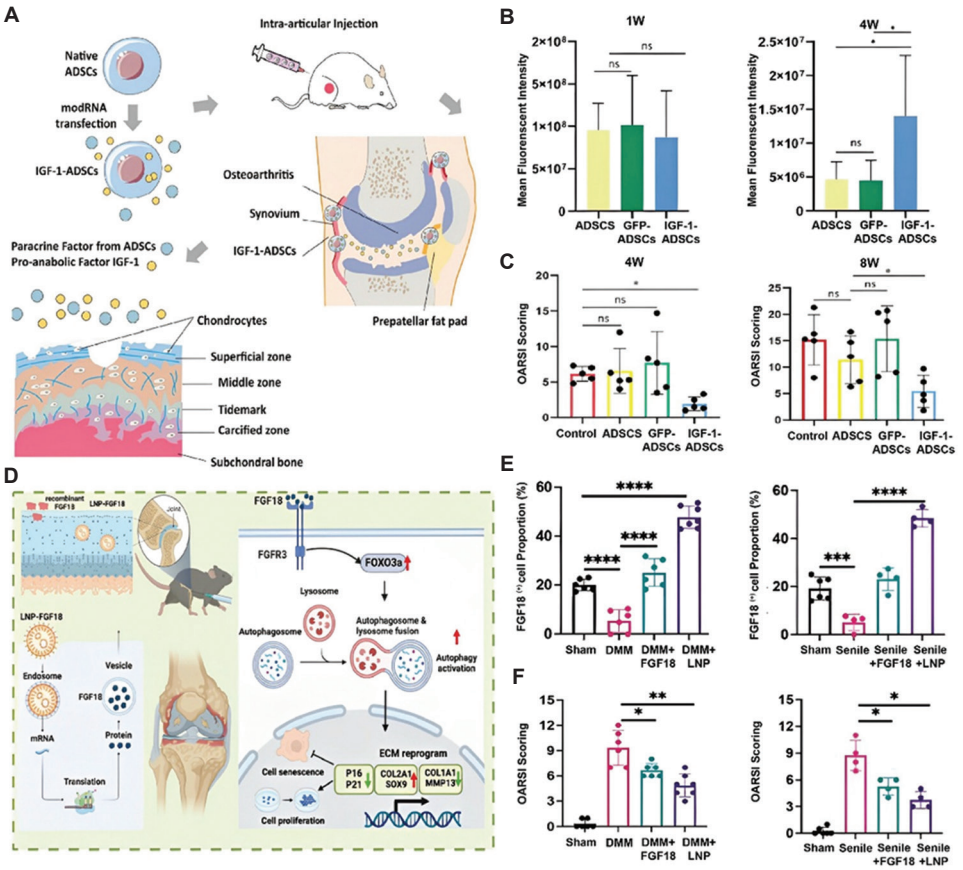

Figure 6. Applications of messenger RNA-based therapy in osteoarthritis. (A) Use of engineered adipose-derived stem cells containing IGF-1 modified mRNA (modRNA) to treat osteoarthritis. Intra-articular transplantation of IGF-1-ADSCs improved cartilage degradation and effectively delayed osteoarthritis progression. (B) Survival rate of transplanted knee joint cells at 1 week (1W) and 4 weeks (4W) after intra-articular transplantation of IGF-1 modRNA-transfected ADSCs. (C) Cartilage degeneration in mice was assessed using OARSI scores for knee joints at 4 and 8 (8W) post-surgery. Reproduced with permission from Wu *et al.*[224] Copyright © 2022 Authors. (D) Intra-articular delivery of LNP-encapsulated FGF18 modRNA for osteoarthritis therapy. Injection of LNP-FGF18 into the mouse joint cavity significantly increased FGF18 expression *in vivo*, activating the FOXO3a-mediated autophagy pathway, thereby protecting cartilage from degeneration by attenuating chondrocyte senescence and degeneration. (E) After intra-articular administration of FGF18 protein or LNP-FGF18 mRNA in both DMM-induced and senile osteoarthritis mouse models, the proportion of FGF18-positive cells in knee cartilage was evaluated. (F) Cartilage degeneration was assessed using the OARSI score following intra-articular injection of FGF18 or LNP-FGF18 mRNA in DMM and aged osteoarthritis models. Reproduced with permission from Kong *et al.*[225] Copyright © 2022 Authors.
Abbreviations: ADSCs: Adipose-derived mesenchymal stem cells; COL1A1: Collagen, type I, alpha 1; COL2A1: Collagen, type II, alpha 1; DMM: Destabilization of medial meniscus; ECM: Extracellular matrix; FGF: Fibroblast growth factor; FGFR: Fibroblast growth factor receptor; FOXO3a: Forkhead box O3; GFP: Green fluorescence protein; IGF-1: Insulin-like growth factor-1; LNP: Lipid nanoparticle; MMP13: Matrix metalloproteinase 13; modRNA: Modified mRNA; OARSI: Osteoarthritis Research Society International; SOX9: SRY-box transcription factor 9.

A herniated disc is also a common degenerative disc disease that typically occurs in the cervical and lumbar regions. It is often caused by the protrusion of the disc's nucleus, which moves through the disc's outer ring (annulus fibrosus) to compress adjacent nerve roots, causing symptoms such as lower back pain, sciatica, sensory loss, and muscle weakness.[232,233] The main causes of herniated disc disease include age-related degenerative changes in the spine, trauma, repetitive strain, improper posture, or sudden heavy lifting.[234] Studies have shown that pregnancy is also a risk factor for herniated discs, associated with increased spinal instability due to hormonal changes, mechanical pressure from the abdomen, and postural and curvature changes that exert additional pressure on the spine.[235] Conservative management remains the primary therapeutic approach, comprising pharmacotherapy, orthotic bracing, and activity modification. Surgical treatment, such as discectomy, is mainly reserved for patients with recurrent symptoms unresponsive to conservative treatment and is recommended for those with severe and intolerable symptoms. However, the surgical risk increases significantly in patients with high-risk factors such as pregnancy or advanced age.[236] In addition, due to the complex anatomy surrounding the IVDs in the neck and waist, surgery can easily damage adjacent tissues and organs, leading to post-operative complications. Moreover, post-operative recovery and rehabilitation remain major concerns, and personalized treatment plans, along with long-term follow-up, have a significant impact on patient prognosis.

mRNA therapy provides multiple potential advantages over traditional treatments, offering a less invasive alternative to surgery by targeting the pathogenesis of herniated discs. For

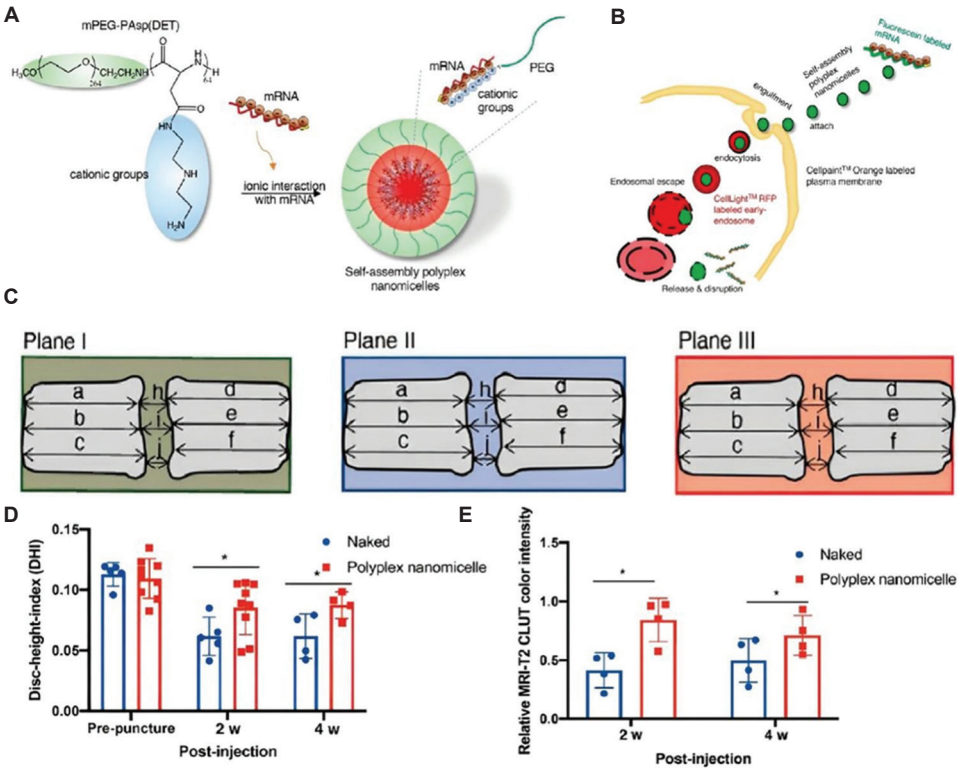

Figure 7. Delivery of Runt-related transcription factor mRNA via multi-chain nanomicelles alleviates disc hydration loss in rats with intervertebral disc degeneration. (A) Schematic illustration of the preparation process for PEG-PAsp (DET) multi-chain nanomicelles loaded with mRNA. (B) Self-assembled nanomicelles deliver mRNA into cells via endocytosis. (C) Schematic of the three sagittal planes used to calculate the disc height index. (D) Assessment of disc height reduction. (E) MRI analysis of hydration changes in punctured rat discs, with relative MRI-T2 CLUT color intensity in the target region calculated using the specified formula. Reproduced with permission from Chang *et al.*[230] Copyright © 2022 Authors.

Abbreviations: CLUT: Color LookUp Table; MRI: Magnetic resonance imaging; mRNA: Messenger RNA; PAsp(DET): Poly (N'-[N-{2-aminoethyl}-2-aminoethyl] aspartamide); PEG: Polyethylene glycol; RFP: Red fluorescent protein.

example, mRNA can encode anti-inflammatory cytokines or growth factors that promote tissue healing and reduce inflammation, which are key therapeutic goals in herniated discs. Bachmeier *et al.*[237] found elevated expression levels of matrix metalloproteinases in patients with degenerative conditions such as herniated discs; histological evidence also showed sustained and significant upregulation of related mRNA levels. These findings indicate that controlling matrix metalloproteinase activity may be a viable therapeutic strategy for herniated discs. Bydon *et al.*[238] sequenced human mRNA from lumbar disc herniation tissues and identified molecular regulatory pathways involving inflammation, cell adhesion, and matrix degradation. Growth factor-related signaling pathways, such as FGF, TGF, BMP, and platelet-derived growth factor, contribute to the physiological functions of disc cells and may serve as potential therapeutic targets. Although mRNA therapy for herniated discs is still in the early stages, with no clinical trials yet confirming optimal delivery methods or long-term clinical efficacy, existing studies have demonstrated its strong therapeutic potential in reducing inflammation and promoting tissue repair.[239]

5.5. Wound healing

As the incidence of high-energy injuries and related open fractures continues to rise, wound management is becoming increasingly important in orthopedics. Unlike the strong repair capabilities of bone, the spontaneous regenerative capacity of tissues surrounding bone injuries is limited. Moreover, with an aging population and a rising incidence of diabetes and vascular diseases, the proportion of patients with chronic wounds and impaired healing conditions is continuously increasing, leading to high post-operative infection and dysfunction rates.[240,241] Therefore, effective wound healing constitutes a pivotal determinant of orthopedic outcomes, affecting not only the repair of the original lesion site but also the overall recovery and quality of life of the patient. Previous studies found that appropriate chemokines,[242] such as stromal cell-derived factor 1α (SDF-1α), applied to the injured site can promote regenerative responses; however, their recombinant proteins are difficult to localize and have a short biological half-life. Gene delivery can achieve targeted, sustained, and potentially regulated expression control to enhance the repair and regeneration of damaged musculoskeletal tissues,[243] among which mRNA therapy is a highly promising therapeutic intervention.

Luo *et al.*[244] integrated SDF-1α modRNA with small skin grafts to treat full-thickness skin defects in diabetic rats. They showed that, compared with small skin grafting alone, simultaneous delivery of modRNA resulted in more effective wound

healing, reduced scar thickness, and increased subcutaneous layer angiogenesis (**Figure 8**). They also indicated[245] the role of SDF-1α modRNA in enhancing angiogenesis and the survival of therapeutic random flaps. Another study[246] loaded vascular endothelial growth factor A (VEGFA) modRNA-transfected cells into a wound-healing scaffold, creating engineered cellular electrospun membrane complexes. The self-secretion of VEGFA significantly promoted wound healing *in vivo* and the formation of vascular networks in the graft area, thereby improving the survival rate of engineered skin. Moreover, ADSCs served as biocompatible vectors for m$^1\Psi$-modRNA delivery to achieve transient, efficient, and pulsatile expression of autologous-derived TGF-β3 and IL-10 proteins.[54] The results showed that ADSCs enriched with modified TGF-β3 and modified IL-10 significantly improved ECM metabolism, myofibroblast regression, and angiogenesis, preventing skin fibrosis by reversing the fibroblast phenotype to reduce scar formation. This strategy overcomes the short *in vivo* half-life and single-mode action of TGF-β3 and IL-10 using modRNA. These examples demonstrate the great potential of mRNA therapy for clinical applications in orthopedic wound healing.

5.6. Osteogenesis imperfecta (OI)

OI is an inheritable disorder characterized by bone fragility.[247] This multisystemic connective tissue disorder primarily manifests in the skeletal system, causing bone fragility and significant growth retardation. Approximately 85% of OI cases result from dominant, autosomal mutations in the type I collagen coding genes (*COL1A1*/*COL1A2*), leading to deficient collagen synthesis or abnormal structural assembly.[248] Extraskeletal manifestations include cardiovascular defects, pulmonary insufficiency, dermal thinning, hearing loss, and dentinogenesis imperfecta.[249,250] However, current therapeutic approaches only slow symptom progression without addressing the underlying *COL1A1*/*COL1A2* mutations responsible for collagen defects. Yang *et al.*[251] utilized recombinant adeno-associated virus to deliver CRISPR-Cas9 to osteoblastic lineage cells, effectively reversing COL1A2-associated osteogenic differentiation defects and ameliorating skeletal phenotypes in

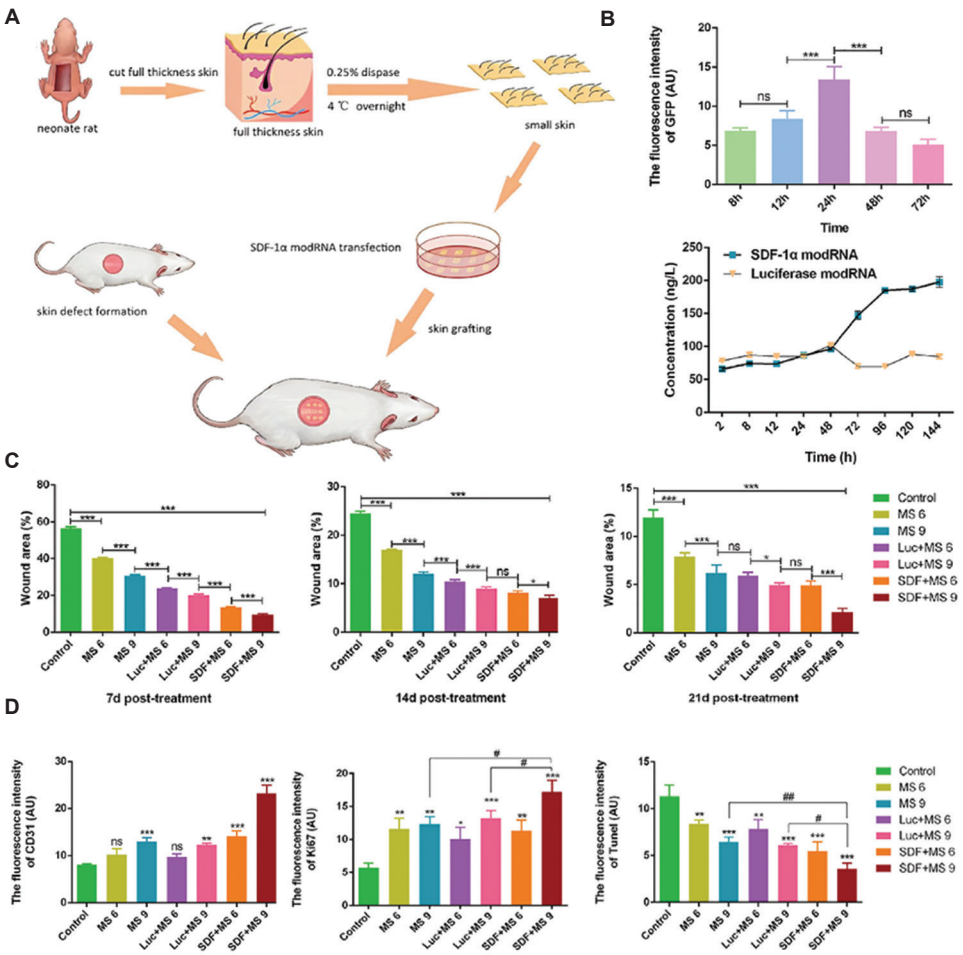

Figure 8. Modified *Sdf1α* mRNA combined with small skin (MS) grafts improves wound healing. (A) Schematic design for transfecting small patches of skin with SDF-1α modRNA and performing *in vivo* experiments. (B) Transfection of small skin grafts with modRNA. GFP fluorescence expression and the cumulative concentration of SDF-1α were quantitatively measured in the grafts. (C and D) Treatment with SDF-1α modRNA promotes the formation of new blood vessels in wound healing and accelerates the proliferation of wound-healing cells. Reproduced with permission from Luo *et al.*[244] Copyright © 2022 Authors.

Notes: Luc + MS = luciferase modRNA transfected into small skin grafts; SDF + MS = SDF-1α modRNA transfected into small skin grafts. The numbers 6 and 9 indicate the number of small skin grafts each group received.

Abbreviations: GFP: Green fluorescence protein; modRNA: Modified mRNA; SDF-1α: Stromal cell-derived factor 1α.

Table 3. Examples of messenger RNA (mRNA) therapy applications in orthopedic diseases

Disease application	Cargos	Delivery vectors	Delivery site	Translation substance	Curative effect	References
Bone defect	BMP-2 modRNA	PEI	Calvarium defect	Osteogenic proteins	Improves bone regeneration ability	199
Bone defect	hBMP-2 modRNA	Transcript-activated collagen matrix	Non-critical rat femoral bone defect	Osteogenic proteins	Stimulates osteoid formation and bone regeneration	200
Bone defect	BMP-2 mRNA	Exosomes-hydrogel	Calvarium defect	Osteogenic proteins	Increases osteogenic gene expression and sustains exosome release	202
Bone defect	BMP-2 mRNA and VEGFA	PEGS-A/t-sEVs	Critical-sized femoral defect	Osteogenic proteins	Achieves efficient bone regeneration in critical-size femoral defects	203
Bone defect	BMP-9 modRNA	PEI and PCM	Calvarium defect	Osteogenic proteins	Enhances osteogenic gene expression	252
Bone defect	BMP-9/BMP-2 modRNA	PEI	Calvarium defect	Osteogenic proteins	BMP-9 modRNA shows superior bone regeneration compared to BMP-2	205
Bone defect	BMP-2 mRNA	BP-LNPs	Bone micro environment	Osteogenic proteins	Enhances BMP-2 expression	142
Ewing sarcoma	Chimeric NKG2D receptor mRNA	-	ESFT cells	Chimeric NKG2D receptor	Recognizes ESFT cells and mediates an anti-ESFT response	215
Osteosarcoma	Allogeneic osteosarcoma mRNA antigen	DCs	Tumor cells	Osteosarcoma antigen	Inhibits tumor growth and induces long-term immune response	216
Bone metastases	modRNA	mRNA-engineered MSCs	Tumor cells	PSGL-1/SLEX and modified CD and OPG	Kills tumor cells and preserves bone integrity with minimal toxicity	218
Sarcomas	$m^1\Psi$ and ATPαS co-modified EphA2-CAR mRNA	CAR-NK cell	Tumor cells	CAR protein	Exhibits antitumor activity in rhabdomyosarcoma and osteosarcoma murine models	219
Osteoarthritis	*Runx1* mRNA	PEG-polyamino acid polyplex nanomicelles	Knee joint	RUNX1	Reduces inflammation and increases expression of cartilage anabolic markers	222
Osteoarthritis	*Il1ra* mRNA	Polyplex nanomicelle	Temporomandibular joint	IL-1Ra protein	Alleviates joint inflammation	223
Osteoarthritis	IGF-1 modRNA	ADSCs	Chondrocytes	IGF-1 protein	Alleviates OA progression	224
Osteoarthritis	Modified *Fgf18* mRNA	LNPs	Chondrocytes	FGF18 protein	Alleviates OA symptoms	225
Vertebral disc degeneration	*Runx1* mRNA	Polyamine-based PEG-polyplex nanomicelle	Damaged disc	RUNX1	Improves disc hydration, disc space, and ECM production	230
Intervertebral disk degeneration	*Runx1* mRNA	Polyplex nanomicelle	Damaged disc tissue	RUNX1	Prevents tissue fibrosis and inflammation	231
Wound healing	SDF-1α modRNA	Small skin	Full-thickness skin wounds	SDF-1α protein	Improves wound healing with reduced scar thickness	244
Wound healing	SDF-1α modRNA	Fibroblasts	Ischemic skin flaps	SDF-1α protein	Enhances vascular formation and flap survival	245
Wound healing	*Vegfa* mRNA	CEMCs	Transplantation	VEGFA	Promotes *in vivo* wound healing	246

Abbreviations: ADSCs: Adipose-derived mesenchymal stem cells; ATPαS: Adenosine-5′-(α-thio)-triphosphate; BMP: Bone morphogenetic protein; BP-LNPs: Bisphosphonate-lipid nanoparticles; CAR: Chimeric antigen receptor; CD: Cytosine deaminase; CEMCs: Cellular electrospun membrane complexes; DCs: Dendritic cells; ECM: Extracellular matrix; EphA2: Ephrin type-A receptor-2; ESFT: Ewing's sarcoma family of tumors; CAP-Exo: Chondrocyte-targeting exosomes; Cas13: Cas13 ribonucleic-protein complex; IGF-1: Insulin-like growth factor-1; IL-1Ra: Interleukin-1 receptor antagonist; modRNA: Modified mRNA; MSCs: Mesenchymal stem cells; $m^1\Psi$: N1-methylpseudouridine; NK: Natural killer; NKG2D: Natural killer group 2D; OA: Osteoarthritis; OPG: Osteoprotegerin; PCM: Perforated collagen membranes; PEG: Polyethylene glycol; PEGS-A: PEGylated poly (glycerol sebacate acrylate); PEI: Poly (ethyleneimine); PSGL-1: P-selectin glycoprotein ligand-1; Runx1: Runt-related transcription factor 1; SDF-1α: Stromal cell-derived factor 1α; SLEX: Sialyl-Lewis x glycan structure; t-sEVs: Therapeutic small extracellular vesicles; VEGFA: Vascular endothelial growth factor A.

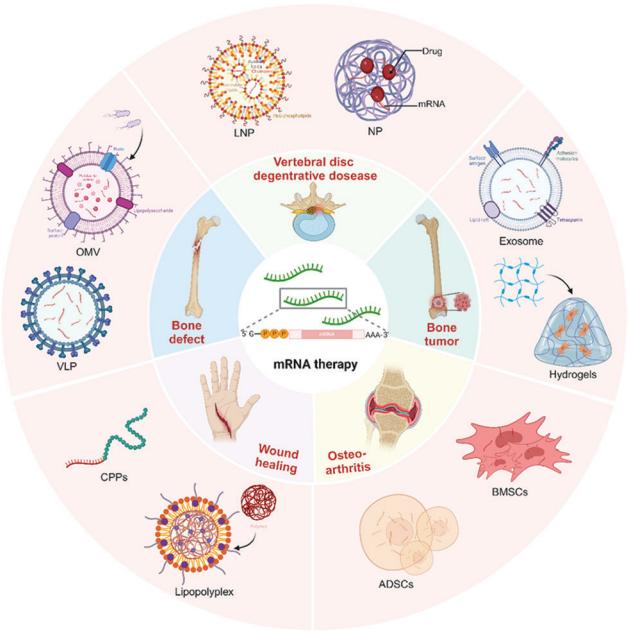

Figure 9. Overview of the applications of messenger RNA therapy in bone and joint diseases. Figure created by the authors.
Abbreviations: ADSCs: Adipose-derived mesenchymal stem cells; BMSCs: Bone marrow-derived mesenchymal stem cells; CPPs: Cell-penetrating peptides; LNP: Lipid nanoparticle; NP: Nanoparticle; OMV: Outer membrane vesicle; VLP: Virus-like particle.

OI murine models. Hence, bone-targeted delivery systems hold promise for precise transport of *COL1A1*/*COL1A2* CRISPR-mRNA, increasing COL1a production (**Table 3**).

6. Conclusions and outlook

Since the concept of mRNA therapy emerged, significant efforts have been made to overcome major technical challenges, including enhancing mRNA stability, reducing innate immunogenicity, and improving delivery efficiency. Through global or local modifications of the mRNA structure, modRNA exhibits favorable expression kinetics, increased stability, and reduced immunogenicity/toxicity. Using various delivery systems, therapeutic mRNA can be effectively introduced into cells, greatly enhancing the stable expression of target molecules and enabling the compensation or repair of abnormal functions.

As a result, mRNA therapy is being actively explored in areas such as regenerative medicine, protein replacement therapy, cancer immunotherapy, and gene editing. The FDA is expected to approve mRNA-based treatments for indications such as cancer and human immunodeficiency virus 1, and this technology is likely to become a key pillar in drug discovery and development. The emergence of new technologies such as CRISPR-mRNA further enables the activation, inhibition, and knockout of target genes, significantly expanding the available tools and scope of gene therapy. Genetically engineered immune cells expressing CARs have become a promising approach in cancer immunotherapy. Modifying immune cells with mRNA-encoding CARs allows for the rapid, large-scale generation of CAR cells *in vivo* without the risk of transgene integration. This approach has successfully entered clinical trials, showing promising results in patients with refractory blood cancers, including acute myeloid leukemia and lymphoma,[253-255] through CAR T-cell infusions.

Given the clinical success and safety of mRNA vaccines against SARS-CoV-2 and their ongoing application in inducing cross-neutralizing antibodies against variants, mRNA is expected to significantly impact clinical treatments for other diseases, including bone and joint disorders. However, ethical concerns persist regarding the clinical approval of these strategies, as they involve a series of costly and complex procedures. For example, CRISPR-mRNA-based gene editing research remains predominantly in the pre-clinical stage, lacking human trial data and long-term genomic surveillance. The associated ethical controversies encompass technical safety, intergenerational impacts, and societal equity challenges, all of which require careful consideration across multiple dimensions. Establishing interdisciplinary governance frameworks and implementing dynamic regulatory mechanisms, such as adaptive ethical reviews of clinical trials, are necessary for risk mitigation and providing actionable guidance for clinical translation.

In addition, enhancing translational efficiency and prolonging the duration of protein expression remain critical challenges in current research. To address these limitations, emerging technologies are being explored, including sustained-release mRNA delivery systems and mRNA sequence optimization to extend expression duration. Further exploration of alternative mRNA design, such as self-amplifying RNA, trans-amplifying RNA, and circular RNA, may enhance expression duration and therapeutic efficacy, potentially reducing the need for repeated dosing. Other challenges include cold chain storage requirements and improving delivery efficiency and tissue targeting. High-throughput screening of materials and formulations, such as advanced nanocarrier formulation development, can support large-scale production of functional carriers and enhance therapeutic delivery. Targeted nanoplatforms can improve mRNA delivery to specific organs and tissues, including the spleen, brain, lungs, lymph nodes, and kidneys. Future research may explore alternative administration routes, such as nasal inhalation, oral delivery, or microneedle patches. Individualized delivery strategies may also offer greater efficiency compared to systemic administration.[256] In addition, the rational design of mRNA constructs and delivery systems can be accelerated through quantitative structure-activity relationship modeling, artificial intelligence, and machine learning.[257]

In orthopedics, mRNA therapy has achieved early success in several diseases (**Figure 9**). However, many technical barriers remain to be overcome. Although mRNA vaccines demonstrate outstanding efficacy in inducing robust humoral and cellular immune responses, the innate immunogenicity triggered in orthopedic applications remains unclear. To address the unique requirements of orthopedic therapies, mRNA modification with $\Psi/m^1\Psi$ may be employed to reduce TLR recognition sensitivity. Moreover, the anatomical and physicochemical properties of bone tissue impose substantial challenges for

mRNA delivery. Specifically, the intricate blood–interstitial fluid dynamics and the dense, negatively charged ECM with high hydroxyapatite content create formidable diffusion barriers for conventional carriers. The targeting specificity of mRNA therapy for bone cells is not yet fully understood, and enhancing intracellular uptake of mRNA in osteoblasts or chondrocytes remains a significant challenge. The absorption efficiency of mRNA varies in hard tissues, and changes in concentration can severely disrupt cellular function. Thus, it is crucial to determine appropriate dosing concentrations and delivery systems for orthopedic diseases.[258] Engineering delivery vectors with bone/cartilage-targeting aptamers can help enhance tissue specificity. The synergistic optimization of tissue-specific delivery and innate immune regulation is also important for enhancing the safety profile of mRNA-based orthopedic therapies.

Future mRNA therapies can be developed based on the mechanisms underlying different orthopedic diseases. For example, in OA, m^6A modifications of pro-inflammatory factors could target apoptosis inhibition and promote the synthesis of cartilage ECM, thus alleviating OA progression. However, research on cell-specific mRNA modifications in osteocytes and chondrocytes remains in its early stage. More importantly, large animal studies and well-designed clinical trials are needed to validate the efficacy and long-term safety of mRNA therapy in orthopedic diseases. Finally, the clinical application of mRNA therapy must account for individual patient differences and pathological conditions. Variations in the physiological and pathological states among patients may result in differential responses to mRNA therapy. For example, studies have shown that inflammatory factors such as tumor necrosis factor α play a complex role in bone healing, exerting both promotive and inhibitory effects.[259,260] Such complexity necessitates a more personalized approach to designing mRNA therapy protocols. Although mRNA-based therapies still face many challenges, continued research and technological advancements will undoubtedly accelerate the development of next-generation mRNA therapies, unlocking their full therapeutic potential to treat bone and joint diseases.

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