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Organoid extracellular vesicle-based therapeutic strategies for bone therapy

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Key Words:
bone therapy; engineering modifications; extracellular vesicles; nanotechnology; organoid extracellular vesicles

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ABSTRACT

With the rapid development of population ageing, bone-related diseases seriously affecting the life of the elderly. Over the past few years, organoids, cell clusters with specific functions and structures that are self-induced from stem cells after three-dimensional culture in vitro, have been widely used for bone therapy. Moreover, organoid extracellular vesicles (OEVs) have emerging as promising cell-free nanocarriers due to their vigoroso physiological effects, significant biological functions, stable loading capacity, and great biocompatibility. In this review, we first provide a comprehensive overview of biogenesis, internalisation, isolation, and characterisation of OEVs. We then comprehensively highlight the differences between OEVs and traditional EVs. Subsequently, we present the applications of natural OEVs in disease treatment. We also summarise the engineering modifications of OEVs, including engineering parental cells and engineering OEVs after isolation. Moreover, we provide an outlook on the potential of natural and engineered OEVs in bone-related diseases. Finally, we critically discuss the advantages and challenges of OEVs in the treatment of bone diseases. We believe that a comprehensive discussion of OEVs will provide more innovative and efficient solutions for complex bone diseases.

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Introduction

The most significant issue arising from population ageing is the gradual decline in the function of tissues and cells as age advances. Various degenerative diseases continue to develop, affecting multiple tissues and organs within the body, and this process is largely irreversible.[1, 2] The skeletal system, a component of the human musculoskeletal system, undergoes numerous physiological changes as the body ages. The deterioration of physiological functions leads to conditions such as osteoporosis, osteoarthritis, and osteoporotic fractures due to the fragile bone microstructure, resulting in diminished bone strength and susceptibility to fractures.[3, 4] Additionally, the ageing of cartilage cells reduces lubrication, cartilage degeneration, and subchondral bone sclerosis leading to joint discomfort and even pain during movement. Overall, ageing has adverse effects on the musculoskeletal system.[5-7] Currently, effective treatment options for conditions like osteoporosis and osteoarthritis caused by ageing are lacking. Long-term use of anti-osteoporosis drugs and non-steroidal anti-inflammatory drugs places a substantial burden on the body and carries severe side effects.[8-10]

Extracellular vesicles (EVs) have emerged as a focal point in biomedical research in recent years due to their nanoscale structure, low immunogenicity, favourable biocompatibility, and drug delivery potential.[11-14] Moreover, EVs have been shown to effectively in improving the progression of osteoporosis and osteoarthritis,[15-21] and have also been shown to accelerate the healing of osteoporotic fracture.[22-25] Although EVs have shown great advantages in bone ageing diseases, their inherent limitations, such as low yield and low therapeutic efficiency, hinder their further development.

With the advancement of stem cell technology, a three-dimensional (3D) model of organ-like structures that closely mimic native tissue architecture and physiological functions has emerged. Organoids are cell clusters with specific functions and structures that are self-induced from human adult stem cells or pluripotent stem cells after 3D culture *in vitro*.[26, 27] Moreover, organoid EVs (OEVs) have also received much attention. Compared with traditional EVs, OEVs contain more quantity, better biological properties, and better therapeutic effects.[28] We innovatively propose the concept that not only organoids but also OEVs can be used for the treatment of complex diseases.[29] The discovery of OEVs might yield unexpected benefits for the improvement of age-related bone diseases. It is firmly believed that OEVs represent a novel research paradigm, harboring immense scientific exploration and clinical utility [25].

Here, a comprehensive overview of biogenesis, internalisation, isolation, and characterisation of OEVs was provided. Then, the differences between OEVs and traditional EVs were comprehensively highlighted. Subsequently, the applications of natural OEVs in disease treatment were presented. Furthermore, the engineering modifications of OEVs were summarised. Moreover, an outlook on the potential of natural and engineered OEVs in bone-related diseases was provided. Finally, the advantages and challenges of OEVs in the treatment of bone diseases were discussed. We hope that a full understanding of OEVs will promote progress in the field of biomedical field and provide new strategies for the treatment of complex bone diseases (**Figure 1**).

Figure 1. Schematic illustration of organoid extracellular vesicle (OEV)-based bone disease treatment strategy. OEVs have emerging as promising cell-free nanocarriers for bone therapy due to their vigoroso physiological effects, significant biological functions, stable loading capacity, and great biocompatibility. Created with BioRender.com.

Overview of Organoid Extracellular Vesicles

Organoids are cell clusters constructed through *in vitro* 3D cultivation using stem cells, recapitulating the spatial architecture and physiological functions of the source tissue.[30-32] As 3D models composed of living cells, they are also capable of secreting OEVs, which exhibit significant advantages in quantity and physiological function compared with traditional two-dimensional (2D) cultured EVs.[33] Currently, organoid research is in its infancy, where extensive investigation on the OEV biogenesis is limited. However, traditional EVs and OEVs are essentially derived from mammalian cells.[13, 34] The general principles underlying the biogenesis of these two types of EVs are similar. Therefore, we exploited the biogenesis, internalisation, and isolation of mammalian EVs (MEVs) to characterise OEVs. Although the biogenesis of OEVs may be similar to that of EVs, the unique properties of the organoids may introduce subtle differences to OEVs. As OEVs continue to advance, it is expected that the biogenesis of OEVs will be further elucidated.

Biogenesis and internalisation of organoid extracellular vesicles

Here, the biogenesis of OEVs is summarised (**Figure 2**). The cellular plasma membrane undergoes invagination to absorb cell surface proteins and soluble proteins from the extracellular environment, resulting in the formation of early sorting endosomes (ESEs), sometimes synthesised in conjunction with pre-existing ESEs; ESEs engages in cargo exchange with the endoplasmic reticulum and Golgi apparatus, maturing into late sorting endosomes, ultimately giving rise

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to multivesicular bodies. The multivesicular body membrane undergoes secondary invagination to form intraluminal vesicles, subsequently guided by relevant proteins, the multivesicular body elects either to merge with lysosomes for degradation or to fuse with the cell plasma membrane to release intraluminal vesicles into the extracellular space, thereby releasing OEVs.[25, 35-37]

Released EVs primarily function in maintaining intercellular communication and can be absorbed by recipient cells through three modes: membrane fusion, receptor-ligand binding, and endocytosis (**Figure 2**). Benefiting from the fact that both OEVs and recipient cells possess phospholipid bilayer membrane structures, when they can overcome the instability of phospholipid bilayer interactions and the high activation energy barrier, membrane fusion between the two becomes plausible.[38] During membrane fusion, a partially fused connecting segment forms between two adjacent distinct phospholipid bilayer membranes. As they draw closer, this connecting segment expands, evolving into a semi-fused double-layered membrane structure that progressively enlarges until OEVs fuse with recipient cells.[39] On the other hand, the ligand-receptor binding pathway heavily relies on the presence of lectins, polysaccharides, integrins, and other cell adhesion molecules.[40] The presence or absence of these substances significantly impacts the internalisation of OEVs through this pathway, although the specific mechanisms remain indeterminate in current research.[41] Some studies indicate that protein-coated internalisation is a typical route for OEV uptake, and inhibiting the coating proteins can notably diminish cellular uptake of OEVs.[42, 43] Moreover, owing to the distinctive 3D structure of organoids, the efficiency of OEVs internalisation is enhanced. The intricate cellular interactions and spatial arrangement within organoids create a unique microenvironment that facilitates efficient uptake of OEVs by neighbouring cells.[44]

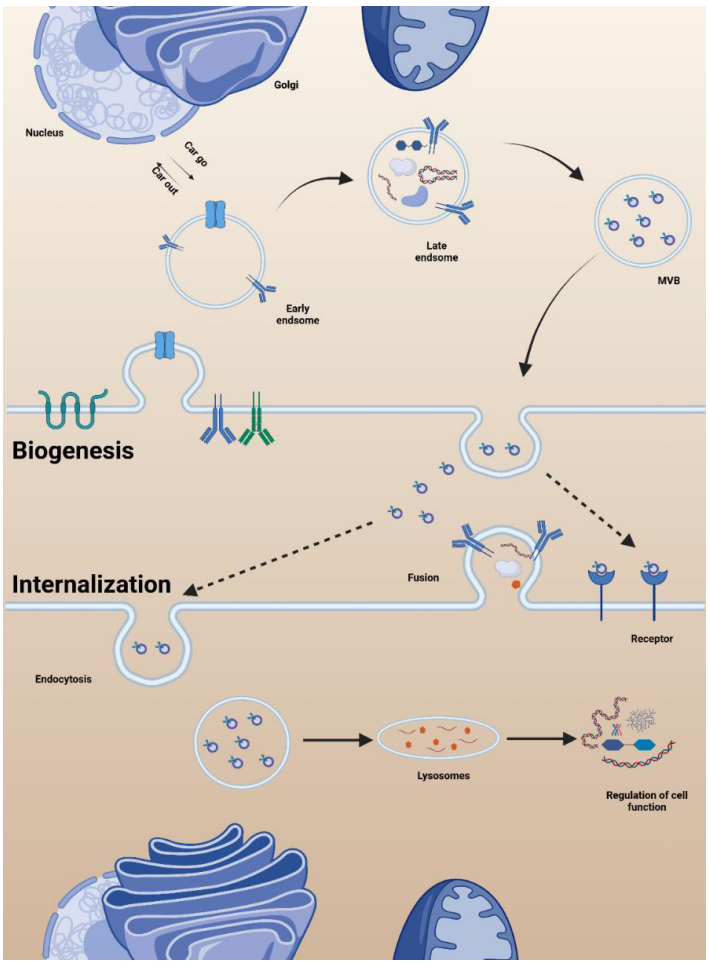

Figure 2. Biogenesis and internalisation of organoid extracellular vesicles (OEVs). The early endosome was formed by the absorption of extracellular proteins by the plasma membrane through endocytosis. The early endosome gradually matured into the late endosome by exchanging goods with the endoplasmic reticulum and Golgi apparatus. In the late endosomes, a second plasma membrane invasion occurs to form multivesicular body (MVB), which is finally selected to fuse with the plasma membrane of the cell to release OEVs or to fuse with lysosomes with the participation of sorting proteins. The free OEVs arrive at the recipient cell and are absorbed by endocytosis or receptor ligand binding. Created with BioRender.com.

Isolation and characterisation of organoid extracellular vesicles

Currently, methods for the isolation of EVs primarily encompass density gradient centrifugation, differential centrifugation, size-exclusion chromatography, and kit-based extraction. Each approach possesses its distinct advantages and limitations. Here, we summarised extraction methodology for OEVs (**Figure 3**). First, the separation of organoids and matrix gel is achieved by subjecting the mixture to low-speed centrifugation at 1,000 × *g* for 5 minutes at 4°C. Subsequently, the supernatant is subjected to a low speed centrifugation at 10,000 × *g* for 10 minutes at 4°C, followed by filtration through a 0.22 μm sterile filter. Then, employing ultracentrifugation at 150,000 × *g* for 90 minutes at 4°C, the sediment at the bottom is identified as OEVs, which are subsequently resuspended in sterile phosphate buffered saline. For purification, a repeat ultracentrifugation at 150,000 × *g* for 90 minutes at 4°C is conducted, yielding the purified OEVs as the sediment at the bottom. Sterile phosphate buffered saline is employed to resuspend the collected purified OEVs, which are then stored at –80°C for future use.[14, 45-47] Other OEVs extraction schemes are shown in **Table 1**.[48-52]

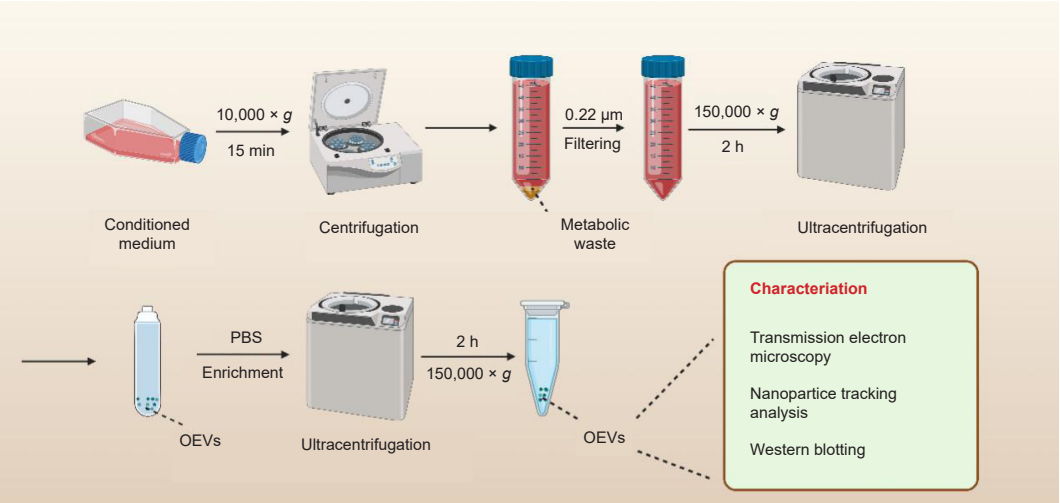

Figure 3. Isolation of organoid extracellular vesicles (OEVs). After three-dimensional (3D) cultivation, the organoid culture is collected and centrifuged at 10,000 × *g* for 15 minutes at 4°C. The supernatant is then filtered by 0.22 μm sterile filter to remove impurities. Subsequently, OEVs precipitate are collected by the ultracentrifugation for 2 hours at 150,000 × *g*. The collected OEVs are purified with phosphate buffered saline (PBS) and ultracentrifuged at 150,000 × *g* for 2 hours. The obtained OEVs can be characterised and verified using nanoparticle tracking analysis, transmission electron microscopy, and Western blotting to represent the size, shape, concentration, and specific markers of OEVs. The collected OEVs are used immediately or stored at –80°C until use. Created with BioRender.com.

Table 1. The extraction methods of organoid extracellular vesicles

Method	Principle	Advantage	Disadvantage	Reference
Gradient ultrafast centrifugation	Different settlement coefficient	High purity; Separable subgroup	Time-consuming; High equipment requirements	48
Volume exclusion chromatography	Different particle size	High purity; Fast preparation	Expensive; Low output	49
Immunoaffinity capture	Specific binding	High purity; Specific exosomes	Expensive; Need to optimise ligand; Low yield	50
Microfluidic technology	Immunoaffinity, particle size and density	High efficiency; No chemical pollution	Low yield; Expensive	51
EVs extraction kit	Immune magnetic bead capture	Simple method	Low output; Expensive	–
Sucrose density gradient centrifugation method	Centrifugal force	High purity	Low output; Long time; Tedious process	52

After the isolation of OEVs, transmission electron microscopy, nanoparticle tracking analysis, and Western blotting are the most used characterisation methods.[16, 53, 54] Generally, transmission electron microscopy and nanoparticle tracking analysis are used to represent the size, shape, and concentration of OEVs. In addition, Western blotting is used to characterise specific markers such as transmembrane proteins CD9, CD63, CD81, and tumour susceptibility gene 101[55, 56] (**Figure 3**).

In summary, this isolation and characterisation protocol provides a clear procedure to obtain pure OEVs suitable for subsequent research. As organoid technology matures and more people realise the existence and ability of OEVs, it is necessary to propose a standardised set of guidelines for the isolation and characterisation of OEVs.

The Differences between Organoid Extracellular Vesicles and traditional Extracellular Vesicles

In contrast to 2D monolayer cell culture models within culture dishes, organoids exhibit advanced 3D physiological structures, facilitating intricate intercellular communication.[50, 57] A study investigated EVs released by gastric cancer cells from the same source cultured in both 2D and 3D conditions.[58, 59] Subsequent observations revealed a substantial advantage in the quantity of EVs collected under 3D conditions. Furthermore, a decrease in adenosine diphosphate-ribosylation factor 6 expression and an overall elevation in microRNA expression were detected within EVs collected from 3D conditions.[58, 59] A recent study on 3D EVs has shown that, unlike 2D EVs, many miRNAs choose to be highly expressed in 3D EVs rather than in 2D EVs.[60] In addition, when stimulated by amyloid-β42, 3D EVs showed stronger anti-inflammatory effects, while 2D EVs showed reduced pro-inflammatory factors.[60]

Additionally, studies explored the transplantation of mesenchymal stem cell derivatives cultured in 2D and 3D conditions into mice with brain injuries.[61-63] Mice injected with derivatives from 3D culture exhibited superior improvements in angiogenesis and neural recovery. This potentially arises from the more natural physiological structure of cells from 3D culture, leading to the secretion of EVs with advantages in both quantity and physiological functionality. At present, many preclinical and clinical studies have demonstrated the therapeutic effect, drug delivery capacity and diagnostic potential of EVs, but the production and clinical conversion technology of EVs are the main challenges limiting the application of EVs.[48, 64-66] Monolayer cell culture mode leads to reduced cell-cell interaction, which seriously affects the production and function of EVs. Fortunately, OEVs from 3D solved these problems perfectly. 3D culture not only preserves the cell phenotype, but also brings more high-yield and efficient OEVs.

Overall, OEVs derived from 3D culture conditions surpass traditional EVs in terms of quantity and physiological effects, rendering them more suitable for therapeutic applications in disease treatment[67] (**Figure 4**).

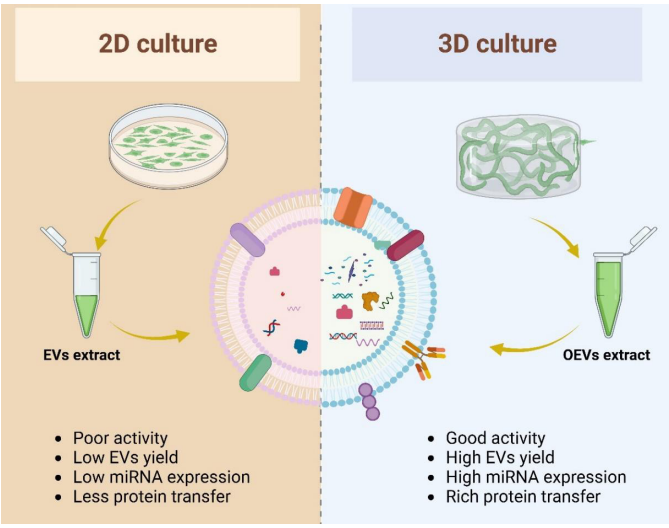

Figure 4. The differences between organoid extracellular vesicles (OEVs) and traditional extracellular vesicles (EVs). Two-dimensional (2D) cultured cells produced fewer EVs, poor bioactivity, and less protein and nucleic acid (left), while three-dimensional (3D) cultured cells produced more OEVs, more active, and more protein and nucleic acid (right). Created with BioRender.com. miRNA: microRNA.

Applications of Organoid Extracellular Vesicles

Currently, the literature related to OEVs is very scarce.[55, 68] However, existing research results show that OEVs have significant therapeutic effects, which makes scientists and clinicians full of interest and confidence in the huge therapeutic potential of OEVs.[69] In addition to disease treatment, it is foreseeable that OEVs will prosper in fields such as liquid biopsy, pharmacological testing, toxicity testing, drug testing, and genetic research (**Figure 5**). Here, we summarise the applications of OEVs in immunomodulation and epithelial repair.

Organoid extracellular vesicles for immunomodulation

Many studies have shown that EVs are effective mediators of intercellular communication between intestinal cells and immune cells.[70, 71] Current studies show that EVs derived from human intestinal organoids can modulate the

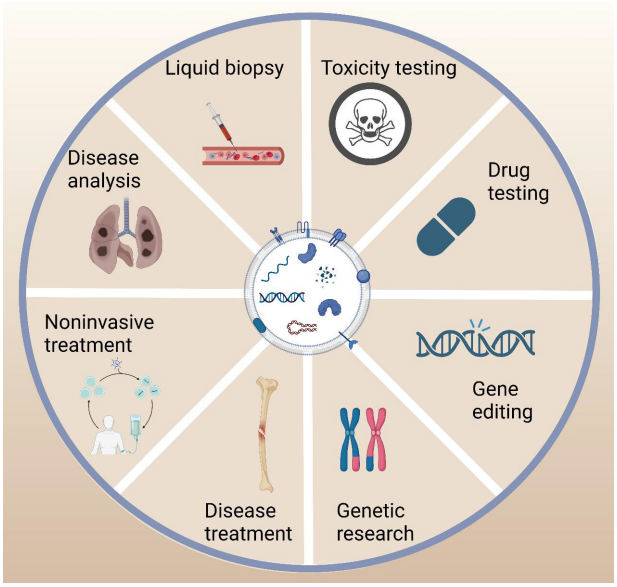

Figure 5. The application diagram of organoid extracellular vesicles (OEVs). OEVs have a wide range of applications, including liquid biopsy, pharmacological testing, toxicity testing, disease treatment, customised personalised medicine, genetic research. Created with BioRender.com.

inflammatory response of multiple immune cells.[72] OEVs from organoids derived from murine intestinal crypt stem cells exhibit pronounced immunomodulatory capabilities (**Figure 6A**). Notably, OEVs inhibit lipopolysaccharide-triggered cytokine production in immune cells. However, this immunomodulatory function is susceptible to suppression by opiate drugs. Specifically, upon intervention with opioid analgesics on intestinal organoids, the immunomodulatory prowess of OEVs becomes nullified.[73, 74] Through microarray analysis, a multitude of microRNAs, particularly Let-7 (an inflammation-regulating factor), were found to regulate OEVs-mediated immune regulation.

In *in vivo* experiments, Zhang et al.[72] found that injection of EVs derived from intestinal organoids significantly reduced lipopolysaccharide-induced systemic inflammation and improved the symptoms of dextran sulfate sodium-induced colitis. Similarly, EVs derived from intestinal organoids under morphine treatment failed to suppress immune responses. Intestinal organoids are essential *in vitro* tools that bring new research opportunities to intestinal stem cell research. Watanabe et al.[75] used intestinal organoids to treat inflammatory bowel disease. The medical team at Tokyo Medical and Dental University in Japan announced that they successfully completed a colon transplant by using tissue taken from the patient's intestine to create organoids to treat inflammatory bowel disease.[76] This is the first time in the world that organoid technology has been used to perform transplantation for patients with the refractory disease ulcerative colitis. We have reason to believe that EVs derived from intestinal organoids will also be a powerful weapon in the treatment of inflammatory bowel disease. In the future, the combination of organoids and EVs derived from intestinal organoids may achieve better therapeutic effects.

Organoid extracellular vesicles for epithelial repair

Radiation therapy can cause significant damage to the salivary glands (SGs).[76, 77] Stem cell technology stands as a prospective strategy for repairing SG injuries. Adine et al.[78] has employed a magnetic 3D bio-assembly platform to construct SG-like organs (SGOs), aiming to introduce novel therapeutic approaches for SG injury restoration. However, subsequent experiments revealed suboptimal reparative outcomes of SGOs, achieving only 25% efficacy.

In addition, during the cultivation of SGOs, it was observed that conditioned media obtained by continuous centrifugation of the cultures could yield oligodendrocyte-derived EVs derived from SGOs (**Figure 6B**). These EVs derived from SGOs were validated through nanoparticle tracking analysis, transmission electron microscopy, and Western blot analysis. Chansaenroj et al.[79] found that EVs derived from SGOs had significant effects on SG injury, specifically stimulating SG epithelial cell mitosis and promoting the growth of associated neurons with an efficacy of 60% (**Figure 6B**). To a certain extent, the therapeutic potential of OEVs greatly surpasses that of organoids.

Engineering Methods for Modifying Organoid Extracellular Vesicles

Although OEVs have better physiological effects than EVs, OEVs still have limitations, such as lack of organ targeting.80-82 Currently, numerous articles have reported that modified EVs can endow them with more powerful functions.[83-86] Given the similarities between OEVs and EVs, engineering strategies can also be used on OEVs to enhance their functionality and achieve specific goals. Here, we summarise different engineering approaches including engineering parental cells to create therapeutic OEVs and engineering OEVs after isolation (**Figure 7** and **Table 2**).[11, 53, 87-90]

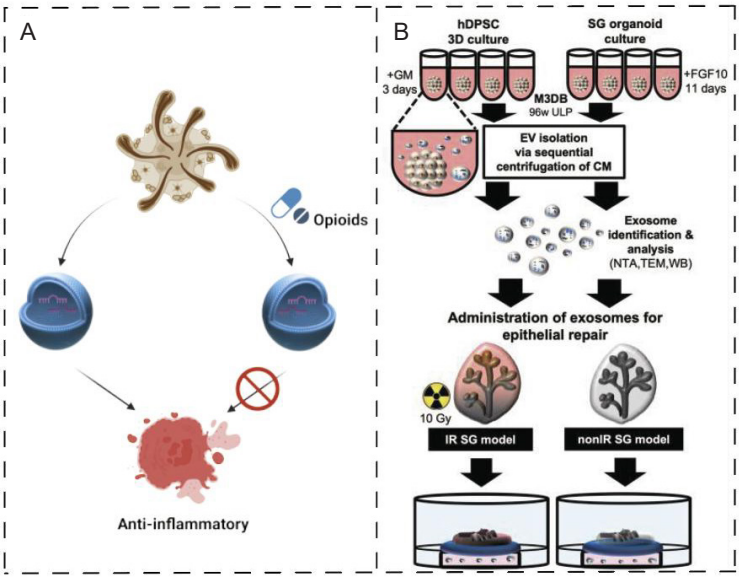

Figure 6. Organoid extracellular vesicles (OEVs) for disease treatment. (A) OEVs secreted by intestinal organoids can exert anti-inflammatory effects, while the anti-inflammatory effects of secreted OEVs are lost after the use of opioids acting on organoids. Created with BioRender.com. (B) Cultivation and collection of SG-like organ (SGO) by magnetic 3D bio-assembly (M3DB) system for the treatment of radiation-induced epithelial damage. Reprinted from Chansaenroj et al.[79] 3D: three-dimensional; 96w ULP: 96-well ultra-low attachment plate; CM: conditioned media; EV: extracellular vesicle; FGF10: fibroblast growth factor 10; GM: growth media; hDPSC: human dental pulp stem cell; IR: irradiated; NTA: nanoparticle tracking analysis; SG: salivary gland; TEM: transmission electron microscopy; WB: Western blotting.

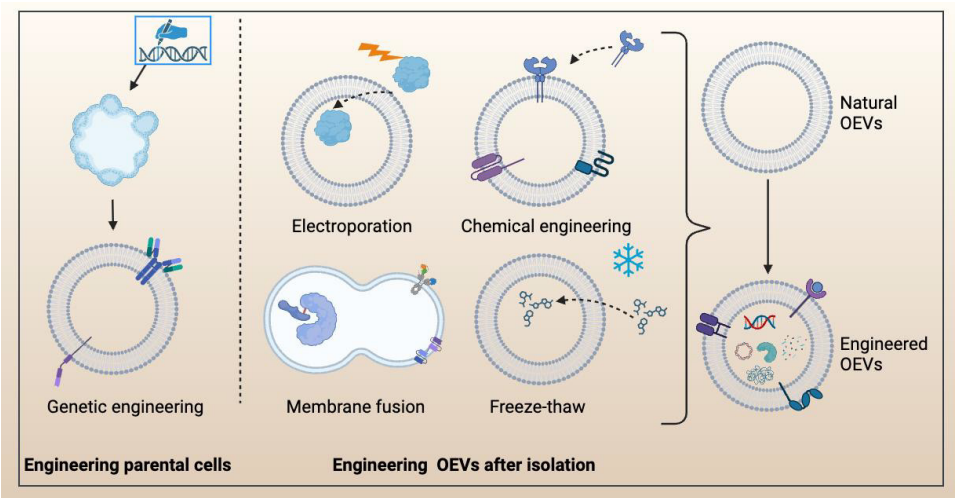

Figure 7. The engineering approaches for modifying organoid extracellular vesicles (OEVs), including the engineering parental cells and engineering OEVs after isolation. Engineering parental cells, such as using clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9 (CRISPR-Cas9) to modify cells to obtain engineered organoids. Engineering OEVs after isolation are mainly Electroporation, chemical engineering, membrane fusion, and freeze thaw. Created with BioRender.com.

Engineering parental cells

When parental cells are genetically engineered to overexpress proteins, secreted EVs can also carry such proteins. For example, Hu et al.[53] genetically engineered NIH-3T3 cells to generate engineered MEVs with C-X-C motif chemokine receptor 4 (CXCR4) on their surface (**Figure 8A**). Moreover, Liu et al.[11] used synthetic biology methods to construct probiotic *Escherichia coli* Nissle 1917 (ECN) containing recombinant plasmid pClyA-CXCR4 (ClyA, a bacterial surface protein,[91, 92] thereby obtaining a large number of engineered bacterial EVs (BEVs) displaying CXCR4 on the membrane surface (**Figure 8B**). In addition, organoid can also be genetically modified to generate engineered OEVs with powerful functions.

Table 2. Engineered retrofit solutions for organoid extracellular vesicles

Engineering approach	Strategy	Method	Purpose	Reference
Engineering parental cells	Genetic engineering	Direct modification of parent cells	The protein was displayed on the surface of extracellular vesicles to enrich its physiological function	53
	Synthetic biology	Shuttle plasmid	Giving new functionality to bacterial extracellular vesicles	11
Engineering after isolation	Membrane fusion	Co-incubation	Loading of exogenous cargo into the membrane	87
	Chemical engineering	Non-covalent reaction	Increased extracellular vesicles targeting	88
	Chemical engineering	Click chemistry	Loading of azides onto the membrane surface	89
	Freeze-thaw	Freeze-thaw cycle	Loading extracellular vesicles with exogenous substances and ensuring normal morphology	90
	Electroporation technique	High voltage electric field	Transfer of DNA, or/and RNA into extracellular vesicles	11

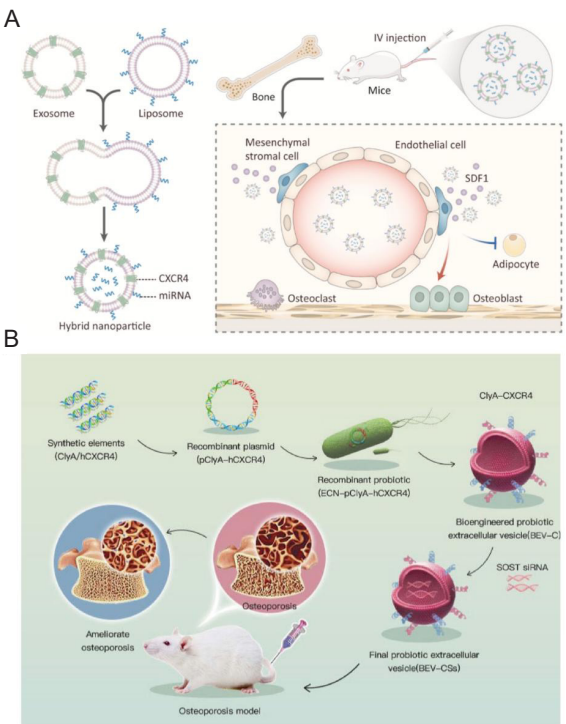

Figure 8. Engineering parental cells to endow their extracellular vesicles (EVs) with powerful functions. (A) Schematic illustration of exosome-guided microRNA (miRNA) blocking. Reprinted from Hu et al.[53] (B) Schematic illustration of the construction of bioengineered bacterial EVs (BEVs). Reprinted from Liu et al.[11] Copyright 2023, with permission from Elsevier. BEV: bacterial extracellular vesicle; BEV-C: BEVs-hCXCR4; BEV-CS: BEVs-hCXCR4-SOST siRNA; ClyA: A bacterial surface protein; CXCR4: C-X-C motif chemokine receptor 4; ECN: construct probiotic *Escherichia coli* Nissle 1917; hCXCR4: human C-X-C motif chemokine receptor 4; IV: intravenous; p: plasmid; SDF1: stromal cell-derived factor 1; siRNA: small interfering RNA; SOST: sclerostin.

Engineering organoid extracellular vesicles after isolation

Membrane fusion

OEVs with a phospholipid bilayer structure can spontaneously fuse with other phospholipid bilayer materials, thereby endowing OEVs with new functions.[87, 93, 94] In general, EVs can fuse with liposomes after 12 hours of incubation at 37°C.[87] Moreover, polyethylene glycol can accelerate the fusion of EVs and functionalised liposomes.[95] Chen et al.[96] used membrane fusion technology to construct BEVs-cancer EVs hybrid membranes and achieved tumor targeting and immunogenicity. Lin et al.[87] constructed hybrid nanoparticles for delivering the CRISPR-Cas9 system to MSCs by incubating fused MEVs and liposomes. The same principle can be applied to induce fusion between OEVs and liposomes, thereby augmenting the functions of OEVs.

Chemical engineering

Covalent and non-covalent reactions are commonly employed techniques to modify the membrane surface of EVs.[97, 98] This modification strategy is equally applicable to OEVs. For instance, direct co-incubation utilizing hydration forces can integrate targeting peptides into the phospholipid bilayer of OEVs, enhancing their targeting capabilities.[88] Furthermore, the amine properties on the membrane of OEVs can be modified with alkyne groups and click chemistry can be employed for surface modification of OEVs. When alkyne groups label the amines on the OEVs' membrane, copper-catalysed azide-alkyne cycloaddition reactions can impart new properties to the EVs' membrane by orthogonal reaction with azide compounds.[89]

Freeze thawing

The freeze thawing approach is a straightforward method for loading exogenous substances into OEVs. For example, Haney et al. [99] constructed a new MEVs-based delivery system to treat Parkinson's disease. Catalase was loaded into MEVs *ex vivo* using incubation, freeze-thaw cycles, sonication, or extrusion. In addition, Hajipour et al.[100] isolated MEVs from uterine fluid and loaded human chorionic gonadotropin by freeze-thaw cycle and sonication methods. Shi et al.[90] have evaluated six drug loading methods, including incubation, sonication, extrusion, freeze-thaw cycles, saponin-assisted, and electroporation method, for milk derived MEVs drug delivery. Drug-loaded MEVs obtained through freeze-thaw cycles showed minimal morphological changes.

Electroporation technique

The electroporation technique involves applying high-intensity electric fields to transiently enhance the permeability of the phospholipid bilayer, facilitating the uptake of exogenous substances from the surroundings.[101] Hence, electroporation technique enables the introduction of DNA, RNA, proteins, and more into the membrane of OEVs. An advantage of electroporation lies in its ability to preserve the physical characteristics of small interfering RNA during the transfer process.[102] Alvarez-Erviti et al.[102] loaded exogenous small interfering RNA into brain-targeted MEVs (Lamp2b-MEVs) through electroporation for the treatment of brain diseases, such as Alzheimer's disease. Moreover, Liu et al.11 also used electroporation to load exogenous small interfering RNA into bone-targeted BEVs (BEVs-CXCR4) for the treatment of bone diseases such as osteoporosis.

The Potential Role of Organoid Extracellular Vesicles in Bone Therapy

Due to the limited development of organoids and OEVs, the therapeutic applications of OEV (especially OEV-derived from bone organoids) in the treatment of bone diseases still need to be further explored. However, the existing research on MEVs and BEVs in the treatment of bone diseases has laid the foundation for the treatment of OEVs in bone diseases.[54, 103-105] Importantly, OEVs have surpassed traditional MEVs in quantity and physiological effects, making them more suitable for therapeutic applications in disease treatment.[56] Therefore, OEV-based bone therapies, including the natural OEVs and engineered OEVs, have huge potentials.

The nanoscale size of OEVs as well as their cell-free properties and high safety make them excellent nanocarriers.[85, 106] Natural OEVs have been shown to have promising therapeutic effects against complex diseases.[68, 79, 107, 108] In addition, engineering natural OEVs can endow them with specific organ targeting and stronger therapeutic effects. For example, loading targeting elements on the membrane surface of OEVs can enhance specific targeting. In addition, encapsulating therapeutic agents in OEVs not only enhances drug stability and extends half-life *in vivo* but also reduces the risk of adverse effects.

However, realising the applications of OEVs for bone disorder treatment presents challenges that need to be surmounted, such as organoid preparation, OEV isolation, engineering modifications, drug loading, and release mechanisms. As advances in organoids and OEVs continue, we can foresee that the use of OEVs to treat bone diseases will lead to more effective and personalised treatment options, ultimately improving bone health and improving overall quality of life.[109, 110]

Advantages and Challenges

OEVs represent a novel technological branch that extends from the development of organoid technology. Conceptually aligned with MEVs, BEVs, and plant derived EVs, OEVs are nanoscale vesicles released by living cells into the extracellular environment. Particularly, the similarities between OEVs and MEVs in terms of biological mechanisms are striking. However, the advantages of organoids (similar spatial structure to human tissue) compared to MEVs endow OEVs huge advantages in terms of yield and functionality.[69, 111, 112] Moreover, OEVs inherit the low immunogenicity and efficient crossing of biological barriers of MEVs, thereby showing broad prospects in medical applications and drug delivery vehicles. Furthermore, OEVs have the potential to be engineered to enhance their targeting specificity and confer complementary physiological functions, thus enhancing their potential applications.

Nevertheless, OEVs still face several obstacles in moving them from lab to clinic. The biggest obstacle to bring OEVs from lab to clinic is the construction of organoid. As organoid models are still in their nascent stages, currently generated organoids predominantly exhibit singular functions and lack comprehensive functional integration. For example, currently reported bone organoids mainly represent isolated bone functions, such as bone formation, resorption, or haematopoiesis, rather than integrated multifunctional bone organ systems.[26, 113] These divergences prevent OEVs from perfectly replicating the diversity of human cells. Furthermore, while OEVs possess a pronounced production advantage over MEVs, they still cannot meet the demands of research and clinical applications. In addition, OEV extraction methods lack standardised standards, and their mechanism of action remains unclear. Here, we summarise the advantages and challenges of OEVs (**Figure 9**).

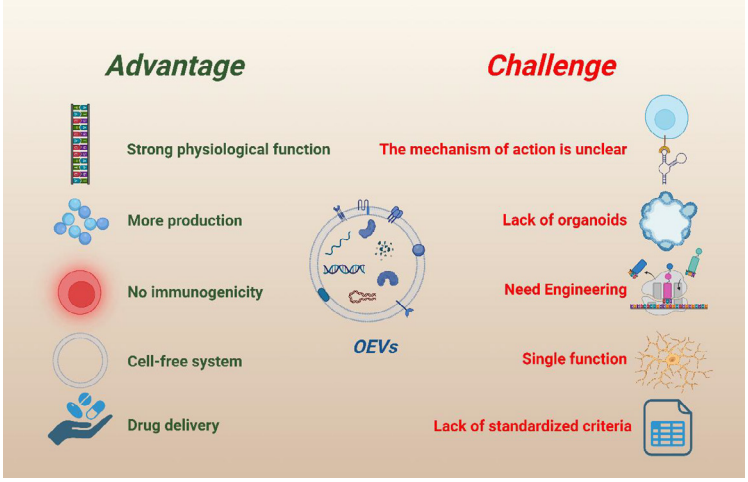

Figure 9. Advantages and challenges of organoid extracellular vesicles (OEVs). OEVs have the advantages of strong physiological function, high yield, low immunogenicity, cell-free system, and good delivery potential. At the same time, OEVs also have several obstacles, including unknown functional mechanism, lack of source, need engineering transformation, single function, and lack of standardised extraction course. Created with BioRender.com.

Conclusions and Perspectives

OEVs and traditional EVs are essentially derived from mammalian cells. Therefore, the biogenesis, internalisation, isolation, and characterisation of OEVs are similar with that of MEVs. However, OEVs exceed traditional MEVs in terms of quantity and physiological effects. Therefore, OEVs are more suitable for therapeutic applications in disease treatment. Subsequently, the applications of natural OEVs in disease treatment, such as immunomodulation and epithelial repair, have been summarised. Although OEVs have better physiological effects than EVs, OEVs still faces several obstacles. We then summarise the engineering modifications of OEVs, including engineering parental cells and engineering OEVs after isolation (membrane fusion, chemical engineering, freeze thawing, and electroporation technique). Furthermore, the potential of natural and engineered OEVs in bone-related diseases is prospected. Finally, the advantages of OEVs (including strong physiological function, high yield, low immunogenicity, cell-free system, and good delivery potential) and challenges of OEVs (including unknown functional mechanism, lack of source, need engineering transformation, single function, and lack of standardised extraction course) have been critically discussed. The comprehensive discussion of OEVs will provide more innovative and efficient solutions for complex bone diseases (**Figure 1**).

Although the research on organoids is very hot, there are only few studies on OEVs, and there are almost no applications of OEVs in diseases, especially bone-related diseases. In essence, we hope that this review can propose a novel concept, where OEVs constitute a powerful new paradigm in the field of biomedical research and provide new therapeutic avenues for a variety of complex diseases, especially bone diseases. With the continuous improvement of organoid technology and the construction of multifunctional integrated organoids, we will be able to highly simulate the physiological environment and functions of the human body *in vitro*. This will also help extract more effective OEVs to meet the needs of disease treatment. There is no doubt that the potential of OEVs will be further demonstrated in the future to benefit patients. Despite ongoing challenges, the field of organoids and OEVs have significant progress in recent years, driving the realisation of clinical applications.

Author contributions

HL conceptualised the review and drafted the manuscript; JS checked and revised the manuscript. Both authors reviewed and approved the final version of the manuscript.

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Conflicts of interest statement

The authors declare no conflict of interest.

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