Bone-a-petite: Engineering Exosomes towards Osteochondral Repair

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Abstract

Recovery from osteochondral diseases and injuries has always been burdensome owing to the avascular nature of cartilage leading to a lack of nutrients and supplying cells. However, traditional means of treatment such as microfractures and cell-based therapy only display limited efficacy due to the inability to ensure cell survival and potential aggravation of surrounding tissues. Exosomes has recently emerged as a powerful tool for osteochondral repair with its complex content of transcription factors, proteins, and targeting ligands, as well as its unique ability to home in on

target cells thanks to its phospholipidic nature. They are engineered to serve specialized applications including enhancing repair, anti-inflammation, regulating homeostasis, etc *via* means of physical, chemical and biological modulations in its deriving cell culture environments. This review focuses on the engineering means to functionalize exosomes for osteochondral regeneration, with an emphasis on conditions such as osteoarthritis, osteoporosis and osteonecrosis. Finally, future implications for exosome development will be given alongside its potential combination with other strategies to improve its therapeutic efficacy in the osteochondral niche.

1. Introduction

Despite being one of the most frequently used structures in the human body, the osteochondral region cannot recover fully from injuries, diseases or wear due to its lack of self-regenerating capabilities attributed to the absence of blood supply and regenerative moieties [1, 2]. Traditionally, this can be circumvented through surgical techniques like microfractures, which involves drilling into subchondral bone to create influx of mesenchymal stem cells (MSCs) to the defect site to secrete intrinsic signals [3]. However, this treatment method damages the subchondral bone, and often only forms fibrocartilage with reduced smoothness and flexibility instead of the native hyaline cartilage. Injectable/crosslinked polymers with minimum invasiveness made of natural biomaterials (e.g., collagen, gelatin, chitosan, hyaluronic acid) and/or synthetic materials (e.g., poly-L-glutamic acid, polyethylene glycol, polyethylene oxide) are therefore developed which allows for encapsulating native cells such as chondrocytes and MSCs and/or growth factors for osteochondral repair [2, 4-10]. However, going forward, these approaches will face many challenges: (1) crosslinking mechanisms of cell-laden hydrogels are highly limiting as strong heat or UV light can damage the encapsulated cells, (2) autologous chondrocytes commonly used clinically have been reported to be ineffective in elderly patients, and are difficult to procure in large quantities,

(3) the allogenic potential of chondrocytes/MSCs (over 100 types) have not been fully realized, requiring much time in clinical trials to prove the safety and efficacy of cell-laden hydrogels using non-autologous sources [11], and (4) the short half-life and poor intracellular delivery of growth factors limits their potential for the long-term healing of osteochondral injuries.

To overcome these limitations, scientists have long since delve into studying exosomes, a form of extracellular vesicles, for tissue engineering purposes as they originate from living cells and contain bioactive components to modulate recipient cell behaviours, all the while exosomes are non-living and do not require external maintenance or assistance to internalize with host cells [12, 13]. Originated from exocytosis by living cells, their small size (30 – 150 nm in diameter) and unique bilayer phospholipidic shells allow them to home in and fuse with distant cell membranes seamlessly to fulfil their role as messengers of cell-cell interactions [14]. Their envelopment of important biomolecules including growth factors, transcription factors, cytokines as well as ease of modification with other engineered molecules grants them cell-esque carrier properties. Their ease of penetration into cells has been widely explored in the biomaterials field for various therapeutic delivery strategies, while their bioactivity has been demonstrated by many studies where exosomes mediate native proliferation, immune reactivity, and attenuate apoptosis (**Figure 1**). Exosomes have been experimented with many animal models for the treatment of osteochondral injury, osteoarthritis, osteoporosis and osteonecrosis with enhanced osteochondral proliferation and formation of integrated cartilage tissues with host tissues [15-17]. Alongside their ease of application to patients as well as easily quantifiable dosage, exosomes are an indispensable bridge between biological and engineering strategies to treating osteochondral repair.

Currently, comprehensive mapping of exosome trafficking mechanism, content and application have been reported, whereas there remains a lack of reviews on upscaling exosome production and specializing exosomal application towards one specific niche. In this review, we

will focus on novel approaches to engineering exosomes at the cellular level towards osteochondral regeneration, including mechanical, biological and chemical cues for upscaling the inherently small-scale production of exosomes for clinical relevance. We will critically discuss the use of exosomes for treatment of model osteochondral conditions and diseases including osteochondral injury, osteoarthritis, osteoporosis, osteonecrosis, and offer implications as to how this field should progress.

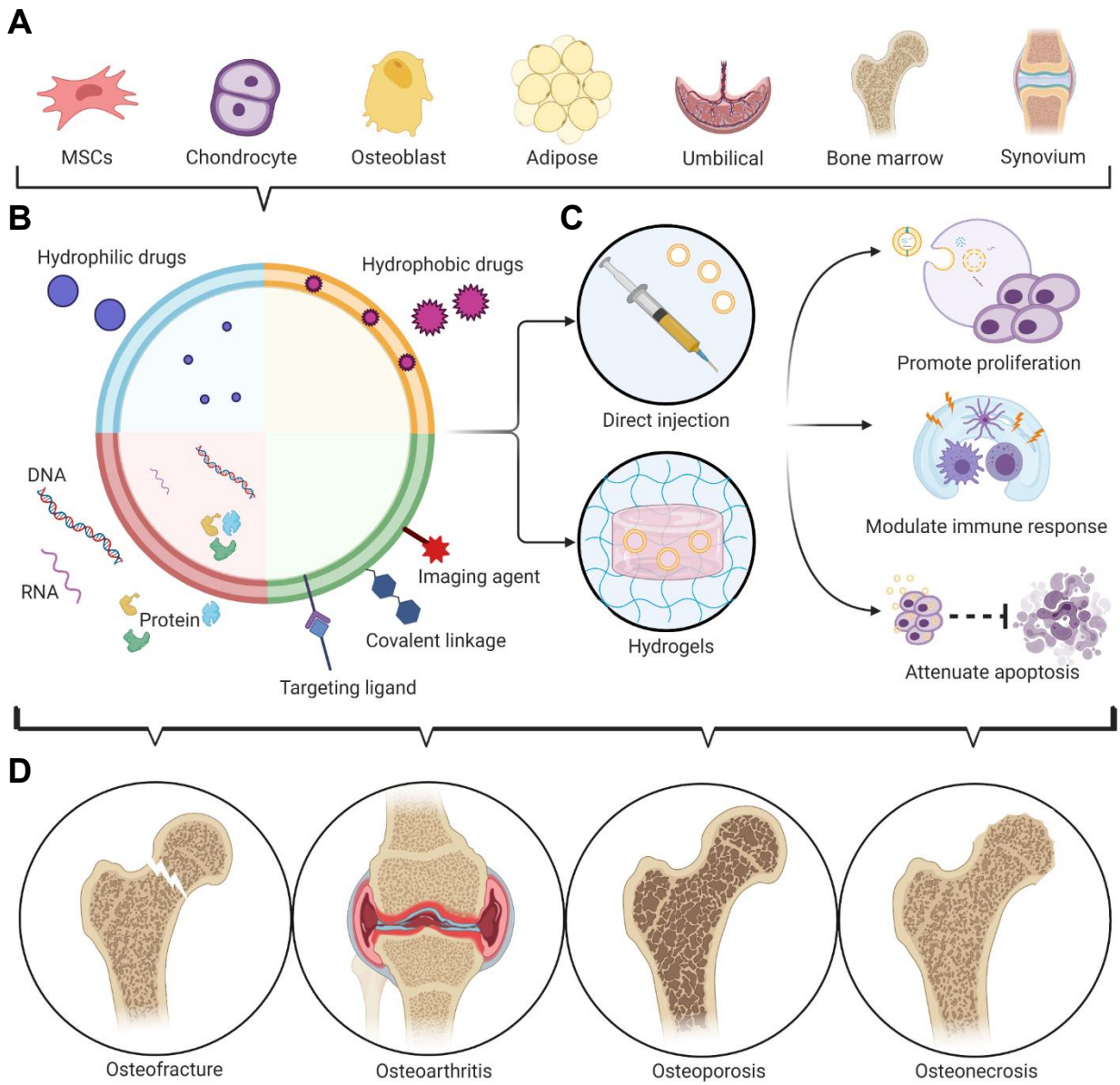

Figure 1. Application of exosomes for osteochondral repair. (A) Sources and exosome-deriving cells researched. (B) Contents of a typical exosome. (C) Application of exosome to defect sites, achieving promotion of cell proliferation, modulation of immune response and attenuation of apoptosis. (D) Osteochondral conditions in the limelight in the field of exosome research. Figures created with BioRender.com.

2. Construction of Exosomes

Through double invagination of plasma membrane, exosomes are secreted through exocytosis and can be taken up and infused into distant cells to elicit biological responses [18]. Extensive research has been put into studying exosomes from various sources including MSCs, monocytes, macrophages and endothelial progenitor cells, which were shown to polarize immune cells, osteoblasts, osteoclasts, chondrocytes and endothelial cells (ECs) into favourable phenotypes for osteochondral regeneration. These physiological changes can be credited to the contents of exosomes, which contain proteins, metabolites and nucleic acids, allowing mediation over inflammatory response, gene expression and induction of signaling pathways via embedded surface ligands [19].

Normally, exosomes are found in abundance in body fluids including saliva, blood and milk, which can be extracted with representation of tissue-specific molecules and serve as disease-specific markers [20]. Established exosome isolation protocols include ultracentrifugation and charge neutralization-based precipitation, both with their distinct advantages and drawbacks [21, 22]. Ultracentrifugation is the simplest and obtains the purest exosomes, but the yield is low (~30% of total isolation kits) and the process is highly time-consuming (3 – 4 h). On the other hand, precipitation-based methods have greater efficiency at exosome isolation (1.5 – 2 h), but the purity is hampered by the co-precipitated proteins alongside exosomes, evidenced by a reduction in zeta

potential of retrieved exosomes. More novel approaches to extraction of exosomes focus on improving extraction speed or yield of exosomes from serum. For example, Shi et al. developed an insulator-based dielectrophoretic (DEP) method for non-specific and rapid entrapment of exosomes. Utilizing the dielectric properties of nano-vesicles, exosomes were trapped and pre-concentrated from plasma of healthy human subjects using the charged nanopipette DEP device in 2 minutes [23]. Results demonstrated that the pipette pore size did not significantly affect isolation efficiency, but different geometry of the same pore size yielded inconsistent results. This shows that there are still underlying factors affecting the exosomes extraction using charge-based methods. On the other hand, immunoaffinity has been experimented with microfluidic approaches by coating a microfluidic chip with exosome capturing antibodies. This allows for highly specific capture of dedicated exosomes through modification of immobilized antibodies. As a proof-of-concept, Tayebi et al. developed a microfluidic chip with microbeads coated with anti-EpCAM and anti-CD63 antibody for hydrodynamic trapping of exosomes in micro channels [24]. The microbeads were first pumped into the microfluidic channels and fixed in the trap mechanism with over 99% efficiency for further collection of exosomes. The localized exosomes collected at the microbeads were then characterized by Western Blot to compare the protein expressions of hydrodynamic trapped exosomes and exosomes extracted using an isolation kit (precipitation-based). Microfluidic chip-extracted exosomes exhibited elevated levels of flotillin-1 and CD63 proteins, suggesting that the exosomes have improved purity compared to commercial extraction kits. These results give exciting prospects for small-scale extraction of multiplexed exosome capture with biomarkers for advancing the field. However, these methods of extraction are size-restricted and not scalable due to their unique reliance on delicate fluid mechanics, and are thus confined to a laboratory setting for research purposes. Henceforth, methods of upscaling exosome production at a cellular level will be illustrated in the following sections **(Figure 2)**.

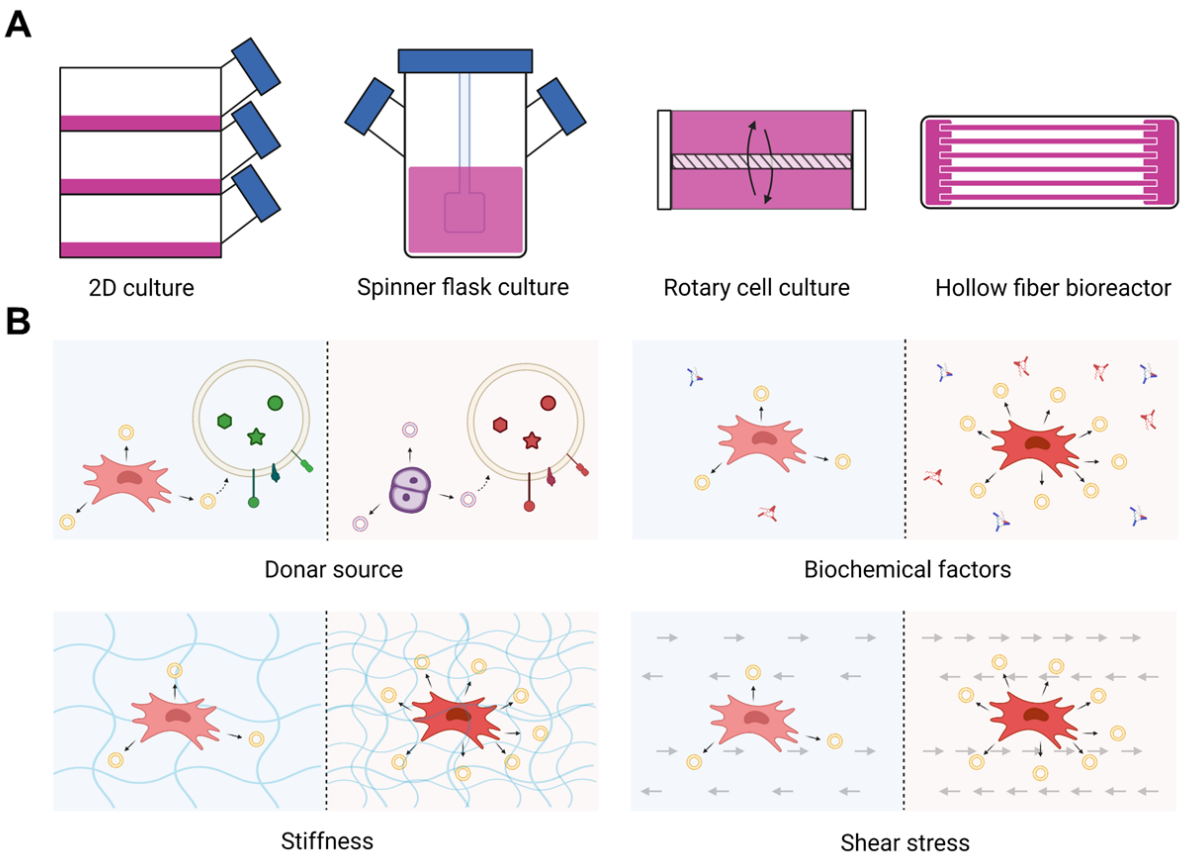

Figure 2. Factors affecting the production rate and quality of exosomes. (A) Culture methods of exosome-deriving cells. (B) External parameters used to upscale exosome production. Figures created with BioRender.com.

2.1 Improved exosome production via biochemical factors

Studies have revealed that the originating cell type regulates the content of secreted exosomes [12]. For instance, selection of cell types determines if the generated exosomes are pro-inflammatory (cancer cells, macrophages, intestinal epithelial cells) or anti-inflammatory (MSCs, dendritic cells), as well as controlling their effectiveness in affecting behaviours of different cell phenotypes. Additionally, exosome-secreting cells respond promptly to environmental changes to alter the encapsulated contents of secreted vesicles, which is an essential process in regulating proper

immune response and achieving homeostasis. Their sensitivity has since been utilized via various stimuli at the cellular level in promoting and specializing exosome production, and to engineer exosomes with a wide spectrum of molecules to favour tissue regeneration **(Table 1)** [25]. This line of approach has more potential for upscaling production of exosomes since the stimuli introduced in the external environment are tunable and quantifiable, while the stimuli-induced exosomal alteration of proteins and nucleic acids can be identified through biopsy of exosome-containing serums. For example, Xu et al. synthesized exosomes through priming MSCs with lipopolysaccharides (LPS), a common pro-inflammatory factor [26]. The derived exosomes were able to significantly attenuate inflammation in Raw264.7 cells under LPS stimulation without influence on cell viability and apoptosis. Western Blot assay revealed that the phosphorylation activation of AKT1 and AKT 2 were excited in M2 and M1 macrophages respectively via the NF-κB signaling pathway, which subsequently downregulated the typical inflammatory factors TNF-α, IL-6 and IL-1, leading to a reduction in inflammation. On the other hand, Domenis et al. attempted to pinpoint the inflammatory stimulation by introducing IFNγ and TNFα inflammatory cytokines to adipose MSCs for upscale exosome production [27]. While the number of exosomes released by adipose MSCs was unchanged, they became more potent at inhibiting T-cell proliferation, as well as up-regulation of immunomodulation genes such as HLA-DRA, CD274B7, IDO, VCAM1, ICAM2, and chemokines such as CCL8, CXCL9 and CXCL10. Additionally, the heightened production of PGE_2, IL-10 and CCL-2 are critical to chemotaxis of monocytes and recruits them to proximity of MSCs, where immunosuppressive factors were directly produced upon and leading to inhibition of inflammation. This dual synergy of immunomodulation by exosomes and small molecule cytokines may offer insight to intracellular communications. In addition to inflammatory factors, regulatory proteins such as bone morphogenetic protein-2 (BMP-2), a protein which potently induces osteoblast differentiation of many cell types, were also

reported to be effective at simulating exosome production. In one study, Wei et al. synthesized exosomes from BMP-2-stimulated macrophages, and discovered that compared with nonactivated macrophages, only exosomes from stimulated macrophages could induce osteogenesis in MSCs [28]. This can be attributed to immune cell-derived exosomes carrying modulatory RNA cargo, which contribute significantly as cellular regulator for osteogenic differentiation of MSCs. In particular, miR233 can stimulate microvesicle secretion, and was shown to modulate behaviour of recipient cells. This interaction may be the missing link between the immune cell stimulation and heightened osteogenic differentiation in osteochondral tissues. In another study, lithium ions were demonstrated to modulate bone marrow stromal cells to secrete exosomes with pro-angiogenic potential [29]. The activation of AKT pathway by these exosomes resulted in upregulation of pro-angiogenic genes in HUVECs, as well as heightened proliferation and tube formation, which may offer implications for recovering from large bone defects. Taken together, tutoring exosome progenitor cells using biochemical factors for generation of enhanced exosomes is a promising strategy for anti-inflammatory treatment.

Table 1. Biochemical factors stimulating production and quality of exosomes.

Exosome source	**Biochemical factor**	**Effect on exosome**	**Reference**
MSCs	Lipopolysaccharides	Anti-inflammation	[26]
Adipose MSCs	IFNγ, TNFα	Monocyte recruitment, anti-inflammation	[27]
Macrophages	BMP-2	Osteogenesis	[28]
BMSCs	Lithium ion	Angiogenesis	[29]
Osteocytes	Myostatin	Osteoblastic differentiation inhibition	[30]

2.2. Improved exosome production via mechanical factors

Mechanical stimulus such as matrix-mediated signals, tensile forces and stiffness has been widely known to affect cell fate [31]. For instance, varied degrees of stimulus will lead stem cells towards particular cell types and function. However, in recent years, it has also been revealed that mechanical cues are able to alter both the quantity and quality of exosome production in secreting cells. For example, Yan et al. synthesized exosomes from umbilical cord MSCs using a rotary cell culture system [32]. The induction of shear stress on the cell membrane increases its surface tension and contraction, which contributes towards exocytosis and trafficking of extracellular vesicles [33, 34]. Through fine-tuning various rotary speeds, they discovered that the rotary system greatly enhanced exosome production of individual MSCs at 36 rpm/min within 196 h. In particular, the expression of LncRNA H19 was highly upregulated, greatly improving the chondral regenerative activities when co-cultured with human chondrocytes. Meanwhile, Kim et al. explored the possibility of utilizing cell-cell interactions for improved exosome production, and cultured MSCs in 3D spheroids [35]. MSCs cultured in spheroids exhibited non-adherent and rounder morphology. Compared with their 2D counterpart, spheroid MSCs significantly increased in production by over 3-folds. Interestingly, the smaller the size of spheroids, the better the efficiency of exosome production became with the same number of cells. Due to the reduction in F-actin expression and tension, the cells exhibited round, non-adherent morphology and were able to dock at favourable fusion site, leading to the elevated exosome production. To more efficiently simulate the 3D culture, Yan et al. cultured MSCs in circulating hollow-fiber bioreactors to improve biological functions and yield of exosomes for cartilage repair [36]. Compared to 2D exosomes cultured in flat flasks, which displayed inferior cartilage surface regularity with morphological disruptions, bioreactor-cultured exosome groups displayed smooth surface, and better integration with the surrounding hyaline cartilage after being applied to rabbit models for 4 weeks. Hollow-fiber bioreactors possess

greatly enhanced volumes of culture medium, can efficiently exchange nutrients, and support the long-term growth of high-density cell cultures attached onto porous hollow fiber supports. The prolonged cell-cell interactions resulted in vastly increased exosome production (7.5 fold of 2D culture flasks). These promising results provided us with great implications for large-scale production of exosomes for tissue engineering purposes.

2.3 Modification of exosomes for specialized applications

While exosomes can be engineered at a cell level through biochemical and physical cues to enhance their efficacy at regeneration, there are tissue engineering tools such as fluorescent probes and siRNAs that cannot be produced naturally within cells [37]. This greatly limits native exosome's clinical potential for *in vivo* imaging and gene therapy. To imbue exosomes with theranostic properties, these molecules must be functionalized into/onto exosomes artificially to attain imaging and gene knockdown capabilities. That is, to combine diagnosis with therapy within one single strategy through engineering of exosome structures.

Exosome cytoskeleton is highly deformable with proteins embedded within the phospholipid bilayer. The abundance of amine groups and alkyl groups on these proteins allows for surface modification of biological macromolecules to its surface by covalent bonds. For instance, to increase targeting of recipient cells, Luo et al. functionalized exosomes with bone marrow MSC-specific aptamers for bone regeneration [38]. Aptamers are oligonucleotides with high specificity and affinity towards selective sequences to recognition and conjugation. Their 5' end can be modified with aldehyde group to react with amino groups of exosome surface proteins by Schiff base reaction. Results showed greatly increased uptake of aptamer-functionalized exosomes into MSCs *in vitro* and accumulation in bone *in vivo*, which shows great potential for treatment of osteoporosis. Additionally, the lipid membrane bilayer can be used for passive loading of

hydrophobic therapeutics. One typical example is curcumin, a highly hydrophobic and unstable molecule in aqueous solvent which limits its clinical potential. To fully realize its antineoplastic, anti-inflammatory and antioxidant properties, curcumin can be loaded by mixing with exosomes and greatly increase in thermal stability and efficacy, successfully providing anti-inflammatory protection in mice models [39]. On the other hand, lumens of exosomes can act as immunotolerant nanocarriers for water-soluble therapeutics and transcription factors. B-cell-derived exosomes can be modified with miRNA-155 inhibitor to achieve gene knockdown and reduce TNF-α production for suppression of negative inflammatory response to diseases such as organ failure and osteonecrosis[40]. While diagnostic level of miRNA-155 is low in exosomes derived from stimulated B cells, electroporation in miRNA-loaded buffer allows for high capacity encapsulation. Taken together, exosomes are highly flexible nanocarriers with both exterior and interior engineerable structures for efficient delivery of small molecule drugs, siRNAs, miRNAs and surface ligands for osteochondral repair.

3. Application of exosomes in osteochondral treatment

3.1 Regeneration

As early as in 2013, researchers have acknowledged the potential of MSC-derived exosomes as therapeutic carriers of transcription factors, drugs and proteins for osteochondral regeneration. They have noted the biocompatibility and immunomodulatory properties of exosomes, but have yet to confirm the therapeutic potential of the extracellular vesicle itself[41]. Zhang et al. attempted to establish the treatment efficacy of human embryonic stem cell exosomes using osteochondral defects in rat models[42]. Exosomes were separated from the hMSC culture using tangential flow filtration, and sequentially filtered through membranes with 100 – 1000 kDa molecular weight cut off. After direct injection of 100 μg exosomes in 100 μL PBS into immunocompetent rat

osteochondral defect model with treatment of 100 μL pure PBS as control for 12 weeks, they observed that injection of MSC exosomes accelerated neo-tissue filling and matrix synthesis of type II collagen and sulphated glycosaminoglycan (s-GAG). By the end of 12 weeks, exosome-treated rats exhibited full recovery of cartilage and subchondral bone with distinct characteristics including a hyaline cartilage with good surface regularity, complete merging with adjacent cartilage, and an ECM deposition that resemble that of the healthy control. Contrarily, the group treated with saline only displayed fibrous repair in contralateral defects. This study unprecedentedly demonstrated the efficacy of human embryonic MSC exosomes in osteochondral regeneration, and the potential of MSC exosomes as an off-the-shelf and 'cell-free' treatment option compared with cell-based therapy. However, the exact regenerative mechanism of the exosomes was not illustrated, leaving the conclusion at a simple cause-and-effect relationship. Additionally, the allogenic potential of the exosomes remains unestablished, not to mention the difficulty in mass producing human embryonic MSCs. Hence, the same research group attempted to analyze the signaling and molecular pathways mediated by MSC exosomes and the mechanism of action behind such favourable responses when applied in cartilage repair **(Figure 3A)** [43]. It was observed that the exosome-mediated repair of osteochondral defects characteristically displayed improved cell proliferation and infiltration within the defect site, enhanced production of matrix proteins and a regenerative immune phenotype. By observing chondrocyte cultures treated with MSC exosomes, they associated the enhanced proliferation and infiltration with exosome CD73-mediated adenosine activation of AKT and ERK signaling. This was further evidenced by the suppression in therapeutic effects by inhibitors of AKT or ERK phosphorylation, as well as the attenuation of AKT and ERK signaling by CD73 inhibitor AMPCP and adenosine receptor antagonist theophylline. Improved infiltration of M2 macrophages than M1 encouraged osteochondral repair, while simultaneously lowering the risk factors for inflammation such as IL-

1b and TNF-α. Together, these observations demonstrated that the efficiency in osteochondral regeneration by MSC exosomes was modulated through a sophisticated network of cell signaling pathway spanning over multiple cell types. Going forward, Gong et al. attempted to elucidate the mechanism behind MSC's benefits towards both osteochondral and ischemic diseases through further characterization of bioactive molecules encapsulated in their derived exosomes **(Figure 3B)** [44]. They cultured spindle-shaped mice MSCs and collected the exosomes using centrifugation, ultra-filtration then precipitation. After introduction to HUVECs, the HUVECs exposed to exosomes exhibited increased tube length compared with HUVECs without MSC exosome exposure. Additionally, to demonstrate the effect of loaded microRNAs on angiogenesis, exosomes with miR-30b overexpressed or knockdown were obtained from MSCs, and transferred to HUVEC cultures. The expression of angiogenic miRNAs significantly increased following treatment with overexpressed exosomes but not in knockdown ones. These results illustrate that the treatment efficacy of MSC exosomes is mainly dependent on their miRNA content. Combining these concepts, Lee et al. transfected miR-140 into rabbit serum derived exosomes for enhanced cartilage repair and regeneration [45]. Exosomes were collected from rabbit serum, filtered and isolated through differential centrifugation at 19, 500 g for 90 minutes, after which the exosomes were loaded with miR-140 through freeze and thaw method. They were then introduced to rabbit bone marrow MSCs (BMSCs) and were found to induce differentiation of BMSCs into chondrocytes. Notably, loading of miR-140 greatly enhanced their efficacy at promoting cell proliferation and expression of differentiation-related genes. This suggests that the combination of well-established miRNA (e.g. miR-210, microRNA-149-5p) therapy with exosomes can be a promising strategy for promoting osteochondral regeneration.

3.2 Osteoarthritis

Osteoarthritis (OA), also known as degenerative arthritis, is a common type of chronic musculoskeletal disease, characterized by slowly progressing joint pain, swelling, stiffness, and limited joint activities. In the case of rheumatoid arthritis, disturbance in miRNA regulation was reported. miRNA17 was upregulated and inhibits induction of regulatory T cells, while miR-155-5p was overexpressed in OA patients, which could downregulate the inhibitory protein of inflammation (SHIP1) [46]. Owing to the underlying disrupted expression levels of transcription factors, biological treatments for OA such as proteins and antibodies only achieve limited therapeutic efficacy. Meanwhile, exosomes had shown promising results in anti-inflammatory treatment owing to their encapsulated content of transcription factors and engineerable structure for multifaceted therapy [47]. Notably, many studies demonstrated the anti-inflammation potential of MSC derived exosomes, and they are currently the gold standard for researching OA treatment. Zhang et al. investigated the role of MSC exosomes in the regulation of inflammatory response in rat model of temporomandibular joint OA **(Figure 3C)**[48]. In this study, human embryonic stem cell-derived MSCs were used to culture exosomes, and separated using tangential flow filtration. Through culturing chondrocyte model of OA (induced by IL-1β) with exosomes, they found that MSC exosomes were able to induce adenosine activation of AKT, ERK and AMPK pathways. Successful suppression of IL-1β were characterized by immunostaining and reduction in nitric oxide production. These results ultimately correlate with reduced expression of pain-associated genes in rats treated with MSC exosomes compared with no treatment, as well as smoother cartilage surface and improved cellularity at the defect site. Taken together, the results well illustrated the potential of unmodified MSC exosomes for OA treatment. On the other hand, MSCs can be transfected initially with transcription factors to produce modified exosomes with elevated gene expressions including siRNAs, miRNAs and lncRNAs[49]. Liu et al. transfected MSCs with lncRNA-KLF3-AS1 to synthesize exosomes with elevated KLF3-AS1 expressions for OA

treatment through the KLF3-AS1/miR-206/GIT1 axis, which greatly promoted the production of essential ECM proteins Col2a1 and aggrecan, while reducing MMP-13 and Runx2 expression, which are typical inflammatory markers [50]. Modified MSC exosome treatment in mouse chondrocytes exhibited attenuation of chondrocyte injury, while introduction of GIT1 knockdown exosomes significantly reversed the enhanced proliferation and apoptosis attenuation achieved by the KLF3-AS1-rich exosomes. This demonstrated that the miR-206/GIT1 axis was a promising mechanism for treatment of OA through cellular delivery of exosomal lncRNA KLF3-AS1. Meanwhile, cell types other than MSCs were also reported to be effective donors for modified exosomal treatment. Utilizing miR- 95- 5p transfected primary chondrocytes, Mao et al. fabricated miR- 95- 5p-rich and miR- 95- 5p-deficient exosomes as control in micromass culture for 14 days [51]. The chondrocyte-derived exosomes were then cultured with hMSCs and exhibited enhanced expression of cartilage-specific proteins including SOX9, COL2A1 and aggrecan through the acetylation of histone H3. Interestingly, downregulation of HDAC2/8, a typical marker of OA initiation and progression, was also observed. To study the potential for targeted gene therapy, the group also transfected siRNAs to downregulate expression of HDAC2/8 in exosomes, and found that the expression of the same cartilage-specific proteins significantly increased. These results suggest that the miR- 95- 5p-rich exosomes derived from chondrocytes could enhance chondrogenesis and prevent OA development by targeting HDAC2/8. However, further studies are required to fully elucidate the mechanism of action of utilizing genetically modified exosomes for clinical treatment of OA as molecular pathways are highly complex and often intersect with each other, where side effects to other cell types are hidden in focused pathological studies.

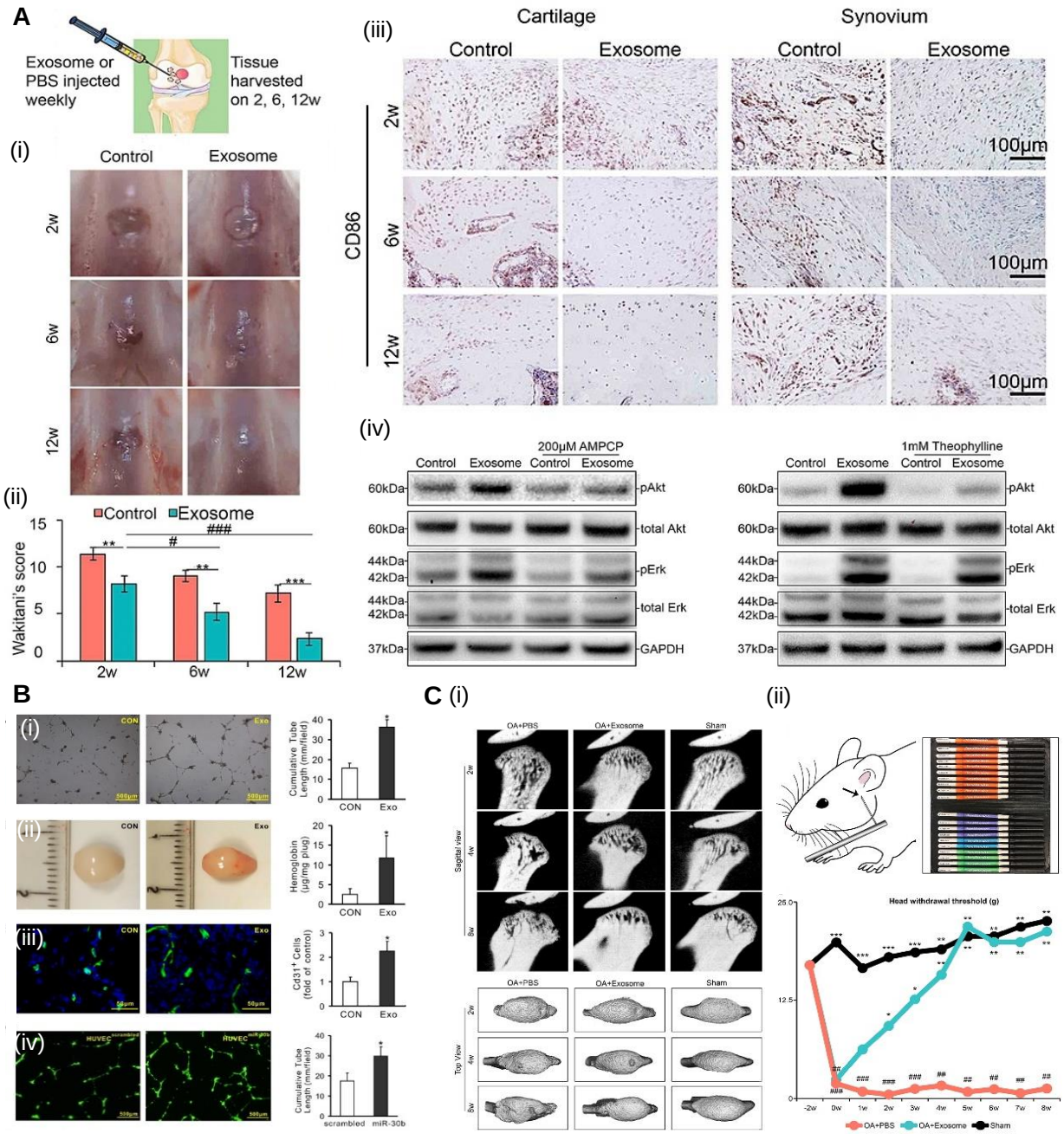

Figure 3. Application of exosomes on osteochondral regeneration and osteoarthritis treatment. (A) Injection of exosome in immunocompetent rat joint model to mediate cartilage repair. (i) Photographs of cartilage repair. (ii) Wakitani scores for histological cartilage sections. (iii) CD86 staining of cartilage and synovium in MSC exosome treated and control groups, indicative of M1 macrophage population. (iv) Western blot indicative of AKT and ERK signalling pathway

activation. Figure modified from [43] with permission. (B) Enhanced angiogenesis in newly forming vasculature by miRNA delivery of MSC exosomes (i) Microscopic image and evaluation of endothelial tube length. (ii) Photograph of endothelialized Matrigel plugs. (iii) Immunofluorescence staining and quantification of $CD31^+$ cells. (iv) Fluorescence image and quantification of endothelial tube length under miRNA or scrambled RNA treatment. Figure modified from [44] with permission. (C) Alleviation of osteoarthritis pain by MSC exosomes. (i) Micro-CT analysis of subchondral bone architecture in temporomandibular joint osteoarthritis rat model. (ii) Illustration and time-response of pain behavior assessment compared with healthy rats. Figure modified from [48] with permission

3.3 Osteoporosis

Osteoporosis is a long-term, age-related systemic bone-weakening condition. It poses a significant burden on an individual's quality of life and is a significant challenge in clinical management. Recent establishments have shed light on bone marrow-derived exosomes as a key regulator of the bone micro-environment, in which disruption in exosomal-regulated bone homeostasis can lead to conditions such as osteoporosis[52]. Since then, studies targeting the use of exosomes as a therapeutic method against the systemic bone weakening in osteoporosis have emerged. Up to now, compelling evidence from preclinical studies collectively indicate the therapeutic role and efficacy of MSC exosomes in improving morphological, biochemical, and histological outcomes in bone regeneration[53].

In one study, Qi et al. demonstrated the effect of human-induced pluripotent stem cells (hiPSC)-derived mesenchymal stem cells secreted exosomes (hiPSC-MSC-Exos) in repairing critical-sized bone defects in an ovariectomized osteoporotic rat model [54]. The authors extracted the exosomes from the conditioned medium culturing the hiPSC-MSCs and implanted directly to

the defect site *in vivo*. The treatment group demonstrates significant enhancement in bone regeneration and angiogenesis compared to the sham group (**Fig. 4Ai & ii**). Further *in vitro* investigation showed enhanced cell proliferation, alkaline phosphatase (ALP) activity, and up-regulated mRNA and protein expression of osteoblast-related genes in BMSCs derived from the ovariectomized rats, collectively validating the efficacy of xenogeneic exosome treatment in stimulating the proliferation and differentiation of BMSC into osteoblasts. Accordingly, the implanted exosomes successfully restored the disrupted bone homeostasis and promoted bone repair in osteoporotic animals. Additionally, the authors also demonstrated the feasibility of coupling the hiPSC-MSC-Exos with a scaffold for direct implantation in improving osteogenesis and angiogenesis at critical-sized calvarial bone defect in ovariectomized osteoporotic rats (**Fig. 4Aiii**). In addition, exosomes could also act as an impediment in inhibiting osteoclast activity. A recent study by Song et al. demonstrates the ability of exosomes in targeting remote bone tissues through delivering EC-secreted exosomes (EC-Exos) indirectly to an ovariectomized osteoporotic mouse model [55]. The authors extracted the exosomes from cell medium of culturing mouse vascular endothelial cells (bEND.3) and delivered intraperitoneally to the animals. Incorporation of EC-Exos was found in the bone defect sites and led to favorable outcomes in enhancing bone repair (**Fig. 4Bi & ii**). Follow-up *in vitro* experiment showed that the EC-Exos act by reversing osteoporotic activity through inhibiting osteoclast activity, potentially due to the high induced expression of microRNA miR-155 in the EC-Exos treated bone marrow-derived macrophages (BMMs; precursor of osteoclasts) (**Fig. 4Biii**).

The above studies demonstrated the role of exosomes in facilitating bone repair and regeneration, specifically in the ovariectomized osteoporotic animal models. The exosomes modulate the bone homeostasis pathway by incorporating its contents, upregulating bone deposition or downregulating bone absorption processes. Given the flexible nature of actions of

the exosomes (to be delivered directly or indirectly), it essentially opens the door to a manifold of clinical applications. For example, the direct implantation of exosomes with scaffolds may be ideal in complementing open surgeries and repair for facilitating bone regeneration, which can be difficult given the osteoporotic nature of the defect site. Whereas the remote bone targeting properties seen in EC-Exos delivered intraperitoneally may allow a systemic reversal of osteoporotic defects in the future. To date, there are no known clinical trials studying exosomes as a potential method for repairing osteoporotic defects. As the essential exosomal biomolecules responsible for mediating the bone remodelling pathway are being further identified, synthetic exosomes can then be engineered and harvested to tailor an effective treatment in reversing the systemic process of osteoporosis. However, critical questions on the safe dosage and long-term effect of using exosomes need to be answered prior to clinical use.

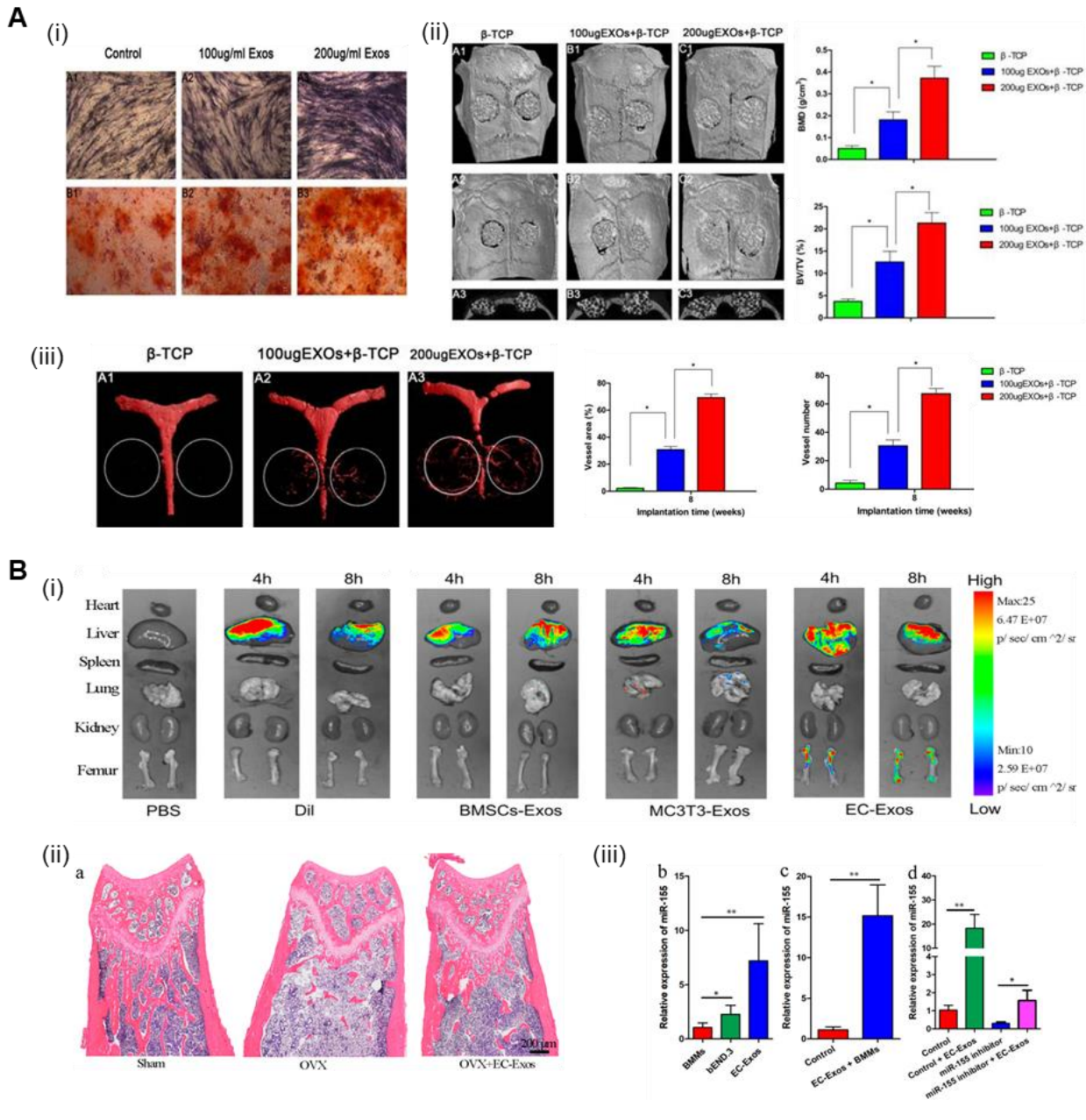

Figure 4. Application of exosomes on osteoporosis treatment. (A) Direct injection of hiPSC-MSC-Exos reversed osteoporotic activity through enhancing osteogenesis and angiogenesis in ovariectomized osteoporotic rats. (i) ALP staining at 14 days (top row); and alizarin red S staining at 21 days (bottom row); (ii) Micro-CT demonstrating the effects of hiPSC-MSC-Exos with β-TCP scaffolds in promoting bone regeneration in calvarial bone defects; (iii) Quantitative analysis of bone mineral density (BMD; left) and bone volume/total volume (BV/TV; right) at 8 weeks post-

operation (*p < 0.05); (iii) Microfil perfusion demonstrating the effects of hiPSC-MSC-Exos with β-TCP scaffolds in promoting angiogenesis in calvarial bone defects; (iv) Quantitative analysis of vessel area (%; left) and vessel number (right). Figure modified from [54] with permission. (B) Indirect (intraperitoneal) delivery of EC-Exos reversed osteoporotic activity through inhibiting bone resorption. (i) Biophotonic images demonstrating the bone targeting properties of EC-Exos *in vivo* when delivered intraperitoneally; (ii) Hematoxylin and eosin staining of distal femur sections (scale bar: 200 μm); (iii) qRT-PCR quantification of miR-155 in EC-Exos (left), in EC-Exos-treated cells (middle), and in different groups (right) (**p <0.005). Figure modified from [55] with permission.

3.4 Osteonecrosis

Femoral head osteonecrosis (FHON) is a joint femoral head ischemia condition that can be caused by hip trauma, misuse of alcohol, overuse of corticosteroids or some hematological diseases; which ultimately leads to the collapse of the femoral head[56]. The screening of exosomes in patients and healthy donors revealed that in patients with steroid-induced FHON, the amount of circulating exosomes was lower than that in healthy donors, suggesting that the amount of serum exosomes for steroid-induced FHON had moderate diagnostic precision[57]. It is even conceivable that the exosome level transition can also precede the MRI change, such as the 'double-line' symbol being seen. These findings indicate that it would be significant to know if the circulating exosomes for steroid-induced FHON can be used as 'super early' diagnostic markers. Additionally, experiments have validated that serum exosomal miRNAs can be screened as novel serological biomarkers for femoral head osteonecrosis caused by steroids (SFHON), which is accentuated by not only collapse of the femoral head, but also extreme OA and inevitable artificial joint arthroplasty [58]. Consistent with the results of miRNA sequencing, the qRT-PCR results demonstrated that hsa-miR-135b-5p

expression was remarkably up-regulated in the SLE-SFHON community. These findings offer much-needed insight in both screening and targeting miRNA sequences to achieve homeostasis for treatment of osteonecrosis.

Apart from screening exosomes in circulation, several studies highlighted that early therapy with human synovial-derived MSCs secreted exosomes had demonstrated good clinical relevance in FHON treatment. Pinpointing the elevated levels of miR-135b in osteonecrotic tissues, Zhang et al. treated FHON in a rat model with pluripotent stem cell induced MSC exosomes alongside miR-135b injections[59]. Results showed that exosomes alone could significantly improve bone repair evidenced by the heightened BV/VT score, while the addition of miRNAs further elevated the osteogenesis to a comparable level to the disease-less control. This suggests that the overexpression of miR-135b in malforming tissues is a response by the host body attempting to restore homeostasis. This opened many opportunities for targeting host response for treatment of degenerative diseases including FHON. Utilizing encapsulated miRNAs, Kuang et al. treated glucocorticoid (GC)-induced osteonecrosis in rat femoral heads, and mapped out the regulation of Wharton's jelly umbilical cord MSC exosomes on miR-21-PTEN-AKT signaling pathway[16]. After culturing umbilical MSCs for 2 days, exosomes were collected using centrifugation, filtration and membrane-based affinity binding isolation kit and analyzed with RNA sequencing. They reported that exosomes were able to reverse apoptosis of GC-induced osteocytes via the AKT pathway, while injection of exosomes into osteonecrosis-induced rat femoral heads was able to inhibit formation of necrotic tissues. Other studies have revealed that miR-21 is the major effector in treatment of osteonecrosis owing to its resemblance to PTEN expression, which upregulates downstream p-AKT to attenuate apoptosis. This demonstrates that the miRNAs in exosomes are valuable tools for reprogramming apoptotic tissues in degenerative diseases. In another study, Liu et al.[60] did an investigation to verify that iPSC-MSC-Exos could assist angiogenesis in FHON; a

model of steroid-induced rat osteonecrosis was injected intravenously with iPS-MSC-Exos, collecting the femoral head samples about 3 weeks after all the injections. The *in vivo* analysis illustrated that iPS-MSC-Exos administration greatly avoided bone loss and improved femoral head microvessel density in contrast to the control group. The proliferation, migration and tube-forming capacities of ECs were evidently enhanced *in vitro* by iPS-MSC-Exos. In ECs, iPS-MSC-Exos can activate the PI3K/Akt signaling pathway. In addition, the promotional effects of iPS-MSC-Exos on ECs were eradicated after the blockade of PI3K/Akt. By encouraging local angiogenesis and mitigating bone degradation, iPS-MSC-Exos transplantation exerts a defensive effect on FHON [60]. While exosomes were demonstrated to be effective at promoting angiogenesis and attenuating apoptosis in FHON models, more in-depth studies concerning their molecular mechanism needs to be conducted to fully elucidate their clinical relevance.

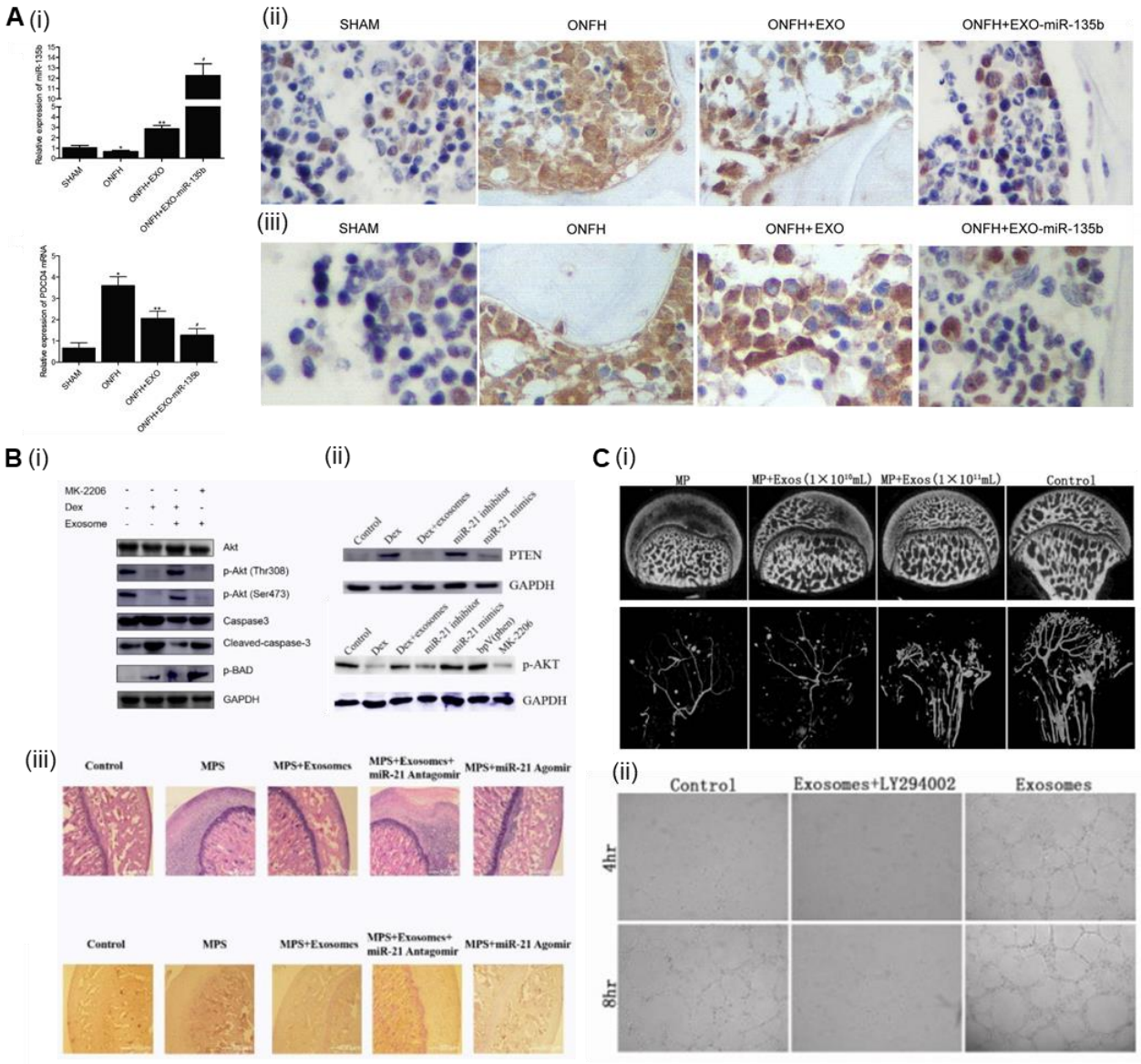

Figure 5. Application of exosomes on osteonecrosis treatment. (A) Treatment of FNOH with mircoRNA- 135b- reinforced exosomes. (i) Real-time PCR quantification of miR- 135b, PDCD4 mRNA/protein expression in exosome groups. (ii) IHC of PDCD4 protein and (iii) TUNEL assay of osteocyte apoptosis in rat animal model of FNOH. Figure modified from [59] with permission. (B) Administration of Wharton's jelly MSC exosomes for treatment of glucocorticoid-induced osteonecrosis. (i) Western Blot of AKT, p-AKT, caspase 3, cleaved caspase 3 and p-BAD. (ii) Interaction between miR-21 and PTEN by western blot. (iii) Histological analysis and

immunohistochemical staining of p-AKT in rat femoral head samples. Figure modified from [16] with permission. (C) Treatment of osteonecrosis with iPS-MSC derived exosomes. (i) Micro-CT and 3D microangiography analysis of osteogenesis and angiogenesis in rat FNOH models 3 weeks after joint injection with exosomes. (ii) Capillary network formation assay of exosome treatment with and without blockade of PI3K pathway with LY294002 inhibitor. Figure modified from [60] with permission.

3.5 Other osteochondral symptoms

Osteochondral conditions may arise from various sources other than disruption of the bone or cartilage homeostasis. In bone metastasis, recent findings revealed that the migration of tumour cells is not autonomous, as demonstrated by a study that treatment of lung metastatic tumour-derived exosomes would direct tumour cells to transfer to the lungs instead of the immediate region such as bone tissues[61]. This proves that the metastatic nature of bone cancer can be manipulated to develop novel treatment strategies, as well as the vital role of exosomes in controlling its behaviours. For instance, Zhang et al. was able to target TRA2B, an overly expressed gene in osteosarcoma tissues, and inhibit the development of osteosarcoma by delivering BMSC exosomal miR-206 to bone tumours[62]. After transfecting BMSCs with siTRA2B, miR-206 mimic, miR-206 inhibitor respectively, osteosarcoma cells transfected with siTRA2B and miR-206 mimic exosomes exhibited reduced proliferation as well as apoptosis, which the miR-206 inhibitor was able to partially mitigate the inhibition. In xenograft models of osteosarcoma, more lung metastases were observed in the treatment-less group while almost no visible metastases could be observed after treatment of miR-206 mimic exosomes, demonstrating the metastatic suppression of BMSC-derived exosomal miR-206 *in vivo*. On the other hand, diabetes mellitus was well demonstrated to negatively affect the turnover rate of bone tissues, leading to conditions such as osteoporosis and

osteochondrosis. It was revealed that Wnt signaling pathway in type 2 diabetes differed from healthy individuals, and that miR-322-3p expression was elevated in exosomal miRNA and blocked the expression of Wnt/β-catenin pathway genes[63]. In another study, Sabry et al. derived exosomes from MSCs for the treatment of induced type 1 diabetes[64]. Exosomes were extracted from supernatants of cell culture by differential and ultracentrifugation, then introduced to diabetic rat models. MSC exosomes was able to reverse both the elevated serum glucose levels and downregulated plasma insulin over 3 weeks, as well as mediation of genes contributing to insulin production. Another disease that may negatively impact bones and joints in human body is Systemic Lupus Erythematosus (SLE), an autoimmune disorder that causes immune cells to attack connective tissues, leading to patients being five times more likely to experience osteoporotic fracture. The steroids used normally for treatment of SLE contributes towards bone loss, as well as inducing pain and fatigue which results in inactivity. Dong et al. identified the significantly reduced miR-146a levels in the circulating exosomes of SLE patients, and explored the possibility of encapsulating external miR-146a in MSC exosomes for treatment of SLE[65]. Without functionalization, patient-derived exosomes caused disorganization of cytoskeletons and reduced growth rates of BMSCs, whereas miR-146a encapsulated exosomes were able to reverse senescence of cultured cells, as evidenced by the lowered expression of SA-β-gal positive cells and TRAF6, a protein mediator of autoimmune inflammation. Together, these studies offer implications to treating osteochondral conditions affected by external factors using exosomes.

Conclusion and future perspectives

Much headway has been achieved in utilizing exosomes for osteochondral repair, where its mechanism, application strategies and elevated productions have been widely explored. As of recent years, researchers have pinpointed multiple molecules including transcription factors,

receptors, enzymes, lipids, and nucleic acids (DNA, mRNA, and miRNA), as well as identifying certain cell-specific or conserved differences in lipid content of exosomes. However, full utilization of all properties of exosomes could not be achieved due to the complexity of its loaded cargo, and most studies could only focus on singular functional molecules within exosomes. A better understanding in exosome biogenesis aids in improving its production in laboratory settings owing to the emergence of various bioreactors specialized for scaling up exosome production, but the limited production scale greatly limits its clinical potential. These include altering physical conditions such as hollow fiber cultures, mechanical stimulations using shear stress, as well as stimulating deriving cells with biochemical factors to induce cellular stress for improved exosome secretion.

Alternatively, the methods for administering exosomes are dubious as well. The injection of exosomes into the osteochondral interface (e.g., joint) is the most direct approach in delivering exosomes to the target site, and is able to initiate osteochondral repair within 2 weeks in most animal models. However, exosomes are endocytosed quickly by chondrocytes within a day with intracellular concentrations peaking at 12 h [42]. This requires frequent injections which may prove too onerous for patients' day-to-day lifestyle and increase risk of infection. A high dosage injection to reduce frequency is inapplicable either as exosomes may present cytotoxicity at high concentrations due to the elevation of cell death associated genes [66]. Thus, researchers have switched their focus to the macro-structural application of exosomes using hydrogels to improve exosomes retention. As aforementioned, hydrogels including collagen, gelatin and hyaluronic acid are widely used in osteochondral regeneration due to their physiochemical properties. Through encapsulation of exosomes with hydrogels, they can compensate for each other's shortcomings: exosome's release and consumption can be greatly extended through entrapment within tendrils of the hydrogel network, while hydrogels attain much needed bioactivity for recruitment of host cells

[67]. Studies have shown that co-crosslinked gelatin/hyaluronic acid tissue patches were fabricated and shown to exhibit excellent exosome retention capabilities with only ~10% loss after immersion in PBS for 14 days [68]. Additionally, scaffolds can be designed with the morphology of defect sites in mind to aid in supporting regeneration and prevention of stress shielding. For instance, 3D printed ECM/GelMA/exosome scaffold with radially oriented channels using sterolithography was shown to enhance cartilage and subchondral bone regeneration, as well as reversal of mitochondria oxidative stress and dysfunction from osteoarthritis [69]. Yet in their study, no control regarding pattern-less scaffold was included, limiting its authority. Strategies revolving around the combination of exosomes with clinical surgeries are on the horizon as well. For treatment of severe osteochondral conditions, total joint replacement is performed to replace the necrotic tissues. Most scaffold frames were made out of titanium, and studies have shown that there is promising therapeutic potential in combining titanium nanotubes with exosomes for osteochondral regeneration[28]. Exosomes were loaded into titanium nanotubes through dip coating with dopamine then exosome solution, and were shown to promote osteogenic activity of BMSCs. This offers opportunity for functionalizing osteochondral implants with exosome-incorporated titanium nanotube surfaces, of which the bioactivity from exosomes and improved surface roughness may improve osseointegration. Herein, while exosomes' therapeutic potential is indisputable, their clinical delivery in respect of macro structures still require further development for long-term and localized treatment at defect sites.

Exosome research has slowly progressed towards clinical application for osteochondral repair. For instance, clinical trials for exosomes were well underway (as of May 2020, 170 clinical studies using exosome therapy are available. Source: ClinicalTrials.gov), while guidelines for exosome production in compliance with good manufacturing practices have been established [70]. This includes Minimal Information for Studies of Extracellular Vesicles (MISEV) from The

International Society for Extracellular Vesicles (ISEV), which offered much information and instructions for researchers to follow. However, these are often general and too vague for exosomes endowed with specific and exquisite properties, as discussed in this review. Much care needs to be taken when producing exosomes under biological stimulus such as proinflammatory factors as the related side effects were currently unknown. Additionally, the use of exosomes in clinical settings create ethical dilemma and safety concerns as the allogenic potential of exosomes were well demonstrated only in laboratory settings but not in clinical trials. In an autologous setting, timely preparation of exosomes is in high demand as early treatment of osteochondral conditions are critical. Thus, techniques such as mechanical stimulation and rotary cultures may be employed to stimulate exosome secretion to reach the target levels of concentrations. These parameters must be tuned rigorously to avoid overexpression of apoptotic factors and shedding of non-exosomal vesicles. While doubtless an indispensable tool for osteochondral repair, as a relatively novel medicine in the tissue engineering field, more studies must be performed especially concerning the clinical efficacy and production of exosomes before they can be safely applied in human body.

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